Impact of Environmental Factors on Key Functional Groups involved in Nitrogen Cycling in Agricultural Ecosystems

Kumulative Dissertation
zur Erlangung des Doktorgrades der
Naturwissenschaften an der Fakultät für Biologie
der Ludwig-Maximilians-Universität München

vorgelegt von
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aus Landshut

Dezember 2009

Dissertation eingereicht am 01.12.2009

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Mündliche Prüfung am 09.03.2010

Abstract

The investigation of biochemical cycles in different environmental habitats is becoming more and more vital to improve and sustain the productivity as climate change and management practices drastically change speed, intensity and balance of nutrient cycling. The conversion of nitrogenous compounds in terrestrial ecosystems, mainly mediated by microorganisms, is regarded as a central turnover process in ecology, as nitrogen is one of the limiting factors for plant growth. The different processes of the nitrogen cycle are controlled by several biotic and abiotic factors. The adaptation mechanisms of functional microbial groups in response to governing factors, however, are still poorly understood.

In this thesis, nitrogen-fixing, ammonia-oxidizing and denitrifying communities were examined with regard to their responses in gene abundances to different agricultural treatments in a long-term agro-ecosystem in semi-arid Burkina Faso (Westafrica). Data were obtained from the rhizosphere of Sorghum over three plant development stages to follow developmental and seasonal effects. The different microbial groups were targeted via respective functional genes that encode the nitrogen-transforming enzymes. The observed changes in community abundance along the entire growth period lasting over the rainy and the dry season indicated that climatic factors and plant development stages interacted in diminishing nitrogen inputs and related effects. As compared to continuous straw and/or urea amendment, manure emerged to be the most important driver increasing gene abundances and shaping the ratios of nitrogen-fixing, ammonia-oxidizing and denitrifying microbes. As a result, coherences between community proportion shifts, amendments and productivity could be observed.

Another long-term agricultural experimental site, located in a moderate climate region (Theix, France) and managed as grassland, was chosen to study switches in grazing and their effects on two phylogenetically different but functionally similar microbial groups: ammonia-oxidizing archaea (AOA) and their bacterial counterparts

(AOB). Urine application altered abundance, activity and composition of both targeted nitrifying communities. The microbial populations adapted quickly to the new management regimens and nitrifier activity was always higher in grazed sites as compared to sites that were not exposed to urine. Nitrifier activity correlated with nitrifier community size, however, a coupling between community structure and activity was not found.

Altogether, both long-term experiments provided new insights into the ecology of nitrogen-transforming microorganisms and how they are affected by different management practices in regard to sustainable agriculture, in soils that strongly depend on anthropogenic nitrogen input.

Zusammenfassung

Die Intensität von Bewirtschaftungsmethoden und klimatischen Veränderungen haben Einfluss auf das Gleichgewicht von Nährstoffkreisläufen. Deshalb ist die Untersuchung biochemischer Umsetzungsprozesse in ökologischen Systemen von großer Bedeutung. Die mikrobielle Stickstoffumsetzung spielt gerade in terrestrischen Ökosystemen eine zentrale Rolle, da Stickstoff ein limitierender Faktor für das Pflanzenwachstum ist. Außerdem können N-Metabolite negative Einflüsse auf Umwelt und Klima ausüben. Die verschiedenen am Stickstoffkreislauf beteiligten Prozesse werden jedoch von unterschiedlichen biotischen und abiotischen Faktoren gesteuert, wobei die Adaptionsmechanismen funktioneller mikrobieller Gemeinschaften als Reaktion auf die genannten Faktoren noch nicht vollständig nachvollzogen werden können. In der vorliegenden Arbeit wurde die Abundanz von zentralen funktionellen Gruppen des N-Kreislaufs mit der Methode der quantitativen PCR erfasst und mit physiologischen Messungen des Umsatzpotentials verglichen. Verschiedene Prozesse des Stickstoffkreislaufs wurden in zwei unterschiedlichen Böden zweier unterschiedlicher Klimazonen untersucht, um die mikrobielle Stickstoffumsetzung in Reaktion auf verschiedene Bewirtschaftungssysteme und Umweltfaktoren besser verstehen zu können.

Der erste Teil der Arbeit beschäftigt sich mit dem Einfluss verschiedener Dünger auf drei wichtige Umsetzungsprozesse des Stickstoffkreislaufs (Stickstofffixierung, Ammoniumoxidierung und Denitrifizierung) in der Rhizosphäre von Sorghum in einem semi-ariden Ackerboden in Burkina Faso (Westafrika). Das Verhältnis von Stickstofffixierern, Ammoniumoxidierern und Denitrifizierern war bestimmt durch die jeweiligen ausgebrachten Dünger. Im Vergleich zu langjähriger Zugabe von Streu und Harnstoff, war nur bei Stalldung der Ernteertrag gesteigert. Die Veränderungen der Genabundanzen entlang des gesamten Pflanzenentwicklungs-zyklus gekoppelt mit Ertrags- und Bodendaten geben allerdings Hinweise darauf, dass der Düngeeffekt stark von klimatischen Faktoren beeinflusst war, da die zum

Teil extremen klimatischen Bedingungen den ertragssteigernden Effekt des eingetragenen Stickstoffs reduzierten.
Der zweite Teil der Arbeit befasst sich mit einem Agrarboden der gemäßigten Klimazone (Theix, Frankreich), welcher als Weideland genutzt wurde. Hierbei wurde untersucht, wie sich die Umstellungen eines bestehenden Beweidungssystems auf die Dynamik zweier mikrobieller Schlüsselgruppen in der Nitrifikation (bakterielle und archaeelle Ammoniumoxidierer, bzw. AOB und AOA) auswirkt. Die erfassten mikrobiellen Gemeinschaften passen sich schnell an neue Beweidungssysteme an. Unabhängig von der neuen Bewirtschaftungsart wiesen beweidete Flächen höhere Nitrifikationsraten auf als nicht beweidete Flächen. Während die gemessenen Nitrifikationsraten mit den Abundanzen der Nitrifizierer korrelierten, war eine Kopplung zwischen der mikrobieller Zusammensetzung und der Nitrifikationsaktivität nicht ersichtlich.

Zusammengefasst liefern beide Langzeit-Untersuchungen neue Einblicke in die Ökologie der stickstoffumsetzenden Mikroorganismen und geben Aufschluss über den Einfluss verschiedener Bewirtschaftungsmethoden auf mikrobielle Gemeinschaften in Hinblick auf eine nachhaltige Landwirtschaft in Böden, die stark von anthropogenen Stickstoffeinträgen abhängig sind.

Acknowledgements

My deepest gratitude goes to Dr. Michael Schloter. I have been amazingly fortunate to have an advisor who supported me along the thesis with excellent guidance and inspiring discussions and who made science even more exciting by providing scientific glacier tours and the possibility to visit national and international congresses which contributed a lot to broaden my scientific view. His patience and support helped me overcome many crisis situations and finish this dissertation. I hope that one day I would become as good an advisor to my students as Michael has been to me.

I am deeply indebted to Prof. Anton Hartmann without whom this thesis would not have been possible. I had his valued help and advice since my diploma thesis and throughout the duration of this project, always holding me to a high research standard. His contribution and support are invaluable to me.

I would like to thank the collaboration and support of Dr. Jean-Luc Chotte and Prof. Xavier Le Roux who gave me the opportunity to experience and take an active part in a multifaceted research and Prof. Jean Charles Munch for the precious chance to go ahead in research world in the Institute of Soil Ecology at the Helmholtz Zentrum München. I gratefully acknowledge the Agence Nationale de la Recherche (ANR) for funding the “MicrObEs” project.

Many thanks go to Saidou Sall and Hélène Ndeye Diallo for their support at the experimental field site in Burkina Faso and for unforgettable, exciting moments which will be always in my memory.

I’d like to extend special thanks to Dr. Kristina Kleineidam whose talent helped me turn my ideas into something you might actually want to read, but also for inspiring discussions and continuous motivation. You were always a competent and dear colleague.

In addition, I am indebted to my brilliant colleagues with whom I had the honor of working and without whom my investigations would have been much more difficult and less exciting. Special thanks go to Julien for your loving support, to Andrea Bannert who brought the “one song project” into life, an essential compensation to my work and Marion Engel for last minute rescue.

Most importantly, none of this would have been possible without the love and support of my family and Wah-Pui Sam. They have been a constant source of love, concern, support and strength all these years. I would like to express my heart-felt gratitude to them and to God for strength, wisdom and good health to accomplish this thesis.

Contents

Abbreviations

Elements of the microbial nitrogen cycle

Nr	reactive nitrogen
N_2	dinitrogen gas
N_2O	nitrous oxide
NH_2OH	hydroxylamine
NH_3	ammonia
NH_4^+	ammonium
NO	nitric oxide
NO_2^-	nitrite
NO_3^-	nitrate

Enzymes involved in the nitrogen transformation

AMO	ammonia monooxygenase
HAO	hydroxylamine oxidoreductase
MoFe	molybdenum iron protein of nitrogenase
NAP	membrane-bound nitrate reductase
NAR	periplasmic nitrate reductase
NirK	copper-containing nitrite reductase
NirS	cytochrome cd_1 nitrite reductase
NOR	nitric oxide reductase
NXR	nitrite oxidoreductase
NxrA	large or α-subunit encoding the nitrite oxidoreductase
NxrB	small or β-subunit encoding the nitrite oxidoreductase

Functional genes catalyzing processes in the microbial nitrogen cycle

amoA	gene encoding the α-subunit of the ammonia monooxygenase
amoB	gene encoding the β-subunit of the ammonia monooxygenase
amoC	gene encoding the γ-subunit of the ammonia monooxygenase
hao	gene cluster encoding the hydroxylamine oxidoreductase

napA	gene encoding the periplasmic nitrate reductase
narG	gene encoding the membrane-bound nitrate reductase
nifD	gene encoding the dinitrogenase subunit a
nifH	gene encoding the nitrogenase reductase
nifK	gene encoding the dinitrogenase subunit b
nirK	gene encoding the copper-containing nitrite reductase
nirS	gene encoding the cytochrome cd_1 nitrite reductase
nor	gene encoding the nitric oxide reductase
nosZ	gene encoding the catalytic subunit of the nitrous oxide reductase
nxrA	gene encoding the α-subunit of the nitrite oxidoreductase
nxrB	gene encoding the β-subunit of the nitrite oxidoreductase

Grazing regimen

G→G	grazing control
G→U	cessation of grazing
U-G	grazing regimen in un-grazed systems
U-U	un-grazed control

Macronutrients

C	carbon
N	nitrogen
P	phosphorus

Metal ions required for the regulatory function of enzymes

Fe	iron
Mo	molybdenum
Va	vanadium

Plant development stages

EC30	early leaf development of the plant
EC60	flowering of the plant
EC90	plant senescence

Prokaryotes involved in the nitrogen turnover processes

AOA	ammonia-oxidizing archaea
AOB	ammonia-oxidizing bacteria
NOB	nitrite-oxidizing bacteria

Others

ANAMMOX	anaerobic ammonia oxidation
BNF	biological nitrogen fixation
DNA	deoxyribonucleic acid
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
mRNA	messenger RNA

Introduction

Significance of nitrogen for environment and agronomy

Nitrogen occurs in many different forms, which differ in function, reactivity and mobility. The least reactive species, dinitrogen (N_2), is at the same time the most abundant form of nitrogen making up 78% of the atmosphere. N_2 is not directly available for plants, but is introduced as plant available N by lightning, human activities or bacteria, either living in symbiosis with leguminous plants (Bradic *et al.*, 2003; Vance, 1998) or being root-associated and free-living in soil (Vadakattu & Paterson, 2006). Reactive nitrogen (Nr) represents all biologically active nitrogen compounds including inorganic oxidized forms, e.g. NO_2^-, NO_3^-, N_2O, whereby nitrate (NO_3^-) is best assimilated by plants, but most easily lost by leaching due to its high mobility (Gustafson, 1983; Stopes *et al.*, 2002). Furthermore, Nr comprises inorganic reduced forms of nitrogen like NH_3 and NH_4^+, whereby ammonium (NH_4^+) can be directly assimilated by plants and is less easily lost than NO_3^-. Reactive N in form of organic compounds includes e.g. urea, amines and proteins and can be retransformed into inorganic nitrogen by nitrogen-transforming microbes.

Nitrogen is the most important determinant of plant growth and crop yield (Hooper & Johnson, 1999; Marschner, 1995). However, the amount of fixed N in un-managed ecosystems is not sufficient to meet the human dietary demand. Firstly, the Haber-Bosch process provided the needed amounts of Nr to meet the growing demand for food and fibre. Since then, human activities like the "green revolution" have massively increased the inputs of nitrogen to terrestrial ecosystems worldwide over the past century (Smil, 1990). Beside the beneficial effect on human health and welfare through the provision of quality food and renewable energy sources from biomass, the acceleration of Nr has also led to a complex network of environmental impacts. Adverse effects cross all environmental spheres with the main impact on

ecosystem functioning due to losses of biodiversity in terrestrial and aquatic ecosystems and changes in abundance of beneficial soil organisms (Matson *et al.*, 2002). For example, increased NH_x from cattle husbandry often lead to acidification of soils and freshwater ecosystems (Matson *et al.*, 2002; Rabalais, 2002). N saturation in soils increase export of Nr to downstream aquatic environments (Matson *et al.*, 2002), resulting in eutrophication and in some cases hypoxia in coastal ecosystems (Rabalais, 2002). NO_x emissions increase tropospheric ozone, which can result in ozone-induced damage to crop, forest and natural ecosystems. Decreases in stratospheric ozone and changes in global climate can result from increased emissions of the greenhouse gas N_2O from terrestrial and aquatic ecosystems due to excess input of Nr (Matson *et al.*, 2002; Wolfe & Patz, 2002). In conclusion, anthropogenic inputs of nitrogen have caused dramatic ecological imbalances in various ecosystems. In order to effectively monitor these ecological changes, investigations on the microbial scale are urgently needed on the one hand to understand the mechanisms behind the nitrogen cycle and on the other hand because microbial activities and nitrogen turnover are considered to be primary controlling factors. Several attempts have been made to explore the microbial nitrogen cycle and adaptation mechanisms in response to environmental factors and anthropogenic nitrogen inputs. However, surprising findings in molecular ecology like the existence of ammonia-oxidizing archaea (Könneke *et al.*, 2005) have shown that our knowledge of the microbial nitrogen cycle and key players involved is still scarce.

The following sections describe agricultural ecosystems that served as experimental sites in this thesis: (i) arable soils in semi-arid climate regions used for crop production and (ii) temperate grasslands coupled with grazing managements. Both ecosystems depend upon nitrogen input through anthropogenic activities.

Nitrogen budgets in semi-arid tropic agro-ecosystems

Agriculture is the principle sector in Africa upon which the population depends in order to sustain their livelihood. 630 million ha are suitable for cultivation (Cleveland, 2008), however, productivity and food security are limited by high soil fertility degradation, mainly resulting from soil erosion and drought. With an average loss of 660 kg N ha^{-1} from Sub-Saharan Africa (during the last 30 years from 200 million ha of cultivated land) nutrient depletion is enormous (Stoorvogel & Smaling, 1990). In contrast, positive net nutrient balances of 2000 kg N ha^{-1} were reported from temperate regions of similar size (Sanchez, 1994). In order to replenish N stocks in African soils from 0.1 to 0.3%, applications of 160 t ha^{-1} of dry biomass or 8.7 t ha^{-1} of urea ought to be implemented (Sanchez *et al.*, 1997), amounts that are simply impractical and environmentally undesirable. Inorganic fertilizers account for one-third of the N input in Africa (Smaling, 1993), but do not increase C or N stocks in sandy soils when added alone (Pieri, 1989). In combination with organic fertilizers like crop residues, manures and composts, inorganic fertilizers may increase soil N and C stocks in arid ecosystems. The beneficial impact of organic fertilizers on plant growth and productivity has also been reported for semi-arid soils in Europe particularly with regard to microbial turnover processes (Bastida *et al.*, 2009; Ros *et al.*, 2003). However, compared with data from temperate climate regions, our knowledge about the significance of the microbial nutrient cycling in semi-arid ecosystems is far from being sufficient.

To sum up, nutrient depletion in Africa contrasts sharply with nutrient accumulation in temperate regions. Although much is known about the nutrient cycling in temperate climate regions, a generalization of data is not easily possible and hinders the advancement of soil science in the tropics, where research strongly lags behind. There is no question about the need of replenishing nutrient-depleted soils in Africa

by external inputs of nitrogen. But according to Giller (1997), the main issue in N replenishment is not the amount of nitrogen, but the effectivity of nutrient cycling. Therefore, it is important to improve our knowledge on the impact of fertilizing treatments on important processes in microbial nitrogen cycling in order to find effective and sustainable management practices to improve productivity in semi-arid ecosystems.

Nitrogen management in temperate grassland ecosystems

Grasslands occupy more than 40% of the global terrestrial ecosystem and occur in regions that are too dry for forests but have sufficient soil water to support a closed herbaceous plant cover (Chapin *et al.*, 2001). Temperate grasslands therefore usually evolve in areas with 150-1200 mm of annual precipitation and temperatures ranging between 0-25°C, comprising the Prairie of North America, the Pampa of Argentina and the steppes of Europe (Chapin *et al.*, 2001). Grasslands harbour a wide range of forbs and grasses, amounting 30 species in some grasslands of Patagonia (Golluscio & Sala, 1993) to 400 species in grasslands of the Pampa in South America (Cabrera, 1970). However, the majority of grasslands is semi-natural, meaning that plant communities are natural, but their maintenance depends upon anthropogenic activities like low-intensity farming. Such managed grassland ecosystems include the employment of grazing animals as a major farming strategy. Grazing plays an important ecological role, as with the deposition of nitrogen-rich urine and dung, grazing animals contribute largely to nutrient cycling (Risser & Parton, 1982), which in turn influences plant responses (Leriche *et al.*, 2001). Many studies demonstrate the impact of grazing on aboveground traits like plant diversity and shoot density (Coppock *et al.*, 1983; Olff & Ritchie, 1998; Valentine *et al.*, 1997) and below-ground traits like roots and soil organic matter (Johnson & Matchett, 2001). Other studies tried to link above- and belowground interactions with regard to grazing (Bardgett *et al.*, 1998; Bardgett & Wardle, 2003). In this context, grazing may not only have positive effects. For example, grazers feed selectively on plants with high N content leading to a dominance of plants with low N content, whereas litter from those species are less easily degraded. A few studies investigated the effects of grazing on microbial nitrogen cycling, in particular, denitrification and demonstrated enhanced N_2O emissions in urine-affected soils (de Klein & Logtestijn, 1994; Frank

& Groffman, 1998; Monaghan & Barraclough, 1993). Le Roux *et al.* (2003) studied the impact of grazing on two key processes of the nitrogen cycle, nitrification and denitrification. While intensive grazing stimulated the denitrifier activity only slightly, nitrification seems to be promoted by grazing, indicating that nitrification may play a more important role in grazed grasslands. Knowledge about grazing-related nitrification, however, is still scarce.

Taken together, herbivores are of central importance in the regulation of N storage and cycling, impacting various above- and belowground traits of plants. Designing an effective and ecological beneficial grazing practice requires more information about the impact on nitrogen cycle dynamics, especially nitrification, which provides plant-available nitrogen.

The following section describes the different processes of the microbial nitrogen cycle and involved key functional groups in detail. Special focus has been laid on the inorganic nitrogen cycle, in particular nitrogen fixation, nitrification and denitrification, as those processes make the major contribution to nitrogen turnover in agricultural well aerated soils.

Nitrogen cycling in terrestrial ecosystems

The biological nitrogen cycle in terrestrial ecosystems combines two cycles that are interlinked by NH_4^+: the organic and the inorganic nitrogen cycle (Fig. 1). The organic nitrogen cycle describes the conversion of ammonium to organically bound nitrogen and the reconversion of biomass into ammonium. However, formation and degradation of biomass is not addressed in this thesis as associated molecular processes and the microorganisms behind are yet poorly understood.

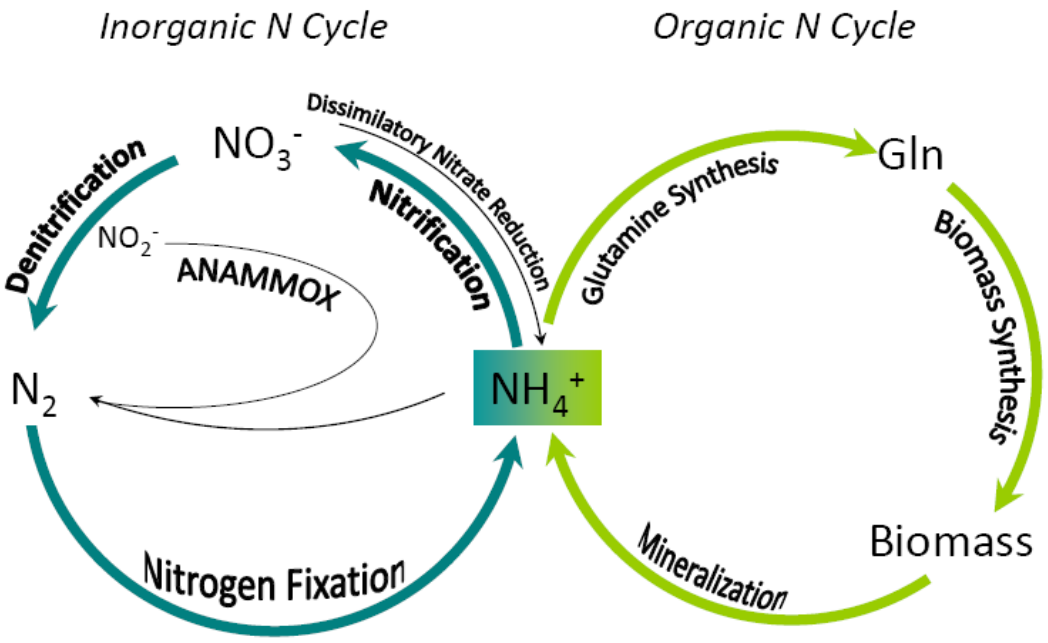

Figure 1. A simplified demonstration of the organic and inorganic nitrogen cycle.

Instead, three key processes of the inorganic nitrogen cycle were focused in this thesis which are closely interlinked and highly impacted by agricultural management practices in aerated soils: (1) biological nitrogen fixation, which is the only way for soil microorganisms to introduce molecular nitrogen into terrestrial ecosystems, (2)

nitrification, a process in which plant-available nitrate is formed under aerobic conditions, and (3) denitrification, the recirculation of inorganic nitrogen to the atmosphere in form of nitrous gases, occurring mostly under anaerobic or low oxygen conditions.

The regulation of these processes and their significance in soil ecology are elucidated in the next chapter. Thereby, the anaerobic ammonia oxidation (ANAMMOX) was neglected as it rather plays a role in paddy than in agricultural aerated soils (Cai, 2002). Evidence for the presence of ANAMMOX bacteria has only been found in aquatic ecosystems (Dalsgaard *et al.*, 2005; Mulder *et al.*, 1995).

The biological nitrogen fixation

The biological nitrogen fixation process describes the conversion of atmospheric nitrogen into ammonia by symbiotic, associative and free-living bacteria collectively called diazotrophs. This process is of tremendous importance to natural ecosystems and agriculture as it replenishes the overall nitrogen content and compensates the losses incurred through denitrification (Vitousek *et al.*, 2002).

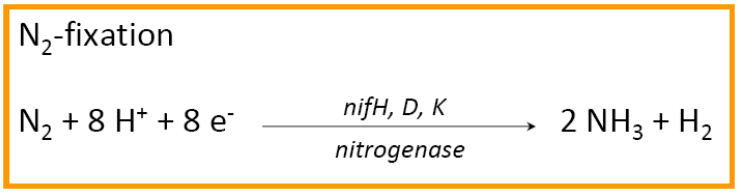

Figure 2. Biological N_2-fixation: the *nifH* gene, encoding the nitrogenase reductase part of the nitrogenase complex (*nifH*, *D*, *K*), is a commonly used marker for studying the diazotrophic community and the gene activity in different ecosystems.

Nitrogenases, which catalyze the biological reduction of dinitrogen to ammonium, are complex metallo-enzymes. There are four genetically distinct nitrogenases of which three are closely related (Newton, 2007): the molybdenum-based (Mo-), vanadium-based (V-) and iron-based (Fe-) nitrogenase. The fourth type of

nitrogenase was described in thermophilic streptomycetes having a completely different structure (Ribbe *et al.*, 1997). The best studied enzyme is the Mo-nitrogenase, which also presents the most efficient catalyst for N_2 reduction, followed by the V- and Fe-nitrogenase. While all N_2-fixing bacteria possess the Mo-nitrogenase – except those which carry the unique fourth nitrogenase – the distribution of V- and Fe-nitrogenase seem to appear completely random (Newton, 2007). The Mo-nitrogenase is composed of two metallo-proteins (Burris, 1991): (1) the MoFe-protein, also called dinitrogenase, is encoded by the *nifD* and *nifK* gene and is responsible for the nitrogen reduction and (2) the Fe-protein, known as dinitrogenase reductase, which is encoded by the *nifH* gene. Activity is only given when both proteins form a complex. *nifH* is one of ~ 20 genes that code for the nitrogen fixation pathway and is commonly used to identify nitrogen-fixing organisms (Jenkins *et al.*, 2004; Rosado *et al.*, 1998; Rösch *et al.*, 2002). BNF requires large amounts of energy (Zehr *et al.*, 2003) and responsible genes are only expressed when almost no fixed nitrogen is available. This is true for symbiotic N_2-fixing bacteria that live in close association, e. g. with legumes. Free-living or root-associated N_2-fixing bacteria are able to fix nitrogen under aerobic, anaerobic or micro-anaerobic conditions (Newton, 2007).

Symbiotic nitrogen fixers, such as *Rhizobium* spp. and *Bradyrhizobium* spp. possess significant economical and agricultural potential, as they supply the leguminous host plant with significant amounts of fixed nitrogen (Elsheikh & Ibrahim, 1999; Yanni *et al.*, 2001). Fixation rates of *Rhizobium* associated with legume forages range between 100 and 200 kg N ha^{-1}, *Rhizobium* associated with seed legumes reach approximately 300 kg N ha^{-1} (Smil, 1999). Vadakattu and Patterson (2006) indicate that non-symbiotic nitrogen fixers may also contribute significant amounts of plant-available nitrogen to arable soil. However, the amounts add up to 25-35 kg ha^{-1} and are therefore usually not comparable with symbiotic N_2 fixation. Highest non-symbiotic N_2 fixation rates were found in regions with high summer rainfall and is promoted by high temperatures in combination with moisture (Vadakattu & Paterson, 2006) indicating that free-living nitrogen fixation may have relevance in tropical soils with cultivation of non-leguminous crops (van Berkum & Bohlool, 1980). However, for large areas where BNF may be important, particularly in tropic regions

of Africa, Asia and South America, there is actually not much data available on natural terrestrial rates of BNF.

The nitrification pathway

Nitrification is a key process in the global nitrogen cycle as it influences N availability to plants and N losses to surface and groundwater. The oxidation of ammonia to hydroxylamine (NH_2OH) is the first and rate-limiting step in nitrification (Fig. 3), catalyzed by the ammonia monooxygenase (AMO). This enzyme consists of at least three subunits (α-, β-, γ-AMO) encoded by the respective genes *amoA*, *amoB* and *amoC*, whereas the *amoA* gene is commonly used as functional marker for studying aerobic ammonia oxidation (Rotthauwe *et al.*, 1997).

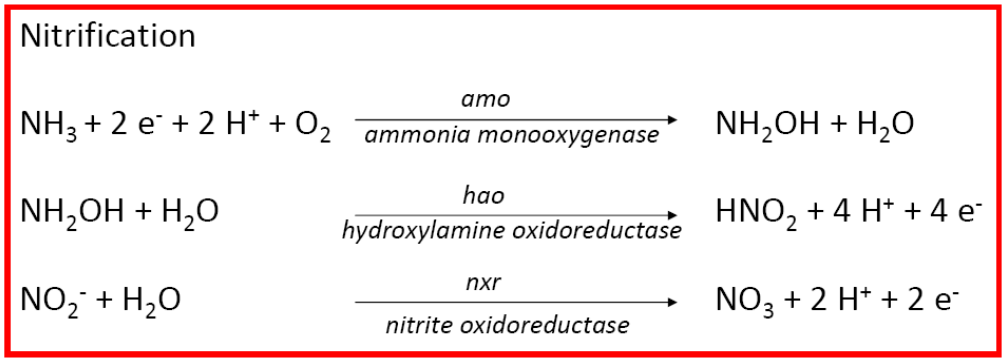

Figure 3. Stepwise oxidation of ammonia to nitrate: indications below the arrow describe enzymes that catalyze respective oxidation processes; indications above the arrow describe corresponding genes.

Autotrophic ammonium oxidation was until now attributed to aerobic (AOB) and anaerobic (ANAMMOX) ammonia-oxidizing bacteria. However, this picture has changed with the detection of a unique ammonia monooxygenase gene on an archaeal-associated scaffold sampled from the Sargasso Sea (Venter *et al.*, 2004) and was enlarged by the identification of archaeal ammonia oxidizers (AOA) through metagenomic studies (Hallam *et al.*, 2006; Schleper *et al.*, 2005; Treusch *et al.*,

2005). The first AOA strain, *Nitrosopumilus maritimus*, was isolated by Könneke and colleagues (2005). It is the first chemolithoautotrophic nitrifier in the domain Archaea and the first mesophilic species in the marine group 1 of crenarchaeota (Könneke *et al.*, 2005). The archaeal *amoA* gene (α-subunit of AMO) is ubiquitous; however, it can be easily differentiated from that of AOB, as they are sufficiently divergent (Rotthauwe *et al.*, 1997; Treusch *et al.*, 2005).

The presence of AOA was demonstrated in various ecosystems: in marine water (Francis *et al.*, 2005), hot spring (Hatzenpichler *et al.*, 2008), coastal sediment (Beman *et al.*, 2007), subterranean estuary (Santoro *et al.*, 2008) and in agricultural, sandy, forest, semi-arid, antibiotic-contaminated and paddy soils (Adair & Schwartz, 2008; Boyle-Yarwood *et al.*, 2008; Chen *et al.*, 2008; He *et al.*, 2007b; Schauss *et al.*, 2009; Shen *et al.*, 2008). Most of the investigations from terrestrial and aquatic ecosystems have shown a predominance of AOA over their bacterial counterparts (Adair & Schwartz, 2008; Chen *et al.*, 2008; He *et al.*, 2007a; Herrmann *et al.*, 2008; Leininger *et al.*, 2006; Schauss *et al.*, 2009). The oxidation rates, however, show a different picture. Known values of AOB (32-83 fmol NO_2^- $cell^{-1}$ h^{-1}) (Okano *et al.*, 2004; Prosser, 1989; Ward, 1987) exceeded by far the values of AOA (0.3 -1.4 fmol $cell^{-1}$ h^{-1}) (de la Torre *et al.*, 2008; Könneke *et al.*, 2005), suggesting that AOA may compensate the higher oxidation rates of AOB by higher abundances, meaning that AOB may still remain the main players in ammonia oxidation (Schauss *et al.*, 2009).

The subsequent dehydrogenation of NH_2OH to NO_2^- is catalyzed by the hydroxylamine oxidoreductase (HAO) (Wood, 1986). HAO is a multimeric protein (Hoppert *et al.*, 1995) binding seven *c*-type hemes and an active-site heme (Arciero & Hooper, 1993) encoded by the *hao* gene (Bergmann *et al.*, 2005). To date, efforts have been made to study the biochemical pathway and genetics of the hydroxylamine oxidation in *Nitrosococcus oceani*, *Nitrosospira multiformis* (Bergmann *et al.*, 2005) and *Nitrosomonas europaea* (Arciero & Hooper, 1993; Bergmann *et al.*, 1994; Hommes *et al.*, 1994; Hoppert *et al.*, 1995; McTavish *et al.*, 1993; Sayavedra-Soto *et al.*, 1994). However, because the ammonia oxidation constitutes the rate-limiting step of the nitrification pathway, ecological studies lay the major focus on the first step in nitrification.

The third step in nitrification, the oxidation of NO_2^- to NO_3^-, is performed by nitrite-oxidizing bacteria (NOB) and catalyzed by the enzyme nitrite oxidoreductase (NXR). The NXR of *Nitrobacter* is an integral membrane-bound enzyme complex, which consists of at least two subunits, the large subunit NxrA and the small subunit NxrB (Meincke *et al.*, 1992; Sundermeyer-Klinger *et al.*, 1984), which are encoded by the genes *nxrA* and *nxrB*, respectively. A few studies have been performed on *Nitrospira* indicating that the NXR of *Nitrospira* differs distinctly from that of *Nitrobacter* being membrane-associated in the periplasmic space (Spieck *et al.*, 1998). Furthermore, the subunits revealed differences in their molecular weight and their phylogenetic affiliation (Bartosch *et al.*, 1999). However, because biochemical properties of other known nitrite-oxidizing genera are scarce, data may not be generalized for all NOB.

Early phenotypic classification, based on cell morphology, the presence of characteristic cytoplasmic membrane structures (Watson *et al.*, 1989) and distinct fatty acid profiles (Lipski *et al.*, 2001), led to the description of four different genera of nitrite oxidizers. This classification has recently been extended to six genera on the basis of 16S rRNA sequence analyses: I) The most intensively studied genus, *Nitrobacter*, belongs to the α-proteobacteria (Navarro *et al.*, 1992). II) "*Candidatus* Nitrotoga arctica", a cold-adapted NOB isolated from a Siberian Arctic soil, is affiliated to the β-proteobacteria (Alawi *et al.*, 2007). III) The γ-subclass of *Proteobacteria* harbours the marine NOB *Nitrococcus mobilis* (Watson & Waterbury, 1971) and IV) a recently isolated strain from Konstanz sewage sludge, which is closely related to *Thiocapsa roseopersicina* (Griffin *et al.*, 2007). V) Two marine strains of *Nitrospina gracilis* (Nb-211, Nb-3) are representatives of the δ-proteobacteria (Teske *et al.*, 1994) and VI) NOB of the genus *Nitrospira* form a distinct phylum within the domain Bacteria (Ehrich *et al.*, 1995).

Data about the distribution of NOB, notably *Nitrobacter* and *Nitrospira*-like sequences indicate a dominance of *Nitrospira* spp. and suggest that *Nitrobacter* spp. may be adapted to high nitrite and oxygen concentrations (Daims *et al.*, 2001; Schramm *et al.*, 1999). Phylogenetic analyses further suggest a high diversity within the phylum *Nitrospira* (Daims *et al.*, 2001), with several 16S rRNA gene sequences isolated from soil environments. However, studies have laid their focus predominantly on the identification of AOB, as nitrification is limited by ammonia oxidation in most environments (Prosser, 1989). Consequently, the environmental

significance of nitrite-oxidizing bacteria (NOB) lags behind and remains to be further resolved.

The denitrification pathway

The denitrification pathway describes the stepwise reduction of NO_3^- to N_2 (Fig. 4). This process occurs under anaerobic conditions, whereby NO_3^- and NO_2^- are reduced to gaseous nitric oxide (NO), nitrous oxide (N_2O) and N_2. As some nitrate-reducing bacteria were misidentified as denitrifiers (bacteria performing the dissimilatory NO_3^- reduction (DNRA), Fig. 1), genetic analysis usually proceed from the reduction of NO_2^- which is the first step of the denitrification process exclusively restricted to denitrifiers (Goregues *et al.*, 2005) (Fig. 4). Mahne and Tiedje (1995) have therefore developed criteria to identify "true" denitrifiers and to distinguish them from NO_3^--respiring ammonia-producers: (1) reduction must be coupled to an increase in growth yield taking the concentrations of NO_3^- and NO_2^- into account, (2) N_2O and/or N_2 are major products of the nitrogenous oxide reduction and (3) the presence of nitrite, nitric oxide, or nitrous oxide reductases must be given.

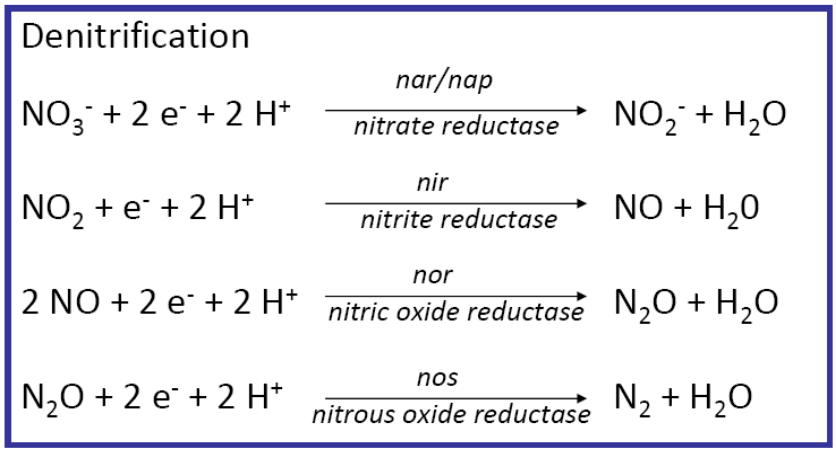

Figure 4. The denitrification pathway. Indicated are enzymes catalyzing respective reduction processes and gene clusters encoding the corresponding enzymes.

The first step in denitrification, the reduction of NO_3^- to NO_2^-, is catalyzed by two types of molybdo-enzymes, NAR and NAP (Zumft, 1997). The membrane-bound nitrate reductase (NAR) is involved in anaerobic NO_3^- respiration and denitrification, while the periplasmic nitrate reductase (NAP) participates in redox balancing and may denitrify under aerobic and anaerobic conditions. The active subunits NAR and NAP are encoded by the genes *narG* and *napA*, respectively, whereas denitrifiers can either possess one or both enzymes (Philippot, 2002; Zumft, 1997).

The reduction of NO_2^- to NO represents the key step in denitrification and is catalyzed by two structural different enzymes: the copper (NirK) (Fenderson *et al.*, 1991; Ye *et al.*, 1993) and the cytochrom cd_1 nitrite reductase (NirS) (Zumft, 1997). Both enzymes are equivalent in terms of functionality (Glockner *et al.*, 1993). However, no bacterial strain has been found to possess both enzymes. Quantification of the respective *nirK* and *nirS* genes in terrestrial ecosystems revealed that *nirK* genes may be more abundant in rhizosphere soil (Avrahami *et al.*, 2002; Huić Babić *et al.*, 2008), while *nirS* genes may be more abundant in bulk soil (Bastida *et al.*, 2009; Kandeler *et al.*, 2006) indicating a niche differentiation between the denitrifying populations (Cole *et al.*, 2004).

Three types of metallo-enzymes (NOR) are involved in the reduction of NO to N_2O (Zumft, 1993): cNOR, which uses soluble *c*-type cytochromes or pseudoazurin and qNOR, which uses hydroquinones as electron donor. qCU_ANOR is bifunctional using two electron donors: MQH_2 serving detoxification and cytochrome c_{551} serving a bioenergetic function. Non-denitrifying organisms may also possess the NOR enzyme due to the detoxifying function being able to cope with the toxic NO. However, Kwiatkowski *et al*. (1997) proposed that the nitric oxide reductase of *R. sphaeroides* uses NO from the environment to gain energy rather than to protect cells from the toxic compound.

The conversion of N_2O to N_2 is the last step of the denitrification cascade, catalyzed by the nitrous oxide reductase (NOS). The enzyme is a periplasmic homodimeric protein, each monomer containing two characteristic copper centres. The catalytic subunit is encoded by the *nosZ* gene. The reduction of N_2O is most important in terms of environmental concerns as it represents a respiratory process where many

denitrifiers grow at the expense of N_2O as sole electron acceptor (Philippot *et al.*, 2007). However, an unknown percentage of denitrifiers lack the genes encoding the N_2O reductase, while others possess them but downregulate the N_2O reduction activity, so that N_2O builds the end product of denitrification (Stouthamer, 1988).

With up to 5% of the total soil bacterial community, denitrifiers rank out other functional groups involved in nitrogen turnover (Henry *et al.*, 2004; Henry *et al.*, 2006). In view of their contribution to greenhouse gas emission, the reduction of denitrification by steering microbial activity is a great challenge in microbial ecology.

Aims and outline of the thesis

The overall aim of this thesis was to improve our knowledge of the microbial nitrogen cycling in terrestrial ecosystems. In this context, two different approaches have been conducted (**Publication II, III**) addressing different ecological questions, on the one hand (i) resolving factors that influence the nitrogen turnover in a semi-arid ecosystem with regard to sustainable land management and on the other hand (ii) studying the ecology of two functional groups that are regarded as key communities for efficient N cycling in a moderate climate ecosystem exposed to different grazing regimens.

Beforehand, particular attention has been paid to the evaluation of different molecular methods that are described to be suitable for the quantification of functional genes (**Publication I**). Quantitative data about genes in soil do not necessarily imply actual or potential turnover rates, but gene abundances provide a microbial basis for understanding substrate fluxes (Sharma *et al.*, 2006b). Therefore, three PCR-based, cultivation-independent methods have been compared and discussed (MPN-PCR, competitive PCR and real-time PCR) with respect to their applicability and reliability. The evaluation served to find a method to resolve ecological questions in **Publication II** and **III**.

Publication II examines key factors steering multiple processes of the microbial nitrogen cycle in a semi-arid agricultural ecosystem. Productivity of semi-arid soils is dramatically influenced by negative N balances (Sanchez *et al.*, 1997) mainly due to the extreme climatic conditions. Many studies have tested the effect of different nitrogen fertilizing treatments on plant and grain yield, but totally neglected the effect on microbial transformation processes on the molecular level. Therefore, the effect of different fertilizing treatments on the most important transformation processes (nitrogen fixation, nitrification and denitrification) was investigated to test

the hypothesis that each fertilizer regimen results in a typical ratio of the targeted functional groups. The experiment was conducted on a field site allowing the consideration of environmental factors and testing the sustainability of the different fertilizing treatments after almost 30 years of continuous application. Finally, grain yields were related to typical ratios of the targeted functional groups assuming coherence between abundance pattern and sustainability of particular fertilizing treatments.

Publication III describes two nitrifying communities (AOA and AOB) in grassland monoliths from moderate climate regions and their adaptation to novel grazing regimens. Grazing-induced enhancement of soil nitrogen dynamics can promote plant productivity by mitigating nutritional deficiencies (Leriche *et al.*, 2001; McNaughton *et al.*, 1997), whereby nitrification is considered as key process for efficient nitrogen cycling. Formation of nitrate, however, may be impaired by management modifications like intensification, de-intensification or abandonment of grazed grasslands mostly occurring at short time scales due to demographic and socio-economic constraints. The microbial response to such disturbances is hardly explored and requires evaluation, for which reason the dynamics of the nitrifying populations were investigated by determining their abundance, activity and distribution in response to switches in grazing management.

Publications

List of publications and contributions

Publication I-III

I. Sharma, S., V. Radl, **B. Hai**, K. Kloos, M. Mrkonjic Fuka, M. Engel, K. Schauss, and M. Schloter. 2007. Quantification of functional genes from prokaryotes in soil by PCR. *Journal of Microbiological Methods* 68:445-452.

II. **Hai, B.**, N. H. Diallo, S. Sall, F. Haesler, K. Schauss, M. Bonzi, K. Assigbetse, J. L. Chotte, J. C. Munch, and M. Schloter. 2009. Quantification of key genes steering the microbial nitrogen cycle in the rhizosphere of sorghum cultivars in tropical agro-ecosystems. *Applied and Environmental Microbiology* 15:4993-5000.

III. Le Roux, X., F. Poly, P. Currey, C. Commeaux, **B. Hai**, G. W. Nicol, J. I. Prosser, M. Schloter, E. Attard, and K. Klumpp. 2008. Effects of aboveground grazing on coupling among nitrifier activity, abundance and community structure. *ISME Journal* 2:221-232.

My contributions to the publications

I. Took part in planning the manuscript and participated in literature research.

II. Participated in the "Microbes" kick off meeting and took part in planning the study. Performed the major part of the laboratory work, including the quantification of all nitrogen cycle genes and the determination of mineral nitrogen, the statistical evaluation of the results and writing the manuscript.

III. Performed the quantification of the ammonia-oxidizing archaeal (AOA) and bacterial (AOB) abundance, participated in analysing the results and participated in writing the manuscript.

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ScienceDirect
Journal of Microbiological Methods 68 (2007) 445–452
Journal of Microbiological Methods
www.elsevier.com/locate/jmicmeth

Review

Quantification of functional genes from procaryotes in soil by PCR

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Received 24 June 2006; received in revised form 8 October 2006; accepted 8 October 2006
Available online 28 November 2006

Abstract

Controlling turnover processes and fluxes in soils and other environments requires information about the gene pool and possibilities for its in situ induction. Therefore in the recent years there has been a growing interest in genes and transcripts coding for metabolic enzymes. Besides questions addressing redundancy and diversity, more and more attention is given on the abundance of specific DNA and mRNA in the different habitats. This review will describe several PCR techniques that are suitable for quantification of functional genes and transcripts such as MPN-PCR, competitive PCR and real-time PCR. The advantages and disadvantages of the mentioned methods are discussed. In addition, the problems of quantitative extraction of nucleic acid and substances that inhibit polymerase are described. Finally, some examples from recent papers are given to demonstrate the applicability and usefulness of the different approaches.

Keywords: Quantitative PCR; Real-time PCR; MPN-PCR; Competitive PCR; Nucleic acid extraction; Microbial activity

Contents

1. Introduction

Soil quality is mainly based on the effectiveness of turnover processes of nutrients and recalcitrant substances under changing environmental conditions (Schloter et al., 2003). The organisms being responsible for these ecosystem services are mainly bacteria, fungi and archaea. However the relationship between microbial community structure and turnover processes (microbial community function) is poorly understood in all environments, as most results are based on studies measuring enzymatic ex situ potentials, ignoring the specific habitat conditions and the composition of the microbial communities being responsible for these processes (Emmerling et al., 2002). Also some other studies using isolation techniques to cultivate bacteria and fungi and to measure their metabolic

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0167-7012/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.mimet.2006.10.001

potential (Amann et al., 1995) are not suitable to understand the regulation of enzymatic activities in soil, as most processes, mainly the degradation of complex substrates, are typically controlled by a guild of diverse organisms and studies of a single isolate are not sufficient to understand in situ turnover rates. Hence, an approach which describes quantitatively the whole microbial community involved in a certain process is warranted. However, the same step of a turnover process can be found in diverse groups of organisms, which makes a simple quantification of ribosomal RNA genes for most functional microbial groups impossible. Even functions that were thought to be distributed only in a narrow phylogenetic group like the oxidation of ammonium into nitrite, have been detected in the last years using metagenomic approaches in very diverse groups of organisms (Leininger et al., 2006). Therefore techniques are necessary to quantify the genes of interest independent from the organism that is responsible for the corresponding enzymatic reaction to understand the potential for a certain turnover process. Moreover, data is needed that describe the relation between environmental factors and the expression of the genes of interest to understand regulation of turnover rates. Besides techniques based on the use of stable isotopes (Friedrich, 2006), which is a more indirect measurement of specific microbial activities, a more suitable approach to quantify the composition of functional guilds is the analysis of the abundance of functional genes by quantitative PCR (qPCR) from whole communities in nucleic acids (DNA and RNA) extracted from soils and other environmental samples.

A growing database of functional gene sequences from an array of environments as well as from genome projects has allowed researchers to develop primer sets using the conserved regions within the gene sequences. These primer sets can be used in quantitative PCR to measure the abundance of each gene and its transcripts in soils. In the following sections we describe some important aspects related to qPCR and its application in soil systems.

2. Quantification of nucleic acids extracted from soil

While qPCR can provide accurate measurements of gene/transcript numbers per unit of soil DNA/cDNA, it is more difficult to convert these values to cell densities and biomass or to set them in direct relation to soil mass or soil volume. A conversion to cell densities requires the knowledge of the copy number per genome and the genome size. Both numbers can be very variable (Fogel et al., 1999). The existence of multiple operons in one organism has been described for genes coding for the 16S rRNA many years ago. Today we know that the number of 16S rRNA genes per cell could be between one (many organisms) and 15 (*Clostridium paradoxum*) (Klappenbach et al., 2001). The number of gene copies for 16S rRNA is often reflecting the ecological strategies of the bacteria (Klappenbach et al., 2000). For functional genes, Mrkonjic et al. (in press) could show for example that the number of genes coding for the neutral metalloprotease (*npr*) can vary from one (many organisms) to seven (*Bacillus cereus* E33L). Additionally some genes encoding for neutral metalloproteases can occur on chromosome and plasmids. From seven copies of the gene encoding for NPR in *B. cereus* E33L, five are chromosomal coding and two are plasmid coding occurring on pZK467 plasmid as it is known from genome analysis (http://www.ncbi.nlm.nih.gov/). However, for other genes there is a good correlation between gene copy number and cell density. The number of genes coding for subtillisin (sub) is in most proteolytic bacteria one copy per genome. Only for *Bacillus amyloliquefaciens* ATCC 23844) two copies of the gene per genome were found.

Scaling to soil mass or volume requires quantification of the nucleic acid extraction efficiency. Hence, the first step in molecular analysis of soils, the nucleic acid extraction, is a very critical one for the later quantitative interpretation of the data.

It is regularly assumed that culture-independent analysis of purified nucleic acids allows a more representative view on environmental microbial communities than culture-dependent characterization of cells isolated from the soil (Amann et al., 1995). However, this perspective implicitly assumes that all microorganisms present in a given soil are amenable to nucleic acid extraction in the same way. To ensure *qualitative analysis*, e.g. microbial diversity, *all cell types must be lysed with equal efficiency. Quantitative analysis*, *in addition*, *requires that total nucleic acids are extracted quantitatively*, *without sequence specific bias*. Considering the morphological and physiological variability of soil microorganisms these assumptions are non-trivial.

Since the first protocols were established, direct extraction of DNA and RNA from soil became almost a routine. Although a lot of effort has been put into the optimization of protocols, there is still no consensus standard defined to ensure quality control for nucleic acid extractions from different soil types and microbial communities. Studies evaluating different extraction protocols mostly tried to optimize the maximum total DNA yield, DNA quality (long fragments and amenability to enzymatic reactions such as PCR and restriction), and maximum diversity of detectable organisms (Bürgemann et al., 2001; Kuske et al., 1998; Miller et al., 1999). Methods also compromise between maximum nucleic acid yield (or recovery rate) and remaining contaminations acceptable for down stream analysis. Numerous methods were used for nucleic acid extraction but also for determination of yields and recoveries (discussed in detail below) limiting a comparison of results. Nucleic acid extraction protocols e.g. consist of two steps: in the first step microbial cells are lysed. In the second step the nucleic acids are purified from the other cell and soil components of the crude extract. Yields, recovery rates and detection limits were measured in a third step either directly as nucleic acid concentrations or sequence specific with PCR or hybridization techniques.

The first step required (*the lysis of the cells*) can already introduce a qualitative and quantitative bias into the preparation. Two major strategies are described for the lysis of microbial cells from soils: cells can be separated from the soil before lysis (Torsvik et al., 1990; Holben et al., 1988) or lysed directly in the soil matrix (Ogram et al., 1985). For either protocol, it is very difficult if not impossible to thoroughly quantify cell lysis based

on the total microbial population as direct microscopic determinations are not possible due to the optical density of the soil. An upstream extraction of cells before lysis (Ozawa and Yamaguchi, 1986; Bakken, 1985) generally gives lower yields of total extracted DNA and is supposed to be selective for bacterial cells (Steffan et al., 1988; Courtois et al., 2001). Mainly cells that may be tightly adhering to soil surfaces or even be enclosed in particles are protected from dissolution even after detergent treatment or bead milling dependent on the type and the physiological state of the cells. Recoveries of 30–42% were reported (Bakken, 1985; Steffan et al., 1988). Harsher treatments for cell extraction may cause a preliminary lysis of less protected cells also resulting in losses or shearing of the nucleic acids. Though it is generally recognized that total DNA yields are higher with direct lysis protocols, they do not warrant that all microorganisms are targeted. Lysis efficiencies vary with the protocol used and the type and size of the cells in the sample (Zhou et al., 1996; Kuske et al., 1998). Microorganisms can form very rigid cell walls or capsules in dependence of their growth state and as a protection against adverse environmental effectors especially then forming survival states. With an SDS-based lysis protocol, Zhou et al. (1996) achieved lysis efficiencies ranging from 26–92% dependent on the soil type. Previous grinding of the soil improved rupture of Gram-positive cells by a factor of 4 (Zhou et al., 1996). Grinding of soil was estimated to lyse 34–75% of the cells dependent on soil type (Frostegard et al., 1999). Frostegard et al. (1999) also observed a significant shift in the surviving actinomycete population comparing different lysis protocols indicating a qualitative bias due to the rupture procedure. Increased DNA yields were observed when soils seeded with Gram-positive and fungal cells were treated by bead milling (~20%, Kuske et al., 1998) before extraction. DNA recovery from seeded spores and mycelia of *Streptomyces lividus* differed by 1–2 magnitudes also indicating differential lysis in dependence of the growth state (Frostegard et al., 1999). Additionally, the extraction methods largely influence the quality of the DNA: sheared DNA can lead to incorrect results in the down stream analysis by hybridization or PCR (Picard et al., 1992; Tebbe and Vahjen, 1993).

As contaminating soil components, e.g. humic acids, inhibit the activity of nucleic acid amplifying or modifying enzymes (Steffan et al., 1988; Tebbe and Vahjen, 1993; Wilson, 1997), the purification of nucleic acids (*step two*) is of central importance for qPCR. Tebbe and Vahjen (1993) showed that the MIC (minimum inhibition concentration) for humic acids is varying with the source and composition of the humic acid fraction (Tebbe and Vahjen, 1993). Further extraction and purification of nucleic acids (*step two*) is influenced by the soil matrix and soil type. Nucleic acids can bind to soil particles and colloids by ionic interactions, hydrophobic and van der Waals forces (Aardema et al., 1983; Lorenz and Wackernagel, 1987). Breaking these bonds during lysis and extraction leads to coextraction of extracellular DNA. Inversely, this also results in binding of the nucleic acids from lysed cells resulting in significant losses of up to 90%. It has been observed that this binding can introduce a bias for fragment size (Ogram et al. 1985; Frostegard et al., 1999). Blocking of binding sites with tRNA or phoshphate increased nucleic acid yields (Frostegard et al., 1999; Miller et al., 1999). Further purification steps mostly use either organic solvents or column purification. Both methods may be disturbed by soil components and have a limiting capacity that cannot be trespassed. Steffan et al. (1988) using a ^{14}C-thymidine label determined recoveries of 22–90% dependent of the purification protocol. Zhou et al. (1996) estimated the DNA recovery after different purification steps based on total cell counts and a theoretical DNA content of 1.6 or 5 fg cell^{-1} ranging from 10 to 300%. Investigating DNA yields from crude extracts before and after purification by densitometric measurements of fluorescence in agarose gels, Zhou et al. (1996) determined recoveries varying from 56–80% with solvent extraction. Tsai et al. (1991) estimated losses of 40% using Elutip Columns, supposedly due to selectivity for fragment sizes (Picard et al., 1992).

To overcome the problem of inhibiting substances, the addition of polymerase stabilizing substances or substances binding selectively the inhibitor to the PCR reaction is a solution if PCR products are analyzed qualitatively (e.g. by fingerprint analysis). If genes should be quantified, however, additives such as BSA (Poussier et al., 2002) or T4 protein (Jiang et al., 2005) are not suitable as they also bind extracted DNA or cDNA, resulting in lower gene copy numbers compared to the original extract of nucleic acids. Therefore, often the only possibility if a nucleic acid extract still contains inhibiting substances is to dilute the extract, which consequently reduces the target number. This is mainly for low-abundance target sequences often a problem as they cannot be quantified using qPCR techniques. In many studies using inoculated microbes, the detection limit of PCR techniques was a matter of research. Tracing a gene marker for labelled *Escherichia coli*, *C. glutamicum* and *H. polymorpha* cells, detection limits of 10^4–10^5 cells g^{-1} soil by hybridization and 10^1 cells g^{-1} soil with PCR were found (Tebbe and Vahjen, 1993). A detection limit of 10^5 cells g^{-1} dry soil was reported for *Bradyrhizobium* using hybridization with specific marker genes as probes (Holben et al., 1988), and a detection limit of 10^4 cells g^{-1} soil and 20–100% recovery with *Agrobacterium tumefaciens* tracking *vir* sequences with MPN-PCR (Picard et al., 1992). Using spores and mycelia from *Streptomyces* species, Frostegard et al. (1999) showed that the detection limit depends on the growth state and the soil type varying from 10^3–10^7 spores g^{-1} soil. Kuske et al. (1998) reported variations of the detection limit over two orders of magnitude (10^3–10^5 g^{-1} soil) dependent on the soil type but not on the cell type. However, it remains questionable if inoculated cells are truly representative for soil bacteria in terms of extractability. Mostly, log phase cultures are used for inoculation that is not comparable to the morphological and physiological state of the indigenous cells in soil.

For ecological investigations that require an appreciable number of samples to be analysed, it is not possible to optimize protocols for every soil or soil treatment. To compare different probes for sample variations but also variations in sample preparation and analysis, external extraction standards have been used. Only few attempts were made to define standard procedures to determine extraction

efficiencies that allow the correction of quantitative data for losses in nucleic acid extraction. There is no natural internal standard sequence that can be used in analogy to housekeeping genes widely used for pure culture or tissue analysis. Mumy and Findlay (2004) used cells of *E. coli* DH5α labelled with a specific pBR322-lambda construct as an extraction standard. Added as cells or purified plasmid, they determined recoveries of 37.5 or 26.7%. Similarly, Park and Crowley (2005) used plasmid or plasmid containing *E. coli* JM109 cells carrying specific genes (EGFP-N1) to normalize qPCR and DGGE data and to correct for extraction losses. For the same purpose, Widda et al. (2001) used a *Pseudomonas* labelled with a minitransposon construct that could be targeted in addition to the catabolic gene of interest and used as internal extraction standard. Lebuhn et al. (2004) compared samples spiked and non-spiked with the target sequence to achieve this correction for their qPCR and RTqPCR data. An alternative to avoid problems connected to DNA recovery and extraction efficiency is to quote gene abundance data to 16S rRNA gene considering that recovery efficiency for each of the target gene present in a soil sample is identical. This approach may help to overcome many of the mentioned problems. However, due to the huge overall heterogeneity in the number of genes encoding for the 16S rRNA an approach like this has to relay on the fact that bacteria which are responsible for the same turnover process belong at least to the same group of r- or k-strategists to ensure a similar number ribosomal operons (see above).

3. Methodologies for quantitative PCR

Quantitative PCR can be performed using three different methods: most probable number (MPN)-PCR, competitive PCR and real-time PCR. A comparison of the advantages and limitation of each method is provided in Table 1. Whereas for relative quantification, there is no requirement of a standard, it is needed for absolute quantification. As standards, mostly the gene of interest is cloned in a high copy vector system which is transformed to a suitable host. After growing of the host, the plasmid is extracted, purified, quantified and used in serial dilutions as reference. Occasionally, even genomic DNA has been used as standards. If quantification of transcripts is performed, the extracted RNA should be reversely transcribed using unspecific hexamers as primers (Sharma et al., 2004).

4. MPN-PCR

Simple end-point quantification of PCR products can be used for gene quantification if all the samples under comparison are in the logarithmic phase of the PCR reaction. However, employing the technique of quantitative PCR, this limitation of end-point PCR can be minimized. A first step towards real quantification was the combination of PCR with the statistical MPN procedure developed by Mc Crady (1915) and further improved by other authors (Cochran, 1950). This method consists of serial dilutions of DNA/cDNA samples until extinction and replicated PCR reactions for every dilution. The result of each reaction is scored positive or negative after gel electrophoresis analysis. The number of gene copies is calculated using the MPN statistics. The following assumptions are necessary to support the method: (1) the template should be randomly distributed within the sample; (2) a single copy of the target provides a signal. The accuracy of the assay will depend on the number of replicates and dilution rates. The coefficient of variation is inversely correlated to the replicate number used per dilution. The average accuracy is almost identical for dilution rates between 2 and 10. Nevertheless, coefficients of variation are more stable and slightly lower for 2 fold dilution assays (Cochran, 1950). The straightforwardness of the approach is its main advantage in relation to other qPCR methods, as the development of special probes, primers or internal/external standards is not required. Mainly the possibility to use also primer systems that amplify longer amplicons (>500 bp) and the possibility to use the same fragment for a qualitative (fingerprint or cloning/sequencing) and a quantitative analysis makes the technique quite interesting to be used. However, the amplification of longer fragments reduces the efficiency of PCR and consequently the detection limit of MPN-PCR. Additionally, the high number of reactions per DNA sample associated with post processing steps (agarose gel electrophoresis) turns the MPN-PCR into a laborious technique. Moreover, this method bears the high risk for contaminations, as many individual PCR reactions are necessary. This fact also relates to the relative high methodological error if replicates are compared.

Table 1
Advantages and disadvantages of techniques employed for quantification of genes and transcripts in soil

qPCR technique	Advantages	Disadvantages
MPN-PCR	—No special primer requirement	—Relative quantification
	—Relatively inexpensive	—Post-PCR processing
Competitive PCR	—Highly precise and accurate	—Post-PCR processing
	—Control of amplification efficiencies ensures accurate quantification	—Primers may not have same affinity for target and competitive sequences
Real-time PCR	—Reproducible	—Requirement of special cycler and software
	—Highly precise and accurate	—Primers usually unable to capture sequence-divergent species in environmental samples (high specificity)
	—High-throughput	—Requirement of special oligonucleotides (for TaqMan, Molecular Beacons etc)
	—Specific and reproducible	—Non-specific binding (SYBR-Green analysis)
	—Low detection limit (due to fluorescence technology)	—Amplicon length limiting
	—Additional information on amplification efficiency	
	—Measures template abundance over six orders of magnitude	
	—Accurate quantification of genes and transcripts	
	—Possible to target different genes in one step using probes labelled with different reporter dyes and multiplex PCR	

MPN-PCR has been introduced into microbial ecology first by Mäntynen et al. (1997). They aimed to develop a simple, fast and reliable method for the quantification of enterotoxin C producing *Staphylococcus aureus* NCTC 10655 from fresh cheese. Comparing their results with classical cultivation dependent methods, they could show that by amplifying the single copy chromosomal enterotoxin C gene fragment, it was possible to detect as little as 20 cfu g^{-1} cheese. Leung et al. (1997) were able to monitor by using an MPN-PCR protocol the survival of the pentachlorophenol-degrading *Sphingomonas* sp. UG30 by targeting a 753-bp fragment of the tetrachlorohydroquinone reductive dehalogenase gene (*pcpC*) of UG30 in soil. The MPN-PCR protocol had a detection limit of 3 cfu g^{-1} dry soil. They could show that the cell density of the UG30 inoculum decreased from 1.8×10^8 to 1.9×10^5 cells g^{-1} soil in the first 20 days of incubation and stabilized at 1.9×10^4 cells g^{-1} soil after 50 days. When the soil was autoclaved prior to inoculation, UG30 cell density remained at 6.7×10^7 cells g^{-1} of soil after 50 days of incubation. Degrange et al. (1998) investigated bacterial ammonium oxidation and nitrification in three coniferous forest soils using an MPN-PCR approach. The results indicated not only the presence of ammonia and nitrite oxidisers in the soils, but also factors that were involved in the inhibition of nitrification. The study threw light on some important ecological aspects demonstrating that (1) even if autotrophic nitrifiers are present, soil samples submitted to optimal (artificial?) conditions during short periods may be unable to exhibit a nitrifying activity, (2) *Nitrobacter* could survive in soil without expressing (or expressing at a very low level) nitrite-oxidation, which can be related to the fact that "autotrophic" nitrite-oxidisers may survive through a heterotrophic pathway. Apart from ecological contribution, the paper clearly showed that the use of a DNA extraction buffer with pH value similar to soil pH allowed the extraction of only very small amount of soil DNA. Highest yield was achieved with extraction buffer at pH 8.0. Moreover, the efficiency of DNA extraction was negatively correlated to the final purity of DNA. Sharma et al. (2006) used the method successfully also for the quantification of specific transcripts from genes involved in denitrification (periplasmic nitrate reductase gene (*napA*) and cytochrome cd_1 nitrite reductase (*nirS*)) during soil freezing and thawing. Differences in expression levels were observed by MPN-RT-PCR with higher levels of expression occurring just after thawing began, followed by a decrease. Interestingly, these data correlate well with the emission data of N_2O.

Also in other habitats, MPN-PCR was used successfully. Sei et al. (2004) was able to monitor the behaviour of catabolic genes and change of microbial community structures in seawater microcosms during aromatic compound degradation using this technique. Recently, also eukaryotes were tacked quantitatively by MPN-PCR. Carey et al. (2006) were able to develop a MPN-PCR method for quantifying infectious *Cryptosporidium parvum* oocysts in environmental samples.

5. Competitive PCR

In competitive PCR, serial dilutions of a competitor are added to the DNA/RNA sample prior to PCR amplification. The competitor molecule must have the same priming sites, but differ in size from the target gene, making a co-amplification possible during the PCR and distinction of the amplicons by electrophoresis (Mumy and Findlay, 2004). To prevent changes in the amplification rates of both templates, differences in size should not exceed 10 to 15% (Freeman et al., 1999). In competitive PCR, as the copy number of the competitor increases, the signal rises but the target gene decreases. Preferential amplification may occur due to: (1) differences in melting behaviour of the templates; (2) differential annealing of the primers; (3) differences in size; (4) low amount of template; (5) degraded or impure target DNA. Data analysis can be performed at the plateau phase assuming that both templates are amplified with the same efficiency. Nevertheless, as shown by Freeman et al. (1999), at the plateau phase, equal amounts of PCR template exhibited different signal intensity. Moreover, under special conditions such as low template concentration, presence of monovalent salts or degraded template, a difference of 6 base pairs was sufficient to generate amplification bias (Mutter and Boynton, 1995).

Hallier-Soulier et al. (1996) were one of the first who could demonstrate the usefulness of this approach for quantifying target genes from soil. They were able to quantify degradative genes in soil contaminated by toluene. The *xylE* gene coding for catechol 2,3-dioxygenase was chosen as a target gene. Comparing quantification of target DNA by competitive PCR with data obtained by enumeration of degradative microflora, they could prove the usefulness of the method. In the same year, Wikström et al. (1996) were able to quantify catechol 2,3-dioxygenase genes from different soil types using the same approach. They demonstrated a nice correlation between the concentration of PAHs and catechol 2,3-dioxygenase DNA in the samples. Philips et al. (2000) used a competitive PCR for a quantitative analysis of ammonia oxidizing bacteria, based on the specific amplification of 16S rRNA genes for the β-subgroup of proteobacterial ammonia oxidizing bacteria, as culture-based methods for enumeration, such as most probable number (MPN) methodologies, have proven to be inefficient due to difficulties in the isolation and cultivation of ammonia oxidizing bacteria in the laboratory. Consequently, they could show that the numbers of ammonia oxidisers were significantly underestimated by conventional MPN and were 1–3 orders of magnitude lower than those obtained by cPCR. However, it was necessary to construct a separate standard curve for each soil type as differences in DNA extraction, quantity and purity had a clear impact on the ease of PCR of both, competitor and target DNA. Mendum and Hirsch (2002) were also interested in quantifying ammonium oxidizing bacteria from different soil samples by competitive PCR. However, they chose the functional *amoA* gene as target for PCR. They could clearly demonstrate changes in the size and composition of β-proteobacterial autotrophic ammonia oxidizers in arable soils in response to agricultural practice. Similarly, Bjerrum et al. (2002) tested the competitive PCR method with respect to equal amplification efficiency of the two templates, degeneracy of the priming site and the importance of flanking regions surrounding the competitive template for the *amoA* gene. Calibration curves

made by addition of known amounts of *Nitrosomonas europaea* to soil samples revealed a detection limit for this technique of less than 1000 cells g^{-1} soil and a linear response over a wide range of cell additions. Cloning and sequencing of *amoA* amplificates have confirmed the specificity of the primers. Qiu et al. (2004) developed a quantitative competitive PCR system to detect and quantify copper-denitrifying bacteria in environmental samples. The primers were specific to copper-dependent nitrite reductase gene (*nirK*). They were able to detect about 200 copies of *nirK* in the presence of abundant, non-specific target DNA and about 1.2×10^3 *Pseudomonas* sp. G-179 cells from 1 g of sterilized soil by PCR amplification. A 312-bp *nirK* internal standard (IS) was constructed which showed a very similar amplification efficiency to the target *nirK* fragment (349 bp) over 4 orders of magnitude (10^3–10^6). The accuracy of this system was evaluated by quantifying various known amounts of *nirK* DNA. The linear regressions were obtained with a r^2 of 0.9867 for 10^3copies of *nirK*, 0.9917 for 10^4 copies of *nirK*, 0.9899 for 10^5 copies of *nirK* and 0.9846 for 10^6 copies of *nirK*. A high correlation between measured *nirK* and calculated *nirK* (slope of 1.0398, $r^2=0.9992$) demonstrated that an accurate measurement could be achieved with this system.

Again, as for MPN-PCR, this technique has been also successfully applied in other ecosystems and with other organisms. For example, Baek and Kenerley (1998) could quantify a genetically modified fungus in different soils by competitive PCR. Also for *Trichoderma*, Weaver et al. (2005) demonstrated the usefulness of the method for quantification of the fungus.

6. Real-time PCR

Real-time PCR technique is based on detection of fluorescence signals emitted due to the synthesis of PCR amplicons by *Taq* polymerase (Lee et al., 1993). The data utilized for the analysis of samples is acquired at the cycle at which the fluorescence signal is higher than the background (normally 10 times the standard deviation of the base line), known as threshold cycle (Wittwer et al., 1997). The quantification at the exponential phase of the PCR, when the efficiency is recognized to be the highest, is one of the greatest advantages of the real-time PCR in comparison to other PCR methods described above. The sensitivity of real-time PCR is depending on the amplicon length. In theory, one single copy can be detected by real-time PCR, however, this requires very short amplicons (shorter than 250 bp; Livak et al., 1995). Consequently, most of the existing primer systems for diversity analysis cannot be used for real-time PCR approaches as they result in amplicons that are too long (Park and Crowley, 2005).

Labelled-probes or dsDNA binding dyes, e.g. SYBR® Green, can be used to monitor amplicon synthesis (Morrison et al., 1998). Many types of probes have been developed for real-time PCR (for detailed information see Wong and Medrano, 2005), however, for environmental samples hydrolysis probes are the most commonly used, e.g. 5′nuclease assay or TaqMan. This method requires a probe labelled with a reporter dye and a quencher at the 5′ and 3′ end, respectively, which binds between the two primer sites to the target DNA (Heid et al., 1996). Ideally, the probe is located 50 bp upstream of the 3′end of the amplicon, to achieve the highest possible sensitivity. Due to the 5′nuclease activity of the *Taq* polymerase the probe is dissolved from the target DNA and the reporter dye is released from the molecule during DNA synthesis, which stops fluorescence resonance energy transfer and liberates a fluorescence signal. Therefore, only specific signals are detected by *Taq*Man assays. In the case of the SYBR® Green method, as the amount of dsDNA increases during cycling, more dye can be bound and more fluorescence will be emitted. This method does not require development of specific probe as for *Taq*Man protocols. Nevertheless, as the dye binds indiscriminately to dsDNA, false positives may occur and the analysis of the dissociation curve of the samples must be carried out after PCR amplification. The presence of different amplicons is reflected by the number of first-derivative melting peaks. In case of environmental samples, divergences in the GC content of a specific gene present in different organisms may also lead to the formations of multiple or blunt peaks. In this case, the specificity of the reaction should be confirmed on an agarose gel.

Several factors have contributed to the transformation of real-time technique into a main stream research tool, some of them being listed in Table 1. Especially for quantification of gene expression which is known to show high variability due to the reverse transcription step, real-time assays prove to have lower coefficients of variation than end-point methods (Schmittgen et al., 2000; Wong and Medrano, 2005).

Employing the various techniques of real-time PCR, researchers have been studying the influence of soil environmental factors such as nutrients, oxygen status, pH, pollutants, agro-chemicals, moisture and temperature on gene abundance, expression and some of the mechanisms involved in the responses of microbes to their environment.

One of the first studies using real-time PCR to describe a functional group of bacteria in soil was carried out by Hermansson and Lindgreen (2001). Again, the quantification of ammonium oxidizing bacteria was in the focus of their interest and were tacked using a real-time PCR assay consisting of a primer pair and an internal probe targeting a 16S ribosomal DNA region of the ammonia oxidizing bacteria. In the fertilized soil, there were approximately 6.2×10^7 ammonia oxidizing bacteria per g of soil, three times more than the number of bacteria in the unfertilized soil. Also studies using the functional gene (*amoA*) as the target for qPCR have been published. Most of these assays also make use of a primer–probe combination for quantification. It was possible to show the big influence of fertilization on the abundance of *amoA* sequences in soil (Okano et al., 2004). Also a strong relationship observed among *amoA* densities, ammonium disappearance, and nitrate accumulation have been clearly demonstrated. However, also results using a SybrGreen assay have been published recently. Demanou et al. (2006) revealed the influence of different fungicide combinations on the abundance of *amoA* genes and the corresponding transcripts. Again, these data nicely correlated to the measured nitrification rates. In the last years also growing interest has been drawn to the role of *Archaea* for turnover processes in soil ecosystems. Leininger et al. (2006) could show that *Archaea* are also key players in some steps of

microbial nitrogen turnover, mainly the oxidation of ammonium, not only in extreme environments but also in agricultural soils from temperate regions. Interestingly, in this study data from real-time PCR were related to other biomarkers data. The lipid crenarcheol, which is only present in Crenarchaea, correlated nicely with the abundance of archaeal *amoA* genes. Furthermore, data from real-time PCR and MPN-PCR were compared using different primer combinations for the quantification of the archaeal *amoA* genes, showing a clear primer independent correlation between both techniques. Also the other steps in nitrogen turnover have been studied by many authors combining real-time PCR and turnover rates (e.g. Lopez-Gutierrez et al., 2004; Bach et al., 2002; Henry et al., 2004; Bürgmann et al., 2005). A review about qPCR and nitrogen cycle was given by Wallenstein and Vilgalys (2005).

Besides genes involved in nitrogen turnover, *pmoA*, the gene responsible for the oxidation of methane, has been quantified in many habitats by several authors (e.g. Seghers et al., 2005; Scheid et al., 2003; Knief et al., 2006). Additionally, many studies have focussed on genes involved in the degradation of polycyclic aromatic hydrocarbons (e.g. Laurie and Lloyd-Jones, 2000).

7. Conclusions and perspectives

Quantitative data about genes and transcripts in soil are becoming more and more important as they help to understand the microbial basis of substrate fluxes. These new quality of data is of high relevance to improve mathematic models of turnover processes. The choice of method for quantitative PCR is highly dependent on the characteristics of individual experiments—and whether relative or absolute quantification is required. Also, the sequence information available and the primers and probes published for any particular functional gene can be a limiting factor in the selection of a method. As in conventional PCR, primer design is critical for achieving specific products that amplify as many versions of each gene as possible. It is already known that primers targeting these functional genes do not likely amplify all variants of these genes, but continued expansion of public databases will allow for improved primers.

While most studies to date have focussed on a single functional gene, analysis of a more complex suite of genes would enable us to better address the role of the community structure in controlling various processes in soil. Furthermore, in the future it will become necessary to relate abundance of genes and transcripts to diversity of the same gene family to understand the role of redundancy. Using a combination of microarray technology and multiplex qPCR might help to solve this challenge in the future.

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Applied and Environmental Microbiology, Aug. 2009, p. 4993–5000 Vol. 75, No. 15
0099-2240/09/$08.00+0 doi:10.1128/AEM.02917-08

Quantification of Key Genes Steering the Microbial Nitrogen Cycle in the Rhizosphere of Sorghum Cultivars in Tropical Agroecosystems[▽]

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Received 22 December 2008/Accepted 1 June 2009

The effect of agricultural management practices on geochemical cycles in moderate ecosystems is by far better understood than in semiarid regions, where fertilizer availability and climatic conditions are less favorable. We studied the impact of different fertilizer regimens in an agricultural long-term observatory in Burkina Faso at three different plant development stages (early leaf development, flowering, and senescence) of sorghum cultivars. Using real-time PCR, we investigated functional microbial communities involved in key processes of the nitrogen cycle (nitrogen fixation, ammonia oxidation, and denitrification) in the rhizosphere. The results indicate that fertilizer treatments and plant development stages combined with environmental factors affected the abundance of the targeted functional genes in the rhizosphere. While nitrogen-fixing populations dominated the investigated communities when organic fertilizers (manure and straw) were applied, their numbers were comparatively reduced in urea-treated plots. In contrast, ammonia-oxidizing bacteria (AOB) increased not only in absolute numbers but also in relation to the other bacterial groups investigated in the urea-amended plots. Ammonia-oxidizing archaea exhibited higher numbers compared to AOB independent of fertilizer application. Similarly, denitrifiers were also more abundant in the urea-treated plots. Our data imply as well that, more than in moderate regions, water availability might shape microbial communities in the rhizosphere, since low gene abundance data were obtained for all tested genes at the flowering stage, when water availability was very limited.

Land degradation is one of the most serious threats to food production on the African continent. Soil erosion, nutrient depletion, low organic matter content, and unfavorable pH values are some of the reasons for a deficient soil fertility (30), mainly in Central African countries. Combined with high variability and irregular distribution of rainfall, these factors contribute to negative nutrient balances. For example, 4.4 million tons of nitrogen (N) are lost per year in African soils, but only 0.8 million tons are reapplied by fertilization (12, 34). Since nitrogen is a key nutrient determining the productivity of agroecosystems (7, 11, 43), it is of central importance to optimize the nitrogen balance in these countries, mainly by steering the genetic resources of soil microbes in a way that losses of applied nitrogen are minimized and biological nitrogen fixation is increased. The aim should be to obtain a highly efficient nitrogen turnover, with leaching of nitrate and losses of gaseous products such as nitrous oxide (N_2O) or dinitrogen (N_2) as low as possible.

Despite the importance of this issue, not much data are available on microbial community structure and function related to the nitrogen cycle in agroecosystems of Central Africa, and scenarios from moderate climatic regions cannot simply be transferred to tropical agroecosystems. Furthermore, the few studies published thus far only investigated effects of agricultural management on a single process of the nitrogen turnover (18, 20, 32) and ignore the fact that nitrogen turnover is a network of closely interlinked processes.

Therefore, we sought to investigate the effects of different fertilizer regimens on multiple transformation processes within the nitrogen cycle in agroecosystems from semiarid areas in Central Africa. We investigated nitrogen dynamics on a full-cycle approach, including the most important steps in well-aerated agricultural soils (nitrification, denitrification, and nitrogen fixation). We hypothesized that each fertilizer regimen results in typical abundance pattern of the functional populations. While in moderate agroecosystems the plant (respectively, the plant development stage and the plant performance) plays an important role in shaping microbial community structure and function in the rhizosphere, we further postulated that nitrogen turnover and the corresponding populations are also influenced by the availability of water in semiarid soils. To test these hypotheses on a molecular basis, we quantified bacterial genes encoding the nitrogenase reductase (*nifH*), ammonia monooxygenase (*amoA*), and nitrite reductase (*nirK* and *nirS*), as well as archaeal *amoA* genes, by

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▽ Published ahead of print on 5 June 2009.

TABLE 1. Soil physical and chemical properties, plant biomass and grain yields of the differently amended plots

Sample	Mean ± SD[a]					
	Amt (mg g^{-1})			pH (KCl)	Amt (kg ha^{-1})	
	Total C	Total N	Total P		Grain yield	Plant biomass
Control	2.16 ± 0.33^{A}	0.15 ± 0.05^{A}	0.11 ± 0.0^{AB}	4.58 ± 0.33^{A}	167 ± 174^{A}	480 ± 426^{A}
C+U	2.22 ± 0.77^{A}	0.16 ± 0.05^{AB}	0.10 ± 0.03^{A}	4.09 ± 0.17^{B}	207 ± 124^{A}	627 ± 286^{A}
Straw	2.48 ± 0.38^{A}	0.15 ± 0.11^{A}	0.11 ± 0.02^{AB}	5.19 ± 0.24^{CD}	90 ± 60^{A}	300 ± 192^{A}
S+U	2.69 ± 0.42^{A}	0.17 ± 0.07^{AB}	0.11 ± 0.02^{AB}	4.76 ± 0.23^{AC}	233 ± 154^{A}	600 ± 390^{A}
Manure	3.57 ± 0.2^{B}	0.27 ± 0.12^{B}	0.15 ± 0.02^{B}	5.51 ± 0.30^{D}	730 ± 319^{B}	$1{,}893 \pm 779^{B}$
M+U	3.56 ± 0.43^{B}	0.25 ± 0.05^{AB}	0.15 ± 0.02^{B}	5.14 ± 0.36^{CD}	737 ± 350^{B}	$1{,}900 \pm 806^{B}$

[a] Significant differences between treatments are indicated by different superscript letters ($n = 6$).

real-time PCR and linked the data to soil properties and grain yield.

MATERIALS AND METHODS

Location. The experimental site is located at the Saria Agricultural Research Station (Saria II, 12°16′N, 2°9′W, 300-m altitude) in Burkina Faso. This site represents the North-Sudanese zone, with tropical climate and an average rainfall of 800 mm per year. The climate in Burkina Faso is characterized by two seasons: the dry season from October to April and the rainy season from May to September. The soil is strongly weather-beaten, eroded, and classified as ferric lixisol (13).

Site description and sampling strategy. A long-term fertilizer experiment was established in 1980, including six replicates of six treatments in a randomized plot design. The treatments unamended control, control plus urea (C+U; 60 kg ha^{-1}), straw (sorghum; 8.3 tons ha^{-1}) , straw plus urea (S+U), cattle manure (10 tons ha^{-1}), and cattle manure plus urea (M+U) were applied to a continuous sorghum cropping system [*Sorghum bicolor* (L.) Moench] 2 months before sowing. Rhizosphere samples were collected according to standard procedures (28) in August (EC30, early leaf development), September (EC60, flowering stage), and November 2006 (EC90, senescence stage) by mixing roots of three plants per plot to form one composite sample. The six replicate plots were used as independent replicates. Rhizosphere samples were filled into cryotubes, shock frozen in solid carbon dioxide right after sampling, and stored at −80°C for nucleic acid extraction.

Soil physical and chemical properties. Physical and chemical analyses have been performed in all treatments in soil samples taken at the flowering stage (i.e., EC30). Soil pH values were measured in 2 M KCl suspensions at a soil/liquid ratio of 1:2.5. The total amount of nitrogen and carbon was quantified by using the combustion system ThermoFinnigan Flash EA 1112 (ThermoFinnigan, France), and the colorimetric determination of total and available phosphorus (P) was performed according to the method of Dabin (10). Corresponding data are listed in Table 1.

Determination of ammonium (NH_4^+) and nitrate (NO_3^-) in rhizosphere soil. Prior to chemical analyses, 3 g of rhizosphere soil was overhead shaken for 30 min with 12 ml of 0.01 M $CaCl_2$. Each extract was filtered through a Millex HV Millipore filter (pore size, 0.45 μm). Ammonium and nitrate measurements were performed on a Nanocolor 300D photometer from Macherey-Nagel (Germany) by using the commercial kits Nanocolor Ammonium 3 and Nitrate 50 according to the manufacturer's protocol (data are indicated in Table 2).

Nucleic acid extraction. DNA was extracted from 0.5 g of soil by using the method described by Griffiths et al. (15). Extraction was performed by using Precellys-Keramik kit lysing tubes (PEQLAB Biotechnologie GmbH, Germany) in combination with the Bertin Precellys 24 beat-beading system (Bertin Technologies, France). DNA yield and purity were measured by using a microvolume fluorospectrometer (NanoDrop Technologies, Delaware).

Real-time PCR assay. Absolute quantification of all investigated genes was carried out in triplicate on the ABI Prism 7300 Cycler (Applied Biosystems, Germany). The following reagents were used for the real-time PCR assay: bovine serum albumin (Sigma-Aldrich, Germany), primers (5, 17, 21, 27, 31, 33, 37, 42) (Metabion, Germany), dimethyl sulfoxide (Sigma, Germany), and Power SYBR Green PCR master mix (Applied Biosystems). The composition of each reaction mix is given in Table 3. All PCR runs started with an initial enzyme activation step performed at 95°C for 10 min. The subsequent thermal profile was different for each gene, as indicated in Table 4. The specificity of the amplification products was confirmed by melting-curve analysis, and the expected sizes of the amplified fragments were checked in a 1.5% agarose gel stained with ethidium bromide.

To test possible inhibitory effects on quantitative PCR amplification caused by coextracted humic substances, the optimal dilution for each DNA extract was determined by pre-experiments (data not shown). Dilution series of plasmid DNA targeting the bacterial nitrogen cycle genes (*nifH*, *amoA*, *nirS*, and *nirK*) and the fosmid clone 54d9 targeting archaeal *amoA* genes (referenced in Table 4) were used to generate a standard curve for each of the five target genes (standard dilutions used for creating a standard curve ranged from 10^1 to 10^6 gene copies/μl). The amplification efficiencies were calculated by using the formula Eff = $[10^{(-1/\text{slope})} - 1]$.

Statistical analyses. Data were analyzed by multifactorial analysis of variance (ANOVA; see Tables 1, 2, and 5) with the independent variables plant development stage, organic fertilizers (straw residues, manure) and urea treatment ($3 \times 3 \times 2$). Normal distribution of the residuals was checked by using histograms and the Kolmogorov-Smirnov test. If the requirement was not met, data were log transformed prior to analysis. The homogeneity of the variances was checked by the Levene test. For pairwise comparison of means, the Tukey's test was applied, and if the homogeneity of the variances was not given, a Games-

TABLE 2. Nitrate and ammonium concentrations in rhizosphere soil in different treatments at three different plant development stages

Sample	Mean concn (mg of N kg of $soil^{-1}$) ± SD[a]					
	NO_3^+-N			NH_4^+-N		
	EC30[c]	EC60	EC90	EC30	EC60	EC90
Control	6.4 ± 3.6^{A1}	5.4 ± 1.5^{A1}	68.7 ± 36.9^{B1}	1.9 ± 1.6^{A1}	3.9 ± 2.7^{A1}	1.1 ± 0.7^{A1}
C+U	18.4 ± 13.2^{A1}	7.9 ± 3.4^{A1}	84.6 ± 42.5^{B1}	4.2 ± 3.5^{A1}	3.8 ± 2.1^{A1}	1.6 ± 1.1^{A1}
Straw	5.3 ± 3.5^{A1}	6.2 ± 1.7^{A1}	82.5 ± 44.9^{B1}	1.5 ± 1.0^{A1}	5.1 ± 1.9^{B1}	3.7 ± 6.1^{AB1}
S+U	19.2 ± 13.8^{A1}	8.9 ± 3.0^{A1}	136.9 ± 70.6^{B1}	1.7 ± 1.0^{A1}	5.8 ± 1.9^{B1}	5.7 ± 6.2^{AB1}
Manure	17.4 ± 15.9^{A1}	6.6 ± 2.9^{A1}	97.3 ± 41.1^{B1}	2.4 ± 0.6^{A1}	6.5 ± 2.0^{B1}	2.2 ± 1.9^{A1}
M+U	36.1 ± 20.8^{A1}	(12.4)	74.9 ± 34.1^{B1}	3.1 ± 1.7^{A1}	(5.6)	1.8 ± 2.0^{A1}

[a] $n = 6$; $n = 1$ for data in parentheses. EC30, young leaf development; EC60, flowering; EC90, senescence. Significant differences of one treatment over three different plant development stages are indicated by different superscript letters. Significant differences among treatments at one plant development stage are indicated by different superscript numbers.

TABLE 3. Reaction components of a 25-μl Mastermix assay used for quantification of the functional target genes involved in nitrogen turnover

Target metabolic group	Target gene	Amt (μl) of assay component						
		2× Power SYBR green	3% BSA	Primer (10 pmol μl^{-1})		Dimethyl sulfoxide	DNA	PCR water
				Forward	Reverse			
Nitrogen-fixing bacteria	*nifH*	12.5	0.5	0.30	0.30		2	9.4
AOB	*amoA*	12.5	0.5	0.75	0.75		2	8.5
AOA	*amoA*	12.5	0.5	0.50	0.50		2	9.0
Denitrifying bacteria	*nirK*	12.5	0.5	0.50	0.50	0.625	2	8.375
	nirS	12.5	0.5	0.50	0.50	0.625	2	8.375

Howell test was used. Due to the observed interactions between plant development stage and the different fertilizing treatments, a two-way ANOVA was carried out for each growth stage in order to explain the effects of the treatments (organic fertilizers and urea) and interactions on gene abundances for EC30, EC60, and EC90 separately (3 × 2 for each plant development stage). The statistical significance was set at $P < 0.05$. Statistical analysis was performed by using SPSS 13.0 (SPSS, Inc.).

RESULTS

Soil physical and chemical properties. The pH values and total C, N, and P contents were measured in soil samples in all treatments at all plant development stages (Table 1). The pH values were low in general and revealed clear differences between the urea-treated plots (<4.76) and plots without urea application (<5.51). As expected, total C, N, and P contents were low in all plots compared to agro-ecosystems in moderate climate zones (38). However, a clear influence of fertilizer regimens was visible with the highest C, N, and P concentrations (Table 1) in the manure-amended plots (3.56 to 3.57 mg of C g of soil^{-1}, 0.25 to 0.27 mg of N g of soil^{-1}, and 0.15 mg of P g of soil^{-1}). Since the C and N values responded similarly to the different treatments, the C/N ratios did not vary significantly between the treatments.

Ammonium and nitrate concentrations in the rhizosphere. Ammonium concentrations in the rhizosphere of all treatments were low at the young leaf development (EC30) and the plant senescence (EC90) stages, with less than 5.7 mg kg of soil^{-1}. The highest amounts of ammonium were found at the flowering stage (EC60) and reached 6.5 mg kg of soil^{-1} in the manure amendment. Despite enhanced ammonium values in the straw and manure treatments, significant responses to the treatments were not found (Table 2).

Nitrate concentrations were lowest at EC60, independent of the different treatments. The amounts of nitrate reached 8.9 mg kg of soil^{-1} at the mentioned sampling time point and were significantly increased at EC90 with up to 136.9 mg kg of soil^{-1}. The nitrate values did not show significant responses to the treatments. However, the amounts of nitrate were always higher in the urea-treated plots (136.9 mg kg of soil^{-1}) than in plots not receiving urea (97.3 mg kg of soil^{-1}) (Table 2).

Plant biomass and grain yield. Highest plant biomass yields were obtained from the manure-amended plots ranging between 1,893 kg ha^{-1} (manure) and 1,900 kg ha^{-1} (M+U). Interestingly, straw amendment reduced plant biomass yields (300 kg ha^{-1}) compared to the untreated control (480 kg ha^{-1}). A certain but not significant stimulatory effect of the urea application was visible (C+U, 627 kg ha^{-1}; S+U, 600 kg ha^{-1}). Grain yields followed the same trends described above for overall plant biomass yields (control, 167 kg ha^{-1}; C+U, 207 kg ha^{-1}; straw, 90 kg ha^{-1}; S+U, 233 kg ha^{-1}; manure, 730 kg ha^{-1}; M+U, 737 kg ha^{-1}). Regarding the fitness of the plant, we obtained not only higher plant and grain yields in manure-amended plots but also a more vigorous plant development compared to the other treatments, independent of plant development stage.

Abundance of functional genes involved in nitrogen cycling. Real-time PCR was used to quantify the population size of nitrogen fixers (*nifH*), ammonia oxidizing bacteria (AOB-

TABLE 4. Primer sets and thermal profiles used for the absolute quantification of functional target genes involved in nitrogen turnover

Target gene	Primer set	Reference	Thermal cycling profile	No. of cycles
nifH	*nifH*-F-Rösch *nifH*-R-Rösch	30	95°C/45 s, 55°C/45 s, 72°C/45 s	40
amoA (AOB)	*amoA*-1F *amoA*-2R	32	94°C/60 s, 60°C/60 s, 72°C/60 s	39
amoA (AOA)	19F CrenamoA616r48x	21 36	94°C/45 s, 50°C/45 s, 72°C/45 s	40
nirK	*nirK*-876 *nirK*-5R	17 4	95°C/15 s, 63°C-58°C/30 s, 72°C/30 s	6[a] 40
nirS	*nirS*-cd3af *nirS*-R3cd	26 40	94°C/60 s, 57°C/60 s, 72°C/60 s	39

[a] Touch down.

TABLE 5. Statistical evaluation of gene abundance by multifactorial ANOVA

Factor	P^a				
	nifH	*amoA* (AOB)	*amoA* (AOA)	*nirS*	*nirK*
Total					
EC	0.00016*	0.00187*	0.00000*	0.00000*	0.00000*
Organic	0.00584*	0.00010*	0.00663*	0.01226*	0.00110*
Urea	0.86490	0.00000*	0.76170	0.03195*	0.00897*
EC × organic	0.01611*	0.06726	0.75901	0.00226*	0.02470*
EC × urea	0.97056	0.01246*	0.94151	0.77689	0.26311
Organic × urea	0.88557	0.91020	0.40479	0.48152	0.67149
EC × organic × urea	0.96940	0.34970	0.70371	0.11885	0.20102
EC30					
Organic	0.21544	0.01357*	0.01120*	0.00461*	0.00226*
Urea	0.90946	0.00011*	0.92578	0.08370	0.03994*
Organic × urea	0.95764	0.50181	0.41531	0.54840	0.31779
EC60					
Organic	0.00028*	0.01036*	0.44637	0.00033*	0.12915
Urea	0.91401	0.00004*	0.76804	0.44207	0.79858
Organic × urea	0.75057	0.43477	0.69985	0.98800	0.68548
EC90					
Organic	0.78713	0.06200	0.12951	0.48282	0.72698
Urea	0.62794	0.000109*	0.74827	0.19272	0.01398*
Organic × urea	0.71996	0.30539	0.19268	0.08998	0.07509

[a] The *P* values describe the impact of plant development stages and nitrogen fertilizing treatments on functional genes involved in nitrogen turnover. Asterisks indicate significant effects of plant development stages or fertilizing treatments, respectively, on the functional genes, after analysis by Tukey's test with SPSS 13.0.

amoA) and ammonia-oxidizing archaea (AOA-*amoA*), and denitrifiers (*nirK* and *nirS*) of rhizosphere samples taken at three different plant development stages. All measured microbial genes showed a clear response to the different sampling time points, as well as fertilizer treatments (Table 5 and Fig. 1).

(i) Bacterial *nifH* genes. Independent of treatment, similar *nifH* gene copy numbers were observed at EC30 and EC90, revealing 2.4×10^6 to 9.7×10^6 copies g of soil^{-1} (Fig. 1A). The copy numbers at EC60, however, differed distinctly from EC30 and EC90, with reduced numbers in control and straw-amended plots and enhanced numbers in manure-amended plots (see below). An influence of straw residues and urea on the *nifH* gene abundance was not apparent. Interestingly, *nifH* gene abundance was influenced by manure application. Both manure treatments (manure only and M+U) resulted in the highest *nifH* gene copy numbers, with up to 2.1×10^7 copies g of soil^{-1}, but this was only the case at the flowering stage of the plant. For all other fertilizers, an opposite trend was observed, with low copy numbers measured at EC60 ranging from 9.9×10^4 to 4.0×10^5 gene copies g of soil^{-1}.

(ii) Bacterial and archaeal *amoA* genes. Overall, bacterial *amoA* gene abundance was highest at the young leaf development, revealing 1.5×10^6 copies g of soil^{-1}, and decreased significantly in all treatments toward the flowering stage, amounting to $<4.3 \times 10^5$ copies g of soil^{-1} (Fig. 1B and C). Among the different treatments, urea amendment had the strongest impact on the community size of bacterial ammonia oxidizers. Their number was at any plant development stage significantly increased compared to plots that did not receive urea. Even though organic treatments influenced the community size of bacterial ammonia oxidizers, this effect was less pronounced than the urea effect. The impact of straw was mainly apparent at the EC30 stage, while the manure effect was mainly apparent at EC30 and EC90.

The archaeal *amoA* gene copy numbers were significantly increased at EC30 and EC90 (6.0×10^4 to 6.6×10^5 copies g of soil^{-1}) compared to EC60 (1.5×10^4 to 2.5×10^5 copies g of soil^{-1}). Interestingly, whereas AOB were affected by urea, this was not true for AOA at any plant development stage. In contrast, organic treatments influenced the AOA mainly at the EC30 stage, resulting in higher gene copy numbers (5.3×10^5 copies g of soil^{-1}) compared to the control plots (1.4×10^5 copies g of soil^{-1}). Independent of the plant development stage, AOA outnumbered AOB when urea was not applied, with AOA/AOB ratios of >1 (control, < 5.6; straw, <7.9; manure, <4.8). In contrast, we obtained values of ≤1 for the AOA/AOB ratios in urea-amended plots (data not shown in detail), except for S+U (1.4) and M+U (2.4) at EC90.

(iii) Bacterial *nirS* and *nirK* genes. To investigate the denitrification potential in the rhizosphere, we quantified two complementary genes encoding the nitrite reductase (*nirS* and *nirK*) (Fig. 1D and E). Gene abundances were clearly influenced by the plant development stage, with significantly increased gene copy numbers at the early plant development and the senescence stages. The *nirK* gene copy numbers ranged from 8.8×10^5 to 4.8×10^6 copies g of soil^{-1}, and the *nirS* gene copy numbers ranged from 1.6×10^5 to 7.9×10^5 copies g of soil^{-1} at the mentioned sampling time points. The tendency of significantly reduced copy numbers at the flowering stage, as observed for all targeted microbial communities, remained the same for the denitrifiers, amounting to 4.3×10^4 to 2.5×10^5 *nirK* genes g of soil^{-1} and 1.6×10^4 to 7.7×10^5 *nirS* genes g of soil^{-1}. This phenomenon could be observed in all treatments. However, *nirK* and *nirS* gene abundances were not only

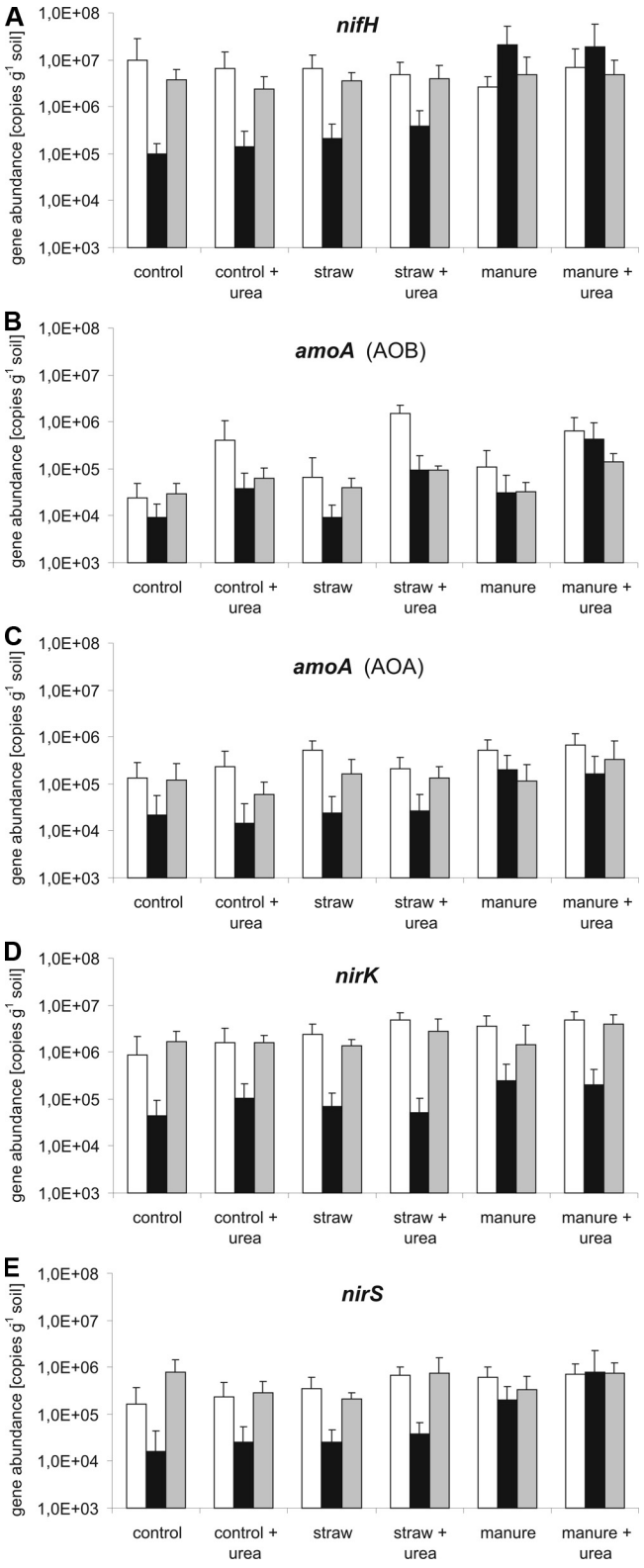

FIG. 1. Copy numbers of functional genes involved in nitrogen cycling per gram of soil under different fertilizing regimens (unamended control, C+U, straw, S+U, manure, and M+U) at three different plant development stages (EC30, young leaf development [□]; EC60, flowering [■]; and EC90, senescence [▨]). (A) *nifH*; (B) *amoA* (AOB); (C) *amoA* (AOA); (D) *nirK*; (E) *nirS*.

influenced by the plant development stage but also by fertilization. Although the abundance of *nirK* genes was enhanced by manure, straw, and urea mainly at EC30, *nirS* genes were increased by manure and straw at EC30 and only by manure at EC60. Overall, the two genes showed similar abundance patterns. However, lower *nirS* gene abundances were found compared to *nirK* in all treatments independent of the plant development stage.

DISCUSSION

The relevance of rhizosphere microorganisms for sustainable agricultural ecosystems was neglected for a long time. Only in the last decade, the impact of exudates on microbial communities in the rhizosphere and their nutrient supply to plants have been studied in more detail (2). However, semiarid ecosystems have yet to be examined with regard to the response of microbial communities in the rhizosphere to agricultural amendments and their role in agricultural sustainability.

Our results reveal that the abundances of all measured microbial groups involved in nitrogen turnover varied distinctly between the different plant development stages ($P < 0.0002$) (Fig. 1). Surprisingly, the abundances of all measured genes were significantly reduced at EC60 in all treatments, a finding which is in contrast to other studies from moderate regions. Most authors relate their results to enhanced root exudation during the flowering stage and high amounts of nutrients in the rhizosphere at that time point of plant development (6, 22), which are optimal conditions for nitrogen fixers (6) and denitrifiers (25, 26). However, in our experiment water availability in the soil was very low, since at the end of the rainy season (August and September 2006) precipitation fell short. Consequently, soil humidity (data not shown) was reduced in all treatments at the flowering stage. Nitrate concentrations at EC60 were also 3-fold reduced in urea-amended plots compared to those at EC30 and up to 15-fold reduced compared to those at EC90, which compromised the plant growth as well as the root exudation. Since the amount and composition of root exudates depend on environmental conditions (23), we suggest a combined effect of plant development stage and climatic conditions, which drive the abundance pattern of the genes under investigation. Since we performed a field experiment and both parameters are interlinked, it cannot be resolved which factor was more important for shaping microbial communities involved in nitrogen turnover. The different fertilization regimens clearly affected the community size of the functional groups as well, although to a lesser extent than the plant development stages.

***nifH*.** Although straw residues and urea did not induce any response from the nitrogen-fixing community, a strong effect of manure ($P < 0.0003$) on the *nifH* abundance became evident at the flowering stage (EC60). Likewise, the most vigorous plant growth was found in the manure-amended plots, where elevated concentrations of total carbon, nitrogen, and phosphorus have also been observed (Table 1), which might have been established over the years due to the continuous fertilizer application. The content of organic matter and the soil structure seem to be improved in a way that the decremental effects of the dry periods were reduced and the high abundance of nitrogen fixers, which can be explained by the increased ammo-

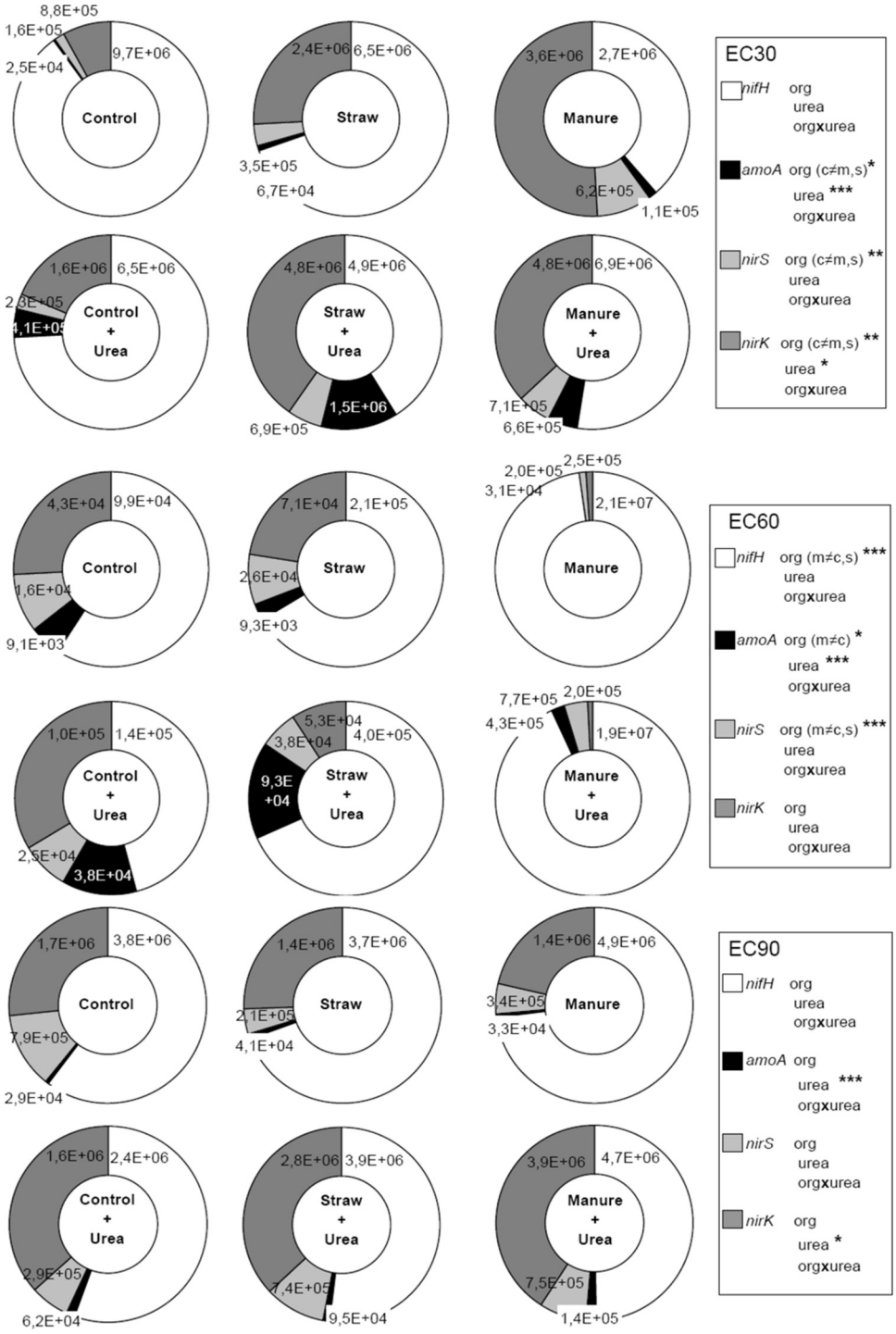

FIG. 2. Effect of one particular fertilizing treatment on all bacterial populations measured in the present study (*nifH*, *amoA* [AOA and AOB], *nirS*, and *nirK*) at three different plant development stages (EC30, young leaf development; EC60, flowering; EC90, senescence). Significance (ANOVA): ***, $P < 0.001$; **, $P < 0.001$; *, $P < 0.05$. Abbreviations: org (organic) = manure + straw, m = manure, s = straw; e.g., c≠m = manure is significantly different from the control.

nium concentrations (Table 2) in the manure-amended plots at EC30, might be based on a higher rhizodeposition. In contrast, low C and P concentrations were found in straw- and urea-amended plots. Furthermore, the same treatments yielded low *nifH* gene copy numbers at EC60, indicating that a lack of macronutrients not only limits the effectivity of biological nitrogen fixation (14, 35) by free-living diazotrophs but also reduces the population size of diazotrophs in semiarid ecosystems.

***amoA*-AOB.** AOB revealed a strong response ($P < 0.0001$) to urea application, as indicated by elevated *amoA* gene abun-

dances in urea-amended plots and reduced gene abundances in plots that did not receive urea. Soil pH value seems to be an important factor for the abundance of bacterial ammonia oxidizers, as observed by Shen et al. (39). In contrast to our study, their experiments were conducted in an alkaline sandy loam with the application of NPK fertilizers in the form of urea. A decrease in the pH by 0.3 increased the bacterial *amoA* abundance 22.5-fold. Similar results were obtained in our study, with 23.1-fold-increased *amoA* gene copies in urea-treated plots, where pH values were reduced by 0.37 to 0.49. The phenomenon can be explained by the fact that, under acidic conditions, urea is assimilated preferentially and consequently nitrate accumulates (24). This is in line with our study, where elevated nitrate concentrations were obtained in urea-amended plots. The amounts of nitrate reached up to 136.9 mg of N kg of $soil^{-1}$ and were thus almost three times higher than the nitrate concentrations in plots that did not receive urea.

***amoA*-AOA.** In contrast to AOB, the abundance of AOA was not affected by urea application. A recent study from Hatzenpichler et al. (16) showed that archaeal ammonia oxidizers were even inhibited by enhanced urea and ammonia concentrations. Determination of the AOB/AOA ratio indicated that AOB were predominant in urea-amended plots, ranging from 1.0 to 7.4, except in S+U-amended soil (0.7) and M+U-amended soil (0.4) at plant senescence. On the other hand, AOA were predominant in plots without urea application, with AOA/AOB ratios ranging from 2.4 to 7.9, independent of the plant development stage. Assuming 2.5 *amoA* gene copies per AOB cell and 1 *amoA* gene copy per AOA cell (21), the AOA/AOB ratio on a cell-based calculation was in the range of 5.9 to 19.7 independent of urea application and plant development stage. This finding is in accordance with recently published data from Chen et al. (9), who obtained ratios from 1.2 up to 69.3. The observation of AOA being predominant over AOB has been confirmed by several studies investigating different soils (1, 21) and in the rhizosphere of terrestrial plants (40, 41). However, even though few studies exhibited the opposite phenomenon, namely, that AOB outnumber AOA in some estuaries (4, 8, 36), it has been shown that AOA are more stable and do not respond as sensitively to environmental differences as their bacterial counterparts (36), as revealed by our study.

***nirS* and *nirK*.** Other than the two functionally redundant populations involved in the oxidation of ammonia (AOA and AOB), which were influenced differently by the investigated treatments, the two functionally redundant groups involved in nitrite reduction (harboring *nirS* and *nirK* as functional genes) showed similar response patterns in all treatments. However, the *nirK* copy numbers were larger in all cases compared to *nirS*. It is generally accepted that nitrogen fertilizers promote denitrification in agricultural soils (19, 29), which was confirmed by our study where organic fertilizers (manure and straw) increased the denitrifier population (*nirS*, $P < 0.0046$; *nirK*, $P < 0.0023$) significantly. However, the *nirK* gene abundance was even more increased when the organic fertilizers were applied in combination with urea. Similarly, Arcara et al. (3) reported low N_2O-N losses from a maize-cropped soil when urea or pig slurry were applied; however, the combination of both fertilizers produced an increase in N_2O emissions due to denitrification, which is in line with our increased gene abundance data.

Our central hypothesis that each fertilizer treatment results in a typical ratio of nitrogen-fixing, ammonia-oxidizing, and denitrifying microbes is confirmed by Fig. 2, which shows clear alterations in the proportions of the different communities due to the fertilizer regimens. Interestingly, the most remarkable and obvious shifts were found in the manure-treated soils at the flowering stage, where the size of the nitrogen-fixing population was increased by a factor of 209. In contrast to the findings with the urea-treated plots, where an increase in nitrogen-fixing bacteria was accompanied by an increase in denitrifiers (C+U, 1.1; S+U, 4.4), in the manure-amended plots the ratio of nitrogen fixers and denitrifiers increased significantly (manure only, 46.2; M+U, 19.5). This might be a reason for the enormous plant growth and grain formation (enhanced plant biomass with 303 to 394%; enhanced grain yield with 354 to 436%) determined in manure-amended plots, since obviously the formed ammonium is not used by bacteria for nitrification and denitrification but by the plant for biomass production.

Summary and outlook. Our data show the influence of different fertilizer treatments on the abundance of selected functional groups and the role of microbes in plant growth promotion in soils from semiarid regions. The presented data describe the sustainability of a particular treatment as population size of functional groups shape soil quality and turnover kinetics in the long term. As indicated by the ring diagrams (Fig. 2), plant and grain yields might benefit from reduced nitrogen losses by denitrification and enhanced biological nitrogen fixation in the manure treatments. However, the measured gene abundances only reflect a microbial potential for metabolizing nitrogen compounds and do not describe actual turnover rates in soils. Therefore, investigations of gene expression and enzyme activity and stability remain to be performed to compare the presence of functional groups with their activities and actual turnover rates. Furthermore, repeating our study in successive seasons and on additional sites would elucidate whether our data could be generalized for semiarid ecosystems. Finally, greenhouse experiments under controlled climatic conditions and using ^{13}C-labeled carbon dioxide could clarify which factor was the main driver for the reduced gene abundances at the flowering stage: the plant development stage or the climate.

ACKNOWLEDGMENTS

The Microbes Project was financially supported by the Agence Nationale de la Recherche.

We gratefully acknowledge Anton Hartmann for constructive comments on the manuscript; Sven Leininger for providing the AOA standard; and the anonymous reviewers, whose suggestions improved the manuscript substantially.

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The ISME Journal (2008) 2, 221–232

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ORIGINAL ARTICLE

Effects of aboveground grazing on coupling among nitrifier activity, abundance and community structure

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The influence of switches in grassland management to or from grazing on the dynamics of nitrifier activity, as well as the abundance of ammonia-oxidizing bacteria, AOB and ammonia-oxidizing archeae, AOA, was analyzed for two years after changing management. Additionally community structure of AOB was surveyed. Four treatments were compared in mesocosms: grazing on previously grazed grassland (G-G); no grazing on ungrazed grassland (U-U); grazing on ungrazed grassland (U-G) and cessation of grazing on grazed grassland (G-U). Nitrifier activity and abundance were always higher for G-G than U-U treatments and AOB community structure differed between these treatments. AOA abundance was in the same range as AOB abundance and followed the same trend. Grazing led to a change in AOB community structure within <5 months and a subsequent (5–12 months) increase in nitrifier activity and abundance. In contrast, cessation of grazing led to a decrease in nitrifier activity and abundance within <5 months and to a later (5–12 months) change in AOB community structure. Activity in G-U and U-G was similar to that in U-U and G-G, respectively, after 12 months. Sequence analysis of 16S rRNA gene clones showed that AOB retrieved from soils fell within the *Nitrosospira* lineage and percentages of AOB related to known *Nitrosospira* groups were affected by grazing. These results demonstrate that AOB and AOA respond quickly to changes in management. The selection of nitrifiers adapted to novel environmental conditions was a prerequisite for nitrification enhancement in U-G, whereas nitrification decrease in G-U was likely due to a partial starvation and decrease in the abundance of nitrifiers initially present. The results also suggest that taxonomic affiliation does not fully infer functional traits of AOB.
The ISME Journal (2008) **2,** 221–232; doi:10.1038/ismej.2007.109; published online 29 November 2007
Subject Category: microbial ecology and functional diversity of natural habitats
Keywords: ammonia oxidizers; ammonia-oxidizing bacteria; ammonia-oxidizing archeae; herbivory; microbial diversity–functioning relationship; nitrogen cycle

Introduction

In grasslands, soil N cycling can be promoted by aboveground grazing pressure (Ruess, 1986; McNaughton *et al.*, 1988; Frank and Groffman, 1998; Bardgett and Wardle, 2003). Negative effects can also be observed in nutrient-poor systems or under high grazing pressure (Bardgett and Wardle, 2003). Because N is the limiting factor for primary production in many ecosystems (Vitousek and Howarth, 1991), grazing-induced enhancement of soil N dynamics is a key mechanism through which herbivores can influence plant productivity in such a way as to alleviate nutritional deficiencies (McNaughton *et al.*, 1997; Leriche *et al.*, 2001). In particular, the regulation of nitrification has often been regarded as the key for an efficient nitrogen cycling in this context, as in contrast to most other steps in nitrogen turnover only a limited number of microbes is able to convert ammonia into nitrate. Enhanced nitrification in response to grazing pressure has been reported in different unfertilized grassland ecosystems (Groffman *et al.*, 1993; Frank *et al.*, 2000; Le Roux *et al.*, 2003; Patra *et al.*, 2005). These changes can be explained by factors such as

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Received 7 August 2007; revised 29 October 2007; accepted 29 October 2007; published online 29 November 2007

urine and dung input by herbivores (Ruess and McNaughton, 1987; Petersen *et al.*, 2004) and changes in competition for N between microorganisms and plants (Busso *et al.*, 2001; Hamilton and Frank, 2001). It has been further shown that grazing-induced differences in nitrification levels in grasslands are associated with changes in the abundance and community structure of ammonia-oxidizing bacteria (AOB) that were thought to be the key players of the first step of nitrification: oxidation of ammonia into nitrite (Patra *et al.*, 2005, 2006). This picture has changed a little in the last years, as it could be shown that ammonia oxidation can be performed not only by AOB but also by ammonia-oxidizing archaeae (AOA) that could be detected in soil (Leininger *et al.*, 2006). However, the role of the latter group in soil N dynamics is still unclear, as factors influencing AOA and AOB abundance and activity are still missing.

Previous field studies have evaluated long-term (years to decades) effects of different grazing regimes on the nitrifier community, often assuming that it had reached a near-equilibrium state determined by the grazing regime. This traditional assumption of near-equilibrium conditions is often divorced from reality, because demographic and socio-economic constraints can lead to management intensification, de-intensification or abandonment in grazed grasslands at timescales much shorter than decades. Studies are thus needed to analyze the kinetics of the response of soil nitrifiers following changes in grazing regime. Such studies could provide insights into the coupling between grazing-induced changes in nitrification and concurrent changes in nitrifier abundance and community structure.

The objectives of this study and associated assumptions were:

(1) to compare the speed and extent of changes in the activity, abundance and community structure of nitrifiers for two years after switches in grassland management to or from grazing, testing the hypothesis that disturbance of a previously undisturbed system would lead to a faster response than cessation of disturbance of a previously disturbed system;
(2) to assess the ability of changes in the community structure and abundance of nitrifiers to explain observed changes in nitrification under these two scenarios, in particular whether changes in nitrification levels first required changes in nitrifier community structure as suggested by Webster *et al.* (2005) and
(3) to evaluate the relative importance of past and current grazing regimes on the activity, abundance and community structure of nitrifiers two years after a change in grazing regime.

Two levels of management intensity, no grazing and intensive grazing, were investigated in mesocosms initially derived from grazed and ungrazed grasslands. Four treatments were compared: (i) grazing applied on previously grazed grassland, (ii) no grazing on previously ungrazed grassland, (iii) application of grazing on previously ungrazed grassland and (iv) cessation of grazing on previously grazed grassland. The abundance of AOB and AOA, and the community structure of AOB were surveyed over 2 years. In addition, the dominant AOB phylotypes in the four treatments after 18 months were characterized by cloning and sequencing. Mechanisms explaining the observed kinetics of changes in activity, abundance and community structure of nitrifiers under the grazing application and grazing cessation scenarios are discussed according to current knowledge of nitrifier ecology.

Materials and methods

Experimental design

In May 2002, grassland monoliths (that is intact soil and plant cover) were sampled from a seminatural grassland located at Theix (France; 3°1′E, 45°43′N). This grassland had been subjected, in a randomized block design, to two contrasted sheep grazing/mowing regimes without inorganic fertilization from 1988 to 2003. Half of the plots had experienced intensive grazing/mowing (four grazing events and one cut per year), while half had experienced light grazing by sheep (one event per year). Basic soil characteristics (including texture and pH) and climate were the same between both plots (Le Roux *et al.*, 2003). Excavated monoliths (104 monoliths; 52 for each plot, size 50 cm × 50 cm, 40 cm deep) were placed in five-sided stainless steel boxes with drainage holes at the bottom. Two simulated grazing regimes were then applied to these grassland mesocosms for 1 year before beginning the experiment (Appendix 1 in Supplementary information), either no grazing (ungrazed U treatment) or grazing (G treatment), which consisted of plant clipping to 5 cm above the soil surface and application of synthetic sheep urine five times per year to mesocosms derived from the intensively managed plot. Urine was prepared as described by Doak (1952), with a total N content of 0.7 g N l^{-1}, including urea (1.12 g l^{-1}), hippuric acid (0.42 g l^{-1}), allantoin (0.18 g l^{-1}) and creatinine (0.09 g l^{-1}) and with pH adjusted to 7 with NaOH. For each simulated grazing event, urine was applied uniformly to the soil surface and at a level chosen such that N as urine represented 80% of N exported by clipped plant material, which is typical of the N budget in these grazed grasslands under field conditions. Soil moisture was monitored and kept around 35–40% (ca. 70% of water holding capacity). After 1 year, that is in late April 2003, eight mesocosms (four grazed and four ungrazed) were sacrificed at the start of the experiment. At the same date, grazing regime was reversed for half of the remaining mesocosms (that is 24 grazed mesocosms were

submitted to a reversion of grazing regime, from grazing to no grazing treatment, G-U; and 24 ungrazed mesocosms were submitted to the reciprocal change from no grazing to grazing treatment, U-G). Grazing regime was left unchanged for the 48 remaining mesocosms (that is 24 grazed controls, G-G; and 24 ungrazed controls, U-U). Details on the frequency of simulated grazing events in each treatment are provided in Appendix 1 in Supplementary information.

Soil sampling

Soil was sampled before reversion of grazing regime (April 2003) and after 1.5 (June 2003), 4.5 (September 2003), 12 (April 2004), 18 (September 2004) and 24 months (April 2005). Soil was sampled at least 1 month after the last simulated grazing event (Appendix 1 in Supplementary information). At each sampling date, four mesocosms per treatment (four G-G and four U-U in April 2003, and then four G-G, four U-U, four G-U and four U-G at each date) were destructively sampled. The 0–10 cm soil layer was sampled on a 10 cm × 40 cm area in the inner part of each mesocosm and sieved (2 mm mesh size). A 50-g subsample was used for measurement of nitrifier activity and a subsample was stored at −18 °C for molecular analysis.

Determination of nitrifier activity

Nitrifier activity was measured according to the method proposed by Lensi *et al*. (1986), in which soil nitrate is measured before and after soil incubation by conversion to N_2O by denitrification. We have previously shown for this soil that this approach to measure nitrate accumulation gives similar results than traditional extraction-colorimetry method but with a higher sensitivity (S Wertz and X Le Roux, unpublished). For each fresh soil sample, two subsamples (equivalent to 10 g oven-dried soil) were placed in 150 ml plasma flasks. One subsample was used to estimate initial soil nitrate content. The atmosphere of this flask was replaced by a 90:10 He–C_2H_2 mixture and was supplied with a suspension of the denitrifying organism *Pseudomonas fluorescens* ($OD_{580\,nm} = 2$) in a solution containing water, glucose and glutamic acid (each at 1 mg C g^{-1} dry soil). N_2O accumulation was surveyed until NO_3^- was fully converted to N_2O. The other subsample was used to determine NO_3^- accumulation after addition of a solution containing water and $(NH_4)_2SO_4$ (200 μg N g^{-1} dry soil) to obtain 80% of the water holding capacity. The flask was sealed with parafilm and incubated aerobically at 28 °C. After incubation for 7 h, the soil subsample was enriched with *P. fluorescens* and incubated as described above to convert nitrate fully to N_2O. During methodological tests, nitrification rate was found to be constant during the first 7 h for this soil. N_2O was analyzed on a Varian STAR 3400 gas chromatograph. Nitrifying activity (μg N h^{-1} g^{-1} dry soil) was computed by subtracting the nitrate content initially present in the soil from that present after incubation.

Quantification of AOB and AOA abundances

The abundance of β-proteobacterial AOB, that is representing known AOB in soil, was measured by quantitative PCR (qPCR) targeting 16S rRNA gene sequences specific for this group (Hermansson and Lindgren, 2001). The final reaction volume was 25 μl, with iQSupermix (Bio-Rad), 1 μM of a 2:1 ratio of primer CTO189fA/B and CTO189fC, 1 μM of RT1r primer, 0.5 μM of TPM1probe, 0.4 mg ml^{-1} bovine serum albumin (BSA), 10 ng of sample DNA or 5.72×10^8–5.72×10^2 copies of the standard DNA (purified AOB 16S rRNA gene PCR product of ATCC19718 *Nitrosomonas europaea*). The samples were run twice on Opticon2 (MJ Research, Waltham, MA, USA) as follows: 3 min at 95 °C, 45 cycles at 95 °C for 15 s and 58 °C for 1 min, and 8 min at 56 °C. No treatment effect was observed on amplification efficiency. To control possible PCR inhibition by material from environmental samples, each standard concentration was co-amplified with several dilutions of pooled samples, but no inhibition was observed.

The abundance of AOA was measured by qPCR targeting arachaeal *amoA* gene. Fragments (624 bp) were obtained using the primer set 19F (Leininger *et al*., 2006) and CrenamoA616r48x (5′-gccatccabckr tangtcca-3′), which was designed on the basis of all available soil-derived AOA *amoA* sequences in GenBank in November 2006 (S Leininger, personal communication). Each 25 μl reaction mixture contained 0.06% BSA (Sigma-Aldrich, St Louis, MO, USA), 5 pmol of each primer (Metabion, Bayern, Germany), × 1 Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and 2 μl of DNA template or standard, respectively. The PCR reactions were carried out on the ABI Prism 7300 Cycler (Applied Biosystems) performing an initial enzyme activation at 95 °C for 10 min, followed by 40 cycles at 94 °C for 45 s, 50 °C for 45 s and 72 °C for 45 s. As standard, a serial dilution of the crenarchaeal fosmid clone 54d9, described in Treusch *et al*. (2005), was used. Melting curve analysis confirmed the specificity of the amplification, whereas amplification efficiencies of 82–89% were obtained. Possible inhibitory effects of co-extracted humic compounds in soil extracts were checked by dilution series, but no inhibition was observed.

Characterization of the structure of the AOB community

For each sample, DNA was extracted from 0.5 g sieved and frozen soil using the FastDNA SPIN Kit for Soil (BIO 101 Systems; Qbiogene, Carlsbad, CA, USA) yielding approximately 6 μg DNA g^{-1} soil. The

AOB community structure was characterized at five of the six sampling dates: 0, 4.5, 12, 18 and 24 months (that is 72 samples), and was analyzed by PCR-denaturing gradient gel electrophoresis (DGGE). Amplification of 16S rRNA gene fragments from extracted soil DNA was achieved by primary amplification with CTO189f and CTO654r primers (Kowalchuk *et al.*, 1997) that are specific for the majority of β-proteobacterial AOB, and with a secondary nested amplification using bacterial 357f-GC and 518r primers (Muyzer *et al.*, 1993). CTO and bacterial primers amplified 465- and 161-bp fragments, respectively. PCR was carried out in 20 and 50 μl reaction volumes for primary and secondary amplifications, respectively, with a Biometra thermal cycler (Goettingen, Germany). The reaction mixture contained 8 ng of template DNA, 0.2 and 0.1 μM of each primer for the first and second PCR, respectively, 1 × PCR buffer (Bioline, London, UK), 250 μM deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 20 ng BSA for the first PCR only, and 0.016 U μl^{-1} of Biotaq DNA polymerase (Bioline). The thermocycling conditions were modified from Freitag and Prosser (2003): 28 cycles instead of 35 for the second PCR and a final elongation time of 10 min for each PCR. The PCR products were examined by electrophoresis on 2% agarose gels (Invitrogen, Cergy Pontoise, France) stained with ethidium bromide. DGGE analysis of PCR products was carried out using the D-Code Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with 8% polyacrylamide gels containing a gradient of 30–60% denaturant, prepared using a gradient maker (Roth, Karlsruhe, Germany), 100% denaturing solution being defined as 7 M urea and 40% formamide. Gels were run for 16 h at 75 V in 1 × Tris acetate EDTA (TAE) buffer at 60 °C. Following electrophoresis, gels were silver stained and photographed with a digital camera (Sony DSC-F77).

Characterization of major AOB phylotypes by cloning–sequencing

The major AOB phylotypes present in each treatment 18 months after the change in grazing regime were determined by cloning and sequencing. Clone libraries were generated from PCR products obtained by amplification with CTO189f and CTO654r primers from all the soils sampled after 18 months, that is four replicate monoliths for each of the four treatments. Cloning was carried out using the pGEM T-Easy vector system (Promega Ltd, Southampton, UK) and JM109 supercompetent *Escherichia coli* cells (Stratagene Inc., Maidstone, UK) and 26–27 clones, randomly selected from each per soil sample (total of 418 clones), were sequenced (Genome Express, Meylan, France).

Sequences were aligned with partial AOB 16S rRNA gene sequences previously published and used to define phylogenetic groups (Appendix 2 in Supplementary information) using Muscle alignment of SeaView software (Galtier *et al.*, 1996). Phylogenetic analysis was performed using three treeing methods: (i) parsimony method, (ii) neighbor joining method with Jukes and Cantor model of substitution and (iii) maximum likelihood tree, using four categories of substitutions with an estimated gamma distribution parameter and JC69 model of substitution. The first two methods were performed with Phylowin software (http://pbil.univ-lyon1.fr/software/phylowin.html) (Galtier *et al.*, 1996), while the third method was performed with Phyml software (http://atgc.lirmm.fr/phyml/) (Guindon and Gascuel, 2003). Then a consensus tree was computed with Consense in the PHYLIP package 3.66 (http://evolution.genetics.washington.edu/phylip.html). The consensus tree was visualized using NJ plot (http://pbil.univ-lyon1.fr/software/njplot.html) (Perriere and Gouy, 1996)). For each monolith, the percentage of AOB phylotypes belonging to the main AOB phylogenetic clusters identified in the literature (Stephen *et al.*, 1996; Purkhold *et al.*, 2000; Koops and Pommerening-Röser, 2001; Kowalchuk and Stephen, 2001; Wagner *et al.*, 2002) was determined. Sequences obtained in this study have been deposited in the GenBank database (Appendix 2 in Supplementary information).

Data analysis

Two-factor analysis of variance was performed to determine the effects of grazing treatment, time and grazing × time interaction on the activity and abundance of nitrifiers. For each variable, Duncan's and Fisher's tests were then used to determine if means differed significantly between treatments at each date. The effect of grazing treatment on the percentage of AOB phylotypes related to previously defined AOB phylogenetic groups was also tested using Duncan's and Fisher's tests.

To analyze genetic fingerprints, the intensity and relative position of each DNA band in all lanes were determined according to a reference lane using GelCompar software (Applied Maths, Kortrijk, Belgium). The total band intensity for each lane was normalized among lanes, and data were square root-transformed. Bacterial community matrices were analyzed using PRIMER software (version 2, PRIMER-E Ltd, Plymouth, UK). For each date, ANOSIM (one-way analysis of similarities) was performed to compare community structure among each pair of treatments, and the SIMPER (SIMilarity PERcentages) procedure was used to compute the percentage of dissimilarity between community structure for each pair of treatments. Changes in AOB community structure after reversion of grazing regime were quantified by determining the percentage dissimilarity of community structure between (i) soils that had experienced reversion of grazing regime and (ii) control soils under the past grazing regime (that is U-G vs U-U, and G-U vs G-G). A similar approach was adopted to determine the

effect of the new grazing regime on the structure of the AOB community.

Results

Changes in nitrifier activity

The effects of grazing, time and grazing × time interaction on nitrifier activity were all significant ($P \leqslant 0.001$). Activity was always higher for G-G than U-U treatments, except 1.5 months after change in grazing regime when variance for activity values was high for G-G and G-U treatments (Figure 1). Cessation of grazing led to a rapid decrease in nitrifier activity as compared to G-G treatment (detected as soon as 4.5 months), and activity for G-U treatment became similar to that for U-U treatment after 12–18 months (Figure 1). Application of grazing did not lead to any change in nitrifier activity during the first 5 months (no change between U-G and U-U treatments). Activity then strongly increased for the U-G treatment as compared to the U-U treatment and became similar to that for the G-G treatment within $\leqslant$12 months (Figure 1).

Changes in the abundance of AOB and AOA

The effects of grazing and time on the abundance of AOB were significant ($P = 0.0001$ and 0.0005, respectively), whereas the effect of grazing × time interaction was not. The abundance of AOB always tended to be higher for G-G than U-U treatments. However, due to the high variance of abundance data (Figure 2) this was significant only for 4.5, 12 and 18 months after the change in grazing regime. Cessation of grazing led to a rapid decrease in AOB abundance as compared to G-G treatment, and AOB abundance for G-U treatment became similar to that for U-U treatment within 12–18 months (Figure 2). Application of grazing led to an increase in AOB abundance as compared to U-U treatment, although this increase was not significant due to large variance in abundance values (Figure 2).

Abundances of AOA and AOB were in the same order of magnitude (Figure 2), and the abundance of AOA was significantly and linearly related to that of AOB ($y = 0.75x + 0.798$ in 10^6 copies g^{-1}; $R^2 = 0.35$). Interestingly, dynamics of AOA gene copy numbers were similar to the AOB abundance dynamics described above (Figure 2). Again, cessation of grazing tended to decrease AOA abundance as compared to G-G treatment after 12 months, whereas application of grazing resulted in an increase of AOA abundance as compared to U-U treatment after 12 months (Figure 2). A tendency for higher abundance of AOA in G-G than U-U treatments was observed, but this was significant only 1.5 and 4.5 months after the change in grazing regime ($P = 0.06$ at day 0), probably due to the high variance of abundance data (Figure 2).

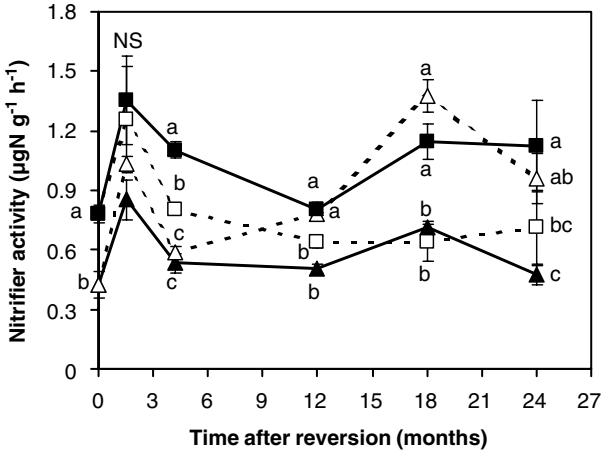

Figure 1 Temporal changes in nitrifier activity in grassland mesocosms subjected to different grazing treatments: (■) grazed controls, G-G; (▲) ungrazed controls, U-U; (□) reversion from grazed to ungrazed, G-U and (Δ) reversion from ungrazed to grazed, U-G. Bars are standard errors ($n = 4$). Values with different letters differ significantly with $P < 0.05$.

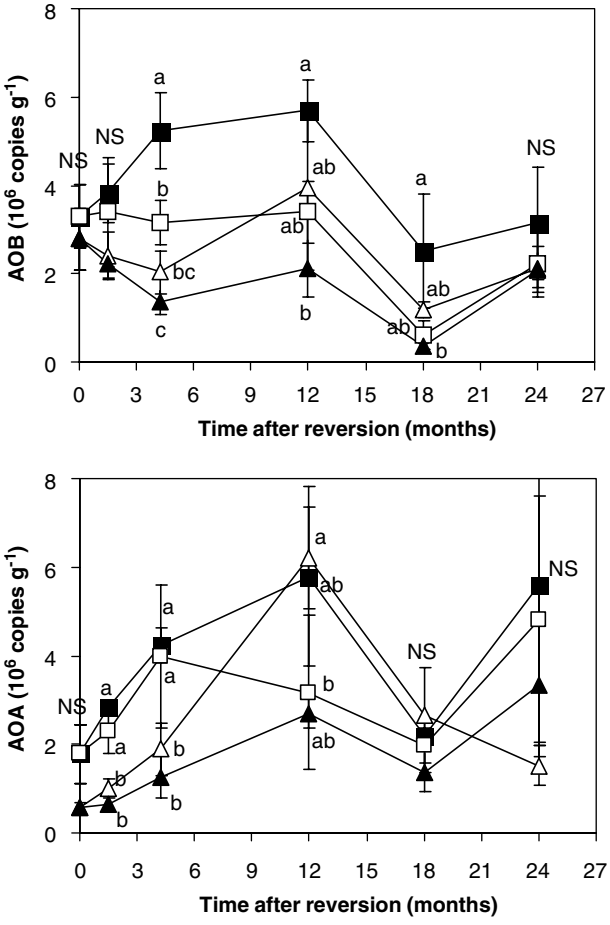

Figure 2 Temporal changes in (top) abundance of ammonia-oxidizing bacteria (AOB) and (bottom) abundance of ammonia-oxidizing archeae (AOA), in mesocosms subjected to different grazing treatments. Symbols as in Figure 1. Bars are standard errors ($n = 4$). Values with different letters differ significantly with $P < 0.05$.

All data were calculated on the basis of grams of soil. However, the results did not change significantly if the values were based on nanograms extracted DNA (data not shown), indicating the robustness of the obtained results.

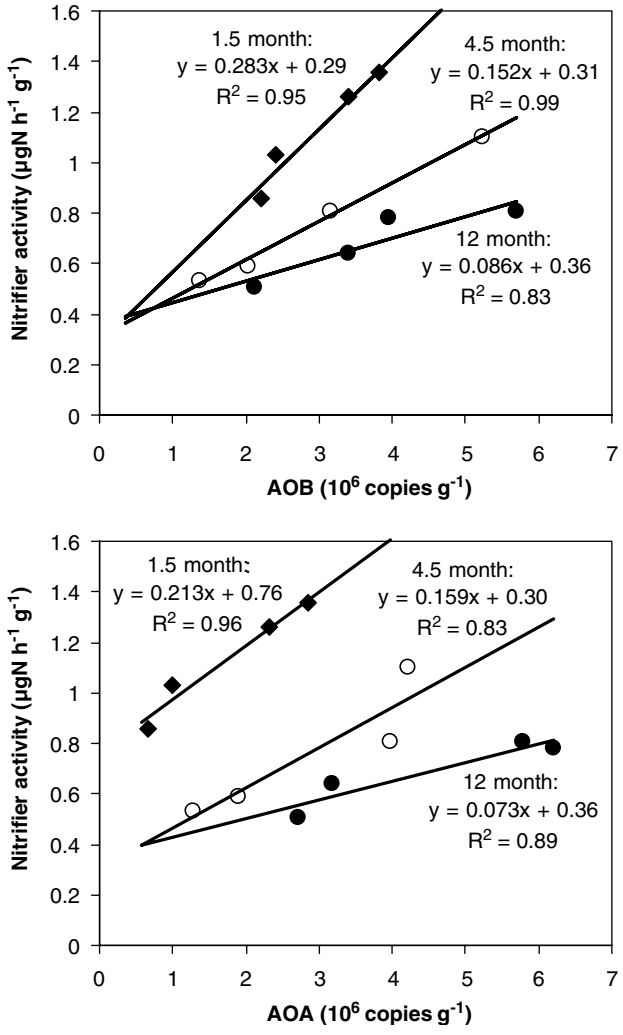

Figure 3 Correlations between nitrifier activity and (top) ammonia-oxidizing bacteria (AOB) abundance and (bottom) ammonia-oxidizing archeae (AOA) abundance, observed 1.5 (◆), 4.5 (○) and 12 months (●) after reversion of grazing regime. Each point corresponds to mean treatment values ($n = 4$). Relationships for both AOB and AOA were not significant at 18 and 24 months.

Relationships between nitrifier activity and AOB or AOA abundance

Strong correlations were observed between mean treatment values of nitrifying activity and both AOB and AOA abundances 1.5, 4.5 and 12 months after the change in grazing regime (Figure 3). At the later time points, no significant correlation was observed for AOB and AOA (data not shown).

Changes in AOB community structure

For each treatment, the major bands observed by DGGE analysis of amplified bacterial ammonia oxidizer 16S rRNA genes changed substantially with time (not shown), indicating dynamics of major AOB populations. However, the percentage of dissimilarity for the AOB community structures between G-G and U-U control treatments remained constant (around 50%) over 2 years (Table 1; Figure 4). Cessation of grazing did not lead to any detectable change in AOB community structure over the first 5 months (no change between G-U and G-G treatments). However, AOB community structure was strongly modified from 12 to 24 months (Table 1; Figure 4). In contrast, the application of grazing led to a rapid change in AOB community structure (detected as soon as 4.5 months) (Table 1; Figure 4).

With increasing time after change in grazing regime, AOB community structure in treatments where grazing regime had been changed did not resemble that for control treatment corresponding to the new grazing regime, that is G-U vs U-U and U-G vs G-G (Table 1; Figure 5). The only exception was for U-G treatment at 24 months, indicating that the AOB community structure at this timescale became mainly determined by the new management regime for the grazing application scenario.

Relative abundance of AOB phylotypes related to different phylogenetic groups

Of the 418 retrieved partial sequences, 95% were related to previously defined β-proteobacterial

Table 1 Results (*P*-values) of one-way ANOSIM for comparisons of the AOB community structure assessed by DGGE profiles between the different treatments

	Before reversion	*4.5 months*	*12 months*	*18 months*	*24 months*
U-U vs G-G	0.03	0.03	0.05	0.03	0.05
U-U vs U-G	—	0.05	0.05	0.05	0.03
G-G vs G-U	—	NS	0.05	0.03	0.03
U-G vs G-G	—	0.03	0.03	0.03	NS
G-U vs U-U	—	0.03	0.03	0.05	0.05
G-U vs U-G	—	0.03	0.03	0.03	0.05

Abbreviations: ANOSIM, analysis of similarities; AOB, ammonia-oxidizing bacteria; DGGE, denaturing gradient gel electrophoresis; G-G, grazed controls; G-U, change from grazed to ungrazed; NS, not significant; U-G, change from ungrazed to grazed; U-U, ungrazed controls.

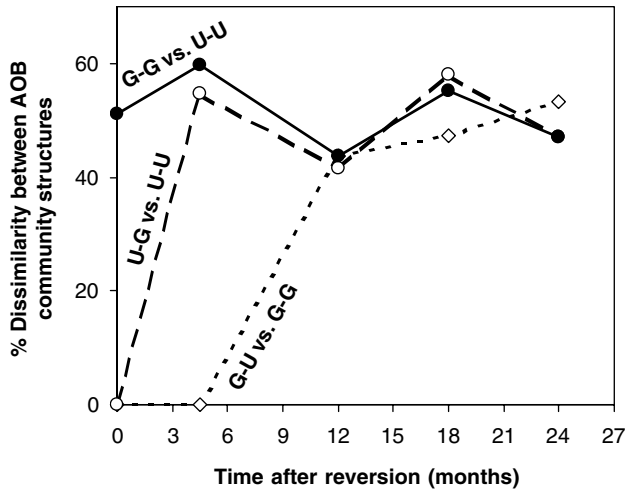

Figure 4 Percentage of dissimilarity between the structure of ammonia-oxidizing bacteria (AOB) community in mesocosms subjected to a reversion of grazing regime and in control mesocosms subjected to the initial grazing regime, that is (○) U-G vs U-U, and (◇) G-U vs G-G. Percentage of dissimilarity between the AOB community structure in the two controls (●) is presented for comparison.

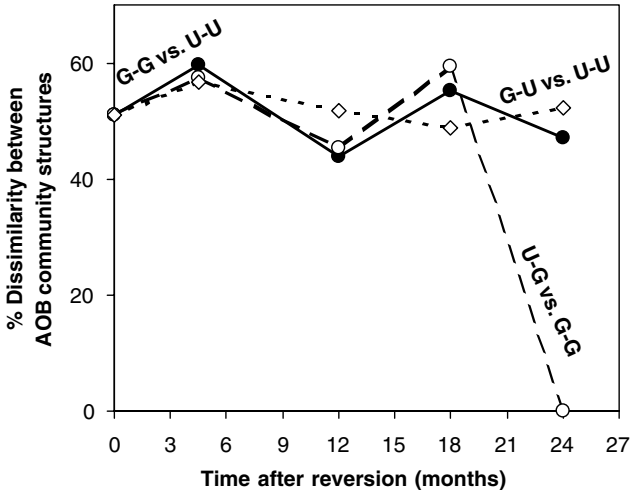

Figure 5 Percentage of dissimilarity between ammonia-oxidizing bacteria (AOB) community structure in mesocosms subjected to reversion of grazing regime and in control mesocosms subjected to the new grazing regime, that is (○) U-G vs G-G, and (◇) G-U vs U-U. Percentage of dissimilarity between the AOB community structure in the two controls (●) is presented for comparison.

ammonia oxidizers (Appendix 3 in Supplementary information): 1 related to *Nitrosomonas*, and 396 to *Nitrosospira*. Of the 21 other sequences, 20 were affiliated to Methylophilaceae and 1 was a bacterial sequence not related to any known groups.

Of the AOB phylotypes retrieved after 18 months from G-G soils, 82% were related to *Nitrosospira* cluster 3a, whereas the AOB phylotypes retrieved from U-U soils were distributed in clusters 0, 3a, 3b and 4 (15–39% for each cluster) (Figure 6). Cessation of grazing led to a significant decrease in the relative abundance of AOB phylotypes related to cluster 3a (G-U as compared to G-G treatment), whereas a marginally significant increase in the relative abundance of phylotypes related to clusters 0 and 4 was observed (Figure 6). Application of grazing led to a significant decrease in the relative abundance of AOB phylotypes related to cluster 3b (U-G as compared to U-U treatment), whereas a marginally significant increase in the relative abundance of phylotypes related to cluster 0 was observed.

Discussion

Differences in nitrifier activity, AOB/AOA abundance and AOB community structure between control grazed and ungrazed mesocosms

Nitrifier activity, AOB and AOA abundances, and AOB community structure exhibited important variations with time. Important temporal variations in nitrifying activity and abundance of AOB have already been reported in grassland and cropping systems (Berg and Rosswall, 1987). In such systems, this could be due to multiple forcings by disturbance regime, climate and plant physiology and phenology acting at a range of scales. However, the activity and abundance of nitrifiers were always higher on G-G than U-U soils and AOB community structure always differed between these treatments throughout the 24-month period studied. Previous analyses of grazing effects on AOB communities in grasslands were generally based on snapshot characterization at only one or two times (Webster *et al.*, 2002; Patra *et al.*, 2005). Comparison of G-G and U-U treatments show that grazing-induced enhancement of nitrifier activity and of both AOB and AOA abundances was remarkably conserved with time. Similarly, a high grazing-induced difference in AOB community structure was maintained between grazed and ungrazed conditions, despite changes in relative abundance of dominant AOB phylotypes for a given grazing regime with time. The data therefore indicate that, despite temporal variations, the effect of grazing on nitrifiers was remarkably stable from a functional rather than structural point of view.

Importance of changes in AOB and AOA abundance and AOB community structure for changes in nitrification after change in grazing regime

It was hypothesized that disturbance of a previously undisturbed system would lead to a faster response than suppression of disturbance on a previously disturbed system. The observed responses were actually more subtle because nitrifier activity responded even more quickly to cessation of grazing (within <5 months) than to application of grazing (5–12 months). The temporal changes in nitrifier activity after change in grazing regime could result from (i) changes in nitrifier abundance, (ii) changes

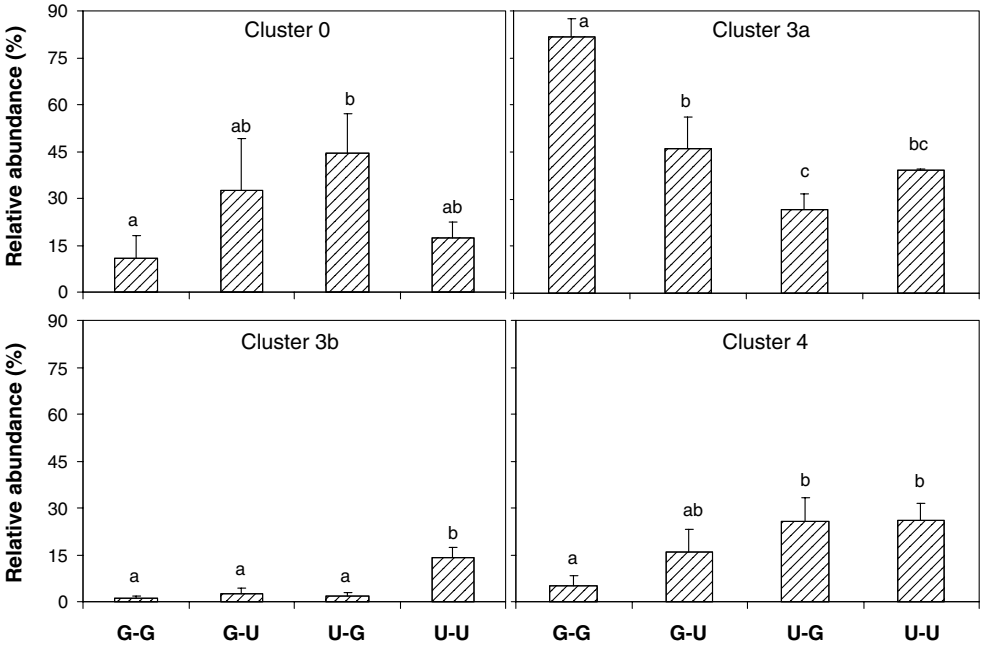

Figure 6 Relative abundances of partial ammonia-oxidizing bacteria (AOB) sequences retrieved from soils experiencing the four grazing treatments, 18 months after change in grazing regime, as related to different *Nitrosospira* groups defined in the literature. Relative abundance of sequences related to cluster 2 was always $<3\%$ without significant grazing effect. Means of four replicates (mesocosms) are presented with standard errors. Values with different letters differ significantly with $P<0.05$.

in the physiological activity of individual nitrifying organisms (that is mean activity per cell) and/or (iii) changes in nitrifier community structure. AOB and AOA abundances were measured by qPCR, so that results were not influenced by cultivation biases that can be high (Kowalchuk *et al.*, 2000; Phillips *et al.*, 2000a, b). Because all known β-proteobacterial ammonia oxidizers are thought to carry only a single 16S rRNA gene per genome (Kowalchuk and Stephen, 2001; see also genome data available for *N. europaea* ATCC19718, *N. eutropha* C91 and *Nitrosospira multiformis* ATCC25196), the measured gene copy numbers should be close to cell numbers for AOB. Similarly, based on the existing data for AOA (Könneke *et al.*, 2005; Treusch *et al.*, 2005) it can be assumed that per organism only one *amoA* copy exists. Thus, the measured AOA *amoA* gene copy numbers should be close to the cell numbers for AOA. For both grazing application and grazing cessation scenarios, changes in nitrifier activity were strongly correlated with changes in the abundance of both AOB and AOA, at least during the first 12 months. The relationship between activity and AOB abundance is consistent with the correlations obtained between nitrification levels and the abundance of cultivable AOB at the same study sites under field conditions (Patra *et al.*, 2005). Similarly, strong correlations were obtained between nitrification and the abundance of AOB assessed by qPCR in salt marsh sediments (Dollhopf *et al.*, 2005), among various soil depths in arable cropping systems (Berg and Rosswall, 1987), and in soil amended with or without ammonium (Hesselsøe *et al.*, 2001) (but see Mendum *et al.*, 1999; Phillips *et al.*, 2000a; Wheatley *et al.*, 2003). Whereas some data have been available on the expected behavior of AOB, almost no results have been published so far on abundance of AOA in grassland soils and possible interaction with grazing. The data published so far has shown a higher abundance of AOA to AOB (Leininger *et al.*, 2006; He *et al.*, 2007). However, it has been shown in these studies that in deeper soil layers the AOA/AOB ratio increases, which may be an indication that AOA/AOB ratio increases with increased nutrient limitation. This could explain that AOA abundance was comparable to AOB abundance in the fertile grasslands studied here. Given that transformation rates for ammonium into nitrite by AOA is currently assumed to be lower than for AOB (Könneke *et al.*, 2005), it can be postulated that the major part of nitrification in the investigated grassland soils was related to AOB activity.

Application of grazing led first (within <5 months) to a change in AOB community structure, with subsequent increases in activity and abundance. The timescale for the grazing-induced change in the structure of the AOB community is consistent with conclusions of Avrahami *et al.* (2003) that changes in the AOB community structure can be detected a few weeks after addition of a fertilizer or urea-amended slurry. Given that particular AOB populations can be selected under high- vs low-ammonia conditions (Kowalchuk and Stephen, 2001) and that urine input leads quickly to increased NH_3 availability in grassland soil (Luo *et al.*, 1999),

changes in ammonia availability were likely the key factor explaining the observed changes in AOB community structure for U-G treatment. This is consistent with results of Patra *et al*. (2006) who observed that grazing-induced changes in ammonia availability explained changes in nitrifier activity and community structure at these study sites under field conditions. Furthermore, the preliminary shift in community structure observed before any change in activity is consistent with results reported by Webster *et al*. (2005), who observed that, in natural, ungrazed soils, a change in AOB community structure was often required before enhancement of nitrification following sheep urine application. The authors suggested that, when AOB communities were dominated by strains sensitive to high ammonia concentration, a shift toward ammonia-tolerant AOB populations was needed to allow nitrification increase. Our results show that, under more realistic conditions (that is planted grassland soils experiencing both urine addition and plant defoliation events), selection of particular nitrifying populations adapted to the new environmental conditions, in particular persistent high urea supply, was a prerequisite for nitrification enhancement in the U-G treatment. Characterization of the physiological traits of dominant AOB populations under grazing vs no grazing conditions, particularly their sensitivity to urea/ammonia levels, is thus a priority. The ability to produce urease that is not encountered in all AOB (Koops and Pommerening-Röser, 2001) could also be an important physiological attribute in this context.

In contrast, cessation of grazing led first (within <5 months) to a decrease in nitrifier activity and AOB abundance, and only later to a change in community structure. Similarly, Griffiths *et al*. (2003) reported important physiological effects of water stress on the activity of microbial community in grassland soil without any detectable effect on the structure of the microbial community. Thus, the decrease in nitrification observed early for the G-U treatment was likely due to partial starvation and decrease in the abundance of AOB initially present.

For both G-U and U-G treatments, nitrifier activity became similar to that observed for control treatments corresponding to the new grazing regime after ca. 12 months. In contrast, this was not observed for the community structure, except for U-G treatment after 24 months. This shows that similar nitrifier activity can be insured by AOB communities differing in their community structure, which is consistent with the high functional redundancy of soil bacterial communities including nitrifiers (Wertz *et al*., 2006, 2007). Investigating the composition of the active nitrifier community using mRNA rather than the genetic community structure could partly explain the decoupling observed between nitrifier activity and community structure.

Phylogenetic affiliation of dominant AOB phylotypes according to grazing regime

We assessed AOB community structure using two methods—DGGE analysis of amplified 16S rRNA genes and quantification of clones belonging to 16S rRNA-defined phylogenetic groups, following sequencing. Both approaches potentially suffer from biases associated with cell lysis, DNA extraction, choice of primers, PCR amplification and cloning, as many other molecular techniques applicable on a large number of samples, but both are accepted methods for assessment of relative abundance and community composition and the biases were probably the same for all samples here and thus did not prevent comparison of treatment effects. DGGE was used to provide a more general picture of community structure at lower cost, whereas cloning and sequencing were performed to gain greater fine-scale taxonomic resolution.

Sequencing results show that CTO primers adequately amplified fragments of the 16S rRNA gene from *Proteobacteria* β-subgroup AOB, and only 5% of the retrieved sequences were not related to *Nitrosospira* or *Nitrosomonas*. In contrast, up to 80% of the clones retrieved from activated sludge and sediment samples with the CTO primer set fell outside AOB lineages in a previous study (Nicolaisen and Ramsing, 2002).

Nearly all AOB sequences (396 among 397) retrieved from soil of these grassland mesocosms after 18 months were affiliated to *Nitrosospira*. Dominance of the AOB community by *Nitrosospira* has already been reported for soil environments (for example, Kowalchuk *et al*., 2000; Wheatley *et al*., 2003). Although the various *Nitrosopira* phylogenetic clusters have been shown to sometimes yield unstable tree topologies (Head *et al*., 1993; Purkhold *et al*., 2000), the assignation of the AOB clones to particular clusters in our study was found to be reliable, that is robust when comparing the assignation obtained with the three different treeing algorithms. The only exception was cluster 2 that was differentiated from cluster 3b only with the maximum likelihood method, but only eight sequences were affiliated to this 'cluster 2'. Both cessation and application of grazing strongly modified the relative abundance of AOB phylotypes as related to *Nitrosospira* clusters defined in the literature, whereas the distribution of AOB phylotypes among clusters for treatments corresponding to changes in grazing regime did not resemble that for control treatment corresponding to the new grazing regime. This effect of change in grazing regime and importance of both past and new grazing regimes are consistent with DGGE data for the same samples.

Several authors have reported possible links between the phylogenetic position of AOB and their ecophysiological traits. For instance, based on physiological measurements made on pure culture and enrichment culture representatives of *Nitrosospira*

clusters 3a and 3b, Webster *et al*. (2005) suggested that AOB related to cluster 3a could be sensitive to high ammonia concentration, while AOB related to cluster 3b could be tolerant. Because (i) AOB related to cluster 3a were strongly dominant in G-G soils experiencing urine inputs, (ii) the relative abundance of AOB related to cluster 3b was highest in U-U soils and (iii) the relative abundance in AOB related to cluster 0 increased in both U-G and G-U soils, our results suggest that AOB adapted to both high and low N levels exist within each of these three clusters. Similarly, Kowalchuk *et al*. (2000) observed a predominance of *Nitrosospira* cluster 4 in late successional fields, likely explained by a low ammonia availability in unfertilized grasslands. Consistently, we observed a higher relative abundance of AOB related to cluster 4 in U-U than G-G soils, and an increase in their relative abundance following cessation of grazing/urine input; however, application of grazing did not affect the relative abundance of AOB related to cluster 4 in U-G soils. All these results suggest that functional diversity of AOB as regards to N levels exists within *Nitrosospira* clusters and that taxonomic affiliation does not fully infer functional traits of these organisms.

Urine input: the key driver of changes in nitrification, AOB/AOA abundance and AOB community structure
Because aboveground grazing was mimicked by plant clipping and urine addition on grassland mesocosms, the results do not reflect exactly the response of grassland soil to change in grazing regime under field conditions. Indeed, changes in dung deposition and trampling regime can also influence soil microbiota (Abdelmagid *et al*., 1987; Ruess and McNaughton, 1987). However, changes in urine input, root functioning and competition between plants and microorganisms are thought to be the key factors mediating linkages between grazing of aboveground plant parts and soil microbiota (Hamilton and Frank, 2001; Bardgett and Wardle, 2003). More generally, more or less severe experimental manipulation of environmental conditions, disturbance regimes and ecological systems is needed to understand better the behavior and driving factors of microbial communities (Kowalchuk and Stephen, 2001). The extent to which aboveground grazing regime was adequately mimicked can be appreciated by the fact that differences in the nitrifier activity between grazed and ungrazed control mesocosms were maintained during the two-year period studied and were consistent with values measured under field conditions (Patra *et al*., 2005). Furthermore, the fact that nitrifier activity and AOB and AOA abundances were determined mainly by the new management regime after 12 months for both scenarios of change in simulated grazing regime showed that urine input and/or plant clipping were the key drivers of the observed effects of grazing on nitrifiers. In addition, changes in botanical composition may explain differences in nitrifier activity, abundance and community structure between long-term grazing regime (Patra *et al*., 2006), but this could not explain the early changes in AOB characteristics in the present study where no major change in plant species composition was observed during the first 6 months in all mesocosms (K Klumpp, personal communication).

Conclusion

These results are an important step forward to understand the influence of different management techniques on microbial communities involved in nitrification. In particular, they demonstrate that AOB and AOA respond quickly to changes in aboveground grazing regime and that, at least for AOB, the mechanisms involved differ between grazing application and grazing cessation scenarios. Selection of particular AOB populations adapted to the new environmental conditions, in particular persistent high urea supply, was a prerequisite for nitrification enhancement in the U-G treatment. In contrast, the decrease in nitrification observed early for the G-U treatment was likely due to a partial starvation and decrease in the abundance of nitrifying organisms initially present. Nitrifier activity and AOB and AOA abundances became similar to those observed in control mesocosms experiencing the new grazing regime after ca. 12 months. In contrast, both past and new management regimes influenced the AOB community structure and distribution of AOB among phylogenetic groups, which shows the extent of decoupling between community function and composition following change in grassland management regime.

Acknowledgements

This study was funded by the French Institute of Agronomic Research (INRA, projet innovant Département EA) and was also supported by the Alliance program. We thank V Degrange for comments on the manuscript and L Darmedru for help during cloning– sequencing. We deeply thank Sven Leininger and Christa Schleper from the University of Bergen/Norway for providing the unpublished new primers for AOA quantification.

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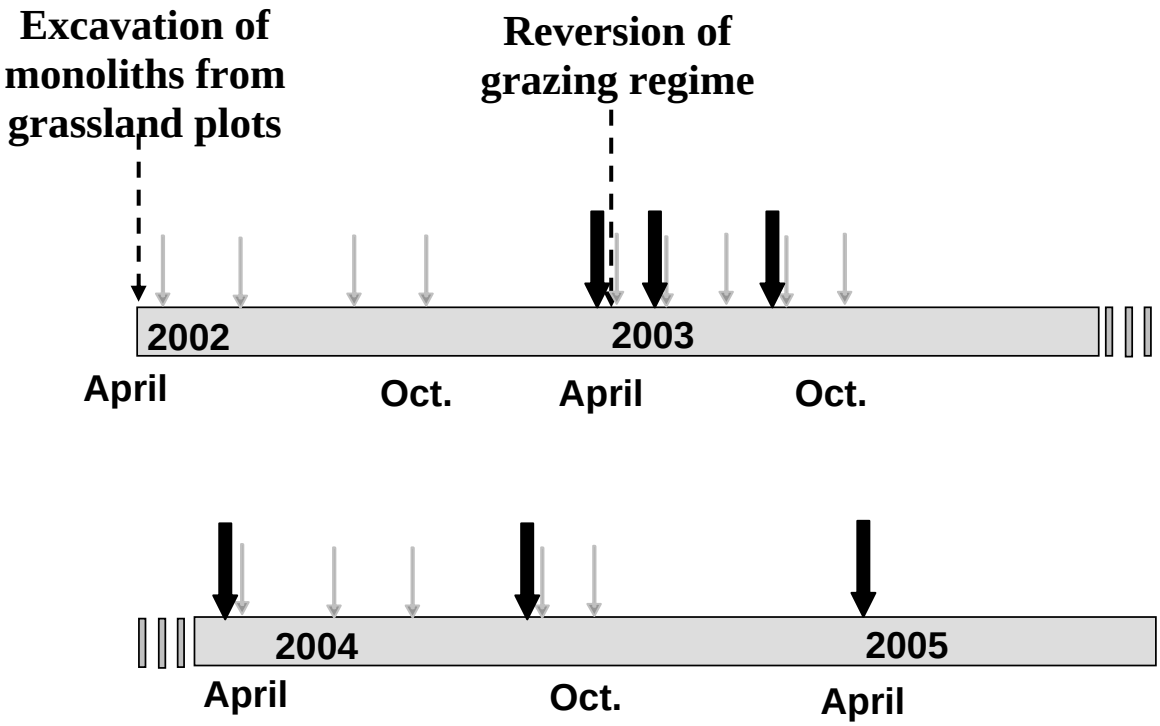
Supplementary Information accompanies the paper on The ISME Journal website (http://www.nature.com/ismej)

Appendices for the manuscript "Effects of changes in grassland management through aboveground grazing on coupling between nitrifier activity, abundance and community structure" by Le Roux X. et al. (*ISME Journal*)

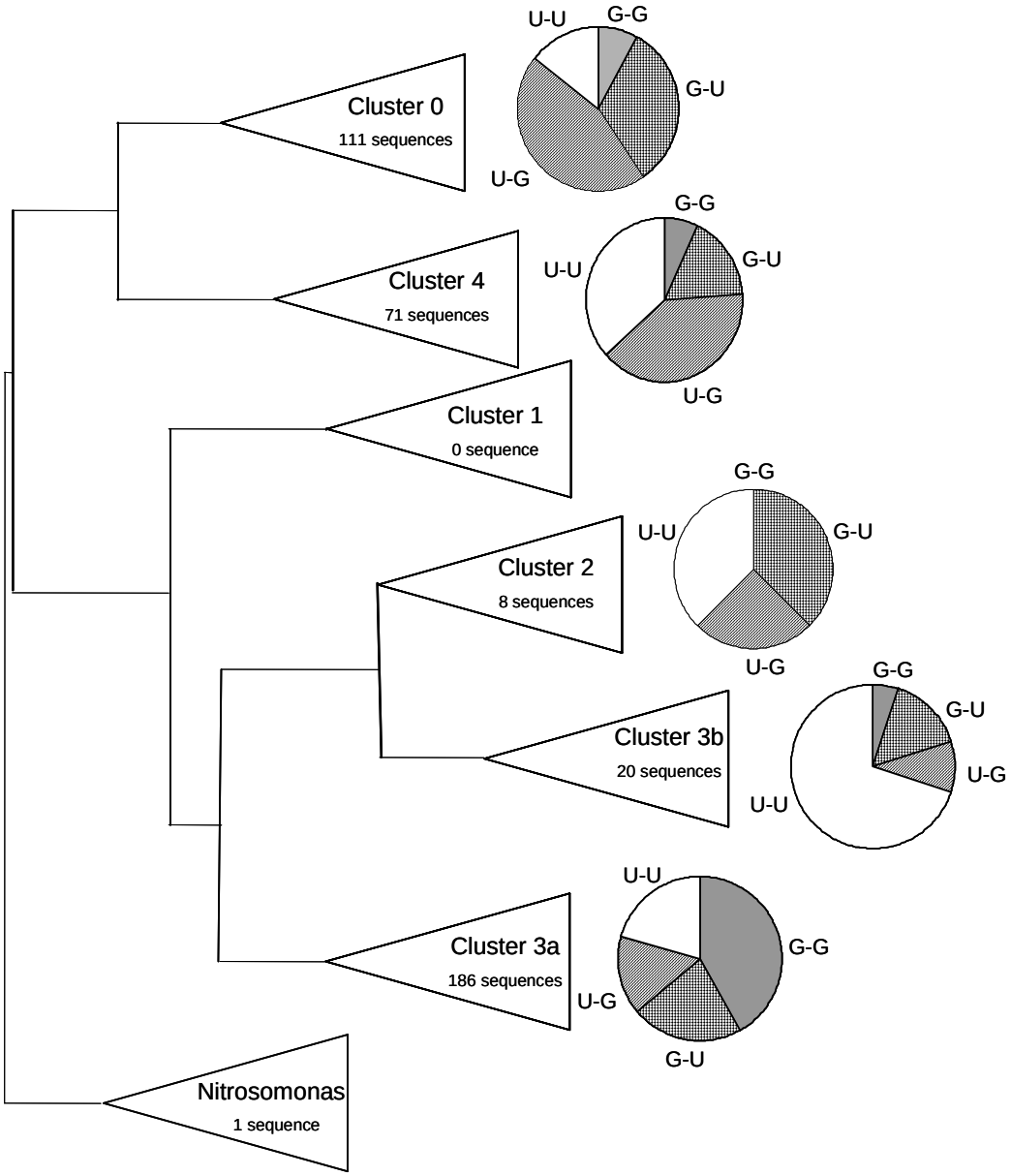
Appendix 1: Scheme presenting the dates of simulated grazing events ( ) and the dates of soil sampling ( ) after set up of grassland mesocosms. All the simulated grazing events were applied to G-G mesocosms, whereas grazing events were stopped after reversion for G-U mesocosms, and grazing events were applied to U-G mesocosms only after reversion.



Appendix 2: GenBank accession numbers of AOB 16S sequences (i) from strains and environmental samples previously used in the litterature to define phylogenetic groups of ammonia-oxidizing β-proteobacteria, and (ii) retrieved from grassland soils in this study.

Nitrosospira **clusters**	**Sequence origins**	**Accession numbers**
	Nitrosococcus oceani isolate Nc1	AJ298727
	Nitrosomonas aestuarii isolate Nm36	AJ298734
	Nitrosomonas communis	AF272417
	Nitrosomonas communis isolate Nm2	AJ298732
	Nitrosomonas cryotolerans isolate Nm55	AJ298738
	Nitrosomonas halophila isolate Nm1	AJ298731
	Nitrosomonas oligotropha isolate Nm45	AJ298736
0	Nitrosospira sp isolate GM4	X84659
0	Nitrosospira sp isolate D11	X84660
0	Nitrosospira sp isolate 40KI	X84656
1	Nitrosospira LD1-A40	AY114342
1	Nitrosospira LD1-B20	AY114344
2	Nitrosospira sp AHB1	X90820
2	Nitrosospira sp En271	AY727031
2	Nitrosospira sp III7	AY123809
2	Nitrosospira sp isolate B6	X84657
2	Nitrosospira sp O13	AJ012108
2	Nitrosospira sp O4	AJ012107
2	Nitrosospira sp T7	X84661
3	Nitrosospira briensis	AY123800
3	Nitrosospira multiformis	AY123807
3	Nitrosospira sp En13	AY856079
3	Nitrosospira sp KAN8	AY631271
3	Nitrosospira sp AF	X84658
3a	Nitrosospira sp 24C	AJ298724
3a	Nitrosospira sp A16	AJ005544
3a	Nitrosospira sp A4	AJ005543
3a	Nitrosospira sp BF16c46	AF386754
3a	Nitrosospira sp En284	AY727032
3a	Nitrosospira sp L115	X84662
3a	Nitrosospira sp NpAV	Y101272
3a	Nitrosospira sp Nsp17	AJ298743
3a	Nitrosospira sp Nsp2	AJ298745
3a	Nitrosospira sp R3c5	AF3867561
3a	Nitrosospira sp R5c20	AF3867551
3b	Nitrosospira briensis Nsp10	AJ2987411
3b	Nitrosospira briensis C-128	L355051
3b	Nitrosospira sp 39-19	AJ2987251
3b	Nitrosospira sp Nsp1	AJ2987441
3b	Nitrosospira sp EnMG6	AY727033
3b	Nitrosospira sp EnU4a	AY727034
3b	Nitrosospira sp Nv6	AJ2987471
3b	Nitrosospira tenuis Nv1	AJ2987461
4	Nitrosospira sp Ka4	AJ012106
4	Nitrosospira sp Ka3	AY1238061
4	Nitrosospira sp Clone SBMSPh11	AY293113
4	Betaproteobacterium sp Clone EnrichZD5	Z69146
4	Betaproteobacterium sp Clonep H7C/56	Z69197
	U-U treatment	EU069931 to EU070031
	U-G treatment	EU070032 to EU070145
	G-U treatment	EU070146 to EU070247
	G-G treatment	EU070248 to EU070348

Appendix 3: Distribution of the 397 partial AOB sequences retrieved from soils of the 16 grassland mesocosms (4 grazing treatments x 4 replicates) among the main phylogenetic groups previously defined in the literature. The total number of retrieved partial sequences related to each cluster and the fraction of this number corresponding to each treatment are presented. Soils were sampled 18 months after change in grazing regime.



Discussion

Molecular tools to study microbial ecology in terrestrial ecosystems

The cultivation of microbes on artificial media under laboratory conditions as well as the use of microscopy were classic methods to identify and quantify microorganisms before the introduction of molecular techniques in microbial ecology. In fact, these methods allow a rough morphological and physiological characterization, but information about biological variety, composition of microbial populations and specific activity in soils cannot be obtained. Firstly, Woese's (1987) advances in molecular phylogeny coupled with developments in molecular biology provided the necessary methods to allow the identification of uncultivated bacteria.

Many of the current molecular detection methods target either DNA or RNA depending on the research objective. Due to its high stability, DNA has been targeted most in nucleic-acid-based studies. It provides primarily information on the presence of specific microorgansims based on specific target DNA sequences or on the presence of functional microbial groups based on functional target genes. Latter constitutes an attractive target to assess specific biochemical processes (Adair & Schwartz, 2008; Coelho *et al.*, 2009; Kandeler *et al.*, 2006).

Differently, RNA molecules provide a measure of expressed genes (mRNA) (Sharma *et al.*, 2006a) and, to some extent, cellular growth and activity (rRNA). The problematic with studies on transcriptome level is the recovery of intact high quality RNA. Most bacterial mRNAs possess short halftimes ranging from a few minutes up to hours (Hambraeus *et al.*, 2003). Therefore, transcriptome studies are snapshots of a very narrow time window as they monitor current concentrations of the mRNA.

The isolation of proteins provides insight into the third level of the genome-transcriptome-proteome cascade. Total protein extraction however, is rather difficult due to the heterogeneity of proteins, suggesting the isolation of specific proteins which has already been conducted for the dissimilatoric copper nitrite reductase

(Metz *et al.*, 2003). When genome, transcriptome and proteome data are combined, they would provide a great potential for understanding the entire complexity of microbes and their contribution to ecosystem functioning.

Culture-independent methods on the basis of nucleic acids require the extraction of DNA or RNA from environmental samples, for which various commercial kits and protocols are available (DNA/RNA co-extraction (Griffiths *et al.*, 2000), DNA extraction (Yeates *et al.*, 1998), RNA extraction (Sessitsch *et al.*, 2002)). A challenge of DNA/RNA extraction from soil samples, notably with high organic content, is the avoidance of co-extraction of humic substances. Such compounds are potential PCR-inhibitors, which can be reduced by the application of electrophoresis or purification steps subsequent to nucleic acid extraction (Harry *et al.*, 1999). Usually, extracts of nucleic acids from soil do not contain sufficient copies of the target genes for direct analysis and therefore require synthesis of the gene fragments of interest by PCR-amplification. This method can be differentiated into non-quantitative and quantitative PCR, but both methods may be biased by e.g. lack of primer specificity or formation of secondary structures of the template (Meyerhans *et al.*, 1990; Pallansch *et al.*, 1990). Moreover, the misincorporation rate of nucleic acids by the *Taq* polymerase (Eckert & Kunkel, 1991) or the formation of chimeric molecules during PCR (Wang & Wang, 1997) and heterogeneity of 16S rDNA sequences (Wintzingerode *et al.*, 1997) were reported to bias PCR product formation.
The introduction of new molecular methods like shot gun sequencing has revolutionized microbial ecology, as it abandons PCR amplification and thus causes minimum bias in studying bacterial community structure (Schlüter *et al.*, 2008) or entire genomes of specific organisms (Reinhardt *et al.*, 2009; Woyke *et al.*, 2009).

In this thesis, a polyphasic approach was chosen (Fig. 5) to gather both potential and activity of the targeted functional microbial groups to get a better insight into their dynamics. The next chapters describe commonly used molecular tools with the emphasis on methods applied in this study and their application in microbial ecology.

Experimental Approach

Soil Samples → *Potential activity: Functional activity (Nitrification)*

DNA Extraction → *Community size: qPCR (nifH, AOB-amoA, AOA-amoA, nirK, nirS)*

PCR amplification → *Community structure: DGGE (AOB-amoA)*

Cloning/Sequencing

Figure 5. Molecular tools applied in this study: **Publication II** (see pictures above experimental approach) involves the quantification of functional genes representing three processes of the nitrogen cycle: nitrogen fixation, ammonia oxidation and denitrification. Experiments were conducted in a semi-arid agricultural field. **Publication III** (see pictures below experimental approach) involves the determination of the nitrifier abundance, activity, community composition and phylotype characterization targeting AOB and AOA. Experiments were conducted in temperate grassland mesocosms each consisting of 24 monolithes.

Assessing the abundance of functional genes

In order to determine the abundance of gene copies or transcripts in an environmental sample real-time PCR is a commonly used method as it enables the specific detection of PCR products in real-time during the exponential amplification stage of the reaction. This method is based on the detection of fluorescence signals and distinguishes between probe-based chemistries (TaqMan®, Molecular Beacons, Scorpions®, FRET Probes) and the SYBR® green chemistry (Fig. 6).

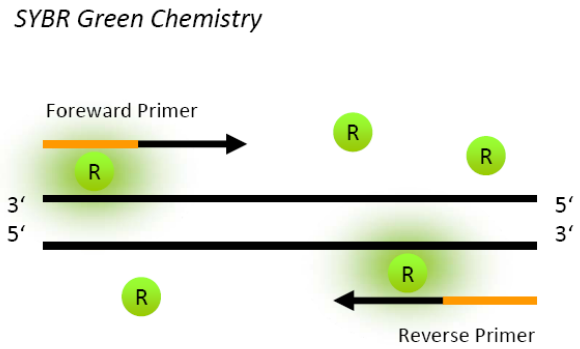


Figure 6. SYBR® green chemistry: Reporter dye (R) starts fluorescing as soon as it intercalates double-stranded products.

When SYBR® green (**Publication II, III**) is added to a reaction, it intercalates all double-stranded DNA present in the sample and binds to each new copy of double-stranded DNA generated during the amplification process (Fig. 6). To achieve an absolute quantification, standard curves have to be created by amplifying known amounts of DNA in a parallel group of reactions run under identical conditions to that of the sample. Additionally, a reference dye, called ROX, serving as internal standard, is used in each sample to normalize the well-to-well difference arising through artefacts like pipetting errors and instrument limitations. The primary disadvantage of the SYBR® green assay is that unspecific products may be detected as well as SYBR® green intercalates any double-stranded product (Wittwer *et al.*, 1997).

However, the performance of a dissociation curve analysis, generating melting peaks of the amplicons, allows the distinct differentiation between the targeted product and undesired products like contaminations and primer dimers (Fig. 7) (Rasmussen *et al.*, 1998). Furthermore, in case of environmental samples, divergences in the GC content of a specific gene, present in different organisms, may lead to the formation of multiple peaks (Fig. 7). Specificity of the reaction must then be confirmed on an agarose gel (Fig. 8).

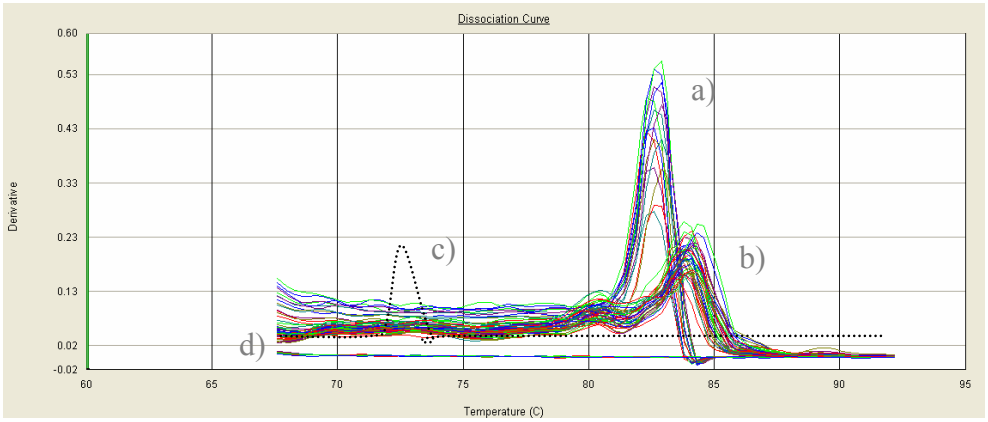


Figure 7. Melting profiles of a typical PCR run (in this case *nirK* gene quantification; **Publication II**) showing a) specific products of the standard, b) multiple peaks of the samples due to divergences in the GC content in the different organisms of a sample and c) the negative controls, giving no melting peaks. Furthermore, indicated is the melting profile of a primer dimer (d) which may occur between 70°C and 75°C.

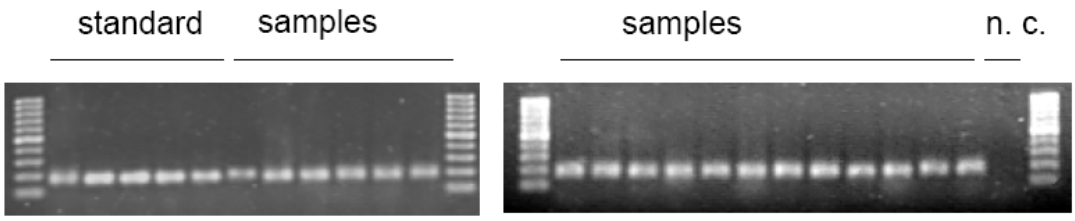


Figure 8. Agarose gels of the amplified *nirK* genes from Fig. 7, showing the specificity of the reaction with fragments of the targeted length (164 bp): standards in position 1-5, samples in position 6-23 and the negative control in position 24.

Further pitfalls include secondary structures in primers, secondary structures in amplification products, length and GC content of amplification products, to name a

few. All these factors have the potential to influence the specificity of the PCR reaction. Low efficiencies increase the number of needed cycles and may lead to the amplification of unspecific products inhibiting the amplification of the targeted product due to competition. Therefore, the selection of an appropriate primer system is of high importance. Ideal primer sets generate amplicons of less than 150 bp (Tichopad *et al.*, 2002). Using the PCR efficiency as quality criterion ($E = (10^{(-1/slope)} - 1) * 100\%$; (Pfaffl, 2001)), short fragments may exhibit values between 90% – 110%. However, primer systems targeting functional genes from environmental samples usually produce amplicons of 150 bp – 500 bp yielding PCR efficiencies between 80% – 120% (-3.9 > slope > -3.0). Due to the mentioned requirements for amplification products, most of the existing primer systems cannot be used; only a few primer sets are verified and approved for the quantification of functional genes from environmental samples, which have been used in this thesis (**Publication II** and **III**).

Another consideration includes the possible existence of multiple gene copies per cell, which affects the actual community abundance and thus aggravates the interpretation of qPCR results. Experimental data indicate multiple *nifH* gene copies in *Paenibacillus azotofixans* (Rosado *et al.*, 1998) and in *Anabaena oscillarioides* (Kirshtein *et al.*, 1991). But due to little information on genome copy variability per cell in different microorganisms, one *nifH* copy per microbial genome was assumed in this thesis (**Publication II**). Likewise, little is known about the genome structure of archaeal ammonia oxidizers. Based on existing data for AOA (Könneke *et al.*, 2005; Treusch *et al.*, 2005), one *amoA* copy per organism was assumed (**Publications II** and **III**). Research on AOB revealed two *amoA* copies in *Nitrosomonas* spp. and three copies for *Nitrosospira* spp. (Norton *et al.*, 2002), for which reason 2.5 *amoA* copies per AOB were calculated in this thesis (**Publication II**). Concerning the denitrifier population, gene copy numbers can be directly correlated with the community size, as no organism has been found yet to possess multiple copies of corresponding nitrogen cycle genes, except nitrate reducers which may harbour two *narG* copies per organism (Philippot *et al.*, 2002). The *nirS* and *nirK* copy numbers were therefore directly referred to the number of nitrite reducers in this study.

The quantification of functional microbial communities in the environment by real-time PCR has been applied in many studies, which proves the potential for quantitative applications in the environment: e.g. quantification of nitrifiers in terrestrial (Adair & Schwartz, 2008; Boyle-Yarwood *et al.*, 2008) and aquatic ecosystems (Wuchter *et al.*, 2006), quantification of denitrifiers (Henry *et al.*, 2008; Kandeler *et al.*, 2006) and nitrogen fixers (Coelho *et al.*, 2009) in soil. In **Publication II**, key genes involved in nitrogen cycling (*nifH*, AOB-*amoA*, AOA-*amoA*, *nirS* and *nirK*) were detected using the SYBR® green assay reflecting the nitrogen-fixing, ammonia-oxidizing and denitrifying communities. In **Publication III**, key genes of the nitrification pathway (AOB-*amoA*, AOA-*amoA*) have been quantified and referred to community structure and activity in order to assess the adaptation to a new management system.

Assessing the activity of functional groups

Methods estimating microbial activities may be classified into field and laboratory experiments, into actual and potential activity measurements and into *in situ* and *ex situ* measurements. Incubation experiments in the field often require long periods of incubation in order to detect significant changes in product concentration. Furthermore, field experiments are affected by soil variation due to permanently varying environmental conditions like temperature, aeration and humidity. In contrast, laboratory experiments have the advantage of standardized and optimized conditions, enabling the comparison of soils of different geographical regions and data from different laboratories.

Methods determining microbial activities distinguish between soil respiration rates and functional microbial activities. Soil respiration rates describe the oxygen uptake and CO_2 release by heterotrophic consumptions (bacteria, fungi, micro- and mesofauna as well as plant roots). However, the problem with soil respiration measurements is that a separation of the activity of microorganisms and other organisms, which vary distinctly in different systems throughout the season (Dilly *et al.*, 2000), cannot be made. In contrast, functional activities give information about functional groups and help understanding their metabolic mechanisms in response to surrounding factors. Several methods have been developed to measure the functional microbial activity in soils using molecular and non-molecular approaches, most of them referring to nitrogen cycling. Quantification of transcripts and *in situ* gas emissions (N_2O, NO, N_2) represent actual turnover rates (Sharma *et al.*, 2006b). While approaches on transcript level reflect a snap-shot, *in situ* measurements provide temporal information of an active transformation process. The determination of enzyme activities (Lensi *et al.*, 1986; Martin *et al.*, 1988; Weaver & Danso, 1994) and the measurement of nitrogenous gas emissions under controlled laboratory conditions provide potential turnover rates, meaning that the maximum capacity of functional microbial populations to carry out nitrogen transformation is determined by the addition of substrates.

In the present study (**Publication III**), potential nitrification rates have been determined according to Lensi *et al*. (1986) in response to urine application. In this procedure, the conversion of NO_3^- to N_2O was measured in soil under anoxic conditions after inoculation with the denitrifier *Pseudomonas fluorescence*. In a parallel approach, another aliquot of the same soil was incubated under oxic conditions for seven days with an ammonium-containing solution and subsequently treated like the soil in the first approach to determine the produced N_2O. A subtraction of N_2O obtained in the first approach from N_2O obtained in the second approach allowed a calculation of the produced NO_3^- during the aerobic incubation and thus the determination of the potential nitrifier activity. The use of the transformation of NO_3^- to N_2O turned out to be a very sensitive method for measuring the native nitrate content of the soils and the level of the nitrification process. It allowed the demonstration of non-negligible nitrifying activity and the rate of activity in response to changes in grazing regimen.

Assessing the community structure of functional groups

Fingerprint analysis

Genetic fingerprinting comprises a set of methods that are appropriate for the assessment of microbial community structure. Commonly used methods rely on sequence-specific restriction analyses (RFLP, T-RFLP) (Liu *et al.*, 1997) or on the separation of double-stranded DNA using denaturing (TGGE, DGGE) (Muyzer *et al.*, 1993) or non-denaturing gels (SSCP) (Lee *et al.*, 1996). These methods allow to address complex ecological questions about spatial, temporal and nutritional interactions that microbial communities are facing in the soil environment (Nakatsu, 2007).

In this thesis (**Publication III**), DGGE has been applied to determine the most dominant species of ammonia-oxidizing bacteria in response to changes in grazing management. This method separates double-stranded PCR products of identical length with different nucleotide sequences by electrophoresis in a gradient of increasing denaturing strength (Muyzer *et al.*, 1993). The fragments migrate according to their melting behaviour under different denaturing conditions using urea or formamide as denaturing chemical. Migration occurs under the influence of an electric current until the DNA fragment reaches the condition that causes its melting leading to a progressive branching of the molecules. To avoid the entire melting of the DNA, GC-clamps of 30-40 bases are usually added to one of the primers. Visualization of DGGE patterns, mainly by silver or SYBR® dye staining, allow the determination of the genetic diversity, which provides a rough estimate of richness and abundance of predominant microbial community members.

However, there is clear evidence that procedures carried out before electrophoresis like PCR or nucleic acid extraction protocols (Martin-Laurent *et al.*, 2001) strongly influence the observed phylotype abundance and composition of the bacterial community. Therefore, methods such as touchdown (Muyzer *et al.*, 1993) or nested PCR (Heuer *et al.*, 1997) have been used to increase specificity. Yet, DGGE has

been approved and successfully applied to study microbial diversity in terrestrial ecosystems (Heuer *et al.*, 1997; Jensen *et al.*, 1998; Kowalchuk *et al.*, 1997a). Due to the immense complexity of soil microorganisms primers are applied to target specific functional groups such as diazotrophs (Piceno & Lovell, 2000), nitrifiers (Enwall *et al.*, 2007; Nicolaisen & Ramsing, 2002) and denitrifiers (Enwall *et al.*, 2005; Hallin *et al.*, 2006; Sharma *et al.*, 2005; Throbäck *et al.*, 2007). The advantage over other fingerprinting methods, such as tRFLP, is the possibility to apply follow-up procedures, such as excision of selected bands, followed by their re-amplification, cloning and sequencing in order to affiliate ribotypes that make up a particular band in the fingerprint.

Clone-library analysis

In recent years, cloning and sequencing of specific PCR amplificates was the most powerful technique to determine the microbial diversity. Thereby, 16S rRNA gene sequences of Bacteria and Archaea and the 18S rRNA genes of Eukarya are primary targets for monitoring microbial community dynamics following natural and anthropogenic environmental changes (Fierer *et al.*, 2007; Horner-Devine *et al.*, 2004; Kang & Mills, 2006). With the advances in sequencing, the rate of Genbank entries for cultivated and especially uncultivated microorganisms has increased dramatically (Rappe & Giovannoni, 2003) and enabled the identification of sequences that were not even assumed to be present in soil, such as Archaea (Leininger *et al.*, 2006). Functional genes have also been used to study changes in community structure, as for example the *nifH* genes which play a role in N_2 fixation (Deslippe & Egger, 2006). Clone libraries have also been used to study the nitrifier (Purkhold *et al.*, 2000; Schmid *et al.*, 2007) and denitrifier diversity (Enwall *et al.*, 2005; Priemé *et al.*, 2002; Throbäck *et al.*, 2007). A functional gene library can provide increased resolution of important organisms that may not be detected within a 16S rRNA clone library due to low abundance, but which may represent important active community functions.

Using this technique, the gene of interest is PCR-amplified and inserted into plasmid vectors. In the cloning process, the fragment is inserted into another gene, either a suicide gene or a gene encoding a colour reaction, whose disruption enables a positive screening of the clone. The plasmid is provided with an antibiotic resistance gene which, once it is transferred into the host (mostly *E. coli*), allows the growth of bacteria on media containing this particular antibiotic. Each colony contains a unique DNA fragment, whereas the collection of the antibiotic-resistant *E. coli* cells is called a clone library. The polymorphism of the clone library can be directly sequenced and subsequently evaluated by phylogenetic analysis tools: e.g. parsimony and neighbour joining method (Galtier *et al.*, 1996) and generation of maximum likelihood tree (Guindon & Gascuel, 2003). A few grams of soil give raise of a huge number of different clones. The coverage of the diversity is strongly dependent on the number of clones selected. Although clone libraries provide more information than other fingerprinting methods, it has to be kept in mind that even a large number of clones may not always cover the actual diversity (Priemé *et al.*, 2002). In this study (**Publication III**), cloning and sequencing was used to characterize phylotypes of the ammonia-oxidizing community 18 months after changing the grazing regimens. Small libraries of 26-27 randomly selected clones per soil were constructed resulting in altogether 418 clones. Phylogenetic groups have been defined and sequences were clustered.

Factors driving microbial turnover processes in the nitrogen cycle

Various biotic and abiotic factors influence the structural and functional diversity of microbial communities in terrestrial ecosystems (Fig. 9). In particular, soil type and vegetation have been described to play key roles in governing turnover processes (Berg & Smalla, 2009). In terms of microbial abundance and activity, the rhizosphere provides an important hot spot, because microbes profit from the carbon supply through rhizodeposition (Lynch & Whipps, 1990) and in turn provide the plant with nutrients (Morgan *et al.*, 2005). This interaction, however, depends strongly on other factors which have been investigated in this thesis and are discussed in the following sub-chapters. These steering factors are differentiated into given factors that are not manipulable by human actions (soil and climate), that can conditionally be influenced (vegetation) and into those that can be effectively influenced by management practices like fertilization and grazing.

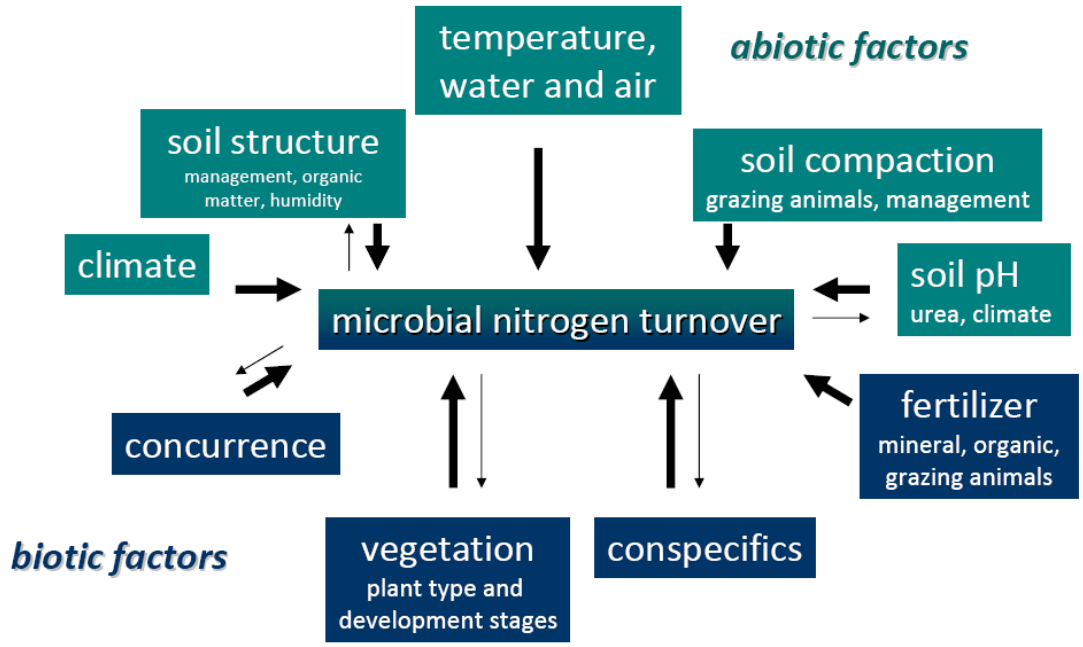


Figure 9. Biotic and abiotic factors influencing the microbial nitrogen turnover in terrestrial ecosystems.

Soil properties

Soil pH

The pH value in soil decreases with time in humid regions due to eluviation and increases with soil depth as most of the H^+-ions are produced through microbial degradation of dead biomass (Scheffer & Schachtschabel, 2002a). Soil acidity affects plant growth directly or indirectly by steering nutrient availability and nitrification (Scheffer & Schachtschabel, 2002b). Most crops and the majority of bacteria thrive at neutral pH values (McKenzie, 2003). However, many microbes can tolerate extreme pH surroundings such as *Halomonas* and *Archaea* (Alva & Peyton, 2003; Ciaramella *et al.*, 2005; Fuetterer *et al.*, 2004; Schleper *et al.*, 1995). Transformation processes like nitrification were observed in acid surroundings (Boer & Laanbroek, 1989; De Boer *et al.*, 1991; Islam *et al.*, 2007; Islam *et al.*, 2006) although it was

thought that nitrifying organisms were dependent on alkaline microsites being acid-sensitive (Hankinson & Schmidt, 1988; Prosser, 1989).

In **Publication II**, soil pH turned out to be an important factor steering the abundance of AOB as significantly increased *amoA* copy numbers were found in urea-treated acidic plots. A variety of AOB possess the capability to hydrolyze urea (Koops & Pommerening-Röser, 2001) and interestingly, these organisms have been detected in various acidic soils (De Boer & Laanbroek, 1989; Klemedtsson *et al.*, 1999; Martikainen *et al.*, 1993; Stephen *et al.*, 1998). These data suggest that low pH values favour the use of urea as an energy source and hence autotrophic nitrification (Burton & Prosser, 2001).

Nutrients (N, C, P)

The experimental site in the tropic ecosystem (**Publication II**) was poor in terms of nutrients under the different fertilizing treatments. Only long-term application of manure enhanced the nutrient content considerably (Table 1). Nitrogen is the limiting factor for plant growth and therefore BNF is of central importance in tropical regions. A lack of water, carbon (C), phosphorus (P) and other macronutrients, however, limits the effectivity to fix nitrogen (Giller & Wilson, 1991). Therefore, nitrogen fixation is highest near decomposition of plant residues (Rebafka *et al.*, 1993) or in the rhizosphere of crops (Coelho *et al.*, 2009) where more carbohydrates are available. In our study (**Publication II)**, highest *nifH* gene abundances were observed in plots where the nutrient content was increased, as well as distinctly increased crop yields. In contrast, the C-rich additive "straw" did not have any improving effect on the low soil nutrient content and hence the community size of the diazotrophs was not increased. Probably, only a small fraction of the straw-C was available for soil microbes.

TABLE 1. Soil physical and chemical properties, plant biomass and grain yields[a] of the differently amended plots

	Amount (mg g^{-1})			
	Total C	Total N	Total P	Grain yield
Control	2.16±0.33^{b}	0.15±0.05^{b}	0.11±0.0^{bc}	167±174^{b}
Control + Urea	2.22±0.77^{b}	0.16±0.05^{bc}	0.10±0.03^{b}	207±124^{b}
Straw	2.48±0.38^{b}	0.15±0.11^{b}	0.11±0.02^{bc}	90±60^{b}
Straw + Urea	2.69±0.42^{b}	0.17±0.07^{bc}	0.11±0.02^{bc}	233±154^{b}
Manure	3.57±0.2^{c}	0.27±0.12^{c}	0.15±0.02^{c}	730±319^{c}
Manure + Urea	3.56±0.43^{c}	0.25±0.05^{bc}	0.15±0.02^{c}	737±350^{c}

[a] Significant differences between treatments are indicated by different letters (values are mean values ± sd; n=6).

Table 1. Abstract of **Publication II**: nutrient content of semi-arid soil in Burkina Faso. Illustrated are the mean values of six replicates. Significant differences are indicated by different letters.

Climate

It could be demonstrated in this thesis that in tropical agro-ecosystems nitrogen cycling is highly affected by extreme environmental factors as for example prolonged drought and thus scenarios of moderate climate regions cannot be transferred (**Publication II**). Overall, factors mentioned in this chapter may impact the nitrogen cycle in tropical ecosystems in the same way as in moderate ecosystems. However, as soon as extreme climatic factors such as prolonged drought and heavy rainfall occurred, plant fitness was highly affected and this in turn affected all investigated microbial functional groups in the rhizosphere.

Sorghum, the cultivated crop at the experimental site in Saria (Burkina Faso), was undergoing a drought period during the flowering stage (Fig. 10). This led to a decreased rhizodeposition and to a reduced community size of the microbes. Thereby, ammonia oxidizers and denitrifiers were reduced to the same extent. It could be demonstrated, that continued manure application was the only soil management that could diminish the climatic influence leading to higher plant

fitness, rhizodeposition at the flowering stage and even to an increase in diazotrophs. Usually, active microbial biomass can be used as indicator for soil functioning (Snakin *et al.*, 1996), but the negative climate effects on productivity were also reflected by the microbial abundance data obtained in this thesis.

Figure 10. Comparison of plots at two different plant development stages: row I) EC30 (young leaf development, August 2006) and row II) EC60 (flowering stage, September, 2006). Although August belongs to the rainy season, this month suffered a lack of rain and resulted in a reduction of plant fitness (**Publication II**). A) control, B) straw application and C) manure application.

Vegetation

Plant species in combination with soil type substantially influence structure and function of microbial populations in the rhizosphere (Berg & Smalla, 2009; Hartmann *et al.*, 2009). Due to enhanced substrate availability (rhizodeposition), microbial biomass and activity are enhanced as compared to the surrounding bulk soil (Sørensen, 1997). Therefore, biotic interactions are more common in the rhizosphere than in bulk soil. Most soil microorganisms are heterotrophic meaning that they depend on plant exudates to obtain carbon for their growth and activity. Studies have shown that 64–86% of the carbon released into the rhizosphere by exudation are respired by microorganisms (Hütsch *et al.*, 2002) leading to a 10- to 100-fold increase in microbial population size as compared with the surrounding bulk soil (Weller & Thomashow, 1994).

Root exudates are not only an important source of carbohydrates but include organic acids, amino acids, sugars, phenoles and vitamins for soil microorganisms (Hütsch *et al.*, 2002; Lynch & Whipps, 1990; Marschner, 1995). The amount and composition of exudates, as well as the solubility of nutrients and toxic elements can dramatically change activity and composition of microbial communities (Diallo *et al.*, 2004; Grayston *et al.*, 1998; Griffiths *et al.*, 1998).

Quantity and quality of exudates varies with plant species, genotype, age, physiological status and root morphology (Lynch & Whipps, 1990). Therefore, a reduced quality and quantity of rhizodeposition due to loss of plant diversity and activity can modify abundance, activity and composition of microbial communities (Hooper *et al.*, 2009; Wardle & Lavelle, 1997) in the rhizosphere.

Publication II describes the combined impact of climate and vegetation on the targeted functional microbial groups. Since severe drought coincided with the flowering stage, a distinct breakdown of all targeted genes at the flowering stage was observed. The severely impaired plant fitness (Fig. 10) due to the unexpected drought at the flowering stage strongly indicates that the observed reduced gene abundances may be attributed to reduced rhizodeposition.

Effects of fertilization

The implementation of agricultural management practices, including fertilization and cropping, affects soil structure (Marinari *et al.*, 2000), soil nutrient content (Tiquia *et al.*, 2002), productivity (Seliga & Shattuck, 1995) and microbial activity (Drury *et al.*, 1998). Especially research on denitrification provided numerous data about the impact of fertilizer application. Various studies reported that organic fertilizers promote denitrification more than mineral fertilizers (Dambreville *et al.*, 2006b; Enwall *et al.*, 2005; Rochette *et al.*, 2000; Wolsing & Prieme, 2004). A most probable reason for this stimulation is, that organic fertilizers provide readily available carbon (Christensen & Sorensen, 1985). But also activity and community structure of denitrifiers were affected by organic fertilizers (Dambreville *et al.*, 2006a; Rochette *et al.*, 2000). Sewage sludge, swine and cow manure promote microbial activity in general (Ritz *et al.*, 1997; Šimek *et al.*, 1999). The problem with the use of pig manure as fertilizer is, that antibiotics such as sulfadiazine are frequently used in veterinary medicine. However, sulfadiazine is poorly metabolized and absorbed in the animal gut, so that it reaches the soil via manuring, inhibiting the growth of many bacteria. Therefore, the impact of antibiotic-contaminated pig manure on ammonia-oxidizing archaea and their bacterial counterparts was recently investigated by Schauss and colleagues (2009). Based on a newly developed mathematical model, they could quantify the separate contribution of AOA and AOB to ammonia oxidation concluding that AOA are less sensitive to antibiotic stress than AOB in terms of activity.

Commonly, fertilization studies are assessed to improve plant productivity. Therefore, many short-term experiments have been conducted to resolve this concern, but short-term yields do not prove the sustainability of the system. In contrast, long-term experiments are necessary for testing the sustainability of farming practices. Experiments at Rothamsted (England) showed that grain yields can be sustained and even increased for almost 150 years in monocultures of wheat and barley given organic or inorganic fertilizer annually (Jenkinson, 1991). The experimental site investigated in **Publication II** (Saria II) is located in semi-arid Burkina Faso, a region that is confronted with soil exploitation, abandonment and

extreme climatic conditions. All these factors contribute to soil infertility and therefore these semi-arid ecosystems have a high demand on resources for soil nutrient replenishment (Sanchez *et al.*, 1997). Serving as experimental site since 1980, Saria II constitutes an ideal model system to test the sustainability of different fertilizers in semi-arid ecosystems and how the microbial nitrogen cycle is affected by long-term fertilization.

Six different fertilizers (control +/- urea, straw residues +/- urea, manure +/- urea) were applied on a sorghum field (Fig. 11) and their effect on the nitrogen-fixing, nitrifying and denitrifying microbial groups was studied. All three processes of the nitrogen cycle were impacted by the mentioned fertilizers (Figs. 1 and 2 in **Publication II**). Manure was the only fertilizer that improved soil nutrient content and productivity. The abundance of diazotrophs was significantly increased as well as the diazotroph:denitrifier ratio. These positive effects of organic fertilizer may be attributed to the improved soil–water–plant relationship and soil structure by the extension of pores (Marinari *et al.*, 2000).

Straw affected mainly the nitrifier and the denitrifier population. However, these effects were small and occurred at a single plant development stage (Fig. 1 in **Publication II**). Nitrate values were low under straw and no inductive effect on productivity could be observed. The impact of straw on nitrogen cycle genes is scarcely studied. Yet, Cox *et al*. (2001) reported about limited application rates of straw due to its bulky structure and due to low effects on productivity.

The application of urea considerably increased the nitrifier community and nitrate values increased. Our results match the result of Chu *et al*. (2007), who reported a strong increase in nitrifier activity in response to urea application. However, urea amendment in this thesis (**Publication II**) increased plant and grain yield only slightly, assuming that the urea effect is less pronounced in semi-arid soils as compared to moderate regions.

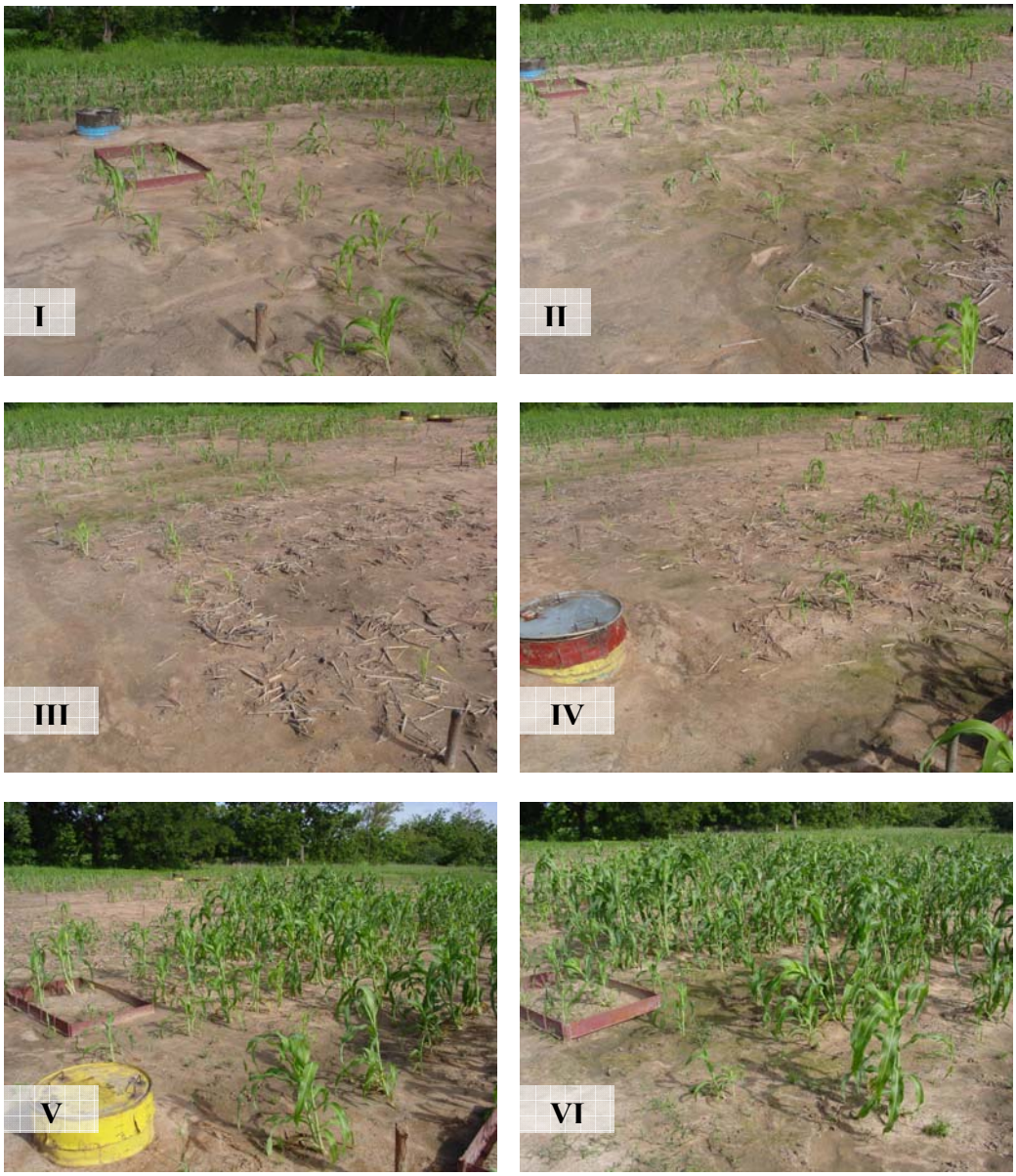


Figure 11. The experimental site Saria II located in Burkina Faso. Shown in these pictures are the differently treated plots at EC30 (young leaf development) in August 2006: I) control, II) control + urea, III) straw residues, IV) straw residues + urea, V) manure, VI) manure + urea.

Grazing

Grazing has a strong impact on the soil nitrogen cycling in grassland ecosystems (Gao *et al.*, 2008; Risser & Parton, 1982). It increases the soil nutrient content (Mathews *et al.*, 1994) and decreases the C/N ratio in aboveground plant biomass, roots and organic matter (Coppock *et al.*, 1983; Holland *et al.*, 1992). Increased nitrogen mineralization in response to grazing (Holland *et al.*, 1992; Molvar *et al.*, 1993) improves the N availability to plants (McNaughton *et al.*, 1997; Risser & Parton, 1982). In turn, above- and belowground components, such as shoot density and productivity, are stimulated (Hik & Jefferies, 1990; Malkamäki & Hæggström, 1997; Valentine *et al.*, 1997).

Le Roux *et al*. (2003) stressed the question, whether the impact on processes of the nitrogen cycle like nitrification or denitrification activity is attributed to the decomposition of urine or to changes in vegetation and found that grazing was the driving force for enhanced microbial activity. Experimental data revealed that intensive grazing enhanced nitrifier activity as compared to slight grazing and that denitrification was less affected than nitrification.

The experimental data in **Publication III** clearly demonstrated that the exposure of grassland soil to urine affected the nitrifier abundance, activity and composition. More importantly, temporal microbial variations in response to novel grazing regimen gave new insight into the nitrifier dynamics, which is important to understand consequences of modifications of grazing intensity. Intensive grazing, however, can cause soil compaction leading to anaerobic conditions and to changes in soil functioning. The stimulation of CH_4 production in response to grazing (Radl *et al.*, 2007) indicated that methanogens may also play a significant role when grazing animals are introduced as management strategy.

Conclusions

The major findings of this thesis can be summarized as follows:

- The evaluation of quantification methods (**Publication I**) revealed that qPCR is a suitable method for studying the community abundance of functional microbial groups. Especially in terms of high throughput this method is superior to competitive and MPN PCR. Quantitative PCR provided reproducible results due to provision of standardized protocols and allowed a distinct differentiation between specific and unspecific products (**Publications II** and **III**).

- Long-term fertilization influences soil pH and nutrient content in nutrient-poor soils, which in turn affects microbial abundance and crop yields (**Publication II**). Long-term experiments allow the observation of the rate, quantity and quality of microbial responses as well as the comparison of past and current effects on the microbial communities (**Publication III**).

- Urea application predominantly impacts the nitrifying community, which is able to adapt quickly to switches in urine management. Nitrifier abundance and nitrate production are stimulated in urea-treated soils with low pH (**Publication II**). Abundance correlates with activity, but nitrifier composition and functioning are decoupled between two consecutive grazing management events (**Publication III**).

- Independent from plant development stage and fertilizing treatment, ammonia-oxidizing archaea predominate in semi-arid soils as compared to their bacterial counterpart (**Publication II**). While AOB respond distinctly, AOA are less responsive to different fertilization treatments.

- Soil pH turns out to be a strong driver for the bacterial nitrifier abundance in semi-arid ecosystems as well as for denitrifiers, but to a lesser extent (**Publication II**). Ammonia-oxidizing archaea and diazotrophs seem to be less affected by low pH values and a correlation AOA and AOB to plant growth could not be observed.

- Manure diminishes degradation and enhances the nutrient content in soils being amended for many years (**Publication II**). Plant and grain yields are improved thereby even under severe climate scenarios (prolonged drought). In turn, manuring affects the nitrogen-processing microbial communities and notably stimulates the nitrogen-fixing bacteria.

- Climatic factors play a more important role in semi-arid soils than in moderate regions due to their extreme occurrence. Field experiments complicate the differentiation of factors influencing the size of microbial groups. Both, climate and plant development stage affect grain yield and microbial abundance. Yet, it cannot be resolved from the present data set, which of these factors is the main driver for the breakdown of the microbial groups at the flowering stage of the plant (**Publication II**).

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- Hai, B., Diallo, N. H., Sall, S., Haesler, F., Bonzi, M., Assigbetse M., Chotte, J.-L., Munch, J. C., Schloter M.: Quantification of the microbial nitrogen cycle in the rhizosphere of sorghum cultivars in tropical agro-ecosystems. *Applied and Environmental Microbiology (2009)*,15:4993-5000.

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- Sharma, S., Radl, V., Hai, B., Kloos, K., Mrkonjic Fuka, M., Engel, M., Schauss, K., Schloter, M. (2007): Quantification of functional genes from prokaryotes in soil by PCR. *J Microbiological Methods (2006)*, 68: 445-452.

Presentations

- Hai, B., Bonzi, M., Sall, S., Diallo, H., Assigbetse, K., Brauman, A., Chotte, J. L., Munch, J. C., Schloter, M.: Effect of different soil management on the bacterial nitrogen turnover in tropical agro-ecosystems. Jahrestagung der VAAM, Frankfurt 2008

- Hai, B., Bonzi, M., Sall, S., Diallo, H., Assigbetse, K., Brauman, A., Chotte, J. L., Munch, J. C., Schloter, M.: Effect of different soil management on the bacterial nitrogen turnover in tropical agro-ecosystems. International Conference, Rhizosphere 2, Montpellier 2007

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