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Diversity, abundance and activity of microbes involved in nitrogen turnover in the rhizosphere of different plants grown on sites contaminated with the antibiotic sulfadiazine or heavy metals

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List of publications and contributions

Publications:

I. **Ollivier J.** *,* Kleineidam K.*,* Reichel R.*,* Thiele-Bruhn S.*,* Kotzerke A.*,* Kindler R*,* Wilke B.-M., and Schloter M. (2010) Effect of sulfadiazine-contaminated pig manure on the abundances of genes and transcripts involved in nitrogen transformation in the root-rhizosphere complexes of maize and clover*. Applied and Environmental Microbiology* 76*,* 7903–7909*.* doi: 10.1128/AEM.01252-10

II. **Ollivier J.**, Wanat N., Austruy A., Hitmi A., Joussein E., Welzl G., Munch J.-C., and Schloter M. (2012) Abundance and diversity of ammonia-oxidizing prokaryotes in the root-rhizosphere complex of *Miscanthus* x *giganteus* grown in heavy metal contaminated soils. *Microbial Ecology* in press. doi: 10.1007/s00248-012-0078-y

III. **Ollivier J**., Schacht D., Groeneweg J., Engel M., Wilke B.-M., Kleineidam K., and Schloter M. Effects of repeated application of sulfadiazine-contaminated pig manure on the abundance and diversity of ammonia- and nitrite oxidizers in the root-rhizosphere complex of pasture plants under field conditions. Submitted to *Frontiers in Microbiology* on November 23, 2012

IV. **Ollivier J.***, Töwe S.*, Bannert A., Hai B., Kastl E.-M., Meyer A., Su M. X., Kleineidam K. and Schloter M. (2011) Nitrogen turnover in soil and global change. *FEMS Microbiology Ecology* 78, 3–16. doi: 10.1111/j.1574-6941.2011.01165.x

*Authors contributed equally to this work.

My contributions to the publications:

I. I was involved in planning and conducting the experiment and the sampling. Only the measurement of sulfadiazine in soil was performed by Rüdiger Reichel, University of Trier. I analyzed the data and the manuscript is mainly based on my input.

II. I performed qPCRs and T-RFLP analysis, and analyzed the data. The manuscript is mainly based on my input.

III. I was involved in planning and conducting the experiment and the sampling. I performed the qPCRs and analyzed the data obtained. I contributed to the analysis of data obtained from the clone library with Dr. Marion Engel, Helmholtz Zentrum München. The manuscript is mainly based on my input.

IV. I was responsible for the chapter “Xenobiotics”.

Summary

Industrial processes and agricultural practices can result in the release of potential toxic chemicals and trace elements into the environment, and consequently impact microbial communities responsible for important soil functions such as nutrient turnover. Whereas most studies focused on bulk soil so far, we studied the impact of contaminants, the antibiotic sulfadiazine (SDZ) and heavy metals (HMs), on the rhizosphere microbial communities, as they differ in term of activity and diversity from those in bulk soil, and play a major role in plant growth, mainly through nutrients mobilization. Particularly nitrogen is of key importance for plant health and yield.

SDZ can reach the soil ecosystem by application of manure, which is commonly used as organic fertilizer in agriculture, from antibiotic-treated animals. We surveyed the potential impact of this broad spectrum antibiotic on the nitrogen-transforming microbial communities in the root-rhizosphere complexes (RRCs) of agricultural plants (i) in a greenhouse experiment and (ii) under field conditions. In the greenhouse experiment, we measured in the RRCs of Maize (*Zea Mays*) and clover (*Trifolium alexandrinum*) the abundance of functional genes and transcripts involved in nitrogen fixation, ammonia oxidation and denitrification using *nifH*, *amoA* (in both ammonia-oxidizing bacteria and archaea), *nirK*, *nirS*, and *nosZ*, respectively, as molecular markers, after a single application of SDZ-contaminated pig manure. Sampling was performed 10, 20, and 30 days after the application. SDZ affected the abundance pattern of all investigated genes in the RRCs of both plant species (with stronger effects in the RRC of clover) 20 and 30 days after the addition. However, effects on the transcript level were less pronounced, which might indicate that parts of the investigated functional groups were tolerant or resistant against SDZ or, as in the case of *nifH* and clover, have been protected by the nodules. In the field experiment, we investigated the impact of repeated applications of SDZ-contaminated pig manure on functional microbial communities involved in ammonia and nitrite oxidation in the RRCs of diverse plants composing a pasture. We assessed the abundance of ammonia-

oxidizing archaea (AOA) and bacteria (AOB) as well as *Nitrobacter*- and *Nitrospira*-like nitrite-oxidizing bacteria (NOB) and the diversity of *amoA* AOA and *Nitrobacter*-like *nxrA* partial sequences. Whereas the first SDZ-contaminated manure application caused only slight effects on the investigated microbial communities and did not change diversity and abundance pattern significantly, the second application of SDZ-contaminated manure induced pronounced effects compared to the control treatment where non-contaminated manure was applied, and resulted in an up to 15 fold increased ratio of AOA:AOB and a reduction of *nrxA* genes. Diversity of *amoA* AOA increased after the second application of SDZ-contaminated manure compared to the control treatment whereas a clear reduction of *nrxA* OTUs was visible in the same samples.

HM contamination, such as by abandoned mine wastes, can result in severe pollution in the local environment and negatively impact important ecosystem services. Whereas HM-contaminated soils are unsuitable for food production, energy crops can allow the commercial exploitation of these soils by establishing biofuel feedstock production systems. In addition, the cultivation of these plants offers opportunities for site stabilization and phytoremediation of contaminated soils. In a pot experiment, we investigated the response of ammonia-oxidizing microbes in the RRC of *Miscanthus x giganteus*, a perennial grass with large annual biomass production potential, grown in soils with different levels of long-term arsenic (As) and lead (Pb) contamination. We measured the abundance of AOB and AOA at two different points of plant growth. Furthermore, bulk soil samples before planting were analyzed. In addition terminal restriction fragment length polymorphism (T-RFLP) analysis was used to investigate the diversity of archaeal *amoA* amplicons. Whereas high concentrations of As and Pb in soil (83 g/kg respectively 15 g/kg) resulted independent from the plant growth in a clear reduction of AOA and AOB compared to the control soils with lower HM contents, in soils with contamination levels of 10 g/kg As and 0.2 g/kg Pb, only AOB were negatively affected in bulk soil samples. Diversity analysis of archaeal *amoA* genes revealed clear differences in T-RFLP patterns, in response to the degree of HM

contamination. Therefore our results could clearly prove different response patterns of AOA and AOB in HM contaminated soils and the development of archaeal *amoA* phylotypes which are more tolerant towards HMs in soil samples originating from the areas the most impacted by the mining waste, which could contribute to functional redundancy of ammonia-oxidizing microbes in soils and stability of nitrification pattern.

Zusammenfassung

Industrielle Prozesse und landwirtschaftliche Praktiken können bei der Eintragung von potenziell toxischen Chemikalien sowie Spurenelementen in die Umwelt beitragen. Sie nehmen damit Einfluss auf mikrobielle Gemeinschaften, welche für essenzielle Bodenfunktionen, wie etwa den Stoffkreisläufen, verantwortlich sind. Während die meisten Studien bisher Fokus auf die Bodenmasse legten, untersuchten wir die Auswirkungen von Kontaminationen mit dem Antibiotikum Sulfadiazin (SDZ) sowie mit Schwermetallen (HMs) auf die mikrobiellen Gemeinschaften der Rhizosphäre. Sie unterscheiden sich sowohl in Aktivität wie Diversität von den mikrobiellen Gemeinschaften im Bodenkörper und tragen wesentlich zum Pflanzenwachstum, hauptsächlich durch die Mobilisierung von Nährstoffen, bei.

Sulfadiazin kann über die Ausbringung von Gülle antibiotikabehandelter Tiere, welche in der Landwirtschaft für gewöhnlich als organischer Dünger Verwendung findet, in den Boden eingetragen werden. Wir untersuchten die möglichen Auswirkungen dieses Breitbandantibiotikums auf die mikrobiellen Gemeinschaften des Stickstoffkreislaufs im Bereich des Wurzel-Rhizosphären-Komplexes (RRC) von Nutzpflanzen (i) im Gewächshausexperiment und (ii) im freien Feld. Im Gewächshausexperiment haben wir für Mais (*Zea Mays*) und Klee (*Trifolium alexandrinum*) im Bereich des Wurzel-Rhizosphären-Komplexes die Abundanz funktioneller Gene sowie Transkripte gemessen, welche bei der Stickstofffixierung, Ammonifikation und Denitrifikation eine Rolle spielen. Nach einmaligem

Auftragen SDZ-kontaminierter Schweinegülle wurden die Gene *nifH*, *amoA* (sowohl in Ammonia-oxidierenden Bakterien als auch Archaeen vorkommend), *nirK*, *nirS* und *nosZ* als molekulare Marker herangezogen. Die Probennahme erfolgte jeweils 10, 20 sowie 30 Tage nach der Auftragung. SDZ beeinflusst die Abundanzmuster aller untersuchten Gene im RRC beider Pflanzenarten (mit stärkerem Effekt auf den RRC von Klee) 20 und 30 Tage nach dem Einsatz. Allerdings waren die Auswirkungen des Antibiotikums auf Transkriptebene weniger stark ausgeprägt, was darauf hindeutet, dass ein Teil der funktionellen Gruppen tolerant bzw. resistent gegen Sulfadiazin war. Oder sie wurden, wie im Fall von *nifH* und Klee, durch Knöllchen geschützt. Im Freifeldexperiment untersuchten wir den Einfluss von mehrfach aufgetragener, SDZ-haltiger Schweinegülle auf funktionelle mikrobielle Gemeinschaften der Ammonia- und Nitritoxidation im RRC diverser Weidepflanzen. Wir maßen die Abundanz Ammonia-oxidierender Archaeen (AOA) und Bakterien (AOB) sowie die der *Nitrobacter*- und *Nitrospira*-ähnlichen Nitrit-oxidierenden Bakterien (NOB). Des Weiteren untersuchten wir die Diversität von *amoA* AOA sowie die der Teilsequenzen *Nitrobacter*-ähnlicher *nxrA* Gene. Während die erste Ausbringung SDZ-kontaminierter Gülle nur geringe Auswirkungen auf die untersuchten mikrobiellen Gemeinschaften hatte und weder die Diversitäts- noch Abundanzmuster signifikant veränderte, führte die zweite Gülleausbringung zu deutlichen Effekten. Ein Vergleich mit Werten aus Proben eines Kontrollbodens, welcher mit nicht-kontaminierter Gülle behandelt wurde, zeigte ein bis zu 15-fach höheres Verhältnis von AOA:AOB sowie eine Abnahme der *nrxA* Gene. Die Diversität von *amoA* AOA war nach der zweiten Ausbringung der SDZ-haltigen Gülle höher als beim Kontrollboden, wohingegen eine deutliche Abnahme der *nrxA* OTUs zu verzeichnen war.

Schwermetall-Kontaminationen, wie sie beispielsweise aus Abfällen stillgelegter Minen hervorgehen, können zur erheblichen Belastung der Umgebung führen und sich negativ auf wesentliche Ökosystemdienstleistungen auswirken. Während HM-kontaminierte Böden für die Lebensmittelerzeugung ungeeignet sind, können Energiepflanzen die kommerzielle Nutzung dieser Böden durch die Etablierung von Biokraftstoff erzeugenden

Produktionsverfahren ermöglichen. Zusätzlich wirkt sich der Anbau solcher Pflanzen positiv auf Bodenstabilität und Schadstoffabbau (mittels Phytoremediation) aus. In einem Gefäßexperiment untersuchten wir die Reaktion von Ammonia-oxidierenden Mikroorganismen im RRC von *Miscanthus x giganteus*, einem mehrjährigen Grases mit jährlich hohem Biomasseproduktionspotenzial. Dieses wuchs auf Böden mit langfristiger Arsen (As)- und Blei (Pb)-Behandlung unterschiedlicher Konzentration. Wir führten eine Abundanzmessung von AOB und AOA zu zwei verschiedenen Stadien des Pflanzenwachstums durch. Darüber hinaus wurden vor der Bepflanzung Bodenproben entnommen und analysiert. Des Weiteren wurde mittels terminaler Restriktionsfragment-Längen-Polymorphismus (T-RFLP)-Analyse die Diversität archaealer *amoA* Amplikons untersucht. Während unabhängig vom Pflanzenwachstum hohe Konzentrationen von As und Pb im Boden (83 g/kg bzw. 15 g/kg) zu einer deutlichen Reduktion von AOA und AOB führten (im Vergleich zu Kontrollböden mit einer niedrigeren Schwermetallbelastung), wurde in Proben aus Böden mit einem Kontaminationsgrad von 10 g/kg As und 0,2 g/kg Pb nur AOB negativ beeinflusst. Diversitätsanalysen archaealer *amoA* Gene zeigten zudem klare Unterschiede in ihren T-RFLP-Mustern bezüglich des HM-Kontaminationsgrades. Demnach können unsere Ergebnisse als Beweis für die Ausbildung unterschiedlicher Abundanzmuster von AOA und AOB als Reaktion auf die Schwermetallbelastung dienen. Zudem belegen sie die Entstehung archaealer *amoA* Phylotypen, welche in Bodenproben aus Regionen, die am stärksten durch Tagebauabfälle belastet sind, eine höhere Toleranz gegenüber Schwermetallen zeigen und zur funktionellen Redundanz Ammonia-oxidierender Bodenmikroorganismen sowie zur Stabilisierung von Nitrifikationsprozessen beitragen können.

Introduction

1. Nitrogen transformations in soil

1. 1. Nitrogen management in agricultural ecosystems and the environment

Nitrogen (N) is essential for the synthesis of nucleic acids and proteins, the two most important polymers of life, and the biogeochemistry of its inorganic forms relies almost entirely upon reduction-oxidation reactions primarily mediated by microorganisms (Canfield et al., 2010). N can be divided into two classes: unreactive and reactive N (Nr). Triple-bonded N_2 makes up 78% of Earth's atmosphere and constitutes the largest N reservoir on earth; N in this form is virtually inert. Nr comprises every other form of the element. Thus, Nr includes inorganic forms of N (e.g., ammonia [NH_3] and ammonium [NH_4^+], nitrogen oxides [NO_x], nitrous oxide [N_2O], and nitrate [NO_3^-]), and organic compounds (e.g., urea, amines, proteins, and nucleic acids). The size of N reservoirs on earth is highly variable, and besides the mains reservoirs (i.e. the atmosphere, the terrestrial mantle and crust) which are evaluated to contain 5.6×10^{20} moles N, about 8×10^{15} moles N are stored in the biosphere (Canfield et al., 2010).

N is of key importance for plant growth and crop yield. In almost all ecosystems, plants take up mainly NH_4^+ and NO_3^-, rather than amino acids or monomers, which apparently only play a role in extremely N-poor and cold ecosystems where N mineralization from soil organic matter is limited (Jackson et al., 2008). However, a few studies have shown that temperate trees have the ability to use amino acid N (Bennett and Prescott, 2004;Hofmockel et al., 2007;Warren and Adams, 2007;Scott and Rothstein, 2011). Supplying agricultural ecosystems with Nr is therefore essential for crop production. Until the end of the nineteenth century, the main agricultural source of N was fixation of N_2 by symbiotic bacteria in legumes, combined with the amount of N contained in animal and green manure; in this respect, N mineralization, the process by which microbes decompose organic N to ammonium, is of major importance (Schimel and Bennett, 2004). By 1900,

industrial processes, e.g., the Haber-Bosch process, were developed to reduce N_2 to NH_3, implementing agricultural practices and boosting crop yields. Thus in the past 4 decades, world food production doubled thanks to an almost 7-fold increase of N fertilization (Tilman, 1999;Tilman et al., 2001). During 2008 alone, the Haber-Bosch process supplied 9.5 x 10^{12} mol N whereas agriculture alone contributes about 2.4 x 10^{12} mol because of cultivation induced N fixation, essentially from fodder legumes (Canfield et al., 2010).

Parallel to the increasing of food production and consequent beneficial effects on human health, N inputs through anthropogenic activities contribute to a host of environmental problems (Galloway et al., 2008). Nitrifying microorganisms can convert ammonia (corresponding to nearly 90% of N fertilizer applied worldwide) to highly mobile NO_3^-, which can leach into rivers, lakes, and aquifers and possibly leads to eutrophication of coastal waters (Diaz and Rosenberg, 2008). Microbial denitrification together with nitrification can form N_2O which is lost to the atmosphere and in absorbing terrestrial thermal radiation, contributes to greenhouse effect; N_2O has 300 time, on a per molecule basis, the warming potential of CO_2 (Schlesinger, 2009). Besides its contribution to climate change, N_2O destroys ozone in the stratosphere (Ravishankara et al., 2009). Agricultural ecosystems account for about one quarter of global N_2O emissions (Mosier et al., 1998). In addition, excessive N fertilizer use results in biodiversity loss and soil acidification (Vitousek et al., 1997). It was recently calculated that excess N in the environment costs the European Union between €70 billion and €320 billion per year, so more than twice the value that N fertilizers are estimated to add to European farm income (Sutton et al., 2011).

Therefore investigations on the microbial scale are necessary to gain better insight into the mechanisms behind the N cycle to (i) develop better N management strategies in agricultural cropping systems and (ii) monitor ecological changes and reduce the negative impact of agriculture on the environment. The following section describes the different processes of the microbial N cycle and involved key functional groups. Special focus has

been laid on the inorganic N cycle, in particular N fixation, nitrification and denitrification, as those processes make the major contribution to N turnover in agricultural soils.

1. 2. Microbial nitrogen cycling in terrestrial ecosystems

The biological N cycle in terrestrial ecosystems consists of two cycles interlinked by NH_4^+: the organic and the inorganic N cycle (Fig. 1). Considering the inorganic part, particularly three processes of key importance in agricultural ecosystems, mainly driven by prokaryotes, will be focused in this thesis: (i) N fixation, (ii) nitrification and, (iii) denitrification. The physiology of these processes and their significance in soil ecology will be addressed in the next chapter.

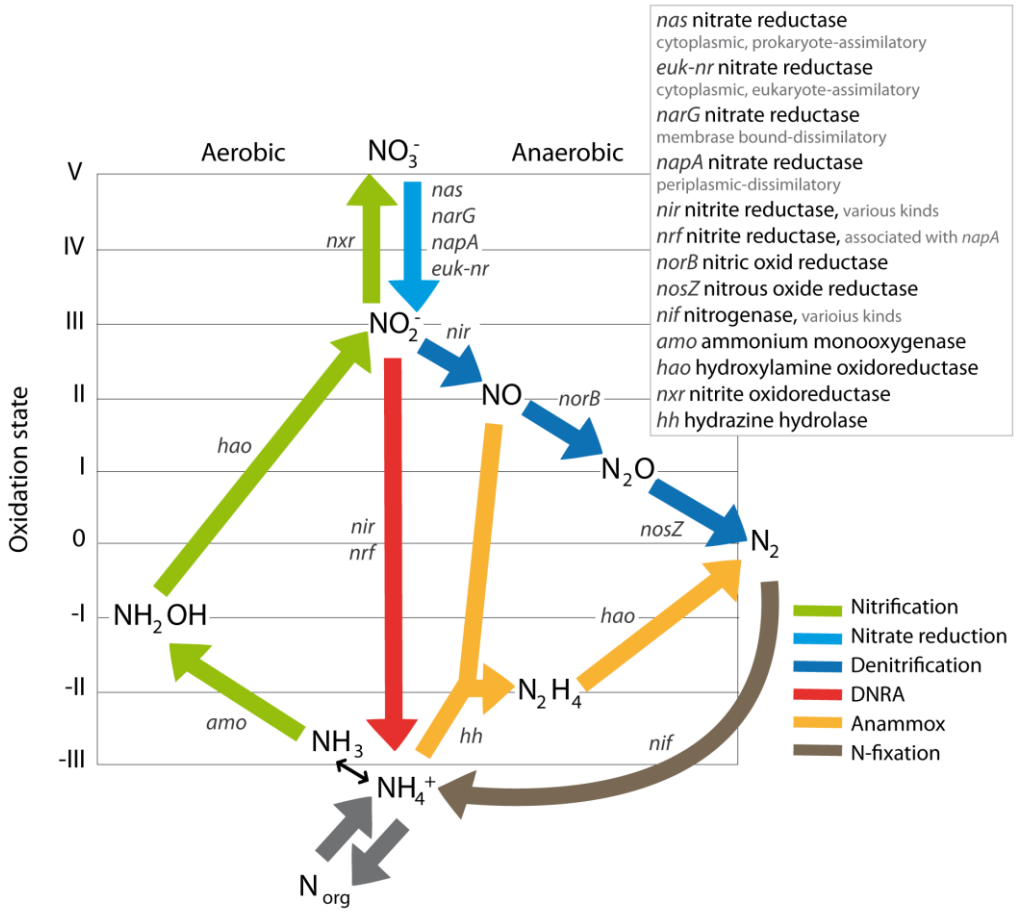

Figure 1. The major biological nitrogen transformation pathways linked to their associated enzymes (adapted from Canfield et al., 2010). Abbreviation: DRNA, dissimilatory nitrate reduction to ammonium.

1. 2. 1. Nitrogen fixation

Biological N fixation is a process where prokaryotes in the bacterial and archaeal domains, collectively called diazotrophs, reduce atmospheric N_2 to NH_4^+ (Fig. 1). Most microorganisms that perform biological N fixation catalyze this reaction with the nitrogenase protein complex, which has been highly conserved through evolution (Howard and Rees, 1996). The heterodimeric enzyme complex nitrogenase is composed of two multisubunit metallo-proteins: (i) the dinitrogenase $\alpha_2\beta_2$ heterotetramer also called MoFe-protein (where α = NifD and β = NifK proteins; component I) and (ii) the dinitrogenase reductase γ_2 homodimer also called Fe-protein (NifH protein; component II). Component I contains the active site for N_2 reduction, typically a $MoFe_7S_9$ metal cluster (termed FeMo-cofactor), whereas component II couples ATP hydrolysis to interprotein electron transfer. Alternative nitrogenases wherein Mo is replaced by either Fe or V (in which case the nomenclature Anf or Vnf, respectively, is used instead of Nif) can be found in a limited subset of diazotrophs. They are closely related to the conventional Mo-based nitrogenase and are present, in all cases studied so far, secondarily to it (Newton, 2007). These enzymes have to some extent different kinetics and specificities; the FeMo nitrogenase has been found to be more specific and more efficient in binding N_2 and reducing it to ammonia than either of the alternative nitrogenases (Burgess and Lowe, 1996;Eady, 1996). The activation energy required to break the N≡N bond is tremendous (16 ATP and 8 electrons per molecule of N_2 fixed), and the enzyme in vitro is sensitive to inactivation by oxygen (Newton, 2007). Moreover, a fourth type of nitrogenase, structurally dissimilar from the others and that is linked to CO reductase activity has been described in *Streptomyces thermautotrophicus* (Ribbe et al., 1997). *nifH,* one of the nitrogenase structural genes, is commonly used as a marker for the detection and identification of potential N fixing microbes in the environment (e.g., Zehr et al., 1998;Hamelin et al., 2002;Rösch et al., 2002;Fong et al., 2008).

Diazotrophs demonstrate diverse lifestyles and N fixation occurs in varied metabolic contexts under both aerobic and anaerobic conditions. *Rhizobia* in symbiosis with legumes

and the actinomycete *Frankia* in symbiosis with a number of plants (e.g., *Alnus*, *Myrica*, *Rosaceae*) assume agricultural importance in performing most of biological N fixation in terrestrial ecosystems (Peoples et al., 1995). However under specific conditions, free-living bacteria (e.g., cyanobacteria, *Pseudomonas*, *Azospirillum*, and *Azotobacter*) may fix significant amounts of nitrogen in soil (Kahindi et al., 1997;Burgmann et al., 2004).

1. 2. 2. Nitrification

Nitrification is a two-step process consisting of: (i) the oxidation of NH_4^+ to NO_2^- by ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) (Kowalchuk and Stephen, 2001;Leininger et al., 2006) and (ii) the oxidation of NO_2^- to NO_3^- by nitrite-oxidizing bacteria (NOB) (Prosser, 1989) (Fig. 1). The nitrification pathway plays a central role in the terrestrial nitrogen cycle. In agricultural ecosystems, this process is responsible for significant losses of N through leaching of nitrate. Moreover, nitrifiers have a further substantial environmental impact as contributors to greenhouse gas emissions; N_2O is a by-product of the nitrification process (Wrage et al., 2001).

NH_3 oxidation was long attributed to aerobic autotrophic chemolithotrophic ammonia-oxidizing bacteria. However, novel microbial players and new metabolisms have been discovered, such as planctomycetes catalyzing anaerobic ammonia oxidation (ANAMMOX) (Strous et al., 2006), and archaea of the phylum Thaumarchaeota (Brochier-Armanet et al., 2008;Spang et al., 2010), ubiquitous in marine and fresh waters, soils and sediments, capable of oxidizing ammonia to nitrite (Könneke et al., 2005;Treusch et al., 2005;Tourna et al., 2011). In the bacterial domain, the oxidation of NH_3 to NO_2^- via hydroxylamine (NH_2OH) is performed by certain organisms belonging to two specific groups of β- and γ-proteobacteria (Bock and Wagner, 2001). To date, most cultured strains belong to the β-subgroup (Kowalchuk and Stephen, 2001). The oxidation of NH_3 to NH_2OH, which constitutes the rate-limiting step of the nitrification pathway (Bock and Wagner, 2001), is catalyzed by the ammonia monooxygenase (AMO). AMO is a membrane-bound protein

consisting of three subunits (α-, β-, γ-AMO) encoded by *amoA*, *amoB*, and *amoC,* respectively, and is evolutionarily and functionally related to particulate methane monooxygenase (pMMO) enzymes of methane-oxidizing bacteria (Holmes et al., 1995). The subsequent dehydrogenation of NH_2OH to NO_2^- is catalyzed by the hydroxylamine oxidoreductase (HAO). HAO is located in the periplasm and is a homotrimer with each subunit containing eight *c*-type hemes, encoded by the *hao* gene (Arp et al., 2002). The discovery of genes encoding proteins with homology to AMO in genome fragments of archaea from soil (Treusch et al., 2005) and in shot-gun sequences of marine environments (Venter et al., 2004), as well as the cultivation or enrichment of archaea from marine waters (Könneke et al., 2005;de la Torre et al., 2008;Hatzenpichler et al., 2008) and soil (Tourna et al., 2011) indicates that AOA are an abundant and predominant group of microorganism (Leininger et al., 2006;Wuchter et al., 2006) and play a key role in global nitrification. Particularly, soil archaea that convert ammonia aerobically to nitrite were recently isolated (Jung et al., 2011;Tourna et al., 2011;Kim et al., 2012), thus confirming that AOA from soil have the capacity of ammonia oxidation. However, the ecological role and metabolism of soil AOA remains mysterious (Schleper, 2010); contrasting results have been thus reported when nitrification rates in soils were directly analyzed in the context of both AOB and AOA populations (Tourna et al., 2008;Di et al., 2009;Jia and Conrad, 2009;Offre et al., 2009;Schauss et al., 2009b). Moreover, It remains to be determined how significant heterotrophy and/or mixotrophy is to AOA in natural environments (Zhang et al., 2010;Jung et al., 2011;Pratscher et al., 2011;Tourna et al., 2011;Kim et al., 2012).

The gene *amoA* is commonly used as functional marker for studying aerobic ammonia oxidation (Rotthauwe et al., 1997). Phylogenetic analysis of both bacterial and archaeal *amoA* shows that archaeal genes are comparatively distant to their bacterial homologues (Nicol and Schleper, 2006). No homology is apparent at the DNA level between AOA and AOB *amo*-like sequences. However ~ 25% sequence identity and 40% sequence similarity can be found at the protein level between archaeal and bacterial variants with

conserved amino acid residues that coordinate potential metal centers. This indicated that these enzymes belong to the same protein family (Nicol and Schleper, 2006). Therefore bacterial and archaeal *amoA* genes can be easily differentiated.

The second step of nitrification is carried out by NOB, which are phylogenetically heterogeneous, and occur in a wide range of aquatic and terrestrial ecosystems. NOB are widely distributed, among α, β, γ and δ classes of proteobacteria and the bacterial phylum *Nitrospirae*, for *Nitrobacter*, *Nitrotoga*, *Nitrococcus*, *Nitrospina* and *Nitrospira* respectively (Orso et al., 1994;Teske et al., 1994;Ehrich et al., 1995;Koops and Pommerening-Röser, 2001;Alawi et al., 2007). However, *Nitrobacter* and *Nitrospira* are the major NOB genera encountered in soil**.** According to studies performed on wastewater (Schramm et al., 1999;Daims et al., 2001;Wagner et al., 2002;Blackburne et al., 2007) and soil environments (Attard et al., 2010), *Nitrobacter* bacteria are commonly characterized as r-strategists, with higher growth rate/specific activity and lower N substrate affinity as compared with *Nitrospira* bacteria, defined as K-strategist. Whereas most study on the physiology of NOB used pure cultures of *Nitrobacter*, the knowledge about *Nitrospira* is relatively scarce; only recently, the complete genome of a *Nitrospira* strain, tentatively named "*Candidatus* Nitrospira defluvii" was reconstructed from a metagenomic librairy of an activated sludge enrichment culture (Lucker et al., 2010). The key enzyme for NO_2^- oxidation by NOB is nitrite oxidoreductase (NXR). In *Nitrobacter*, NXR is an iron-sulfur molybdoprotein (Meincke et al., 1992) located at the inner cell membrane and at the intracytoplasmic membranes. NXR was found to consist of either two (Meincke et al., 1992) or three subunits with a supposed $\alpha_2\beta_2\gamma_1$ stoichiometry (Sundermeyer-Klinger et al., 1984), depending on the purification method applied. The α-subunit (NxrA) is thought to contain the substrate binding site with the molybdopterin cofactor (Sundermeyer-Klinger et al., 1984;Meincke et al., 1992), whereas the β-subunit (NxrB) with [Fe-S] clusters probably channels electrons from the α- to the γ-subunit or directly to the membrane-integral electron transport chain (Kirstein and Bock, 1993). However, *Nitrospira* has been shown to differ distinctly from *Nitrobacter* in the

enzyme NXR, being membrane-bound and located in the periplasm (Spieck et al., 1998;Lucker et al., 2010). Therefore, so far *nxrA* has been only used as a molecular marker to investigate *Nitrobacter*-like NOB communities (Poly et al., 2008;Wertz et al., 2008), whereas the 16S rRNA gene is preferred to survey *Nitrospira*-like NOB communities (Attard et al., 2010;Wertz et al., 2012).

1. 2. 3. Denitrification

Denitrification is a heterotrophic microbial process which consists of four reaction steps by which NO_3^- is reduced to N_2 by the metalloenzymes NO_3^- reductase, NO_2^- reductase, NO reductase, and N_2O reductase, under anaerobic conditions (Fig. 1), by which N oxides serve as terminal electron acceptors for respiratory electron transport. In addition to considerable loss of N, this process contributes to the greenhouse effect through N_2O emission (Schlesinger, 2009) and destruction of the ozone layer (Ravishankara et al., 2009). Denitrifiers include representatives of more than 60 genera of Bacteria and Archaea, as well as some Eukaryotes (Philippot et al., 2007), and can represent up to 5% of the total soil community (Henry et al., 2006). Some microorganisms produce only N_2 as end denitrification product, while others give a mixture of N_2O and N_2, and some only N_2O (Philippot et al., 2007). Also, the dissimilatory NO_3^- reduction to NH_4^+ (DNRA; Fig. 1) should be distinguished from denitrification. Thus, different criteria have been proposed to identify "true" denitrifiers (Mahne and Tiedje, 1995): (i) N_2O and/or N_2 must be the major end product of NO_3^- or NO_2^- reduction, and (ii) this reduction must be coupled to an increased in growth yield that is greater than when NO_3^- or NO_2^- served as an electron sink. Using these criteria, it is possible to distinguish bacteria possessing only the NO reductase as a protection against nitrosative stress (Philippot, 2005).

Two types of molybdoenzymes catalyzing the first step of the pathway, the reduction of NO_3^- to NO_2^- have been described: a membrane-bound (Nar) and a periplasmic (Nap) NO_3^- reductases. Both types of enzymes can be present in the same strain (Carter et al.,

1995;Roussel-Delif et al., 2005). The membrane-bound nitrate reductase is composed of three subunits: (i) a catalytic α subunit encoded by *narG*, containing a molybdopterin cofactor, (ii) a soluble β subunit, encoded by *narH*, containing four [4Fe-4S] clusters, and (iii) the γ subunit, encoded by *narI*, containing two *b*-types hemes. NarGHI is arranged in two domains with the α and β subunits constituting the cytoplasmic domain and the γ subunit constituting the membrane domain required for the attachment of the α and β subunits to the cytoplasmic side of the inner membrane (Philippot, 2002). The periplasmic nitrate reductase is a heterodimer encoded by the *napA* and *napB* genes. NapA is the large subunit containing a molybdopterin cofactor catalytic subunit and a [4Fe-4S] cluster. NapB is a *c* cytochrome (Philippot, 2002).

The reduction of soluble NO_2^- into gaseous nitric oxide (NO), the key step in the denitrification process, can be catalyzed by evolutionary unrelated enzymes that are different in terms of structure and of prosthetic metal: a copper- (NirK) and a cytochrome cd_1- (NirS) NO_2^- reductase (Zumft, 1997). In contrast to the NO_3^- reductases, bacteria carry either the copper or the cd_1 NO_2^- reductase but the two enzymes are functionally equivalent (Glockner et al., 1993). The *nirK* gene and the *nirS* gene encode the copper- and cd_1-NO_2^- reductase, respectively. The ecology of *nirS*- and *nirK*-harboring microbes is still poorly understood. However, it has been shown in several studies that microbes harboring the *nirK* gene form the major part of the NO_2^- reducers in different rhizospheres (Avrahami et al., 2002;Huić Babić et al., 2008;Hai et al., 2009) and show increased activity compared to microbes harboring the *nirS* gene (Sharma et al., 2005), while *nirS* genes may be more abundant in bulk soil (Kandeler et al., 2006;Melero et al., 2011) indicating a niche differentiation between the denitrifying populations in soil (Enwall et al., 2010).

Three types of metalloenzymes are involved in the reduction of NO to N_2O: (i) cNOR, a cytochrome *c* NO reductase which consists in a complex of two subunits encoded by the *norC* and *norB* genes (Zumft et al., 1994;Arai et al., 1995), (ii) qNOR, a quinol NO reductase encoded by *norZ* (Cramm et al., 1997), and (iii) qCu_ANOR, a menaquinol:NO oxidoreductase

which in contrast to the other NO reductases, contains copper in form of copper A (Suharti et al., 2001). The genes encoding qCu_ANOR are still unknown.

The last step of the denitrification cascade, the reduction of N_2O into N_2, is performed by the multicopper enzyme N_2O reductase (NOS), which is composed of two identical subunits and contains eight copper ions and is located in the periplasm. The catalytic subunit is encoded by the *nosZ* gene (Philippot, 2002).

1. 2. 4. Nitrogen transformations in the rhizosphere

The rhizosphere, first defined by Hiltner in 1904 as the volume of soil influenced by plant roots, represents a unique microenvironment in terrestrial ecosystems, where the growth and activity of the root system induce significant modifications in the physicochemical and biological properties (e.g., microbial activity, abundance, as well as structural and functional diversity) of the soil surrounding the roots (Brimecomb et al., 2001;Berg and Smalla, 2009). The so-called “rhizosphere effect” describes the phenomenon that, in comparison with bulk soil, the biomass and activity of microorganisms is enhanced. Roots exert, amongst others, strong effects on the major factors regulating the complex set of N transformations in soil (Fig. 2) (Jackson et al., 2008). Organic compounds are released by plant roots in the surrounding soil through rhizodeposition. They consist in refractory organic matter (e.g., root debris and mucilage), on the one hand, and readily available molecules including sugars, amino acids, organic acids, on the other hand (Brimecomb et al., 2001). Subsequently, depolymerization of refractory organic matter to labile compounds can be performed by extracellular enzymes produced by C-limited fungi and bacteria. Through mineralization, heterotrophic microbes break down organic monomers and release NH_3, which can be used as an energy source by ammonia oxidizers. However, several studies have reported nitrification to be negatively affected in the rhizosphere (e.g., Norton and Firestone, 1996;Priha et al., 1999). It has been explained by (i) the competition between

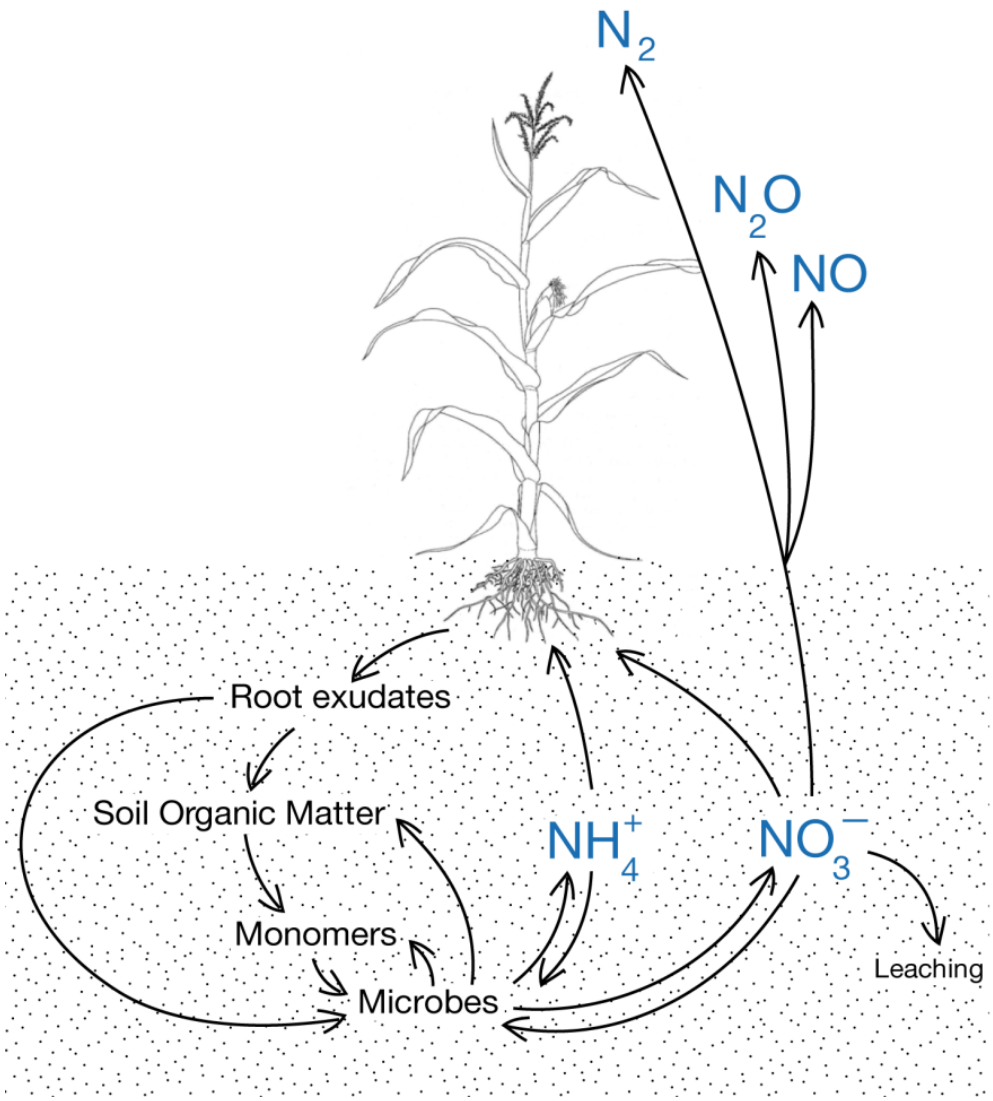

Figure 2. The N cycle in the rhizosphere of maize (*Zea mays*).

plants and soil microbes for NH_4^+; the lower activity of nitrifiers in the rhizosphere can therefore be explained by a decrease in NH_4^+ concentration due to the plant uptake (in cropping systems, plants take up mainly NH_4^+ and NO_3) (ii) the competition between heterotrophic microbes and autotrophic nitrifiers, the firsts being more competitive in this carbon rich environment (Philippot et al., 2009), and (iii) the presence of nitrification inhibitors in root exudates (Subbarao et al., 2007). The release of organic compounds through root exudation can also positively affect denitrification rates: (i) directly by providing an additional source of electron donor, since most denitrifiers are chemoheterotrophs, and (ii) indirectly by increasing overall microbial activity, which lowers the oxygen concentration.

However, factors regulating denitrification in the rhizosphere are strongly interlinked and the stimulating effect of root exudates is only observed under nonlimiting concentrations of NO_3^- and oxygen. It is therefore not possible to state that plant roots always stimulate denitrification (Philippot et al., 2007). Indeed, plant and denitrifiers compete for NO_3^-, and consumption of water by plant roots increases soil gas exchange and oxygen concentration. Finally, leguminous plants are known to exude phenolic compounds of the flavonoid class, which are key signals in initiation of nodule formation in the N fixing symbiosis with compatible rhizobia, under conditions of N limitation (Broughton et al., 2000).

2. The antibiotic sulfadiazine in soil

2. 1. Mode of action of sulfadiazine

The target of the antibiotics forming the class of the sulfonamides, which includes sulfadiazine (SDZ), is the enzyme dihydropteroate synthase (DHPS) catalyzing the condensation of *p*-aminobenzoic acid (PABA) and 1,8-dihydro-6-hydroxymethylpterin-pyrophosphate (DHPPP) to form dihydropteroic acid, which is the penultimate step in the formation of dihydrofolic acid (Fig. 3). Dihydrofolic acid is subsequently reduced to tetrahydrofolic acid (THFA), an essential cofactor for the synthesis of purines, certain amino acids, and thymidine. Sulfonamides competitively inhibit DHPS by their structural analogy to the PABA substrate (Brown, 1962). Sulfonamides can also function as alternative substrates for DHPS forming pterin adducts that cannot participates in folate synthesis and presumably diffuse from the cell (Roland et al., 1979). Higher eukaryotes, like Mammalian, are not dependent on endogenous synthesis of folic acid, and generally lack DHPS; they can use dietary folates by uptake through a transport system, which most prokaryotes and some lower eukaryotes lack. Thus, the latter have to synthesize folates *de novo*, making the basis for the selective effect of sulfonamides on bacteria and for their broad spectrum of

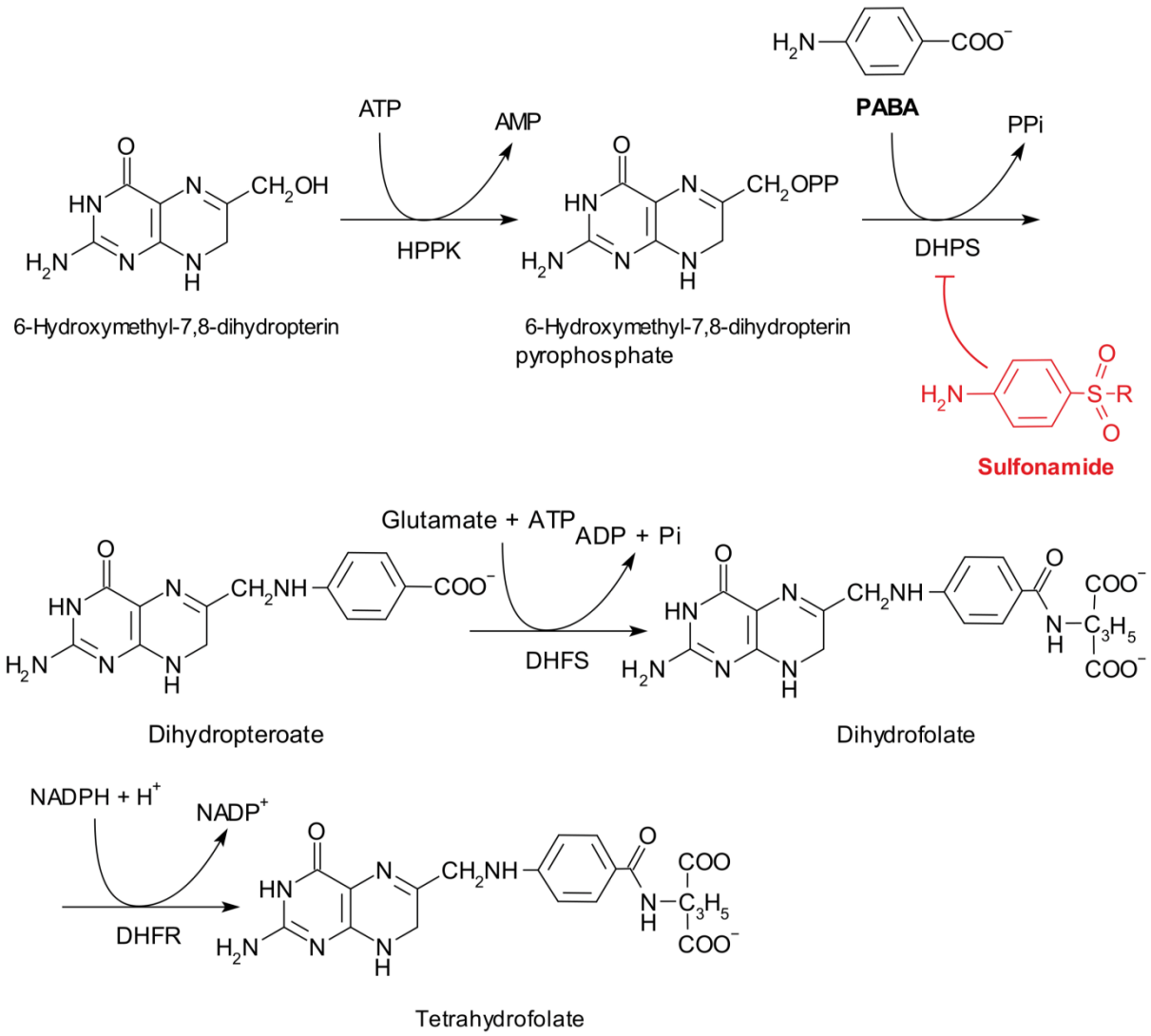

Figure 3. The folate biosynthetic pathway (adapted from Xiao et al., 1999). Abbreviations: HPPK, 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase; DHPS, dihydropteroate synthase; DHFS, dihydrofolate synthase; and DHFR, dihydrofolate reductase, PABA, *p*-aminobenzoic acid.

antibacterial activity (Skold, 2000). Numerous studies have shown that sulfonamides act bacteriostatic on sensitive microorganisms (e.g., Garrett and Wright, 1967;Seydel et al., 1972). Many of investigated sensitive microorganisms are pathogens and belong to the domains of bacteria (e.g., *Mycobacterium spp*. (Nopponpunth et al., 1999), *Staphylococcus spp*. (Hampele et al., 1997), *Streptococcus spp*. (Haasum et al., 2001), but also to the domain of eukaryotes in fungi (Achari et al., 1997) and protozoa (Triglia et al., 1997).

However, effects of sulfonamides on archaea, *a fortiori* AOA (as the first cultures of soil AOA, which may form the basis for such studies, have been isolated only recently; Jung et al., 2011;Tourna et al., 2011;Kim et al., 2012), remain widely unknown.

In bacteria, resistance to sulfonamides is mediated mainly by the genes *sul1* and *sul2*, coding for dihydropteroate synthases which are insensitive to sulfonamides (Skold, 2000). The genes occur in a wide range of species, because they are often located on transposable elements of self-transferable or mobilizable broad-host-range plasmids (Schluter et al., 2003;Heuer et al., 2004;Byrne-Bailey et al., 2009).

2. 2. Occurrence and fate of sulfadiazine in soil

In Europe, antibiotics are nowadays used in animal husbandry to treat infectious diseases; their use as food additives and growth promoters is forbidden since 2006. Sulfonamides constitute one of the major groups of veterinary drugs and are mainly used in pigs (Thiele-Bruhn and Aust, 2004). SDZ is poorly adsorbed in the animal gastro-intestinal tract; manure from SDZ-treated pigs can thus contain considerably high amounts of the parent compound, and, to a lower extent, of the two main metabolites N^4-acetyl-sulfadiazine (Ac-SDZ) and 4-hydroxy-sulfadiazine (OH-SDZ; Fig. 4) (Lamshöft et al., 2007). Concentrations of the SDZ and its metabolites have been shown to be stable during manure

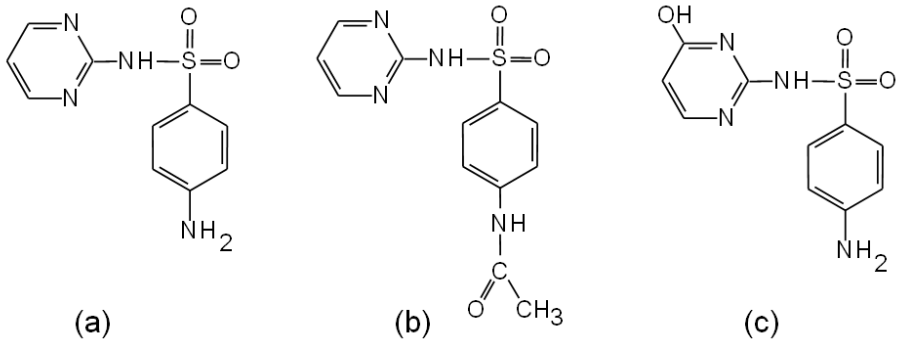

Figure 4. Chemical structure of (a) sulfadiazine and its two main metabolites (b) N^4-acetyl-sulfadiazine (Ac-SDZ) and (c) 4-hydroxy-sulfadiazine (OH-SDZ) (from Zarfl et al., 2009).

storage and the SDZ concentrations even increased over time due to the deacetylation of the Ac-SDZ (Heuer et al., 2008). Thus, the antibiotic and its potentially bioactive metabolites reach the soil ecosystem through the use of manure as fertilizer.

SDZ is known to persist in soil (Burkhardt and Stamm, 2007;Forster et al., 2009;Rosendahl et al., 2011) and its long-term fate is governed by its sequestration into hardly extractable and non-extractable forms (Kreuzig and Höltge, 2005;Forster et al., 2009) – the term sequestration corresponding to the mechanisms decreasing the extractability and thus the bio-availability of the compound in soil (Lueking et al., 2000). Three fractions of SDZ in soil have been described (Zarfl et al., 2009): (i) the $CaCl_2$- and MeOH-extractable fraction corresponding to the bioavailable fraction, (ii) the subsequent microwave-extractable fraction (Forster et al., 2008) identified as the residual fraction, and (iii) the non-extractable fraction consisting of bound residues. Whereas rapid decreased in SDZ extractability with the sequential $CaCl_2$ and MeOH extraction procedure has been described (Kotzerke et al., 2008;Forster et al., 2009), kinetic modelling suggests that the underlying sequestration mechanisms are at least partly reversible and SDZ can be released back into available forms (Zarfl et al., 2009). Moreover, Rosendahl and colleagues (2011) showed that dissipation from both easily extractable and residual SDZ fractions was largely temperature-dependent and soil moisture controlled sequestration, being accelerated in dry soil.

2. 3. Effects of sulfadiazine in soil

SDZ has been reported to affect general and potential microbial activities and the bacteria community structure (Zielezny et al., 2006). However, effects of SDZ have been shown to depend on the addition of an energy source, i.e. to the addition of a substrate to promote microbial growth. This is mainly relevant for manure as the main carrier of antibiotics to soil. Thus, Hammesfarh and colleagues (2008) showed that amending soil with manure that had been spiked with SDZ lowered microbial biomass and altered bacterial

community structure. Soil respiration provides information about the broad status of microbial activity in soil. Kotzerke and co-workers (2008) observed reduced CO_2 production in response to manure contaminated with SDZ. Overall the antibiotic effect of SDZ depends on time, dose and soil (Schauss et al., 2009a). Particularly, effects on N turnover and functional microbial communities have been investigated. Potential nitrification activity remained unchanged under low SDZ concentration conditions in bulk soil when applied in combination with manure (Kotzerke et al., 2008). This might have been due to a substitution of the highly affected AOB by their archaeal counterparts (Schauss et al., 2009b). Moreover, potential denitrification rates decreased in treatments where sulfadiazine was applied (Kotzerke et al., 2008). However, it remains unclear if the observed alterations in potential denitrification rates are caused by a general reduced abundance of denitrifiers, a loss of specific phylotypes, or changes in expression levels of the corresponding genes (Kleineidam et al., 2010).

3. Heavy metals in soil

3. 1. Occurrence and fate of heavy metals in soil

The accumulation of heavy metals (HMs) in topsoil can result from (i) industrial deposition e.g., from mining activities (ii) livestock manure and other organic wastes e.g., sewage sludge and waste waters used as fertilizer, and (iii) pesticides. Besides, HMs can also occur naturally, but rarely at toxic levels (Alloway, 1990). Whereas Zinc (Zn), copper (Cu), nickel (Ni) arsenic (As), chromium (Cr), and cadmium (Cd) can be essential trace elements for living organisms (although they can be toxic if present at excessive levels), , lead (Pb), and mercury (Hg) have no biological function and can be harmful if they enter the environment. Industrial activities can be responsible for atmospheric fallout of HMs; the most important sources include energy production, mining, metal smelting and refining, manufacturing processes, transport and waste incineration (Nriagu, 1990;Martley et al.,

2004;Rodríguez Martín et al., 2007). HM deposited on the soil surface will gradually become incorporated into the soil and will contribute to overall soil concentrations. Atmospheric deposition is ubiquitous, although deposition rates vary depending on proximity to point sources of pollution such as industrial sites or major roads (Nicholson et al., 2003). In some cases, areas far from the source region may be considerably affected (Steinnes et al., 1989;Steinnes et al., 1997;Fitzgerald et al., 1998;Douay et al., 2008). Particularly, the mining industry represents a major source of contamination. Indeed, abandoned mine wastes, e.g., tips and tailings, can result in severe HM pollution in the local environment owing to dust blow, and from the leaching of mineral weathering products. Agricultural practices are also considered as sources of HM contamination, although to a more limited land area. Thus, sewage sludge and livestock manures have been identified as significant sources of HMs (Nicholson et al., 2003). Indeed, HMs are present in sewage sludge as a result of domestic, road run-off and industrial inputs to the urban wastewater collection system. Moreover, HMs, e.g., Cu or Zn are present in livestock diets at background concentrations and may be added to certain feeds as supplementary trace elements for health reasons or as growth promoters. Most of the HMs consumed in feed is excreted in the faeces and urine, and will thus be present in manure (Nicholson et al., 2003). Finally, HMs can be present in agrochemicals. The use of inorganic fungicides with a high Cu content (e.g., Bordeaux Mixture) are regularly applied to vineyards and have been identified as significant source of contamination (Komárek et al., 2010).

The retention of HMs in soil systems depend to a large extent on their chemical speciation and soil characteristics, and is mainly linked to pH and redox potential (Chuan et al., 1996;de Matos et al., 2001;Cappuyns and Swennen, 2008;Wilson et al., 2010). For example, As occurs in the environment mainly as arsenate [As(V)] and arsenite [As(III)] anions, the first dominating in oxidizing environmental conditions and the second being more stable in reduced environments. Moreover, organic As species are known to exist; the methylated As species are the most widespread organic As species know in soil although

more complex forms such as arsenosugars, arsenobetain, arsenocholine and arsenolipids have also been identified (Wilson et al., 2010). As speciation, solubility and bioavailability depends on pH, CEC, texture (clay mineralogy), amorphous Fe-Al oxides, organic matter, sulfur content, phosphorus concentration, and soil redox conditions (Voigt et al., 1996;Fitz and Wenzel, 2002;Moreno-Jimenez et al., 2010;Wilson et al., 2010).

3. 2. Effects of heavy metals in soil

HM pollution has been reported to alter the microbial community structure and decreased diversity (Kandeler et al., 2000;Muller et al., 2001;Li et al., 2006;Macdonald et al., 2007) as well as microbial activities (Frostegard et al., 1993;Kuperman and Carreiro, 1997;Konopka et al., 1999;Dai et al., 2004). However the results obtained depend on the experimental system, e.g., short-term versus long-term incubation, various dosing of a single HM or a combination of HMs, the presence of organic matter. Thus, short-term responses of microbial processes to HMs spiked in soils are, in general, not predictive of long-term effects due to microbial adaptations reactions (Giller et al., 1998). Community adaptation may be explained by selective growth of tolerant populations and selective decay of sensitive groups (Diaz-Ravina and Baath, 1996;Diaz-Ravina et al., 2007;Fernandez-Calvino et al., 2011). The mechanisms of metal resistance of microbes consist in (i) intra- and extracellular metal resistance mechanisms, (ii) metal excretion via efflux transport systems, (iii) sequestering compounds of the cytosol binding and detoxifying metals inside the cell, (iv) the release of chelators into the extracellular milieu, and (v) binding of metal on the cell envelope by sorption thus preventing influx (Haferburg and Kothe, 2007; Fig. 5). Thus, Park and Ely (2008) determined 27 genes that were up-regulated by Zn in the ammonia-oxidizing bacteria *Nitrosomonas europaea*. These included for example mercury resistance genes and inorganic ion transport genes. Furthermore, microbial communities tolerant to a certain HM have been shown to better cope with stress caused by another HM due to, e.g., similar physiological mechanisms (Bruins et al., 2000;Tobor-Kaplon et al.,

2006). For example, Ruyters and colleagues (2012) reported co-tolerance to Zn and Cu of the soil nitrifying community.

Several studies have shown that N fixation, mineralization, nitrification and denitrification are affected to HMs (e.g., Bardgett et al., 1994;McGrath et al., 1995;Giller et al., 1998;Holtan-Hartwig et al., 2002). Especially the nitrification process is altered by HM contamination, making this process one of the most sensitive microbial assays to indicate HM toxicity (Broos et al., 2005). Thus, nitrification is for example highly sensitive to elevated Zn (Smolders et al., 2004). However, recovery of nitrification after Zn exposure occurs gradually and has been attributed to the development of Zn-tolerant AOB communities, AOA being more sensitive (Mertens et al., 2006;Mertens et al., 2009;Ruyters et al., 2010). The adaptation to the contamination is accelerated by the stimulation of the activity of the nitrifying community (Ruyters et al., 2010). Nevertheless, Xia and colleagues (2007) suggested that AOA may play an important role in long-term fertilized soils contaminated with Zn; moreover, Li and colleagues (2009) shown that AOA were more tolerant than AOB to Cu contamination.

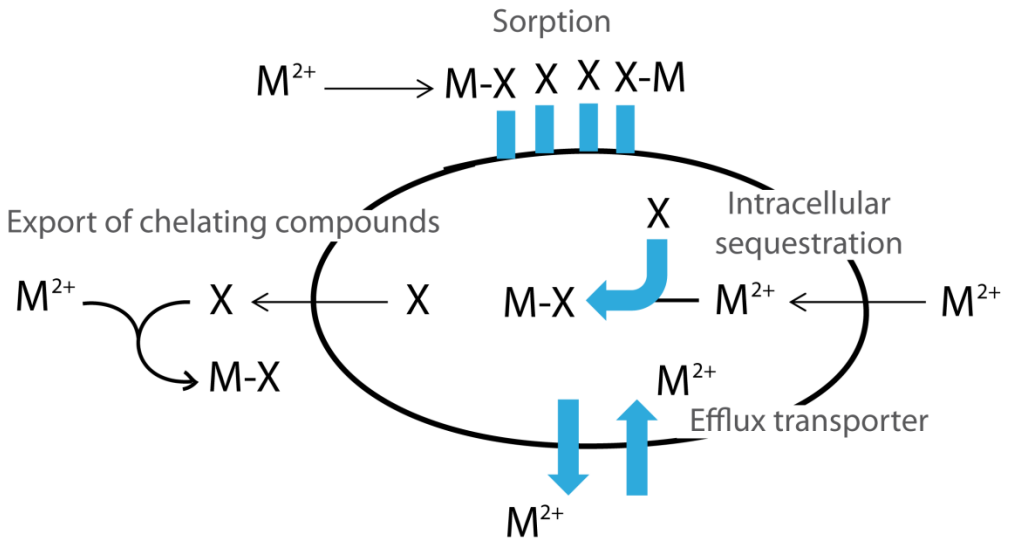

Figure 5. Overview of microbial resistance mechanisms (adapted from Haferburg and Kothe, 2007). X, cell constituents interacting with metal cations; M, metal cation.

4. Molecular tools to study soil microbial communities

The characterization of soil microbial community diversity and function has been long based on cultivation-dependent methods. While leading to remarkable discoveries in the field of microbial physiology and genetics, culturing provides only poor access to many organisms; it is estimated that microorganisms refractory to cultivation represent the vast majority (>85 to 99.999%) of organisms in most environments (Amann et al., 1995). Thus, the application of polymerase chain reaction (PCR) technology since the early 1990's (e.g., Giovannoni et al., 1990;Schmidt et al., 1991;Barns et al., 1994) together with the development of nucleic acids extraction methods from environmental samples (Tsai and Olson, 1991;Zhou et al., 1996;Miller et al., 1999) has been a major breakthrough in microbial ecology. Indeed, the combination of PCR amplification of a target gene and/or its transcripts — the 16S rRNA gene being the predominant target for studying the microbial diversity (Hugenholtz et al., 1998) whereas functional genes are the basis to assess subpopulations with particular physiological capabilities, as these can be widely distributed among different genera or even domains (see chapter 1.2.) – with fingerprinting- (e.g., terminal restriction fragment length polymorphisms [T-RFLP]) or sequencing-based analyses allows the description of the diversity and ecology of the uncultivated majority (Head et al., 1998;Hugenholtz et al., 1998). By targeting a gene, these approaches provide information about the genetic potential of a given environment whereas RNA-based methods give an indication on microbial activity status.

4. 1. Microbial nucleic acids extraction from soil

Two main approaches to microbial nucleic acids extraction from soil are currently available: (i) direct extraction, which relies on direct cell lysis inside the soil matrix – following the lysis, the nucleic acids are extracted and purified – and (ii) indirect extraction, in which microbial cells are first isolated from the soil matrix, and then lysed for subsequent nucleic acids extraction and purification. Although direct extraction is widely in use now,

both methods present contrasting advantages and drawbacks in term of nucleic acids quantity and quality (Lombard et al., 2011). Thus, direct DNA extraction method is often yielding 10—100-fold more DNA whereas the indirect extraction method further releases high-quality DNA in terms of large fragments and a higher DNA purity (Gabor et al., 2003). Therefore, differences in community structures between different extraction methods have been described (Thakuria et al., 2008;Inceoglu et al., 2010).

4. 2. Quantitative PCR

Quantitative PCR (qPCR) allows the estimation of the abundance of a targeted gene and/or transcript in environmental samples. This technique is based on the detection of fluorescence signals corresponding to the synthesis of PCR amplicons (Heid et al., 1996). Quantification of gene and/or transcript numbers is determined during the exponential phase of the PCR amplification when the numbers of amplicons detected are directly proportional to the initial number of target sequences present in the environment. The data utilized for the analysis of samples is acquired at the cycle at which the fluorescence signal is higher than the background, known as threshold cycle (Wittwer et al., 1997). The copy number of the target DNA or cDNA can be accordingly determined using a standard curve generated with target of a known concentration (Smith and Osborn, 2009).

In this thesis, SYBR® green was used as double-stranded DNA (dsDNA) binding dye to monitor amplicons synthesis. It was preferred to labeled-probes for cost considerations as it can be used for any reaction without sequence information. However, SYBR® green assays don't allow the discrimination between amplicons sequences and false-positives may occur. Therefore, a post dissociation curve analysis should be carried out to confirm that the fluorescence signal is generated only from target templates and not from the formation of non-specific PCR products or primer-dimers (Smith and Osborn, 2009).

4. 3. Microbial community profiling

4. 3. 1. Cloning/sequencing approach

Sequencing is the method offering the highest phylogenetic resolution, allowing either species identification or determination of similarity to already known species through the use of extensive and rapidly growing sequence data-bases (Nocker et al., 2007). However, the number of clones required to adequately catalog the majority of taxa in a sample is unwieldy and cultivation, construction and screening of clone libraries are laborious and time consuming. It has been suggested that environmental samples may require $>10^4$ sequencing reactions to document half of the microbial richness (Dunbar et al., 2002). Nevertheless, it is possible to predict richness within microbial communities by using rarefaction and statistical estimators (Schloss and Handelsman, 2005;Schloss et al., 2009). In the last decade, the development of next generation sequencing technologies, e.g., pyrosequencing (Margulies et al., 2005), has revolutionized the field of microbial ecology in permitting a much deeper sampling of microbial communities by providing magnitude more sequence information than Sanger sequencing of PCR clone libraries (Roesch et al., 2007). However, more detailed phylogenetic information can be obtained by the latter, as this very high throughput is achieved with substantial sacrifices in length of the individual reads when compared to Sanger sequencing (Hutchison, 2007).

4. 3. 2. Terminal restriction fragment length polymorphism analysis

Terminal restriction fragment length polymorphism (T-RFLP) analysis is a high-throughput fingerprinting technique allowing the detection of differences in the composition and structure of microbial communities by targeting small-subunit (SSU) rRNA and also functional marker genes (Bruce, 1997;Liu et al., 1997). Thus, fingerprinting of functional communities involved in N cycling in soil, e.g., N fixers (Yeager et al., 2005), archaeal and bacterial ammonia oxidizers (Boyle-Yarwood et al., 2008) nitrite reducers (Wolsing and Prieme, 2004) and nitrous oxide reducers (Stres et al., 2008), have been performed. T-RFLP

analysis consists of the PCR amplification of a target gene using a fluorescently labeled forward primer and subsequent digestion of the amplicons with one or several restriction enzymes typically containing a four base-pair recognition site. *In silico* digestion to evaluate the ability of restriction enzymes to discriminate between sequences can be done, e.g., with Restriction Endonuclease Picker (REPK) (Collins and Rocap, 2007). Finally, only the fluorescently labeled terminal restriction fragments (T-RFs) are visualized by electrophoresis on an automated sequencer, and the differences in the length and abundance of the T-RFs are determined by comparison to an internal standard (Schutte et al., 2008). Thus, each T-RF is assumed to represent a single operational taxonomic unit (OTU) or ribotype. However, an individual T-RF may correspond to several OTU leading to the underestimation of the community diversity (Kent et al., 2003).

PCR based methods for microbial community profiling, include limitations because of the inherent biases of the end-point PCR methodology (Osborn et al., 2000;Lueders and Friedrich, 2003), e.g., difference in gene copy number and preferential amplification of certain templates (von Wintzingerode et al., 1997;Polz and Cavanaugh, 1998;Crosby and Criddle, 2003;Huber et al., 2009). Therefore, they are considered to allow semiquantitative assessment of community population, as the profiles generated are a quantitative reflection of the PCR product pool and not a quantitative reflection of the original community (Nocker et al., 2007).

5. Aims and hypotheses

Rhizosphere microbial communities involved in nutrient turnover are of central importance for plant nutrition, health and quality; this especially in a context of crop production, either in food/feed or biofuel feedstock production systems. However, the soil/plant interface can be exposed to various contaminants through human activities – intentionally or not, when using HM polluted soils for the cultivation of energy crops (Hartley

et al., 2009) or when manure contaminated by antibiotics is used as fertilizer (Halling-Sørensen et al., 1998), respectively – impacting these functional communities, and can affect eventually the plant biomass production.

Contrary to pesticides, which in most cases when applied at the recommended field rate concentration don't have a significant impact on the structure and function of the soil microbial communities (Review of the effects of xenobiotics on N transforming communities in **Publication IV**), antibiotics, such as sulfonamides, are explicitly designed to affect microorganisms. Although changes in turnover rates have been reported for some microbial processes (e.g., Kotzerke et al., 2008), a number of studies in the last decade have shown that the influence of sulfonamides on microbes and their metabolic performance in bulk soil is relatively low (reviewed in Schauss et al., 2009a). These findings have been explained by (i) the large microbial diversity in bulk soil systems and by possible mechanisms of functional redundancy (Nannipieri et al., 2003), (ii) the relatively low activity of microbes in bulk soil that are nearing the dormancy state (Roszak and Colwell, 1987) in which microbes are not affected by sulfonamides, and (iii) the development of resistant populations by horizontal gene transfer (Heuer and Smalla, 2007). Moreover, no results on effects of sulfonamides on functional or structural diversity are available under field conditions.

Most studies published so far concerning the impact of HM contamination on ammonia oxidizers focused on AOB communities (e.g., Mertens et al., 2006) and therefore little is known about the response AOA. Besides, the effects of HMs on soil NOB remains largely unexplored so far. However, heavy metal resistance genes were found in *Nitrobacter hamburgensis* (Starkenburg et al., 2008) and "*Candidatus* Nitrospira defluvii" (Lucker et al., 2010). Although AOA are thought to be more tolerant to chronic stress conditions than bacteria (Schleper et al., 2005;Valentine, 2007), the influence of HM on AOA is discussed controversially in literature (Xia et al., 2007;Mertens et al., 2009;Ruyters et al., 2010). Furthermore the few studies assessing the response of both AOA and AOB to HM contaminations were mostly performed using soils spiked with contaminant, without

including plant effects (e.g., Gremion et al., 2004;Frey et al., 2008;Mertens et al., 2010) and short-term responses of microbial processes to HMs spiked in soils are in general not predictive of long-term effects due to microbial adaptations (Diaz-Ravina and Baath, 1996;Giller et al., 1998).

Thus, the aim of this thesis was to assess the effects of the antibiotic SDZ or HMs on the rhizosphere functional microbial communities involved in the inorganic N cycle; specifically in NH_3 oxidation (**Publication I**, **II**, and **III**), NO_2^- oxidation (**Publication II**), NO_2^- reduction, N_2O reduction, and N fixation (**Publication I**), using genes encoding subunits of the bacterial and archaeal ammonia monooxygenase (*amoA*), the *Nitrobacter*-like nitrite oxidoreductase (*nxrA*), the nitrite reductase (*nirK* and *nirS*), the nitrous oxide reductase (*nosZ*), and the nitrogenase (*nifH*) as molecular markers, respectively. Moreover, since no primers targeting *Nitrospira*-like *nxrA* were available (Attard et al., 2010), the abundance of *Nitrospira*-like NOB was quantified targeting the 16S rRNA *Nitrospira* gene.

The abundance of the functional populations of interest was measured by qPCR (**Publication I**, **II**, and **III**) and their community structure was assessed by cloning/sequencing (**Publication III**) or T-RFLP analysis (**Publication II**). Moreover, changes were surveyed at the gene level (representing the genetic potential for the corresponding pathways; **Publication I**, **II**, and **III**) as well as the transcript level (representing the expression level of the enzymes under study; **Publication I**), in a greenhouse experiment (**Publication I** and **II**) or under field conditions (**Publication III**).

The main hypotheses of this thesis were:

(I) Processes in the rhizosphere are more affected by the application of SDZ compared to bulk soil, as highly active organisms will react more intense to the antibiotics compared to inactive or dormant microbes in the bulk soil. Additionally, due to reduced diversity in the rhizosphere compared to bulk soil, functional redundancy is lower in this soil compartment resulting in lower resilience of turnover rates.

(II) SDZ affects the ratios between archaeal and bacterial ammonia oxidizers as these phylogenetically diverse groups presumably exhibit different life strategies and different susceptibility to the antibiotic. Similarly, AOA and AOB are affected by HM contamination to different extent. *Nitrospira*- and *Nitrobacter*-like nitrite oxidizers are both inhibited by the application of the SDZ-contaminated manure, considering the broad spectrum nature of the antibiotic.

(III) Plant growth will be affected by the contaminants; especially in the case of the legume where the symbiosis between plant and microbes is a major determinant of plant health.

Discussion

The present study was part of the German Research Foundation (DFG) research group 566 (FOR 566) “Veterinary medicines in soils: basic research for risk analysis” which aim to determine the fate and effects of veterinary antibiotics in soils. Such antibiotics can reach the soil environment by the application of manure from antibiotic-treated animals to arable fields and pasture, manure being commonly used as organic fertilizer in agriculture. The major focus of this PhD thesis was to assess the effects of the antibiotic SDZ on microbial communities involved in N cycling in the rhizosphere of plants of agricultural importance, as N is of key importance for plant growth and yield. To this end, functional communities involved in N fixation, nitrification and denitrification were investigated. Besides the contamination by xenobiotics due to agricultural practices, industrial activities can lead to the pollution of the soil ecosystem, notably by HMs, making impossible food/feed crop production. However HM-contaminated soils can be employed to establish biofuel feedstock production systems. In addition to biomass production, the cultivation of bioenergy crops offers opportunities for site stabilization and phytoremediation of contaminated soils. Therefore, the effect of long-term HM contamination on ammonia-oxidizing microbes in the rhizosphere of the bioenergy crop *Miscanthus x giganteus* was investigated.

1. Nitrogen fixation

In **Publication I**, the phenol-chloroform co-extraction of DNA and RNA from soil followed by a column-based separation (Griffiths et al., 2000;Towe et al., 2011) allowed the comparison on the effect level. Whereas the presence of a functional gene (e.g., *nifH*) is not necessarily evidence of the associated ecosystem function but rather provides information

on potential activity, the transcription level of a gene allows a better estimation of the actual activity. Legume roots exude various flavonoid and isoflavonoid molecules that are known to induce development of symbiotic interactions between the plant and N-fixing α-proteobacteria within root nodules (Squartini, 2003). This is consistent with our results showing explicitly higher *nifH* gene expression and, to a lower extent, higher *nifH* gene abundance in the clover RRC, which includes nodules, compared to the maize RRC (**Publication I**). We postulated that legume growth would be more affected by SDZ (Hypothesis III), as legumes need a symbiotic partner for an optimal supply of N. Despite a considerable decrease in *nifH* gene abundance in the clover RRC 20 days after application of the SDZ-contaminated manure, the abundance of transcripts was not significantly affected by the antibiotic, which might be the reason for similar plant quality and yield in both treatments. It is possible that the active N-fixing bacteria within the root nodules are protected from the antibiotic and therefore are not affected. However, it must be noted that external N was introduced to the soil during manure application; thus, N provided by diazotrophs was not needed to maintain a high plant yield.

2. Nitrification

2.1. Ammonia oxidation

The antibiotic tended to abolish the increase of the AOB population in response to the manure application in the RRCs of the different crops investigated (**Publication I** and **III**). Similar results were shown in bulk soil (Schauss et al., 2009b). These results demonstrate that SDZ clearly inhibited the growth of AOB. Moreover, in **Publication I**, lower ammonium values were measured in the SDZ-contaminated manure treatment at the 10-day time point that might be related to an overall inhibition of N mineralization by the antibiotic. This relative ammonia depletion clearly induced lower bacterial *amoA* transcripts in comparison to the control treatment. Therefore, in addition to direct effects on AOB, SDZ

may influence these communities indirectly in impacting processes of the organic N cycle, such as the N mineralization.

Archaea are characterized by their broad-spectrum resistance to antimicrobial agents (Khelaifia and Drancourt, 2012). In particular, their cell wall lacks peptidoglycan (Koga and Morii, 2007) making archaea resistant to the antimicrobials agents interfering with peptidoglycan biosynthesis (e.g., ampicillin and vancomycin; Dridi et al., 2011). However, effects of sulfonamides on archaea remain widely unknown, since mechanistic information about the folic acid requirements of AOA is missing. Still, SDZ inhibition constants of 30 mg/kg have been estimated for AOA in the soil used in our study, in comparison with 0.01 mg/kg soil for AOB, indicating a lower susceptibility to the antibiotic in AOA compared to its bacterial counterpart (Schauss et al., 2009b). Consistently, in **Publication I,** although AOA were significantly influenced by SDZ, they were affected to a lower extent than AOB. Moreover, in **Publication III**, AOA abundance significantly increased in response of the application of SDZ-contaminated manure. Possibly, the reduced susceptibility of AOA to SDZ-contaminated manure can be explained by a shift in the AOA diversity towards more SDZ resistant phylotypes over time, as *amoA* diversity has been shown to well reflect phylogeny of AOA (Nicol et al., 2008; **Publication III**).

Yet the major environmental drivers determining AOB and AOA population dynamics are little understood, despite both groups having a wide environmental distribution. However, parameters e.g., pH (Nicol et al., 2008;Yao et al., 2011;Zhang et al., 2012) or salinity (Moin et al., 2009;Li et al., 2011) have been demonstrated to influence AOA:AOB ratios in soils and sediments. Thus, the relative importance of these two groups in soil nitrification is still debated (Schleper, 2010). Whereas AOB were recently reported to be key players in nitrification in agricultural soils exhibiting relatively high ammonia concentrations (Di et al., 2009;Jia and Conrad, 2009;Di et al., 2010), it was also observed that AOA were actively involved in nitrification (Offre et al., 2009;Zhang et al., 2010) and responded to ammonia as well as organic fertilizer amendments (Schauss et al., 2009b;Verhamme et al.,

2011). Interestingly, the occurrence of functional redundancy under antibiotic stress between the two communities in bulk soil has been shown by Schauss and colleagues (2009b). Inasmuch (i) one copy of an *amoA* AOA gene represents one AOA cell and 2.5 copies of *amoA* AOB, one AOB cell (Leininger et al., 2006), and (ii) effective AOB maximum oxidation rate constants are mostly below 32 fmol NO_2^- $cell^{-1}$ h^{-1} (Prosser, 1989;Jiang and Bakken, 1999;Okano et al., 2004) – although the maximum value has been reported to be 83.3 fmol NO_2^- $cell^{-1}$ h^{-1} for *Nitrosocystis oceanus* (Ward, 1987) – and maximum oxidation rates for AOA range between 0.3 (*Nitrosopumilus maritimus*, Könneke et al., 2005) and 1.4 fmol NO_2^- $cell^{-1}$ h^{-1} (*Nitrosocaldus yellowstonii*, de la Torre et al., 2008), they could demonstrate by model calculations, that AOA can perform a substantial proportion of ammonia oxidation. AOA appears thus to hold a "back-up function" (McCann, 2000) and might serve as insurance for the soil ecosystem to maintain the ammonia oxidation under more unfavorable environmental conditions (Valentine, 2007). According to our results, such a functional redundancy could also be relevant in the rhizosphere under SDZ stress (**Publication I** and **III**).

In **Publication II**, AOA were found to be less sensitive towards Pb and As than AOB. Similarly, a higher tolerance of AOA than AOB in soils contaminated by Zn (Xia et al., 2007) and Cu (Li et al., 2009) has been suggested. However, other studies showed contrasting results and ascribed tolerance development in ammonia-oxidizing communities to AOB rather than AOA populations (Mertens et al., 2009;Ruyters et al., 2010). Nevertheless, as most of these results are based on soils spiked with HMs, a direct comparison to the data presented in our study was not possible, as bioavailability of HMs is different in soils with artificially added HMs, and the time of adaptation of microbes in response to the stressor is missing (Diaz-Ravina and Baath, 1996). The latter argument has been proven to be of high importance in our study, as different AOA phylotypes showing differences in HM tolerance were observed. However, detailed data on the resistance development towards HMs of AOA are still missing, due to the extremely limited and recent availability of cultivated

representative of AOA from terrestrial environments (Jung et al., 2011;Tourna et al., 2011;Kim et al., 2012).

2.2. Nitrite oxidation

As ammonia oxidation is considered to be the rate-limiting step of nitrification, and despite their role in soil functioning, only few studies addressed the physiology and ecology of NOB and their response to disturbance such as those generated by agricultural practice are scarcely known (Attard et al., 2010;Xia et al., 2011;Wertz et al., 2012). However various stress conditions, e.g., steam disinfestation of soil (Roux-Michollet et al., 2008) or drought (Gelfand and Yakir, 2008), inducing a higher nitrite oxidation compared to ammonia oxidation have been reported. In **Publication III**, we hypothesized that *Nitrospira*- and *Nitrobacter*-like nitrite oxidizers are both inhibited by the application of the SDZ-contaminated manure, considering the broad spectrum nature of the antibiotic (Hypothesis II). However, parallel to the inhibitory effects affecting directly the functional communities investigated (e.g., related to their respective activity status and related susceptibility; Lewis, 2007), dissimilar ecological strategies for survival and proliferation among these populations may influence their response to the antibiotic stress. While niche differentiation and competition is known to influence the composition of functional microbial communities, the components of the nitrite oxidizing communities investigated respectively in this study possess different substrate affinities and therefore are adapted to distinct N availabilities. It has been suggested that *Nitrobacter*-like NOB bacteria are r-strategists with higher growth rate/specific activity and lower affinity for nitrite and oxygen, whereas *Nitrospira*-like NOB are K-strategists with a higher substrate affinity (Schramm et al., 1999;Attard et al., 2010). However, Maixner and colleagues (2006) shown that nitrite concentration influences the structure of *Nitrospira*-like bacterial communities, and assumed that sublineages may occupy different positions on an scale reaching from K- to r-strategists within the genus *Nitrospira*. Thus, the reduction of AOB abundance and activity may have resulted in lower

nitrite availability and consequently favorable conditions for *Nitrospira*-like NOB compared to *Nitrobacter*-like NOB, explaining the reduction of *Nitrobacter*-like *nxrA* abundance (P = 0.030) and the increase of *Nitrospira* 16S rRNA gene abundance (P = 0.036) at day 49 with PMSDZ treatment. However the release of organic substrates from the dead microbial biomass under the antibiotic treatment could have also influenced indirectly the abundance of different *Nitrospira* sublineages as some *Nitrospira*-like bacteria are mixotrophic (Daims et al., 2001).

To bring further clarification on putative community structure shift towards SDZ-resistant populations, effects of SDZ contamination on diversity was investigated using a cloning/sequencing approach in **Publication III**, focusing on *Nitrobacter*-like NOB communities. Indeed, antibiotic resistance genes have been found in the genomes of *Nitrobacter hamburgensis* (Starkenburg et al., 2008) and *Nitrobacter Winogradskyi* (Starkenburg et al., 2006). Thus, shifts towards putative SDZ resistant phylotypes were observed, accompanied by a decrease of diversity where SDZ-contaminated manure was applied.

3. Denitrification

As indicated by decreased copy numbers of all three genes involved in denitrification, SDZ had a long-lasting negative effect on the denitrification potential in the RRC (**Publication I**). This is in contrast to results obtained in bulk soil where denitrifiers were only slightly affected by SDZ (Kleineidam et al., 2010) and underlines the assumption of more pronounced antibiotic effects on highly active microbial communities living in hot spots like the RRC (Hypothesis I). We observed that *nirK*-harboring microbes were more affected by SDZ than *nirS*-denitrifying bacteria. This can be explained by different abilities exhibited by the microbes to regulate their internal pH, affecting the accumulation and speciation of the SDZ in the cells (Tappe et al., 2008;Zarfl et al., 2008), and therefore its

antimicrobial effects; in addition to resistance mechanisms mediated by the genes *sul1* and *sul2* (cf. Introduction, chapter 2). Furthermore, the quantification of both genes and transcripts revealed significant impacts on the gene level but to a lower extent on the transcript level, this in contrast to what could be assumed taking into consideration the bacteriostatic mode of action of SDZ. Presumably, microbial subpopulations intrinsically able to cope with the antibiotic stressor could have taken advantage of the altered competitive environment and maintained denitrification, the wide phylogenetic diversity of denitrifying bacteria allowing the maintenance of the process (Wallenstein et al., 2006). However, an indirect antibiotic effect could have contributed to the reduced abundances of *nirK* (and *nosZ*) transcripts via impaired microbial respiration activity in the RRC, hence higher oxygen levels and consequently inhibited gene expression in the PMSDZ treatment.

4. Plant/microbes interactions

In **Publication I**, the differences in gene abundance patterns between the two treatments were more pronounced and long-lasting in the RRC of clover than in the RRC of maize. Presumably, differences in quality and quantity of root exudates and in root morphology known to shape microbial communities and to form the basis for microbial activity in the rhizosphere might have contributed to the different effects observed (Marschner et al., 2001). Whereas maize may have provided primarily recalcitrant organic carbon from decaying root material to the microflora of its RRC (Semenov et al., 1999), clover roots might have excreted more readily available organic compounds (Haichar et al., 2008), resulting in an increase in microbial biomass and activity in this RRC. In contrast, AOA, which might exhibit a rather oligotrophic lifestyle (Jung et al., 2011;Kim et al., 2012), could have been outcompeted by the faster-growing microorganisms in the clover RRC, as indicated by the reduced numbers of AOA *amoA* gene copies found in the clover RRC compared to the maize RRC. As dormancy or reduced activity results in reduced

susceptibility to SDZ, the bacteriostatic antibiotic might have found fewer targets (Balaban et al., 2004;Lewis, 2007), thus explaining the differences observed in the effect of SDZ when both plant species were compared.

In **Publication III**, the extent of the impact of the antibiotic contamination on the rhizosphere ammonia oxidizing populations was greater after the second manure application compared to those observed after the first application. This could be explained by a shift in the community structure of the plants composing the grassland during the experimental period, influencing the response to the antibiotic stress of the rhizosphere microbial communities. Indeed, plant diversity and species composition are known to influence the magnitude and the stability of ecosystem processes over time, as well as the size and composition of associated microbial communities (Hooper and Vitousek, 1997;Kowalchuk et al., 2002;Steenwerth et al., 2002;Johnson et al., 2003;Balvanera et al., 2006;Millard and Singh, 2010). However, interactive effects between contaminants and plant diversity received little attention so far (Eisenhauer et al., 2009). Moreover, the mechanisms through which changes in plant diversity affect soil microbial communities remain unclear, Whereas Zack et al., (2003) observed changes in microbial abundance and composition across a plant diversity gradient in a long term experimental grassland system and concluded that these changes were more related to differences in plant productivity associated with diversity rather than plant diversity *per se*. In contrast, Einsenhauer et al., (2010) suggested that the quality of rhizodeposits rather than plant productivity affects soil microbial community.

Although not investigated in this thesis, N mineralization is of critical importance in crop production systems in supplying available N for crop uptake. In **Publication I**, for both plant species, the yield of the green biomass was not influenced by the presence of SDZ in the manure applied, and the extent of manure N mineralization might have played a key role in maintaining plant health and growth. In addition, the action of the antibiotic on the microbial biomass could have (i) induced the release of organic matter from dead cells into

the soil N pool and (ii) alter the competitiveness of the microbial communities for nutrients, with possible positive effects in plant nutrient acquisition and growth (Jackson et al., 2008). However, such effect would have negative implications for ecosystem nutrient storage, as immobilization of N by microbes has been shown (i) to act as a short-term sink for N in several terrestrial ecosystems (Zogg et al., 2000;Bardgett et al., 2003), thus potentially limiting the export of N to e.g., groundwater and (ii) to be important for longer-term ecosystem N retention, via the transfer of the nutrient form to more stable organic matter pools after cell death (van der Heijden et al., 2008). In **Publication II**, no influence of the degree of HM contamination on plant growth was observed. Possibly, the amount of N contained in the rhizome was sufficient to maintain plant health and growth during the experimental period (Wiesler et al., 1997) as the soils used were not fertilized.

Conclusions

Using molecular techniques, it was possible to apprehend changes in the abundance, activity, and diversity of functional communities involved in N cycling (i) in response to the application of SDZ-contaminated pig manure or (ii) in long-term HM-contaminated soils.

Our data revealed that the application of manure contaminated with SDZ has a lasting impact on the functional microbial biomass involved in N cycling in the RRCs of different plants of agricultural importance under greenhouse (**Publication I**) and field (**Publication III**) conditions. In **Publication I**, effects on the transcript level were less pronounced, which might indicate that parts of the investigated functional groups were tolerant or resistant against SDZ. Moreover, NOB community shifts towards potential resistant phylotypes were observed (**Publication III**). In addition, the antibiotic does not impact AOA and AOB to a similar extent, which could allow functional redundancy between these two groups of ammonia-oxidizing microbes and contribute to the stability of N turnover. The effects of SDZ on the microbial communities involved in the major processes of the inorganic N cycle, i.e. N fixation, ammonia oxidation, nitrite oxidation, nitrite reduction and nitrous oxide reduction, are sum up in Fig. 6. In addition, these effects have been shown to be influenced by plant factors such as rhizosphere effect and the development of root nodules (**Publication I**), and potentially the plant community structure of a pasture (**Publication III**). However, the effect of plant diversity on the microbial response to antibiotic stress needs to be further investigated in future studies.

Based on our data (**Publication II**), it can be postulated that selected phylotypes of AOA tolerate higher concentrations of Pb and As in soil and RRC compared to AOB. However, abundance of a functional group cannot be directly linked to the activity of these microbes. Therefore, it remains unclear if, mainly in soil M, AOA can substitute AOB and if

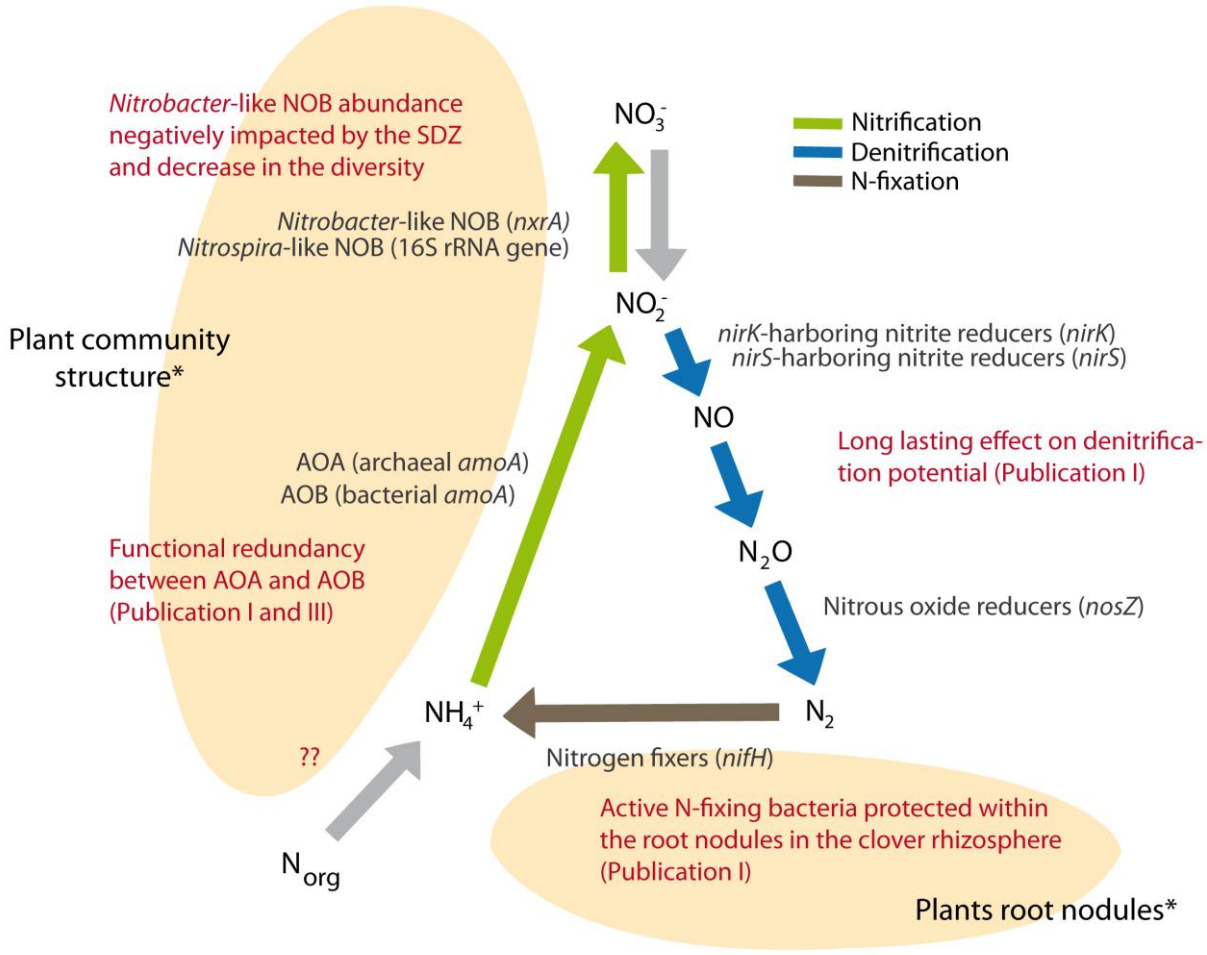

Figure 6. Effects of the antibiotic sulfadiazine on the microbial communities involved in N cycling. Asterisks indicate plant factors influencing the effects of sulfadiazine. Abbreviations: AOA, ammonia-oxidizing archaea; AOB, ammonia-oxidizing bacteria; NOB, nitrite-oxidizing bacteria.

functional redundancy between both groups of ammonia-oxidizing microbes exists under the given conditions. To address these points in detail, further studies are needed, including (i) analysis of mRNA, and (ii) using ^{15}N-labeled ammonium. Moreover, further studies should include the assessment of the impact of HMs on NOB communities and on nitrite oxidation rates to determine whether ammonia oxidation remains the rate-limiting step in the nitrification process in HM-contaminated soils, as an accumulation of nitrite in soils could have further implications on microbial N immobilization due to nitrite toxicity.

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List of abbreviations

amoA	gene encoding the α-subunit of the ammonia monooxygenase
AOA	ammonia-oxidizing archaea
AOB	ammonia-oxidizing bacteria
As	arsenic
ATP	adenosine triphosphate
$CaCl_2$	calcium chloride
CEC	cation-exchange capacity
Cu	cupper
DHPPP	1,8-dihydro-6-hydroxymethylpterin-pyrophosphate
DHPS	dihydropteroate synthase
DNA	deoxyribonucleic acid
e.g.	*exempli gratia*
et al.	*et alii*
h	hour
HM	heavy metal
i.e.	*id est*
MeOH	methanol
mRNA	messanger RNA
N	nitrogen
N2	dinitrogen
N_2O	nitrous oxide
NH_2OH	hydroxylamine
NH_3	ammonia
NH_4^+	ammonium
nifH	gene encoding the dinitrogenase reductase subunit of the
nirK	gene encoding the copper-NO_2^- reductase

nirS	gene encoding the cytochrome cd_1- NO_2^- reductase
NO	nitric oxide
NO_3^-	nitrate
NOB	nitrite-oxidizing bacteria
nosZ	gene encoding the nitrous oxide reductase
Nr	reactive N
nxrA	gene encoding the α-subunit of the nitrite oxidoreductase
OH-SDZ	4-hydroxy-sulfadiazine
OTU	operational taxonomic unit
PABA	*p*-aminobenzoic acid
Pb	lead
PCR	polymerase chain reaction
PM	pig manure
PMSDZ	pig manure contaminated with sulfadiazine
RNA	ribonucleic acid
RRC	root-rhizosphere complexe
SDZ	sulfadiazine
sul1, *sul2*	genes encoding dihydropteroate synthases
T-RF	terminal restriction fragment
T-RFLP	terminal restriction fragment length polymorphism
WEOC	water extractable organic carbon
Zn	zinc

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Effect of Sulfadiazine-Contaminated Pig Manure on the Abundances of Genes and Transcripts Involved in Nitrogen Transformation in the Root-Rhizosphere Complexes of Maize and Clover[▽]†

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The antibiotic sulfadiazine (SDZ) can enter the environment by application of manure from antibiotic-treated animals to arable soil. Because antibiotics are explicitly designed to target microorganisms, they likely affect microbes in the soil ecosystem, compromising important soil functions and disturbing processes in nutrient cycles. In a greenhouse experiment, we investigated the impact of sulfadiazine-contaminated pig manure on functional microbial communities involved in key processes of the nitrogen cycle in the root-rhizosphere complexes (RRCs) of maize (*Zea mays*) and clover (*Trifolium alexandrinum*). At both the gene and transcript level, we performed real-time PCR using *nifH*, *amoA* (in both ammonia-oxidizing bacteria and archaea), *nirK*, *nirS*, and *nosZ* as molecular markers for nitrogen fixation, nitrification, and denitrification. Sampling was performed 10, 20, and 30 days after the application. SDZ affected the abundance pattern of all investigated genes in the RRCs of both plant species (with stronger effects in the RRC of clover) 20 and 30 days after the addition. Surprisingly, effects on the transcript level were less pronounced, which might indicate that parts of the investigated functional groups were tolerant or resistant against SDZ or, as in the case of *nifH* and clover, have been protected by the nodules.

Antibiotics have been used in animal husbandry worldwide to treat infectious diseases. Sulfonamides, including sulfadiazine (SDZ), belong to one of the major groups of veterinary drugs and are mainly used in pigs (10). Sulfonamides are poorly adsorbed in the animal gut; consequently, they are excreted unchanged in urine and feces (1, 12, 16, 26) and reach the soil ecosystem via manuring. Sulfonamides have been characterized as broad-spectrum antibiotics with a bacteriostatic mode of action based on inhibition of folic acid metabolism (9). Therefore, sulfonamides in the environment may impact soil health by changing microbial activity patterns and the kinetics of important turnover processes (6, 25, 48). Although changes in turnover rates have been reported for some microbial processes (e.g., see reference 24), a number of studies in the last decade have shown that the influence of sulfonamides on microbes and their metabolic performance in bulk soil is relatively low (42). These findings have been explained by (i) the large microbial diversity in bulk soil systems and by possible mechanisms of functional redundancy (37), (ii) the relatively low activity of microbes in bulk soil that are nearing the dormancy state (40) in which microbes are not affected by sulfonamides, and (iii) the development of resistant populations by horizontal gene transfer (20).

The impact of sulfonamides on microbial turnover processes may be more pronounced in highly active microbial communities (e.g., in the rhizosphere) than in inactive or dormant microorganisms in bulk soil. The rhizosphere is defined as the soil that is influenced by the plant root system, which strongly affects the surrounding soil chemistry through nutrient depletion, acidification, and the secretion of organic substances (53). Due to the organic compounds they release, plant roots provide suitable ecological niches for microbial growth and activity (3). Furthermore, several studies have indicated changed or even reduced diversity patterns in the rhizospheres compared to bulk soils (29). Therefore, it can be postulated that the effects of antibiotic-contaminated manure on microbes in rhizosphere soil might differ from that in bulk soil. Moreover, the extent of the antibiotic effect together with the manure effect is difficult to predict due to the complexity of plant-microbe interactions in the rhizosphere.

As rhizosphere microbial communities exert strong effects on plant quality (53), the questions of whether and how antibiotics in soil alter microbial activities in the rhizosphere are of interest not only for basic research and ecotoxicology but also for farmers and plant breeders. Supplying plants with nitrogen is of key importance for yield and plant health in agricultural ecosystems.

The aim of this study was to investigate the effects of the antibiotic sulfadiazine in combination with pig manure (PM)

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on the functional microbial biomass involved in nitrogen (N) cycling in the root-rhizosphere complex (RRC) of two different agricultural crops, a C_4 plant (maize [*Zea mays*]) and a legume (berseem clover [*Trifolium alexandrinum*]). As it is well accepted that plant species are a dominant factor influencing the composition of the rhizosphere microbial communities (54), we chose for the cultivation of the two different crops one typical arable soil. SDZ and manure concentrations applied were in accordance with agricultural practice in Germany (48). Ten, 20, and 30 days after application, we analyzed the abundance of functional genes (representing the genetic potential for the corresponding pathways) catalyzing key steps of nitrification, denitrification, and nitrogen fixation as well as transcripts of the same marker genes (representing the expression level of the enzymes under study) in order to reconstruct major parts of the nitrogen cycle in the RRC.

We hypothesized that nitrogen turnover processes in the RRC would be affected by the application of SDZ and that the changes would consequently influence plant growth, especially in the case of the legume where the symbiosis between plant and microbes is a major determinant of plant health. Furthermore, we assumed that the abundance patterns of transcripts would be more affected than the gene copy numbers due to the bacteriostatic mode of action of the antibiotic.

MATERIALS AND METHODS

Experimental design. A silt loam (Orthic Luvisol) from the A_p horizon (0 to 40 cm) of an agricultural field located near Merzenhausen, Germany (50° 56′ 3″ N, 6° 17′ 31″ E; see Table S1 in the supplemental material) that had not been previously fertilized with manure was used for the greenhouse experiment. The experiment was conducted in a randomized block design with four independent replicates per treatment and sampling time point. Polypropylene tubes (height, 70 cm; diameter, 15 cm) containing 14 kg of air-dried soil (sieved at 4 mm) were used. After an equilibration phase of 14 days at 20°C and 50% maximum water holding capacity of the soil, three seeds of maize (*Zea mays*) and 30 seeds of berseem clover (*Trifolium alexandrinum*) were sown per pot. Two weeks after germination, 250 ml each of pig manure (PM) and pig manure contaminated with sulfadiazine (PMSDZ) were applied per tube to the soil surface, resulting in final concentrations of 7.4 mg nitrogen kg^{-1} soil and 20 mg SDZ kg^{-1} soil (in the upper 20 cm). The plants were subjected to a photoperiod of 15 h of light and 9 h of darkness with a constant soil water content. Sampling was performed 10, 20, and 30 days after manure application; each of the four independent replicates was treated separately. A composite sample of roots with the attached soil was taken from the upper 20 cm of each tube. After the roots were vigorously shaken, the roots and attached soil were treated as one compartment called the root-rhizosphere complex (RRC). One part of the RRC was immediately snap-frozen in liquid nitrogen and stored at −80°C for nucleic acid extraction; the other part was directly extracted with 0.01 M $CaCl_2$ for the determination of water-extractable organic carbon (WEOC), water-extractable organic nitrogen (WEON), ammonium-N (NH_4^+-N), and nitrate-N (NO_3^--N) concentrations. Samples of homogenized bulk soil were frozen at −20°C until used for the quantification of SDZ and its metabolites.

Sulfadiazine and metabolites in bulk soil samples. The totally desorbable and hence potentially bioavailable SDZ fraction and its metabolites, *N*-acetyl-SDZ and 4-hydroxy-SDZ, were isolated by a sequential extraction procedure using 0.01 M $CaCl_2$ (soil/solution ratio of 1:2.5) followed by an extraction step with methanol (MeOH) (soil/solution ratio of 1:2.5) (17). Separation and detection of extracted sulfadiazine and its metabolites were carried out using a Shimadzu Prominence LC20 high-performance liquid chromatography (HPLC) system. The stationary phase consisted of a SunFire C_{18} column (3.0- by 100 mm; 3.5-μm particle size; Waters, Germany). The injected volume (10 μl) was mobilized at a flow rate of 300 μl min^{-1} in a gradient program by phase A (0.1% HCOOH in water) and phase B (0.1% HCOOH in MeOH). HPLC-separated fragment ions of sulfadiazine and metabolites were captured using an API 3200 mass spectrometer (Applied Biosystems, Germany). The data were analyzed using the Analyst 1.4.2 application (Applied Biosystems, Germany) with a minimum signal-to-noise ratio (SNR) of 10:1. The limit of detection was determined by the method of Antignac et al. (2) and was in the range of 0.2 ng SDZ, 1 ng hydroxy-SDZ, and 5 ng *N*-acetyl-SDZ g^{-1} soil, respectively.

Nitrogen and carbon content in plants. The total green plant biomass was dried at 65°C for 48 h, ball milled (Retsch MM2; Retsch GmbH, Germany) and transferred into tin capsules (5- by 3.5 mm; HEKAtech GmbH, Germany). Total carbon and nitrogen contents were determined using the elemental analyzer Euro-EA (Eurovector, Italy) (32).

Water-extractable nitrogen and carbon fractions in the RRC. Samples consisting of 5 g RRC were shaken overhead for 45 min in 25 ml of 0.01 M $CaCl_2$. After filtration, water-extractable total nitrogen and organic carbon were measured using a total organic carbon (TOC) analyzer (DIMA-TOC 100; DIMATEC, Germany) equipped with a total bound nitrogen (TNb) module. A continuous-flow analyzer (SA 20/40; Skalar Analytical, Netherlands) was used to determine ammonium-N and nitrate-N. Water-extractable organic nitrogen was calculated as the difference between total nitrogen and ammonium plus nitrate.

DNA and RNA coextraction and separation. DNA and RNA were coextracted from 0.5 g of RRC by the method described by Griffiths et al. (13). Extraction was performed with Precellys-Keramik kit lysing tubes (Peqlab Biotechnologie GmbH, Germany) in combination with the Bertin Precellys 24 bead beating system (Bertin Technologie, France). DNA and RNA were separated using the AllPrep DNA/RNA minikit (Qiagen, Germany) according to the manufacturer's instructions. DNA and RNA yield and purity were measured with a microvolume fluorospectrometer (NanoDrop Technologies, DE). Contamination of RNA samples with coextracted DNA was excluded by PCR assays targeting the 16S rRNA genes using the universal primers 341F (5′-CTGCTGCCTCCCGTAG-3′) and 1401R (5′-CGGTGTGTACAAGACCC-3′) (36).

Single-stranded cDNA synthesis from total RNA. Samples of 2.5 μg total RNA were converted into single-stranded cDNA by reverse transcription using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Germany) according to the manufacturer's instructions. cDNA yield and purity were measured using the microvolume fluorospectrometer.

Abundance of functional genes and their transcripts. Quantitative PCR (qPCR) was used to determine the abundance of functional communities involved in the nitrogen cycle and their activities by targeting genes and their corresponding transcripts encoding key enzymes of nitrogen fixation (*nifH* encoding nitrogenase), ammonia oxidation (*amoA* encoding ammonia monooxygenase) in both ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA), and denitrification (*nirK*, *nirS*, and *nosZ* encoding cytochrome cd_1, copper nitrite reductases, and nitrous oxide reductase, respectively). Absolute quantification of all investigated target genes was carried out in 25-μl samples in triplicate on the ABI Prism 7300 cycler (Applied Biosystems, Germany) with the following reagents: bovine serum albumin (Sigma-Aldrich, Germany), primers (Table 1) (Metabion, Germany), dimethyl sulfoxide (Sigma, Germany), and Power SYBR green PCR master mix (Applied Biosystems, Germany) (21). All PCR runs started with an initial enzyme activation step performed at 95°C for 10 min, but the subsequent thermal profiles differed from gene to gene as indicated in Table 1. The specificity of the amplification products was confirmed by melting-curve analysis. No template controls gave a null or negligible value. To avoid inhibitory effects on quantitative PCR, samples were diluted 10-fold based on results from a previous experiment (data not shown). Dilution series of plasmid DNA with cloned bacterial *nifH*, *amoA*, *nirK*, *nirS*, and *nosZ* genes and archaeal *amoA* functional gene fragments were used to generate standard curves ranging from 10^0 to 10^5 gene copies μl^{-1} for cDNA quantification and from 10^1 to 10^6 gene copies μl^{-1} for DNA quantification with efficiencies ranging from 97 to 100%.

Statistical analysis. Prior to analysis, DNA and cDNA abundance data were log transformed (ln) to achieve normal distribution. Data were analyzed by two-way analysis of variance (ANOVA) with treatment (PM, PMSDZ) and time (10, 20, and 30 days) as independent factors. Homogeneity of the variances was checked by the Levene test. The significance level was set to $\alpha = 0.05$. Furthermore, independent *t* tests were used to test for a significant difference between the two treatments at a given time point with significance level corrected by the Šidák's equation to $\alpha = 1 - (1 - 0.05)^{1/3} = 0.017$. Statistical tests were calculated with SPSS 11.5 (SPSS, Inc., IL).

RESULTS

SDZ concentrations in bulk soil samples. At all sampling time points, the concentrations of $CaCl_2$/methanol-extractable sulfadiazine (SDZ), *N*-acetyl-SDZ, and 4-hydroxy-SDZ in bulk

TABLE 1. Primers and thermal profiles used for real-time PCR quantification of different functional genes

Target gene[a]	Primer set[b]	Reference	Thermal profile	No. of cycles	Amplicon size (bp)
nifH	*nifH*-F-Rosch	39	45 s at 95°C, 45 s at 55°C, and 45 s at 72°C	40	458
	nifH-R-Rosch	39			
AOB *amoA*	*amoA*-1F	41	60 s at 94°C, 60 s at 60°C, and 60 s at 72°C	40	500
	amoA-2R	41			
AOA *amoA*	19F	27	45 s at 94°C, 45 s at 50°C, and 45 s at 72°C	40	624
	CrenamoA616r48x	43			
nirK	*nirK*-876	18	15 s at 95°C, 30 s at 63°C to 58°C, and 30 s at 72°C	6 td[c]	164
	nirK-5R	7		40	
nirS	*nirS*-cd3af	35	60 s at 94°C, 60 s at 57°C, and 60 s at 72°C	40	413
	nirS-R3cd	50			
nosZ	nosZ2F	19	15 s at 95°C, 30 s at 65°C to 60°C, and 30 s at 72°C	6 td	267
	nosZ2R	19		40	

[a] Abbreviations: AOB, ammonia-oxidizing bacteria; AOA, ammonia-oxidizing archaea.
[b] The forward (F) and reverse (R) primers are indicated.
[c] td, touchdown.

soil were below 0.2, 1, and 5 ng g^{-1}, respectively (data not shown).

Nitrogen and carbon content of the plants. For both plant species, the yield of the green biomass was not influenced by the presence of SDZ in the manure applied at the sampling time points. In the clover biomass, increased nitrogen content was found compared to maize and decreased N content was observed with pig manure contaminated with sulfadiazine (PMSDZ) treatment (see Tables S2 and S3 in the supplemental material). A significant treatment effect on the carbon content of the maize plants was revealed by slightly higher carbon concentration in the PMSDZ treatment after 10 days (see Tables S2 and S4 in the supplemental material).

Nitrogen and carbon content in the RRC. The application of SDZ had no significant effect on the amount of water-extractable organic nitrogen (WEON) and carbon in the root-rhizosphere complex (RRC) of either plant type (see Table S3 in the supplemental material). However, plant species- and time-dependent differences, i.e., larger amounts of WEON in the RRC of the legume, were detected (Fig. 1; see Table S2 in the supplemental material). Differences in ammonium and nitrate concentrations between the treatments were observed; in the RRC of clover 10 days after application, there were lower ammonium concentrations in the PMSDZ treatment (20 μg NH_4^+-N g^{-1}) than in the PM treatment (50 μg NH_4^+-N g^{-1}) ($P = 0.044$). In the RRC of maize 30 days after application of the different types of manure, the ammonium concentration was higher in the PMSDZ treatment than in the PM treatment ($P = 0.026$).

Quantification of functional genes and their corresponding transcripts. For all genes and transcripts, clear variations over time were visible in both plant species.

In clover RRC, SDZ contamination of the manure significantly reduced *nifH*, ammonia-oxidizing bacteria (AOB) *amoA*, *nirK*, *nirS*, and *nosZ* gene abundance patterns 20 days after application (Fig. 2A and Table 2). At this time point, the difference in copy numbers between the treatments PM and PMSDZ reached up to 2 orders of magnitude. AOB *amoA*, *nirK*, *nirS*, and *nosZ* copy numbers remained lower in the PMSDZ treatment at day 30. In contrast, ammonia-oxidizing archaea (AOA) *amoA* gene abundance was not influenced at any sampling time point by the SDZ-treated manure. Similar to the observations at the DNA level, AOA *amoA* transcripts did not respond to the contaminated manure. As expected, the reduced number of genes involved in denitrification (*nirS*, *nirK*, and *nosZ*) and nitrification (AOB *amoA*) in the PMSDZ treatment resulted in a reduced number of transcripts. Moreover,

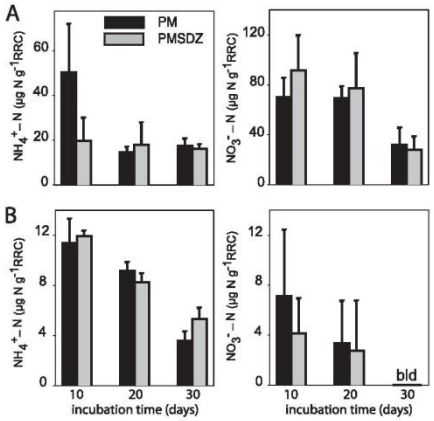

FIG. 1. Ammonium and nitrate concentrations in the root-rhizosphere complex of clover (A) and maize (B) after the addition of pig manure (PM) or pig manure plus sulfadiazine (PMSDZ) at three different time points (10, 20, and 30 days) after application. Error bars represent standard deviations of means ($n = 4$). Abbreviations: RRC, root-rhizosphere complex; bld, below limit of detection.

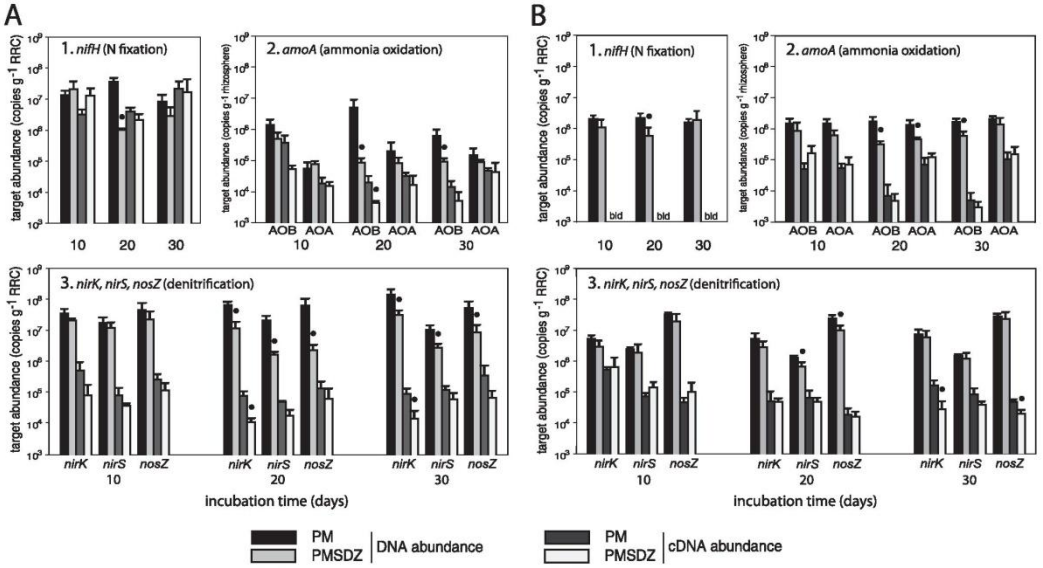

FIG. 2. Quantification of functional gene and transcript copies involved in nitrogen cycling (*nifH*, AOB *amoA*, AOA *amoA*, *nirK*, *nirS*, and *nosZ*) in the root-rhizosphere complex of clover (A) and maize (B) after the addition of pig manure (PM) or pig manure plus sulfadiazine (PMSDZ) at three different time points (10, 20, and 30 days) after application. Significant differences between the two treatments at a particular time point are indicated by solid black circles ($P < 0.017$). Error bars represent standard deviations of means ($n = 4$). Abbreviations: RRC, root-rhizosphere complex; bld, below limit of detection; AOA, ammonia-oxidizing archaea; AOB, ammonia-oxidizing bacteria.

we observed a reduced number of transcripts for AOB *amoA* 10 days after application ($P = 0.023$) and lower abundance values for *nirK* transcripts ($P = 0.032$) 30 days after application of the PMSDZ compared to the control PM treatment. However, despite reduced gene abundance, *nifH* transcripts were not affected by PMSDZ.

In maize RRC, *nifH*, AOA and AOB *amoA*, *nirK*, *nirS*, and *nosZ* gene levels were significantly decreased by PMSDZ treatment, notably 20 days after application (in the range of 0.5 order of magnitude; Fig. 2B and Table 2). Whereas most of the investigated genes in the PMSDZ-treated samples exhibited recovery at a later time point, AOB *amoA* gene copy numbers remained low compared to the corresponding PM-treated samples 30 days after application. The abundance of transcripts for *nirK*, *nirS*, and *nosZ* in maize RCC was not affected by PMSDZ at the early sampling time points. However, 30 days after application, lower levels of *nirK*, *nosZ*, and *nirS* transcript copy numbers were observed in the PMSDZ treatment (Fig. 2B). Abundance patterns of *amoA* (AOB and AOA) transcripts were not affected by the treatment (Table 2), whereas the number of *nifH* transcripts was below the detection limit at all sampling time points.

TABLE 2. Statistical evaluation of gene and transcript abundance by two-way ANOVA

Plant and factor	*P* value[a]											
	nifH		AOB *amoA*		AOA *amoA*		*nirK*		*nirS*		*nosZ*	
	DNA	cDNA	DNA	cDNA	DNA	cDNA	DNA	cDNA	DNA	cDNA	DNA	cDNA
Clover												
Treatment	**0.000**	0.764	**0.000**	**0.000**	0.553	0.094	**0.000**	**0.000**	**0.000**	**0.001**	**0.000**	**0.007**
Time	**0.002**	**0.045**	**0.002**	**0.000**	0.165	0.117	**0.001**	**0.002**	**0.000**	**0.003**	0.055	0.222
Treatment × time	**0.000**	**0.000**	**0.001**	0.244	0.250	0.439	**0.012**	0.984	**0.000**	0.576	**0.009**	0.777
Maize												
Treatment	**0.006**		**0.000**	0.408	**0.001**	0.159	**0.028**	0.263	**0.020**	0.616	**0.006**	0.426
Time	0.493		0.487	**0.000**	**0.014**	0.266	0.174	**0.000**	**0.047**	**0.035**	0.241	**0.008**
Treatment × time	0.120		0.306	0.265	0.605	0.675	0.828	**0.034**	0.747	0.059	0.551	0.201

[a] The *P* values show the impact of the manure treatments and time on functional genes and their corresponding transcripts involved in nitrogen turnover. Boldface values indicate significant effects ($P < 0.05$).

DISCUSSION

The objective of this study was to assess the impact of a single application of SDZ-contaminated manure on the functional biomass involved in major nitrogen turnover processes in the RRCs of two different crops. By targeting marker genes for nitrification, denitrification, and nitrogen fixation, we quantified the functional communities and functionally redundant populations (37, 38, 43) on both the DNA and RNA level to comprehend the effect of SDZ on the genetic potential and activity status. Although molecular methods have been shown to be suitable tools to improve our understanding of microbial community structure and function in soils, they are accompanied by drawbacks, such as biased extractions of nucleic acids from soils. Therefore, it is difficult to compare results that are based on the same extraction protocol but derive from samples of different soil type and texture, as extraction efficiencies of DNA and RNA might differ. In addition, primer selection for PCR as a possible cause of bias should be kept in mind, as not all environmental sequences of the targeted genes might be detected due to the limited number of species used for primer development. For example, the primers used to target *nosZ* in this study are probably specific only for the nitrous oxide reductase gene from Gram-negative bacteria (19), and thus, the response of the functional biomass to the antibiotic could be underestimated. Moreover, metagenomic analysis has demonstrated in the past few years that protein families can cover a much broader sequence diversity than that usually captured (5, 51, 52).

Differences in DNA and RNA levels. As much care was taken to use the same protocol for DNA and RNA extraction, a comparison on the effect level was possible in this study. The quantification of both genes and transcripts revealed, in contrast to our assumption based on the bacteriostatic mode of action of SDZ, significant impacts on the gene level but to a lower extent on the transcript level. Presumably, microbial subpopulations intrinsically able to cope with the antibiotic stressor could have taken advantage of the altered competitive environment and maintained nitrogen turnover. Whether a significant community change took place under the influence of SDZ remains to be clarified.

Delayed microbial response. Ten days after application of PMSDZ, no $CaCl_2$/methanol-extractable SDZ was detected in bulk soil samples. It has been shown that the potentially bioavailable fractions of SDZ quickly decline in bulk soil (17), because sulfonamides have a large potential for rapid adsorption into the soil matrix and manure constituents (23, 49). In both maize and clover RRCs, we observed a time lag between the application of SDZ and its effect on the functional microbial biomass (Fig. 2). Significant effects of the antibiotic-contaminated manure on gene abundance levels were observed 20 and 30 days after application, whereas the concentration of bioavailable SDZ in the surrounding bulk soil had declined below the detection limit. Such a time lag may be related to the slow generation times of microbes in soil and the bacteriostatic action of sulfonamide. However, it must be taken into account that the fate of SDZ in the rhizosphere may differ from that in bulk soil due to differences in chemical, physical, and microbial properties. Decreased pH values by up to two pH units in the rhizosphere compared to bulk soil (8) may affect SDZ bioavailability. This has been demonstrated for other xenobiotics; a faster degradation of selected pesticide residues was observed in rhizosphere soil than in bulk soil (11).

Rhizosphere effects of different plant types. The differences in gene abundance patterns between the two treatments were more pronounced and long-lasting in the RRC of clover than in the RRC of maize. Presumably, differences in quality and quantity of root exudates and in root morphology known to shape microbial communities and to form the basis for microbial activity in the rhizosphere might have contributed to the different effects observed (22, 30). Whereas maize may have provided primarily recalcitrant organic carbon from decaying root material to the microflora of its RRC (44), clover roots might have excreted more readily available organic compounds (15), resulting in an increase in microbial biomass and activity in this RRC. In contrast, AOA, which might exhibit a rather oligotrophic lifestyle (31), could have been outcompeted by the faster-growing microorganisms in the clover RRC, as indicated by the reduced numbers of AOA *amoA* gene copies found in the clover RRC compared to the maize RRC. As dormancy or reduced activity results in reduced susceptibility to SDZ, the bacteriostatic antibiotic might have found fewer targets (4, 28), thus explaining the differences observed in the effect of SDZ when both plant species were compared.

***nifH*.** Legume roots exude various flavonoid and isoflavonoid molecules that are known to induce development of symbiotic interactions between the plant and nitrogen-fixing alphaproteobacteria within root nodules (46). This is consistent with our results showing explicitly higher *nifH* gene expression and, to a lower extent, higher *nifH* gene abundance in the clover RRC, which includes nodules, compared to the maize RRC. We postulated that legume growth would be more affected by SDZ, as legumes need a symbiotic partner for an optimal supply of nitrogen. Despite a considerable decrease in *nifH* gene abundance in the clover RRC 20 days after application of the SDZ-contaminated manure, the abundance of transcripts was not significantly affected by the antibiotic, which might be the reason for similar plant quality and yield in both treatments. It is possible that the active nitrogen-fixing bacteria within the root nodules are protected from the antibiotic and therefore are not affected. However, it must be noted that external nitrogen was introduced to the soil during manure application; thus, nitrogen provided by nitrogen fixers was not needed to maintain a high plant yield.

***amoA* (AOB and AOA).** In the RRC of clover, the antibiotic abolished the increase of the ammonia-oxidizing bacterial population between day 10 and 20 in response to the manure application (PM). Similar results were shown by Schauss and coworkers (43) in bulk soil, although the effect of SDZ was less pronounced. Moreover, 20 and 30 days after application of the manure, the AOB abundance patterns in both plant RRCs were significantly reduced in the PMSDZ treatment, indicating a sustainable reduction in nitrification potential (Fig. 2). Lower ammonium values were measured in the PMSDZ treatment at the 10-day time point that might be related to an overall inhibition of nitrogen mineralization by the antibiotic. This relative ammonia depletion clearly induced lower bacterial *amoA* transcripts in comparison to the PM treatment and consequently resulted in a lower availability of nitrite, which in turn may have affected denitrifiers harboring the *nirK* gene and a lower

transcript abundance of the corresponding gene. Genes and transcripts of archaeal ammonia oxidizers were not affected by SDZ in the RRC of clover, possibly due to their lower abundance compared to AOB. In contrast, in the maize RRC, AOA were as abundant as AOB during the sampling period and although AOA were significantly influenced by SDZ, they were affected to a lower extent than AOB were, indicating a reduced susceptibility of AOA toward the antibiotic. Thus, the functional redundancy between AOB and AOA under antibiotic stress described by Schauss and coworkers (43) for bulk soil could also be a mode of action in the RRC.

***nirK*, *nirS*, and *nosZ*.** As indicated by decreased copy numbers of all three genes involved in denitrification, the denitrification potential in both plant RRCs was reduced 20 days after application of PMSDZ and remained low in the clover RRC even until day 30. This is in contrast to results obtained in bulk soil where denitrifiers were only slightly affected by SDZ (unpublished data) and underlines the assumption of more pronounced antibiotic effects on highly active microbial communities living in hot spots like the RRC.

It has been shown in several studies that microbes harboring the *nirK* gene form the major part of nitrite reducers in different rhizospheres (14, 21) and show increased activity compared to bacteria harboring the *nirS* gene (45). In our study, we confirmed higher *nirK* gene copy numbers than *nirS* gene copy numbers in both treatments at all sampling time points and observed that *nirK*-harboring microbes were more affected by SDZ than *nirS*-denitrifying bacteria. In addition to the differences in activity between *nirS*- and *nirK*-harboring microbes in the RRC samples, which may explain the differences in response to SDZ, an indirect antibiotic effect could have contributed to the reduced abundances of *nirK* (and *nosZ*) transcripts via impaired microbial respiration activity in the RRC, hence higher oxygen levels and consequently inhibited gene expression in the PMSDZ treatment. Furthermore, the uptake of SDZ may vary between different functional populations. Recently, Zarfl and coworkers (55) described a mechanistic model explaining substance-specific and pH-dependent antibiotic effects. In this model, they assumed that differences in the accumulation and speciation of sulfonamides in bacteria are due to different abilities of bacteria to regulate their internal pH value. Tappe and colleagues (47) examined the influence of diverse sulfonamides, including SDZ, on bacterial growth at different pH values and concluded that a possible impact on the microbial population in soil could strongly depend on the method by which bacteria regulate their internal pH value. This could explain the dissimilarities observed in the responses of the denitrifiers in the RRC.

Conclusion. This greenhouse study revealed that a single application of manure contaminated with the antibiotic sulfadiazine has a lasting impact on the functional microbial biomass involved in nitrogen cycling on both the gene and transcript levels in the RRCs of different plants of agricultural importance. However, the data presented are based on constant climatic conditions, and the role of environmental factors like drought periods on the antibiotic effect has been excluded. Besides, influences of the antibiotic in combination with other agricultural management tools, e.g., the application of pesticides (mainly the role of fungicides), have to be assessed before the results can be transferred into practice. Finally, in this study, a single application of SDZ-contaminated manure was performed, but under field conditions manure (possibly contaminated with antibiotics) is applied several times during the vegetation period and consequently, the microbial communities might adapt to the antibiotic stressor.

Furthermore, it remains to be studied how microbial diversity patterns are affected by antibiotics. It might be assumed that for denitrification this issue is not of such high relevance because many soil prokaryotes are able to use nitrate and nitrite as terminal electron acceptors when oxygen is lacking. In contrast, for other processes like nitrogen fixation and ammonia oxidation, which can only be performed by a limited number of soil microbes, this topic seems of interest. In addition, the question of how the antibiotic might influence the organic nitrogen cycle, mainly the process of nitrogen mineralization, needs to be investigated in further experiments.

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Publication II

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SOIL MICROBIOLOGY

Abundance and Diversity of Ammonia-Oxidizing Prokaryotes in the Root–Rhizosphere Complex of *Miscanthus* × *giganteus* Grown in Heavy Metal-Contaminated Soils

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Abstract Mine wastes have been considered as a source of heavy metal (HM) contamination in the environment and negatively impact many important ecosystem services provided by soils. Plants like *Miscanthus*, which tolerate high HM concentrations in soil, are often used for phytoremediation and provide the possibility to use these soils at least for the production of energy crops. However, it is not clear if plant growth at these sites is limited by the availability of nutrients, mainly nitrogen, as microbes in soil might be affected by the contaminant. Therefore, in this study, we investigated in a greenhouse experiment the response of ammonia-oxidizing microbes in the root–rhizosphere complex of *Miscanthus* × *giganteus* grown in soils with different levels of long-term arsenic (As) and lead (Pb) contamination. Quantitative PCR of the ammonia monooxigenease gene (*amoA*) was performed to assess the abundance of ammonia-oxidizing bacteria (AOB) and archaea (AOA) at two different points of plant growth. Furthermore, bulk soil samples before planting were analyzed. In addition, terminal restriction fragment length polymorphism (T-RFLP) analysis was used to investigate the diversity of archaeal *amoA* amplicons. Whereas high concentrations of As and Pb in soil (83 and 15 g/kg, respectively) resulted independent from plant growth in a clear reduction of AOA and AOB compared to the control soils with lower HM contents, in soils with contamination levels of 10 g/kg As and 0.2 g/kg Pb, only AOB were negatively affected in bulk soil samples. Diversity analysis of archaeal *amoA* genes revealed clear differences in T-RFLP patterns in response to the degree of HM contamination. Therefore, our results could clearly prove the different response patterns of AOA and AOB in HM-contaminated soils and the development of archaeal *amoA* phylotypes which are more tolerant towards HMs in soil samples from the areas that were impacted the most by mining waste, which could contribute to functional redundancy of ammonia-oxidizing microbes in soils and stability of nitrification pattern.

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Introduction

The mining industry produces large amounts of waste contaminated by metals and metalloids [1], leading to the accumulation of toxic elements in the environment. Whereas heavy metal (HM)-contaminated soils are unsuitable for food production, energy crops (e.g., *Miscanthus* × *giganteus*, a perennial grass

with large annual biomass production potential) can allow the commercial exploitation of these soils by establishing biofuel feedstock production systems. In addition, the cultivation of these plants offers opportunities for site stabilization and phytoremediation of contaminated soils [2, 3].

However, macronutrients deficiency is one of the growth-limiting factors of plant cultures on contaminated soils [4]. It is therefore of key importance to investigate the response of the functional microbial biomass involved in nutrient turnover to contamination. In this respect, nitrogen (N) cycling is of major concern as contaminated sites are usually not fertilized, and N available to plants is closely linked to N mineralization from dead biomass and subsequent transformation. Several authors have postulated that nitrification responds at a very sensitive level to different types of contamination [5, 6] due to low functional diversity compared to other steps in the N cycle [7]. Nitrification is a two-step process consisting of: (1) oxidation of NH_4^+ to NO_2^- by ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) [8, 9] and (2) oxidation of NO_2^- to NO_3^- by nitrite-oxidizing bacteria (NOB), the first step being assumed as rate limiting [10]. While HM contamination can affect AOB communities [11–13], little is known about how AOA is affected by contamination. Although AOA are thought to be more tolerant to chronic stress conditions than bacteria [14, 15], the influence of HMs on AOA is discussed controversially in literature [16–18]. Furthermore, the few studies assessing the response of both AOA and AOB to HM contaminations were mostly performed using spiked soils [12, 16–21], which do not allow a prediction of the AOA and AOB dynamics in soils with a long history of HM contamination due to adaptation processes of the microflora on the one hand and changes in the amount of bioavailable HMs on the other hand [22, 23].

Therefore, in this study, we investigated in a greenhouse experiment the response of ammonia-oxidizing microbes in the root–rhizosphere complex (RRC) of *Miscanthus* × *giganteus* grown in soils with different levels of long-term combined arsenic (As) and lead (Pb) contamination. We analyzed the abundance of both archaeal and bacterial *amoA* genes in the bulk soils before planting and 6 and 12 weeks, respectively, after planting of the bioenergy crop. Furthermore, we studied the diversity of the *amoA* genes of AOA. We postulated that AOA will be less affected by HM contamination in soil than AOB due to a shift in the AOA diversity towards more HM-resistant phylotypes over time.

Materials and Methods

Experimental Design

Soils classified as Anthroposol Artificiel according to the French classification or Technosol (WRB nomenclature, FAO 2006) from the former gold mining area at La Petite Faye (Limousin, France, 01°34′23″ E, 46°08′37″ N) were chosen for the experiments. This site, which has been abandoned since 1964, presents zones with different As and Pb concentrations and has been colonized by local vegetation (e.g., grasses, ferns, horsetails, birches). Three plots were selected for soil sampling, reflecting zones with severe (S), medium (M), and low (L) levels of HM contamination based on total Pb and As values. The values for Pb ranged from 15,200 mg/kg at plot S to values smaller than 500 mg/kg at plots M and L. Arsenic values ranging from 83,000 mg/kg at plot S to 1,700 mg/kg at plot L were measured. Soils were sampled in January 2010 up to 20 cm soil depth after removing the organic horizon. Sieved soil (2 mm) was transferred into plastic bags and placed into pots (21×21×21 cm) at a bulk density of 1.0 and equilibrated for 1 month at 20 °C and constant water content. Major chemical and physical soil parameters are summarized in Table 1.

After an equilibration phase of 7 days at 50 to 60 % of field water capacity at 20 °C, one pre-grown *Miscanthus* × *giganteus* rhizome (Novabiom, France) was introduced per pot. *Miscanthus* × *giganteus* plants were grown in a phytotron for 3 months, without receiving additional nutrients. The plants were subjected to a photoperiod of 16 h light at 350 μmol m^{-2} s^{-1} and 8 h of darkness at temperatures of 23 and 18 °C, respectively, under constant soil water content (field capacity). Samplings were performed before planting the rhizome (t_0) and after 6 (t_1) and 12 (t_2) weeks. The experiment was conducted in a randomized block design with four independent replicates per soil and per sampling time point. Bulk soil (at t_0) and composite samples of roots with attached soil (at t_1 and t_2) were sampled from each pot. After shaking the roots vigorously, the roots and attached soil were treated as one compartment called the RRC. One part of the samples was immediately shock-frozen in liquid nitrogen and stored at −80 °C for nucleic acid extraction; the other part was directly extracted with 0.01 M $CaCl_2$ for determination of water-extractable As and Pb, organic carbon (WEOC), ammonium–N (NH_4^+–N), and nitrate–N

Table 1 Soil parameters

	Soil S	Soil M	Soil L
Soil type	Silty loam	Silty loam	Silty loam
pH	3.4	3.6	5.6
C_{total} [%]	7.3	5.1	9.7
N_{total} [%]	0.5	0.4	0.7
C/N	13.8	13.5	13.6
As $_{total}$[a]	83,000	9,300	1,700
Pb $_{total}$[a]	1,520	200	300

[a] mg/kg soil

(NO_3^-–N). Bulk soil samples were directly treated by lithium metaborate/tetraborate fusion and nitric acid digestion for HM determination.

Soil Parameters

Samples consisting of bulk soil or RRC were shaken overhead for 45 min in 0.01 M $CaCl_2$ for determination of water-extractable As and Pb (soil/solution ratio 1:10), WEOC, NH_4^+–N and NO_3^-–N (soil/solution ratio 1:4). After filtration, Pb and As were measured by graphite furnace atomic absorption spectrometry (SpectrAA 880 Z, Varian, CA, US) equipped with a Zeeman background correction. NH_4^+–N and NO_3^-–N were determined using a continuous-flow analyzer (SA 20/40, Skalar Analytical, The Netherlands). WEOC was measured using a TOC analyzer (TOC-5050A, Shimadzu Corporation, Japan).

Plant Parameters

The dry weight of rhizome, stem, and leaves of *Miscanthus* plants, respectively, were measured 12 weeks after planting (t_2). As and Pb contents were determined in each organ at t_2 by ICP-MS after nitric acid digestion (Acme Analytical Laboratories Ltd., Canada).

DNA Extraction

DNA of each of 12 samples (four replicates × three time points) was extracted from 0.5 g of bulk soil and RRC, respectively, after a bead beater lysis step (Bertin Technologie, France) using the FastDNA SPIN kit for soil (MP biomedicals, Germany) according to the manufacturer's instructions. DNA concentration was measured by using a microvolume spectrophotometer (NanoDrop, PeqLab, Germany).

Quantitative PCR Assay

Quantitative PCR (qPCR) was used to determine the abundance of functional communities involved in ammonium oxidation by targeting *amoA* genes (encoding the ammonia monooxygenase) in both AOB and AOA using a SYBR® GreenI-based detection system (Applied Biosystems, Germany). Absolute quantification of investigated target genes was carried out in 25-μL samples in triplicate on the ABI Prism 7300 Cycler (Applied Biosystems). The reaction mixture contained 15 μg bovine serum albumin (Sigma-Aldrich, Germany), 0.2 μM of each primer for *amoA* AOA, and 0.3 μM of each primer for *amoA* AOB amplification, respectively (Metabion, Germany), 1X Power SYBR Green PCR master mix (Applied Biosystems), and 40 ng DNA template. PCR conditions and primers used are shown in Table 2. The specificity of the amplification products was confirmed by melting curve analysis and agarose gel electrophoresis. No template controls served as null value. Samples were diluted tenfold as no inhibitory effects on the PCR amplification were detected when known amounts of standard (AOB and AOA) were spiked with tenfold diluted environmental DNA samples (data not shown). Dilution series of plasmids containing cloned DNA of the *amoA* gene from *Nitrosomonas multiformis* ATCC25196 for AOB and of the fosmid clone 54d9 [9] for AOA, respectively, were used to generate standard curves ranging from 10^1 to 10^6 gene copies per microliter. The calculated efficiencies for qPCRs of AOA and AOB were comprised between 90 and 95 %.

Terminal Restriction Fragment Length Polymorphism Fingerprinting

Diversity analysis of archaeal *amoA* gene was carried out by terminal restriction fragment length polymorphism (T-RFLP). Archaeal *amoA* gene amplicons were generated by two successive PCRs using the primers described for qPCR assay, with forward primer labeled with 5′-carboxyfluorescein. The first PCR reaction (50 μL) contained ~100 ng of template DNA, 0.2 μM of each primer, 0.2 mM dNTPs (Fermentas, Germany), 60 μg of BSA (Sigma-Aldrich, Germany), 1 U Top Taq and 1 × PCR buffer (Qiagen, Germany), and nuclease-free water (Promega, Germany). The PCR reaction was achieved according to the following thermal profile: 5 min at 95 °C, followed by 30 cycles of 45 s at 94 °C, 45 s at 55 °C, 45 s at 72 °C, and finally 5 min at 72 °C. Four microliters of PCR products was amplified subsequently in the same conditions as previously described in a final volume of 100 μL. Generated amplicons were checked by standard agarose gel electrophoresis and ethidium bromide staining and purified with the QIAquick PCR purification kit (Qiagen) prior enzymatic digestion with the restriction enzyme MwoI (Fermentas, Germany) according to the manufacturer's protocol. The restriction enzyme was selected based on in silico T-RFLPs using the program REPK (Restriction Endonuclease Picker) [24]. Digested amplicons (~50 ng in 10 μL) were subsequently purified with the MinElute Reaction cleanup kit (Qiagen). Desalted digests (1 μL) were mixed with 13 μL of Hi-Di formamide (Applied Biosystems) containing an 800-fold dilution of a 6-carboxy-X-rhodamine-labeled MapMarker 1000 ladder (Bio-Ventures, TN, US), denatured (3 min at 95 °C), and cooled on ice. Electrophoresis was performed as described previously [25] using an ABI 3730 DNA analyzer (Applied Biosystems). Electropherogram evaluation was performed using the GeneMapper 5.1 software (Applied Biosystems).

Statistical Analysis

Prior to analysis, gene abundance data were ln-transformed to achieve normal distribution. Data were analyzed by one-

Table 2 Primers and thermal profiles used for real-time PCR quantification of bacterial and archaeal *amoA*

Target gene	Primer Set	Reference	Thermal profile	Cycles	Amplicons e, bp
AOB *amoA*	*amoA*-1 F *amoA*-2R	[45] [45]	94 °C/60 s, 58 °C/ 60 s, 72 °C/60 s	40	500
AOA *amoA*	19 F CrenamoA616r48x	[9] [30]	94 °C/45 s, 55 °C/ 45 s, 72 °C/45 s	40	624

way ANOVA with soil (L, M, S) as factor at a given time point for both bacterial and archaeal *amoA* genes with significance level set to α=0.05. Soil parameters data were analyzed similarly. Statistical tests were calculated in SPSS 11.5 (SPSS, Inc., IL, USA). T-RFLP data were imported into T-REX [26] and a data matrix based on peak area was generated. Peaks were inactivated for lack of length (<50 bp) and aligned using clustering threshold of 1 bp. Fragments with relative abundance of less than 1 % were considered as background noise. The data matrix was exported for analysis with the ADE4 package [27] within the R software environment (www.R-project.org) using between-group analysis (BGA) based on correspondence analysis using the function dudi.coa followed by bca. Because correspondence analysis is only the first step in the between-group analysis, no detrended form with down-weighting of variables was used. The between-groups inertia percentage was used for a global test of any difference between the groups. Based on 999 permutations, a P value was calculated. In case of significant results (P<0.05), pairwise tests were performed; the P values were adjusted for multiple comparisons by the method of Hommel [28].

Results

Physical and Chemical Characterization of Bulk Soil, RRC, and Plants

The $CaCl_2$-extractable fraction of As and Pb in all soil samples was lower than 1 % of the total amount of the respective HM and ranged from 10.4 to 2.1 mg/kg for As and from 134.2 to 1.6 mg/kg for Pb, respectively, at t_0. Surprisingly, the clear gradient in total HM in soil samples from S, M, and L was not reflected in the amount of $CaCl_2$-extractable As and Pb. Over the experimental period, the values for the As $CaCl_2$-extractable fraction did not change significantly in all soil samples; for Pb in soil samples from plot S and L, a clear reduction was visible (Table 3).

Initial NO_3^-–N and NH_4^+–N concentrations in bulk soil were independent of the degree of metal and metalloid contamination (Table 3). Higher NO_3^-–N and NH_4^+–N concentrations were found in all samples at t_0. At t_1, significantly higher NO_3^-–N concentrations and lower NH_4^+–N concentrations were measured in RRC samples from soil L compared to soil S. At t_2, however, no differences in NO_3^-–N and NH_4^+–N concentrations were apparent. The amount of WEOC was not influenced by the degree of HM

Table 3 Ammonium-N, nitrate-N, WEOC, and $CaCl_2$-extractable As and Pb in the three studied soils (S, M, and L) at the three time sampling points (t_0, t_1, and t_2)

		Soil S	Soil M	Soil L
NH_4^+–N[a]	*t0*	119.7±63.4 a	82.6±25.3 a	85.9±22.5 a
	t1	55.7±6.7 a	37.1±11.1 a, b	28.0±9.4 b
	t2	47.1±7.3 a	33.3±5.4 a	33.1±7.1 a
NO_3^-–N[a]	*t0*	3.9±2.6 a	4.4±1.4 a	6.1±1.4 a
	t1	0.6±0.2 a	1.4±0.6 a	4.7±0.6 b
	t2	0.5±0.4 a	0.9±0.2 a	2.4±1.9 a
WEOC[a]	*t0*	16.3±5.3 a	16.8±4.5 a	20.1±3.1 a
	t1	29.3±2.4 a	25.0±5.2 a	33.4±8.2 a
	t2	30.6±2.8 a	30.1±4.5 a	44.1±16.1 a
$CaCl_2$-extractable As[a]	*t0*	2.1±0.9 a	7.7±0.7 b	10.4±0.6 c
	t1	1.8±0.2 a	7.0±0.2 b	11.3±0.9 c
	t2	1.7±0.3 a	5.8±0.2 b	9.9±0.3 c
$CaCl_2$-extractable Pb[a]	*t0*	134.2±4.6 a	1.6±0.1 b	18.3±3.4 c
	t1	62.1±6.6 a	1.5±0.1 b	0.0±0.0 c
	t2	63.9±31.3 a	1.6±0.3 b	0.0±0.0 c

Significant differences between soils at a given time point are indicated by different letters (n=4)

[a]mg/kg soil

contamination and was, as expected, higher in the RRC than in bulk soil. Between t_1 and t_2, no changes in WEOC amount were observed.

Biomass of plant leaves, steam, and rhizomes was not affected by the different amounts of HM present in soil (Table 4) at the last sampling time point t_2. All plants accumulated As over time. At t_2, highest As concentrations were measured in the rhizomes independent from the degree of contamination in soil. Surprisingly, the highest accumulation of As was found in plants grown in soil M with total As concentrations of 282.7 mg/kg. For soil S and L, the accumulation of As in plants was comparable (56.2–72.0 mg/kg). The accumulation of Pb in the plants was also observed, and a higher accumulation of this element in leaves and stem was observed in soil S compared to As. Greater Pb accumulations were found at t_2 in plants grown in soil S and M (103.2–121.5 mg/kg). Pb contents in plants harvested from soil L were lower than 10 mg/kg.

Quantification of Bacterial and Archaeal Ammonia Oxidizers

In bulk soil at t_0, a clear influence of the HM contamination on the abundance of AOA and AOB was visible. Whereas *amoA* copy number in the control soil L was, for both AOA and AOB, in the range of $2.9–4.8\times10^5$ copies/g soil, in the severely impacted soil S only $2.2–5.2\times10^4$ copies/g were measured. In soil M, AOA was obviously not influenced by HM contamination and values were comparable to soil L; for AOB, a clear influence of contamination was visible and values were similar to soil S (Fig. 1).

At t_1 in the RRC of plants grown in soil L, as expected, AOB outcompeted AOA by almost one order of magnitude (2.4×10^5 and 4.0×10^4 *amoA* copies/g RRC, respectively).

Table 4 Plant parameters at t_2

Soil	Plant organ	Biomass[a]	As[b]	Pb[b]
S	Rhizome	11.81±5.60 a	49.5	30.6
	Stem	0.73±0.44 a	5.4	29.5
	Leaves	0.88±0.45 a	17.1	43.1
M	Rhizome	9.41±4.63 a	195.7	70.0
	Stem	0.20±0.17 a	66.9	28.6
	Leaves	0.36±0.18 a	20.1	12.9
L	Rhizome	8.82±4.24 a	48.3	3.8
	Stem	0.41±0.31 a	3.6	0.6
	Leaves	0.42±0.33 a	4.3	1.1

Significant differences between soils are indicated by different letters (n=4)

[a] g dry weight

[b] mg/kg

In contrast, in the RRC of plants from soil M, AOA copy numbers were higher (5.7×10^5 *amoA* copies/g) compared to those from soil L, whereas no significant differences in AOB copy numbers between plants from soil L and M were visible. In the RRC from plants obtained from soil S, as expected, *amoA* copy numbers for AOA and AOB showed similar response pattern and were significantly lower compared to plants from the other soils (9.9×10^3—1.7×10^4 *amoA* copies/g).

At t_2, similar copy numbers for *amoA* could be measured. Only in the RRC of plants from soil L were the differences observed at t_1 between AOA and AOB no longer visible and values for both groups of ammonia oxidizers were in the range of 1.5×10^5 *amoA* copies/g.

Diversity Analysis of Archaeal *amoA genes*

T-RFLP analysis of the archaeal *amoA* gene resulted in a detectable T-RF number/sample ranging from two (t_0) to six (t_1) in soil S, from two (t_1, t_2) to three (t_0) in soil M, and from two (t_0) to nine (t_1) in soil L (Fig. 2). T-RF-162 was dominant in bulk soil samples (t_0) for all soils and in the RRC samples (t_1, t_2) for soil S and M, ranging from 66 to 97 % of the total relative community. In the RRC of soil L, T-RFs-162 and -253 had a similar level of relative abundance, contributing for 33 and 30 % of the total *amoA* AOA community at t_1 and 43 and 51 % at t_2, respectively. The contribution of T-RF-253 to the community richness in soil L was comparable at the different time points. However, T-RF-253 was absent in soil S. An increased number of T-RFs was found at t_1 and, to a lower extent, at t_2 compared to t_0 in soil S and L. Statistical evaluation of overall AOA diversity by BGA revealed a clustering according to the different soils at a given time point, with the exception of soils S and M at t_2 which clustered together (Fig. 3; Tables 5 and 6). BGA also indicated significant differences for all soils between t_0 and t_1, as well as between t_0 and t_2 for soil S and t_1 and t_2 for soil M.

Discussion

The objective of this study was to assess the impact of different degrees of As and Pb contamination in soil and the planting of the energy crop *Miscanthus* × *giganteus* on the functional microbial biomass involved in the oxidation of ammonia in soil. Therefore, soils with different long-term As and Pb contamination were used for plant growth. By targeting the marker gene *amoA*, we quantified ammonia-oxidizing bacteria and archaea in bulk soil as well as in the RRC at two different time points during plant development and described the diversity pattern of archaeal ammonia

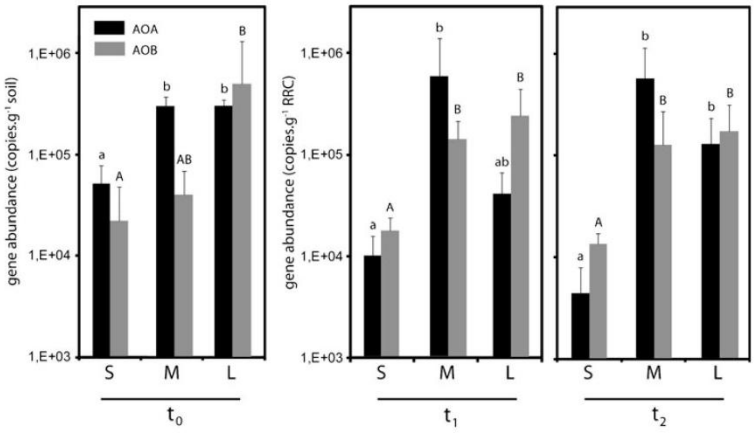

Figure 1 Quantification of *amoA* (AOB and AOA) in bulk soil (t_0) and in root–rhizosphere complex of *Miscanthus* × *giganteus* after 6 (t_1) and 12 (t_2) weeks in three acidic soils contaminated with heavy metals (*S*, *M*, and *L*). Significant differences between the three soils at a particular time point are indicated by *letters*. *Error bars* represent standard deviation of mean (n=4). *RC* RRC root-rhizosphere complex

oxidizers. As shown by Nicol and coworkers [29], there is good evidence that *amoA* diversity well reflects the phylogeny of ammonia-oxidizing bacteria and archaea. Furthermore, Schauss et al. [30] calculated the maximum number of *amoA* genes per cell for AOA and AOB and estimated for both groups of ammonia oxidizers a maximum of three operons per cell; thus, *amoA* has been considered as a good proxy for the total number of ammonia-oxidizing microbes.

Bioavailability of Pb and As in Soils with a Long History of Contamination

In this study, the investigated soils showed different degrees of As and Pb contamination. Remarkably, the total Pb and As contents and the $CaCl_2$-extractable fractions, respectively, did not follow the same trend. This might be related to the pH values of the different soils and the difference in the HM speciation to mineralogy (e.g., respective amount of As- and Pb-bearing phases). Similarly, Cui and colleagues [31] observed an increased availability of As and Pb with higher and lower soil pH, respectively. Total HM concentrations are a poor indicator of the actual concentration in the soil solution to which soil microbes are exposed, and there is no universally acceptable method to assess bioavailable soil metal concentrations [32].

As and Pb as Drivers for Abundance and Diversity of Ammonia-Oxidizing Microbes

In our study, AOA and AOB *amoA* copy numbers in bulk soil, ranging from 4.4×10^3 to 4.8×10^5, were lower than what has been previously reported [16–19, 21] (Fig. 1). However, the investigated soils in this study were of mining waste origin, presumably hostile for microbial populations, low in organic matter content, and not of agricultural provenance. In soil S, *amoA* copy numbers for both groups of ammonia oxidizers were significantly reduced compared to soil M and L, which could be interpreted as a negative impact of the high HM concentration in this soil. These results may indicate low in situ turnover rates of ammonia in response to high HM contamination, as observed in our experiment by the increased NH_4^+–N and reduced NO_3^-–N concentrations at t_1 in soil S compared to soil L. However, potentially lower transformation rates of ammonia into nitrate in soil S might be partly compensated by higher dentrification activities in soil L.

At t_0, while *amoA* AOA gene abundance was significantly higher in soil M than in soil S, no significant difference between these two soils was observed in *amoA* AOB abundance, indicating a higher sensitivity of AOB than AOA towards Pb and As. Similarly, a higher tolerance of AOA than AOB in soils contaminated by Zn [17] and Cu [21] has been suggested. However, other studies showed contrasting results and ascribed tolerance development in ammonia-oxidizing communities to AOB rather than AOA populations [16, 18]. Nevertheless, as most of these results are based on soils spiked with HMs, a direct comparison to the data presented in this study is not possible as bioavailability of HMs is different in soils with artificially added HMs, and the time of adaptation of microbes in response to the stressor is missing [22].

Mainly, the latter argument has been proven to be of high importance in our study as different AOA phylotypes showing differences in HM tolerance were observed. For example, T-RF-253 was below the detection limit in soil S and relatively less abundant in soil M than L, possibly indicating the sensitivity of this genotype to high HM contamination (Fig. 2). Vice versa, T-RF-162 was dominating AOA diversity patterns in soil S and M at all time points, indicating the importance of this phylotype in soils highly contaminated by HMs and possibly its increased resistance against HMs.

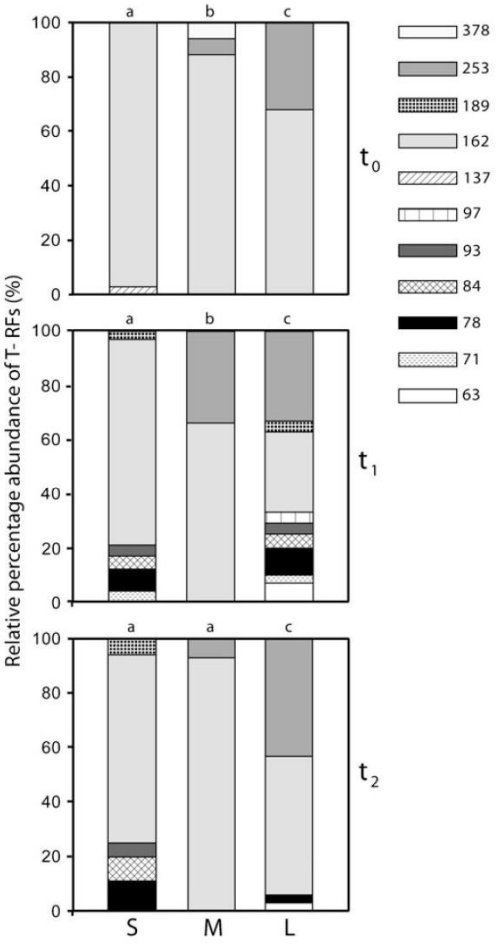

Figure 2 Contributions of T-RFs to total *amoA* AOA gene fragment diversity in bulk soil (t_0) and in root–rhizosphere complex of *Miscanthus* × *giganteus* after 10 (t_1) and 20 (t_2) days in three acidic soils contaminated with heavy metals (*S*, *M*, and *L*). Significant differences between the three soils at a particular time point are indicated by *letters*

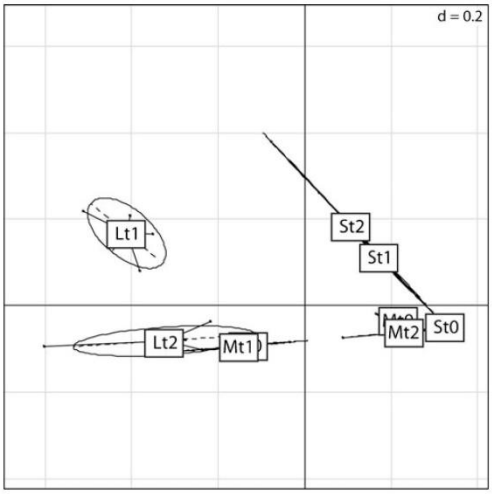

Figure 3 Between-group analysis based on correspondence analysis of the T-RFLP data set for *amoA* AOA gene fragments. The first two axes explain 61 % of variance. Symbols illustrate the four replicates for each soil (*S*, *M*, and *L*) at each time point (t_0, t_1, and t_2). *Ellipses* surround the four replicates for each soil, showing that they cluster together

Although Mertens and colleagues [13] demonstrated that AOB populations from long-term contaminated soil samples were able to tolerate higher Zn concentrations than AOB populations from uncontaminated soil samples and microbial HM, resistance mechanisms for AOB including *Nitrosomonas europaea* [33, 34] have been described in literature in our study; obviously, only a very low tolerance level towards Pb and As of AOB was observed. This might be related to the contamination with two HM in combination with other stressors which affect AOB, like low pH. In contrast, detailed data on the resistance development towards HM of AOA are still missing as the first cultures of AOA from soil, which may form the basis for such studies, have been isolated only recently [35].

Other Factors Driving the Abundance and Diversity of Ammonia-Oxidizing Microbes

When plots for this study were selected, much care was taken on identifying soils which only differ in their As and Pb concentrations. Therefore, soil parameters like soil texture, total C, and total N content were comparable. However, soils differed in their pH values. Whereas soil S and M had a comparable pH (3.5), soil L was less acidic with a pH of 5.3. Soil pH is known to shape the distribution and

Table 5 *P* values of pairwise comparisons for T-RFLP profiles of *amoA* AOA adjusted for multiple comparisons by the method of Hommel. Comparison between two soils at a given time point

Soil	*P* values for comparisons between two time points		
	t_0/t_1	t_0/t_2	t_1/t_2
S	0.028*	0.049*	0.371
M	0.045*	0.371	0.040*
L	0.049*	0.115	0.081

*$P<0.05$ (significant differences)

Table 6 *P* values of pairwise comparisons for T-RFLP profiles of *amoA* AOA adjusted for multiple comparisons by the method of Hommel. Comparison between two time points for a given soil

Time	*P* values for comparisons between two soils		
	S/M	S/L	M/L
t_0	0.049*	0.016*	0.047*
t_1	0.043*	0.018*	0.049*
t_2	0.172	0.048*	0.041*

*$P<0.05$ (significant differences)

activity of archaeal and bacterial ammonia oxidizers [29, 36] and is a driver for selecting different bacterial and archaeal communities [37–40]. Furthermore, nitrification has been suggested to be driven by AOA in highly acidic soils [36, 40]. Therefore, the effects observed in this study may be partly assigned to differences in soil pH.

Although no clear influence of the degree of HM contamination on plant growth was observed, as plant biomass values were comparable from all soils (Table 4), differences were observed in the amount of accumulated As and Pb. Roots are known to shape microbial communities and activity because of the wide variety of organic compounds that they provide [41, 42]. Accumulated HM change overall plant physiology and consequently alter the quantity and quality of root exudates. Taking into account that mainly AOA, as autotrophic microbes, which exhibit a rather oligotrophic lifestyle [43], are often outcompeted by heterotrophic microbes in the rhizosphere due to (1) the increased amounts of available carbon and (2) the competition between plants and microbes for ammonia, resulting in the release of nitrification-inhibiting substances by the plant [44], the change in exudation patterns may explain the shifts in the relative abundance of AOA and AOB, mainly in plants grown in soil M.

Conclusion

Based on the data of this study, it can be postulated that selected phylotypes of AOA tolerate higher concentrations of Pb and As in soil and RRC compared to AOB. However, abundance of a functional group cannot be directly linked to the activity of these microbes. Therefore, it remains unclear if, mainly in soil M, AOA can substitute AOB and if functional redundancy between both groups of ammonia-oxidizing microbes exists under the given conditions. Based on the ammonium levels measured, which are similar between soil L and M, this might be speculated; nitrate concentrations between both soils differ significantly though. Thus, in situ data cannot answer this question in total as it is not clear how nitrite oxidation is influenced by As and Pb. To address these points in detail, further studies are needed, including (1) analysis of mRNA, (2) using ^{15}N-labeled ammonium, and (3) studying the effects of HM on nitrite oxidation.

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Publication III

Effects of repeated application of sulfadiazine-contaminated pig manure on the abundance and diversity of ammonia- and nitrite oxidizers in the root-rhizosphere complex of pasture plants under field conditions

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Running title: SDZ effects on rhizosphere microbes

Abstract

Nitrification is a two-step process consisting of: (i) the oxidation of NH_4^+ to NO_2^- by ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) and (ii) the oxidation of NO_2^- to NO_3^- by nitrite-oxidizing bacteria (NOB). In a field experiment, we investigated the impact of repeated application of the antibiotic sulfadiazine (SDZ)-contaminated pig manure on functional microbial communities involved in ammonia and nitrite oxidation in the root-rhizosphere complexes (RRCs) of diverse plants composing a pasture. We surveyed the abundance of ammonia-oxidizing archaea (AOA) and bacteria (AOB) as well as Nitrobacter- and Nitrospira-like nitrite-oxidizing bacteria (NOB) by quantitative PCR, and the diversity of amoA AOA and Nitrobacter-like nxrA amplicons using a cloning-sequencing approach. Whereas the first SDZ-contaminated manure application caused only slight effects on the investigated microbial communities and did not change the diversity and abundance pattern significantly, the second application of SDZ-contaminated manure induced pronounced effects compared to the control treatment where non-contaminated manure was applied, and resulted in an up to 15 fold increased ratio of AOA:AOB and a reduction of nrxA genes. The diversity of AOA amoA increased after the second application of SDZ-contaminated manure compared to the control treatment whereas a clear reduction of nrxA OTUs was visible in the same samples. Thus, our results indicate that mainly nitrite oxidation by NOB might be affected by the application of SDZ and alternative pathways like nitrite reduction might be favored under these conditions.

Keywords: sulfadiazine, rhizosphere, ammonia-oxidizing microbes, nitrite-oxidizing bacteria, nitrification

1. Introduction

Nitrification rates in soils can be considered as an important indicator for sustainable use. While the base product for this process, ammonium respectively ammonia, is of high importance for plant nutrition and biomass formation, the end product, nitrate, often causes significant losses of nitrogen as well as huge environmental problems including contamination of groundwater by leaching or the formation of the green house gas N_2O by denitrifying microbes (Ollivier et al., 2011). Nitrification is a two step processes including (i) the oxidation of NH_4^+ to NO_2^- via hydroxylamine by ammonia-oxidizing microbes (Kowalchuk and Stephen, 2001;Leininger et al., 2006) and (ii) the oxidation of NO_2^- to NO_3^- by nitrite-oxidizing bacteria (NOB) (Prosser, 1989). Ammonia oxidation and nitrite oxidation are both performed by phylogenetically well separated microorganisms. Thus, ammonia oxidation is performed by autotrophic bacteria belonging to two specific groups of β- and γ-proteobacteria (Bock and Wagner, 2006) and archaea recently assigned to the phylum Thaumarchaeota (Spang et al., 2010); NOB are broadly distributed among the α-, β-, γ-, and δ-proteobacteria as well as the Nitrospira phylum (Spieck and Bock, 2005).

Despite the fact that an efficient nitrification requires the presence of both ammonia oxidizers and nitrite oxidizers, most studies in the past have been dedicated to understand factors driving abundance, diversity and activity of ammonia oxidizers. ISI Web of Knowledge reveals almost 2500 articles using the keywords “ammonia oxidation” and “soil”, whereas only 600 hits were found using “nitrite oxidation” and “soil”. Studies where both processes were investigated using the same samples are rare, and include less than 30 articles in peer reviewed journals. The

69 reason for this strong focus on ammonia oxidation is mainly related to several studies from the
70 last century where the oxidation of ammonia has been considered as rate limiting for the whole
71 process of nitrification (Prosser, 1989). Main findings from that time include varying copy
72 numbers of AOB (Phillips et al., 2000) and nitrite concentrations below the detection limit in
73 many soil samples (Burns et al., 1995), indicating that once nitrite is formed it is more or less
74 quickly further oxidized to nitrate. However at that time, the existence of ammonia oxidizing
75 archaea was not proven and ammonia oxidation in soil was essentially related to some
76 proteobacteria.

77 With the detection of AOA, the paradigm of nitrification changed and a number of new
78 questions has been raised since then, with regards to the role of AOA for nitrification including
79 (i) the transformation of hydroxylamine (NH_2OH) to nitrite by AOA as no homolog of bacterial
80 HAO gene (hao) encoding the enzyme catalyzing the oxidation of NH_2OH to NO_2^- has been
81 found in the genome of AOA so far, (ii) the functional role of the described nirK sequences from
82 AOA, (iii) possible pathways indicating mixotrophy of AOA, and (iv) the interplay between
83 AOA and NOB. These potential differences between AOA and AOB may result in dissimilarities
84 in the response of these communities to inhibitive agents (Schauss et al., 2009). Therefore the
85 aim of this study was to investigate the response of ammonia (AOA and AOB) and nitrite
86 oxidizers (NOB) to a repeated application of antibiotics (sulfadiazine; SDZ) in the root
87 rhizosphere complex (RRC) of a mixture of typical grassland plant species at different plant
88 development stages during the growing season in a field study, and thus to link the data to
89 ammonium and nitrate fluxes in soil. In order to assess changes in the genetic potential, we
90 analyzed the abundance pattern of genes encoding key enzymes of ammonia oxidation (amoA
91 encoding the ammonia monooxygenase) in both AOB and AOA, and nitrite oxidation in

92 Nitrobacter-like NOB (nxrA encoding the nitrite oxidoreductase). Nitrospira-like NOB were
93 quantified based on 16S rRNA genes. Moreover, the functional diversity of archaeal amoA and
94 respectively Nitrobacter-like nxrA, was determined using a cloning-sequencing approach.
95 As the administration of antibiotics to treat infectious diseases is a common practice in
96 animal husbandry, substances like the sulfonamide, which is mainly used in pig production
97 (Burkhardt et al., 2005), are poorly adsorbed by the animal and excreted mostly unaltered in urine
98 and feces together with various metabolites (Elmund et al., 1971;Alcock et al., 1999;Halling-
99 Sørensen, 2001;Lamshöft et al., 2007), and thus reach the soil ecosystem via manuring. In
100 inhibiting the folic acid metabolism, SDZ impairs growth of most Gram-positive and many
101 Gram-negative bacteria (Brown, 1962). The occurrence of SDZ in soil might therefore alter the
102 microbial community structure as well as the activity pattern, and modify kinetics of important
103 turnover processes such as nitrogen (N) cycling. We hypothesized that SDZ affects mainly the
104 ratio between archaeal and bacterial ammonia oxidizers, whereas Nitrospira- and Nitrobacter-
105 like nitrite oxidizers are both inhibited by the application of the contaminated manure, despite
106 their different phylogenetic classification. Therefore, under antibiotic pressure, we postulate that
107 nitrite oxidation might be the rate limiting step of nitrification.
108
109 **2. Materials and methods**
110 **2.1. Experimental design**
111 An agricultural field located near Merzenhausen, Germany, (50° 56′ 3″ N, 6° 17′ 31″ E)
112 which was never fertilized with manure before, and therefore has never been in contact with
113 SDZ, was chosen as experimental site. The soil has been characterized as a silt loam (Orthic
114 Luvisol; Table 1). The experiment was setup as a randomized split plot design with a mixture of

115 pasture plants (47% Lolium perenne, 17% Phleum pratense, 20% Festuca pratensis, 10% Poa
116 pratensis, and 6% Trifolium repens) using manure from untreated pigs (PM) and SDZ-treated
117 pigs (PMSDZ) respectively with four replicates for each variant, resulting in 8 plots in total.
118 Manure was applied twice during the vegetation season (applied total N ranged from 16 to 19
119 g N m^{-2} and from 3 to 6 g N m^{-2}, respectively; Table 2). The first manure application (30 m^3 ha^{-1})
120 was completed in May 2009. The second application (10 m^3 ha^{-1}) was done 48 days later. Pasture
121 plots were cut one day prior the second manure application. The amount of SDZ applied in
122 treatments PMSDZ was equivalent for both applications to 100 mg SDZ m^{-2}. The amount of SDZ
123 recovered from soil during the experimental period declined quickly after each manure
124 application, as already described by Rosendahl and colleagues (2011).
125 Rhizosphere samples were collected from all plots at day 1 (after the first application of the
126 manure) 7, 14, 42, 49 (1 day after the second manure application), 56, 63, and 106 (8, 15, and 58
127 days after the second manure application, respectively). For each plot, ten subsamples were
128 collected, mixed, and homogenized to obtain one sample per plot. Samples taken from plots with
129 the same treatments were used as true replicates. After shaking the roots vigorously, the root-
130 rhizosphere complex samples (RRC; roots and adhering soil) were divided into two sub-samples.
131 One part was immediately shock-frozen in liquid nitrogen and stored at -80°C for nucleic acid
132 extraction, the other part was directly extracted with 0.01 M $CaCl_2$ for the determination
133 ammonium-N (NH_4^+-N) and nitrate-N (NO_3^--N) concentrations.
134
135 **2.2. Inorganic nitrogen fraction in the RRC**
136 Three hundred mg of RRC was shaken overhead for 30 min with 5 ml of 0.01 M $CaCl_2$. After
137 filtration, ammonium-N and nitrate-N measurements were performed on Nanocolor 300D

138 photometer from Macherey Nagel (Germany) by using the Nanocolor Ammonium 3 kit and the
139 Nanocolor nitrate 50 kit, respectively (Macherey Nagel, Germany).
140
141 **2.3. Nucleic acid extraction**
142 RRC DNA was directly extracted after a bead beater lysis step (Bertin Technologie, France),
143 using the FastDNA SPIN kit for soil (MP biomedicals, Germany). Quality and quantity of the
144 extracted DNA were checked with a spectrophotometer (Nanodrop, PeqLab, Germany).
145
146 **2.4. Abundance of functional genes**
147 Quantitative PCR (qPCR) of genes encoding key enzymes of ammonia oxidation (amoA
148 encoding the ammonia monooxygenase) in both ammonia-oxidizing bacteria (AOB) and archaea
149 (AOA), and nitrite oxidation in Nitrobacter-like NOB (nxrA encoding the nitrite oxidoreductase)
150 was used to determine the density of the functional communities involved in nitrification. In
151 addition, the abundance of Nitrospira-like NOB was quantified targeting 16S rRNA Nitrospira
152 gene since no primers targeting Nitrospira-like nxrA were available (Wertz et al., 2012). An
153 absolute quantification of all investigated target genes using a SYBR® Green I-based detection
154 (Applied Biosystems, Germany) was carried out in 25 µL in triplicates on the ABI Prism 7300
155 Cycler (Applied Biosystems). The reaction mixture consisted of 15 µg bovine serum albumin
156 (Sigma-Aldrich, Germany), 0.2 µM of each primer for amoA AOA, nxrA and 16S rRNA
157 Nitrospira gene amplification and 0.3 µM of each primer for amoA AOB amplification,
158 respectively (Metabion, Germany), 1x Power SYBR Green PCR master mix (Applied
159 Biosystems), and 40 ng DNA template. All PCR reactions started with an initial enzyme
160 activation step performed at 95°C for 10 min. The subsequent thermal profile was different for

each gene amplified (Table 3). The specificity of the amplification products was confirmed by melting-curve analysis and migration on 2% agarose gel. No template controls gave null or negligible values. To avoid inhibitory effects on quantitative PCR, samples were diluted 10-fold based on a preexperiment (data not shown). Dilution series of a plasmid with cloned Nitrosomonas multiformis ATCC25196 amoA gene (amoA AOB), the fosmid clone 54d9 (Leininger et al., 2006) for archaeal amoA, Nitrobacter hamburgensis X14 (DSMZ 10229) nxrA gene (Nitrobacter-like nxrA), Nitrospira 16S rRNA gene (Accession No. FJ529918) (Nitrospira-like 16S rRNA gene), were used to generate respective standard curves ranging from 10^1 to 10^6 gene copies μl^{-1} with efficiencies ranging from 94 to 98%, 98% to 100%, 93 to 98%, and 93% to 99%, respectively.

2.5. Cloning and sequencing of archaeal amoA and nitrobacter-like nxrA fragments sequences and phylogenetic analysis

Prior to PCR amplification, replicates corresponding to the treatments PM and PMSDZ at the time points 1, 49 and 106 days after the first manure application, were pooled together to constitute one sample corresponding to one treatment at one time point.

Archaeal amoA and nxrA gene amplicons were generated by PCR using the primers described for qPCR assay (Table 3). The reaction mixture (50 µL) contained 1X PCR buffer, 1X CoralLoad concentrate, 1X Q-solution, 1 U TopTaq (Qiagen, Germany), 200 µM of each dNTP, 0.2 µM of each primer, and 30 ng template DNA. The PCR thermocycling program for nxrA amplification was 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 m, and a final elongation step at 72°C for 10 min. A similar program was used for amoA AOA amplification with an annealing temperature of 50°C. The cloning was carried out using the TA

184 cloning kit (Invitrogen, Germany) in accordance with the manufacturer's instructions. 30 clones
185 were picked randomly for each treatment and time point. Plasmids were extracted using the
186 NucleoSpin plasmid kit (Machery-Nagel, Germany). Inserts from clones amplified with specific
187 primers (M13 forward and M13 reverse) using the BigDye Terminator cycle sequencing kit
188 (Applied Biosystems) were purified by ethanol precipitation. amoA AOA and nxrA fragments
189 were sequenced using an ABI 3730 DNA analyzer (Applied Biosystems). Sequences were run
190 through a mega BLAST search (http://blast.ncbi.nlm.nih.gov/ Blast.cgi) using the nr database and
191 were deposited in the Genbank with the accession numbers KC137376-KC137546 and
192 KC152658-KC152839 for amoA and nxrA, respectively. For further analysis nucleotide
193 sequences were transcribed to aminoacid sequences. These were aligned using clustal W protein
194 alignment (Thompson et al., 1994) implemented in ARB (Ludwig et al., 2004). The nucleotide
195 sequences were realigned according to aligned protein sequences. DNA based maximum
196 likelihood trees were reconstructed applying PhyML (Guindon and Gascuel, 2003) implemented
197 in ARB. Rarefaction curves were created using Mothur for a distance of 0.02 (98% similarity
198 level) (Schloss et al., 2009). The clustering of amoA AOA was done according to Pester and
199 colleagues (2012).
200
201 **2.6. Statistical analysis**
202 Prior to analysis, gene abundance data were ln-transformed to achieve normal
203 distribution. Independent T-tests were used to test for a significant difference between the two
204 treatments at a given time point with significance level corrected by the Šidák's equation to $\alpha = 1$
205 $- (1 - 0.05)^{1/4} = 0.013$ (Šidák, 1967), as each manure application was followed by four sampling
206 time points. Statistical tests were calculated in SPSS 11.5 (SPSS, Inc., Illinois, USA).

207

208 **3. Results**

209 **3.1. Inorganic nitrogen**

210 The first manure application resulted, independent from the level of SDZ contamination in
211 the highest NH_4^+-N concentrations measured during the experimental period shortly after the
212 application at day 1 (up to 22.8 µg NH_4^+-N per gram of dry weight RRC). Only one week later
213 the values dropped to below 10 µg NH_4^+-N g^{-1} RRC in the PM treatments respectively below 5
214 µg NH_4^+-N g^{-1} RRC in the PMSDZ treatments. However differences between the two treatments
215 were not significant. This level remains constant during the experimental period; the second
216 manure application at day 49 did not influence NH_4^+-N concentrations in the RRC (Figure 1).

217 NO_3^--N concentrations peaked independent from the treatment 14 days after the first
218 application of manure (up to 35.4 µg NO_3^--N g^{-1} RRC). Towards day 42, the values dropped
219 sharply and were close to the detection limit in some of the replicates. Similarly to the
220 observation regarding NH_4^+-N concentrations, the second manure application had no effect on the
221 NO_3^--N concentrations in the RRC independent of the treatment (Figure 1).

222

223 **3.2. Gene abundance**

224 The first application of manure did not influence amoA gene copy numbers for AOA and
225 AOB 1, 7 and 14 days after application. Higher copy numbers (in the range of 6.5 x 10^7 copy
226 numbers per gram of dry weight RRC) were measured for AOB; AOA copy numbers were at the
227 same time points slightly lower (in the range of 2.5 x 10^7 copies g^{-1} RRC) resulting in an
228 AOA:AOB ratio of 0.4. No influence of SDZ was visible at these time points either on AOA or
229 AOB. However, 42 days after application, AOB amoA gene copy numbers in the treatments with

control manure (PM) increased up to 1.8 x 10^8 copies g^{-1} RRC, whereas in the plots where contaminated manure (PMSDZ) was applied no changes were visible compared to the earlier time points. AOA amoA gene abundance did not differ 42 days after application of the manure compared to the earlier time points. Therefore, at this time point AOA:AOB ratio in the PM treatment was the lowest measured (0.1). After the second manure application amoA AOA and AOB copy numbers in the RRC of plants from the PM treated plots were comparable, as amoA AOA copy numbers increased compared to earlier sampling time points (ratio AOA:AOB = 1). A clear influence of the antibiotic was visible on both AOA and AOB at all time points investigated. Copy numbers for amoA AOB decreased in PMSDZ plots compared to the control samples. Thus, values were in the range of 2 x 10^7 gene copies g^{-1} RRC in PMSDZ plots compared to 7 x 10^7 g^{-1} RRC in the plots treated with PM. In contrast AOA amoA gene copy numbers increased significantly in the RRC of plants in PMSDZ treated plots compared to control plots, with gene copy numbers in the range of 3 x 10^8 copies g^{-1} RRC, whereas copy numbers in the control treatment were approximately 1 x 10^8 copies g^{-1} RRC. Interestingly, the described effect was stable at day 106. Consequently AOA:AOB ratio increased up to 15 after the second application of SDZ-contaminated manure.

Copy numbers for 16S rRNA genes from Nitrospira ranged from 6.1 x 10^6 to 2.3 x 10^7 gene copies g^{-1} RRC during the experimental period. The highest gene copy numbers were measured after the first manure application at day 1. The lowest gene copy numbers were detected at the end of the experimental period at day 106. The second manure application did not increase 16S rRNA gene copies of Nitrospira. Surprisingly SDZ had no influence on the abundance of Nitrospira over the experimental period. The abundance of nxrA genes from Nitrobacter were 2 orders of magnitude lower compared to 16S rRNA gene copy numbers of Nitrospira. Overall

253 none of the two manure applications changed the nxrA gene abundance significantly. Copy
254 numbers ranged at all time points between 1.0 x 10^5 and 2.3 x 10^5 copies g^{-1} RRC in PM treated
255 plots. We observed a tendency for reduced gene copies 49 days after the first application of the
256 PMSDZ compared to the control PM treatment group (P=0.030). All data are summarized in
257 Figure 2.
258
259 **3.3. Diversity of AOA amoA and Nitrobacter-like nxrA genes**
260 The number of 30 clones per library that have been sequenced per treatment were not
261 enough to cover the total diversity of AOA amoA OTUs present in the samples as indicated by
262 the rarefaction curves (Figure 3). The total number of OTUs for amoA AOA varied from 12 to 14
263 in the PM treated plots, independent from the time point of sampling. In contrast in the PMSDZ
264 treatment, the number of OTUs decreased between day 1 and day 49, and thereafter increased
265 between day 49 and day 106 after the first application. Overall, 85% of the sequences sampled
266 were contained in the Nitrosphaera subclusters 1, 4, and 9; and the Nitrosphaera cluster (soil
267 metagenome fragment 54d9). The Nitrosphaera subclusters 1 and 4 were represented by
268 sequences from all treatments and time points, respectively. However in the Nitrosphaera
269 subcluster 9, the abundance of sequences from the treatment PMSDZ one day after the second
270 manure application (day 49) was higher compared to PM. In contrast, in the Nitrosphaera cluster
271 (soil metagenome fragment 54d9), at time point 106 days, the abundance of sequences from the
272 control treatment was higher compared to PMSDZ (Figure 4, Figure S1).
273 The number of different OTUs for nrxA was lower compared to AOA amoA. Therefore
274 the investigated 30 clones per library nicely reflected the diversity present in the samples and the
275 collector's curves indicated saturation. The total number of OTUs for nrxA ranged between 4 and

11. However the diversity pattern observed followed the opposite trend compared to AOA amoA. The highest number of OTUs was observed in the treatments with control manure one day after the second application (day 49). The lowest number of OTUs was observed at the last sampling time point (day 106) in the PMSDZ treated plots. However at this time point also in the control samples the number of OTUs was already reduced compared to day 49 (Figure 3). Four major clusters containing 89% of the total nxrA sequences sampled were determined (I, II, IV, and V; Figure 4, Figure S2). Each individual cluster was represented by sequences from both treatments and all three time points. The relative abundance of sequences from the treatment where contaminated manure was added one day after the second application of the manure was higher in cluster I compared to the control treatment. A similar picture was observed for the last sampling time point (day 106) for cluster IV. An opposite trend was observed in cluster V where a higher number of sequences was found from the control treatment at the last sampling time point (day 106).

4. Discussion

4.1. Effects on ammonia oxidizers

The antibiotic tended to abolish the increase of the ammonia-oxidizing bacterial population after 42 days in response to the manure application in RRC of the pasture plants (P=0.091) (Figure 2). Similar results were shown in bulk soil (Schauss et al., 2009) and in the rhizosphere of agricultural crops (Ollivier et al., 2010). These results demonstrate that SDZ clearly inhibited the growth of AOB. The response of bacterial and archaeal ammonia oxidizers respectively differed one day after the second SDZ-contaminated manure application. From this time point, the amoA AOB and AOA gene abundance patterns were decreased and increased, respectively (Figure 2).

299 Although it has been already shown that AOA were impacted to a lower extent by SDZ than
300 AOB in greenhouse experiments (Schauss et al., 2009;Ollivier et al., 2010), in this study we
301 observed for the first time a significant increase in amoA AOA gene copies ($P = 0.005$ at day 56)
302 while amoA AOB gene copies were significantly decreased ($P = 0.005$ and $P = 0.013$ at day 49
303 and 63, respectively) with PMSDZ treatment, confirming the potential occurrence of functional
304 redundancy between the two communities under antibiotic stress (Schauss et al., 2009).
305 Possibly, the reduced susceptibility of AOA to PMSDZ can be explained by a shift in the
306 AOA diversity towards more SDZ resistant phylotypes over time, as amoA diversity has been
307 shown to well reflect phylogeny of AOA (Nicol et al., 2008). Thus, whereas 1 day after the first
308 manure application, a similar number of clones from each treatment was observed in the
309 Nitrosphaera subcluster 9, after 49 days a higher relative abundance of clones was sampled in
310 PMSDZ compared to PM (Figure 4). However, the development of antibiotic resistance in AOA
311 has not been described so far, as the first cultures of soil AOA, which may form the basis for such
312 studies, have been isolated only recently (Jung et al., 2011;Tourna et al., 2011;Kim et al., 2012).
313
314 **4.2. Effects on nitrite oxidizers**
315 Because of the broad spectrum nature of the SDZ, we hypothesized that Nitrospira- and
316 Nitrobacter-like nitrite oxidizers are both inhibited by the application of the contaminated
317 manure. However, parallel to the inhibitory effects directly affecting the functional communities
318 investigated, e.g. related to their respective activity status and related susceptibility (Lewis,
319 2007;Ollivier et al., 2010) or to their abilities to regulate their internal pH, which affect the
320 accumulation and speciation of the SDZ in the cells (Tappe et al., 2008;Zarfl et al., 2008),
321 dissimilar ecological strategies for survival and proliferation among these populations may

322 explain their response to the antibiotic stress. While niche differentiation and competition is
323 known to influence the composition of functional microbial communities, the components of the
324 nitrite oxidizing communities investigated respectively in this study possess different substrate
325 affinities and therefore are adapted to distinct N availabilities. It has been suggested that
326 Nitrobacter-like NOB bacteria are r-strategists with higher growth rate/specific activity and lower
327 affinity for nitrite and oxygen, whereas Nitrospira-like NOB are K-strategists with a higher
328 substrate affinity (Schramm et al., 1999;Attard et al., 2010). However, Maixner and colleagues
329 (2006) have shown that the nitrite concentration influences the structure of Nitrospira-like
330 bacterial communities, and assumed that sublineages may occupy different positions on an scale
331 reaching from K- to r-strategists within the genus Nitrospira. In the RRC of the pasture plants
332 from 42 days after the first manure application, the reduction of AOB abundance and activity
333 may have resulted in lower nitrite availability and consequently favorable conditions for
334 Nitrospira-like NOB compared to Nitrobacter-like NOB, explaining the reduction of
335 Nitrobacter-like nxrA abundance ($P = 0.030$) and the increase of Nitrospira 16S rRNA gene
336 abundance ($P = 0.036$) at day 49 with PMSDZ treatment. However the release of organic
337 substrates from the dead microbial biomass under the antibiotic treatment could have also
338 influenced indirectly the abundance of different Nitrospira sublineages as some Nitrospira-like
339 bacteria are mixotrophic (Daims et al., 2001).
340 A community shift due to SDZ was observed for Nitrobacter-like NOB at time point 49 days
341 in cluster I. Moreover, the antibiotic treatment had a long lasting effect on these communities as
342 differences in relative abundance in cluster IV and V between the two treatments were still
343 observed after 106 days after the first application of manure, as well as a decrease in diversity
344 (Figure 3). Therefore, whereas SDZ resistant phylotypes might have developed in response to the

345 antibiotic stress, the studied system was not shown to recover in terms of microbial diversity after
346 the PMSDZ treatment during the experimental period, which may imply negative effects on the
347 ability of the soil to respond to future disturbances (Ives et al., 2000;McCann, 2000).
348
349 **4.3. Consequences for nitrification and N turnover**
350 This field experiment revealed that the application of manure contaminated with the
351 antibiotic SDZ has a lasting effect on the abundance and diversity of nitrifying microbial
352 communities. However, although SDZ impaired the growth of certain microbial populations, no
353 significant effect of the treatment PMSDZ was visible on the concentrations of nitrate and
354 ammonium in the rhizosphere of the plants composing the pasture. Difference in tolerance
355 between the different microbial functional communities, or the development of resistant
356 populations (Heuer et al., 2011) could contribute to functional redundancy of ammonia oxidizing
357 microbes (Schauss et al., 2009) and NOB, and therefore to the stability of nitrification pattern in
358 the rhizosphere. However, the studied system was not shown to recover in terms of nxrA diversity
359 after the PMSDZ treatment during the experimental period, which could indicate that mostly
360 nitrite oxidation by NOB might be affected by the application of SDZ. Possibly, alternative
361 pathways like nitrite reduction might be favored under these conditions; as many soil microbes
362 are able to use nitrite as terminal electron acceptors under anaerobic conditions, the extent of the
363 SDZ-contamination effects on denitrification may be reduced.
364
365
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368

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511 **Figures legends**

512 **Figure 1.** Ammonium and nitrate concentrations in the root-rhizosphere complex (RRC)
513 of the plants composing a pasture after addition of pig manure (PM; full circles, ●) or SDZ-
514 contaminated pig manure (PMSDZ; empty circles, ○) at 4 different time points after the first
515 application (day 1, 7, 14, and 42), respectively the second application (day 49, 56, 63, and 106).
516 Significant differences between the two treatments at a particular time point are indicated by
517 asterisks (*). Error bars represent standard deviation of mean (n = 4).

518 **Figure 2.** Quantification of amoA (AOB and AOA), Nitrobacter-like nxrA and
519 Nitrospira-like 16S rRNA gene in the root-rhizosphere complex of the plants composing a
520 pasture after addition of pig manure (PM, blue bars) or SDZ-contaminated pig manure (PMSDZ,
521 gray bars) at 4 different time points after the first application (day 1, 7, 14, and 42), respectively
522 the second application (day 49, 56, 63, and 106). The arrow indicates the second application at
523 day 48. Significant differences between the two treatments at a particular time point are indicated
524 by asterisks (*). Error bars represent standard deviation of mean (n = 4). Abbreviations: RRC,
525 root-rhizosphere complex.

526 **Figure 3.** Rarefaction curves obtained at three different time points (1, T1; 49, T2; and
527 106 days, T3) after application of pig manure (PM) or SDZ-contaminated pig manure (PMSDZ)
528 for amoA AOA and Nitrobacter-like nxrA sequences, i.e. number of OTUs as a function of the
529 number of the sequences sampled in each library.

530 **Figure 4.** Maximum likelihood trees of partial amoA AOA (A) and Nitrobacter-like nxrA
531 (B) nucleic acid sequences (624 and 322 bp, respectively) obtained at three different time points

532 (1, blue bars; 49, red bars; and 106 days, grey bars) after application of pig manure (PM) or SDZ-
533 contaminated pig manure (PMSDZ) and reference sequences. AOA amoA clustering was done
534 according to Pester and colleagues (2012). Ungrouped trees can be found in Figure S1 and S2.

535

536 **Tables**

537 **Table 1:** Physical and chemical characterization of the soil used in the experiment

Parameter	
Clay (%)	15.4
Silt (%)	78.2
Sand (%)	6.4
WHC_{max} (%)	45.8
C_{org} (%)	2.1
pH ($CaCl_2$)	7.2

538

539

540

541 **Table 2:** Chemical characterization of the pig manures either contaminated with SDZ

542 (PMSDZ) or not (PM) applied in the experiment.

Manure	pH	Total N*	NH_4-N*	P_2O_5*	K_2O*	MgO*	CaO*
first application							
PM	8.0	6.19	3.66	4.21	3.77	2.57	2.89
PMSDZ	7.6	5.43	4.46	3.35	3.30	1.96	2.87
second application							
PM	8.7	5.50	3.13	4.02	3.90	2.61	2.88
PMSDZ	8.3	3.47	2.39	1.05	3.54	0.72	1.16

543 *kg/m^3

544

545

546 **Table 3:** Primers and thermal profiles used for real-time PCR quantification of bacterial

547 and archaeal amoA, Nitrobacter-like nxrA and Nitrospira-like 16S rRNA gene.

Target gene	Primer Set	Reference	Thermal Profile	Cycles	Amplicons size
AOB amoA	amoA-1F amoA-2R	(Rotthauwe et al., 1997) (Rotthauwe et al., 1997)	94°C/60 s, 58°C/60 s, 72°C/60 s	40	500 bp
AOA amoA	19F CrenamoA616r48x	(Leininger et al., 2006) (Schauss et al., 2009)	94°C/45 s, 55°C/45 s, 72°C/45 s	40	624 bp
Nitrobacter nxrA	F1norA R2norA	(Poly et al., 2008) (Wertz et al., 2008)	94°C/30 s, 55°C/30 s, 72°C/30 s	40	322 bp
Nitrospira 16S	Nspra675f Nspra746r	(Graham et al., 2007) (Graham et al., 2007)	94°C/30 s, 64°C/30 s, 72°C/60 s	40	71 bp

548

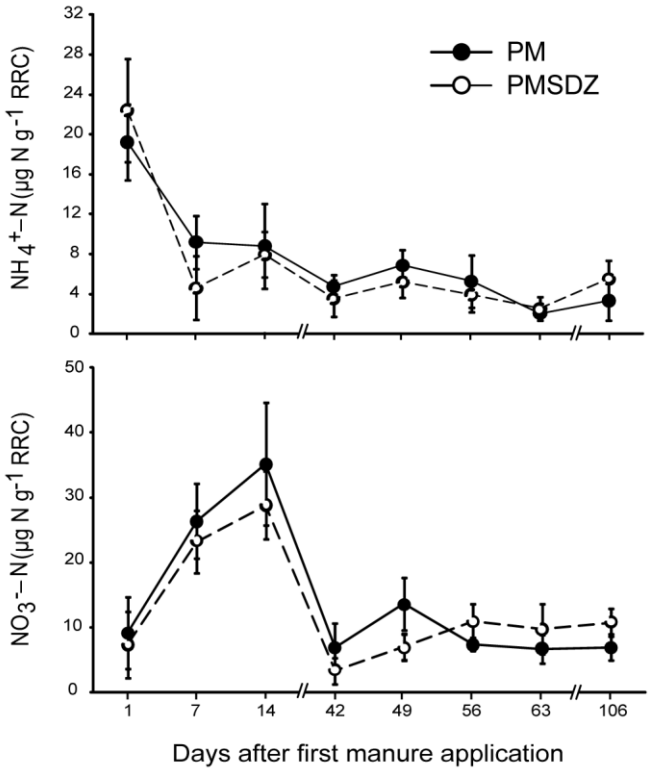
PM
PMSDZ
NH_4^+–N(µg N g^{-1} RRC)
NO_3^-–N(µg N g^{-1} RRC)
Days after first manure application

Figure 1

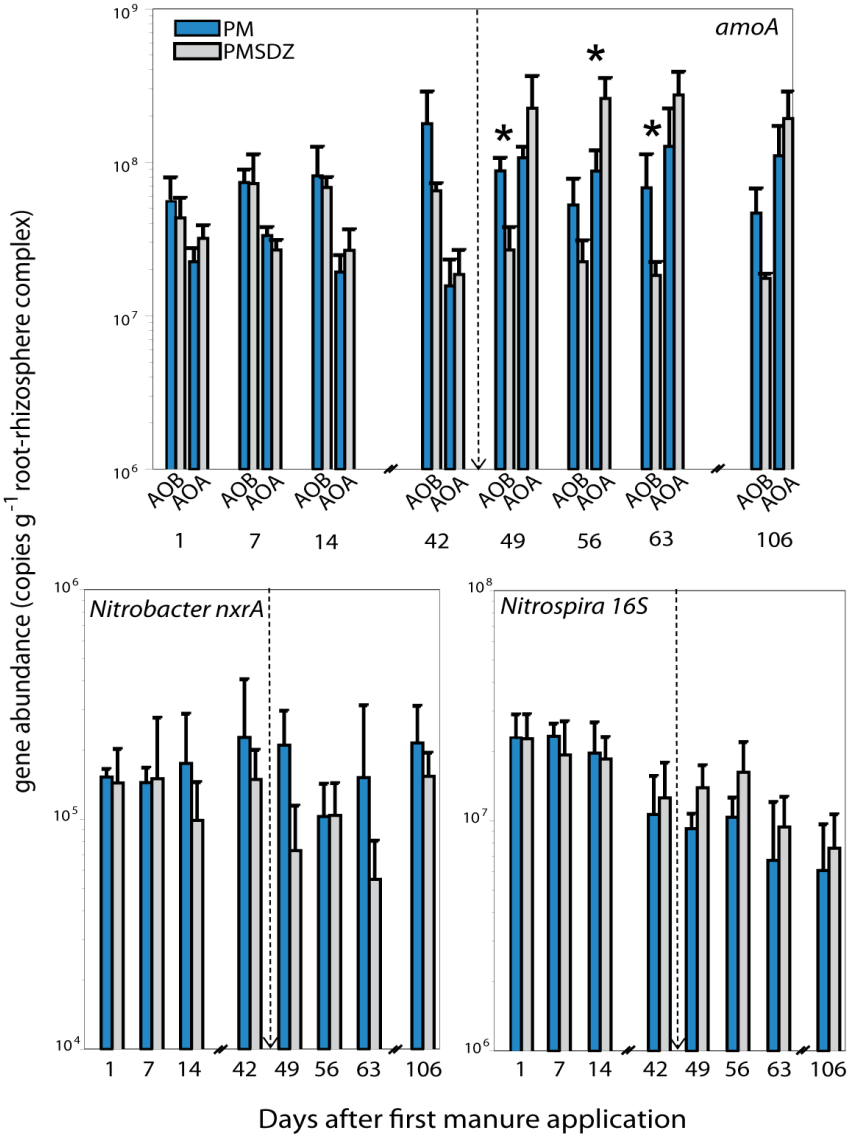
PM
PMSDZ
amoA
Nitrobacter nxrA
Nitrospira 16S
AOB
AOA
gene abundance (copies g-1 root-rhizosphere complex)
Days after first manure application
1
7
14
42
49
56
63
106

Figure 2

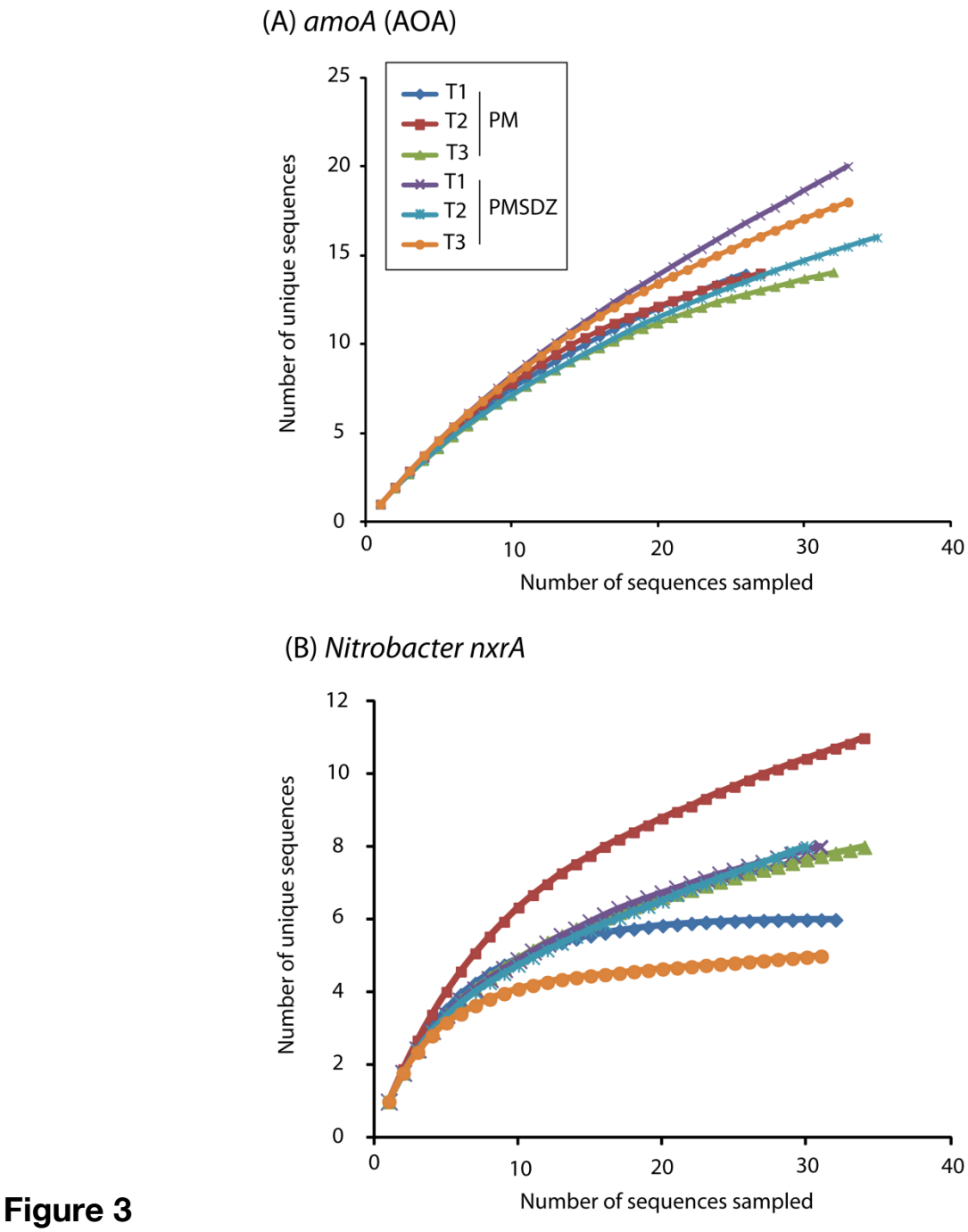
(A) amoA (AOA)
T1
T2
T3
PM
T1
T2
T3
PMSDZ
Number of unique sequences
Number of sequences sampled
0
5
10
15
20
25
0
10
20
30
40
(B) Nitrobacter nxrA
Number of unique sequences
Number of sequences sampled
0
2
4
6
8
10
12
0
10
20
30
40

Figure 3

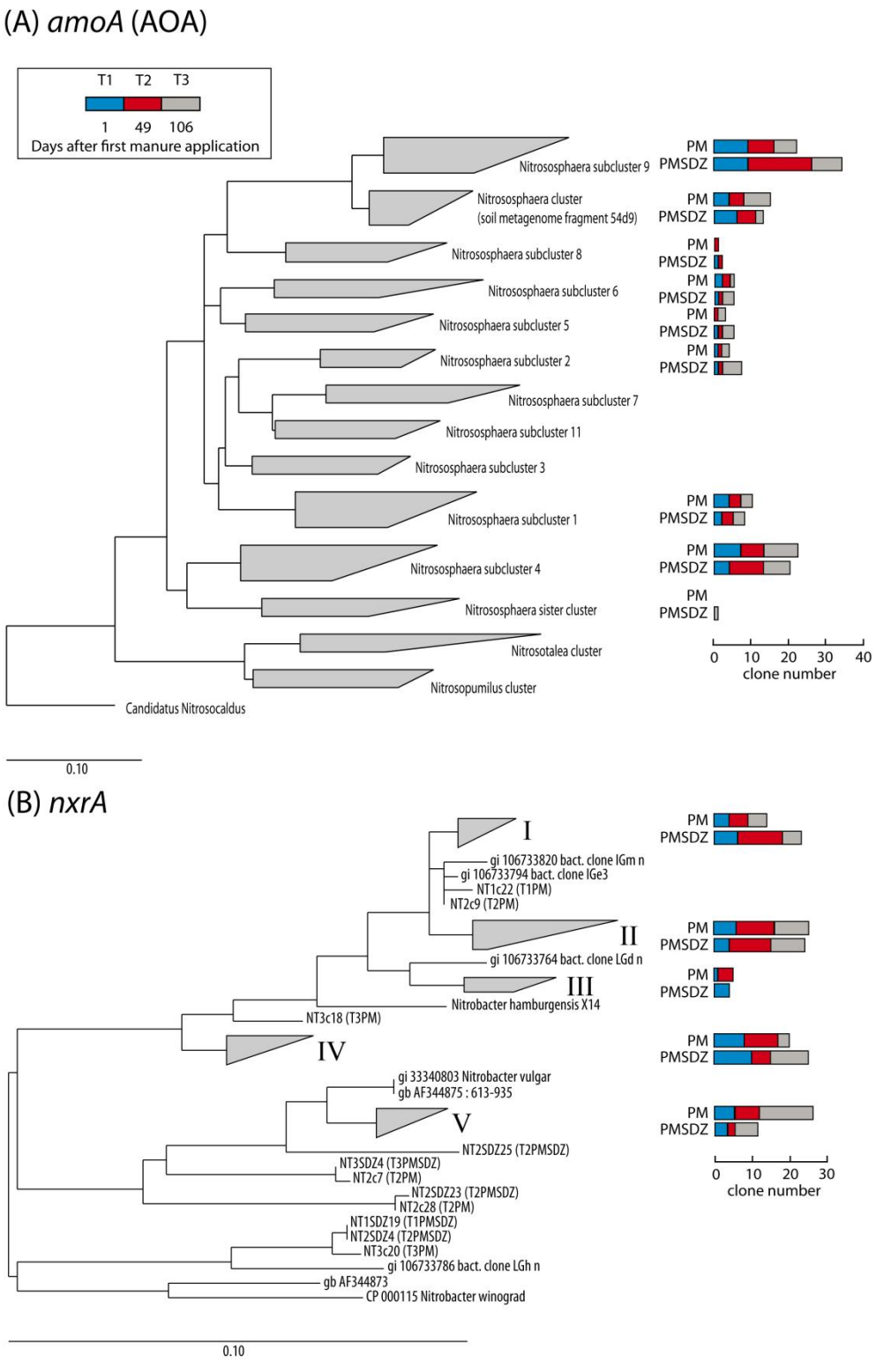
(A) amoA (AOA)
T1 T2 T3
1 49 106
Days after first manure application
Nitrososphaera subcluster 9
Nitrososphaera cluster
(soil metagenome fragment 54d9)
Nitrososphaera subcluster 8
Nitrososphaera subcluster 6
Nitrososphaera subcluster 5
Nitrososphaera subcluster 2
Nitrososphaera subcluster 7
Nitrososphaera subcluster 11
Nitrososphaera subcluster 3
Nitrososphaera subcluster 1
Nitrososphaera subcluster 4
Nitrososphaera sister cluster
Nitrosotalea cluster
Nitrosopumilus cluster
Candidatus Nitrosocaldus
PM
PMSDZ
0 10 20 30 40
clone number
0.10
(B) nxrA
I
gi 106733820 bact. clone lGm n
gi 106733794 bact. clone lGe3
NT1c22 (T1PM)
NT2c9 (T2PM)
II
gi 106733764 bact. clone LGd n
III
Nitrobacter hamburgensis X14
NT3c18 (T3PM)
IV
gi 33340803 Nitrobacter vulgar
gb AF344875 : 613-935
V
NT2SDZ25 (T2PMSDZ)
NT3SDZ4 (T3PMSDZ)
NT2c7 (T2PM)
NT2SDZ23 (T2PMSDZ)
NT2c28 (T2PM)
NT1SDZ19 (T1PMSDZ)
NT2SDZ4 (T2PMSDZ)
NT3c20 (T3PM)
gi 106733786 bact. clone LGh n
gb AF344873
CP 000115 Nitrobacter winograd
0 10 20 30
clone number
0.10

Figure 4

Publication IV

FEMS MICROBIOLOGY ECOLOGY

MINIREVIEW

Nitrogen turnover in soil and global change

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nitrogen cycle; nitrification; denitrification; nitrogen fixation; mineralization.

Abstract

Nitrogen management in soils has been considered as key to the sustainable use of terrestrial ecosystems and a protection of major ecosystem services. However, the microorganisms driving processes like nitrification, denitrification, N-fixation and mineralization are highly influenced by changing climatic conditions, intensification of agriculture and the application of new chemicals to a so far unknown extent. In this review, the current knowledge concerning the influence of selected scenarios of global change on the abundance, diversity and activity of microorganisms involved in nitrogen turnover, notably in agricultural and grassland soils, is summarized and linked to the corresponding processes. In this context, data are presented on nitrogen-cycling processes and the corresponding microbial key players during ecosystem development and changes in functional diversity patterns during shifts in land use. Furthermore, the impact of increased temperature, carbon dioxide and changes in precipitation regimes on microbial nitrogen turnover is discussed. Finally, some examples of the effects of pesticides and antibiotics after application to soil for selected processes of nitrogen transformation are also shown.

Introduction

Nitrogen is one of the crucial nutrients for all organisms (LaBauer & Treseder, 2008), as it is an essential component of important biopolymers. However, most of the N in nature occurs as dinitrogen gas or is fixed in organic compounds, like proteins or chitin, both of which cannot be directly used by plants and animals. Only specialized microorganisms are able to transform the gaseous dinitrogen into ammonia or to make organically bound N bioavailable by mineralization. Not surprisingly, N input by fertilization has always been a key factor for high crop yields and plant quality. Therefore, crop production is by far the single largest cause of human alteration of the global N cycle (Smil, 1999). Whereas in preindustrial times exclusively organic fertilizers had been used, the invention of the Haber Bosch procedure in the 20th century made huge amounts of mineral fertilizer available. The doubling of world food production in the past four decades could only be achieved with a strong land-use intensification including an almost sevenfold increase of N fertilization (Tilman, 1999) as well as wide-ranging land reclamations. These developments have contributed to the doubling of N loads to soil since the beginning of the 20th century (Green *et al.*, 2004). The total global N input in the year 2000 was about 150 TgN (Schlesinger, 2009), whereas supply in croplands via mineral fertilizer was the single largest source accounting for almost half of it. Surprisingly, N entry from N-fixation was the second largest factor and contributed to 16%, while manure and recycled crop residues provided similar amounts and each accounted for only 8–13% of the total global supply. Remarkably, the entry of N via atmospheric deposition was in the same range. In regions with high mineral fertilizer application, the highest N accumulation potential in ecosystems could be observed, whereas the accumulation of N leads to high impacts on environmental quality like loss of diversity (Cragg & Bardgett, 2001), dominance of weed species (Csizinszky & Gilreath, 1987) and soil acidification (Noble *et al.*, 2008). Additionally, land-use intensification also results in an increased use of bioactive chemicals, like pesticides and herbicides as well as antibiotics, which enter the environment via manure (Lamshöft *et al.*, 2007).

According to Liu *et al.* (2010), 55% of the global applied N was taken up by crops. The remainder was lost in leaching

(16%), soil erosion (15%) and gaseous emission (14%). Such N depletion of soils leads to eutrophication (Stoate *et al.*, 2009), surface- and groundwater pollution (Spalding & Exner, 1993) and emission of the greenhouse and ozone-depleting gas nitrous oxide (N_2O) (Davidson *et al.*, 2000), impacting on human health and climate change (Fig. 1).

To reduce these threats, Schlesinger (2009) suggested that policy makers and scientists should focus on increasing N-use efficiency in fertilization, reducing transport of reactive N fractions to rivers and groundwater and maximizing denitrification to N_2.

Because of the use of advanced molecular tools (Gabriel, 2010) and stable isotopes (Baggs, 2008) in recent years, scientists have been able to identify new key players of N turnover for selected processes like nitrification (Leininger *et al.*, 2006) or N-fixation (Chowdhury *et al.*, 2009) as well as completely new processes like anammox (Op den Camp *et al.*, 2006). All these findings have revolutionized our view of N transformation processes in soils, although the relevance for the overall understanding of N transformation is not entirely clear yet and discussed controversially in the literature. However, despite numerous studies and a large amount of collected data, we have to admit that N turnover and factors driving the corresponding populations are not yet completely understood.

Furthermore, according to the *UN Millenium Ecosystem Assessment* (http://www.maweb.org/), global change will highly affect N turnover in soils to a so far unknown extent. According to the definition given in Wikipedia, the term 'global change' encompasses interlinked activities related to population, climate, the economy, resource use, energy development, transport, communication, land use and land cover, urbanization, globalization, atmospheric circulation, ocean circulation, the C cycle, the N cycle, the water cycle and other cycles, sea ice loss, sea-level rise, food webs, biological diversity, pollution, health, overfishing and alteration of environmental conditions including climate change as well as land-use changes and effects of xenobiotic substances. Therefore, there is a need for experimental approaches to study the consequences of altering environmental conditions including climate change as well as land-use changes and the effects of xenobiotic substances on N turnover in soil. In the following review, state-of-the-art knowledge is summarized concerning the impact of selected global change scenarios on microbial N turnover as well as the abundance and diversity of key players. Additionally, implications for future research strategies and priorities are given.

Ecosystem development

Natural and anthropogenic activities lead to new terrain for soil development. In this context, different chronosequences of ecosystem development like glacier forefields, sand dunes, volcanoes or restoration sites have emerged. These are interesting aspects to study the development of N-cycling processes as well as the contributing functional microbial groups. Overall, three phases can be postulated: initial, intermediate and mature phases. Depending on the investigated ecosystem, these phases can range from a few days or weeks (Jackson, 2003) to hundreds of years (Kandeler *et al.*, 2006; Brankatschk *et al.*, 2011), respectively.

Most of the initial ecosystems are characterized by nutrient shortage, barren substrate and scarce vegetation (Crews *et al.*, 2001; Nemergut *et al.*, 2007; Smith & Ogram, 2008; Lazzaro *et al.*, 2009; Brankatschk *et al.*, 2011). The total N concentrations are often far below 0.1% and only traces of ammonia and nitrate can be measured (Brankatschk *et al.*, 2011). Additional N input by the weathering of bedrock material is unlikely as it only contains traces of N. Thus, the colonization with N-fixing microorganisms seems to be the only way for N input, despite the high energy demands for the transformation of N_2 into ammonium. Crews *et al.* (2001) demonstrated that the total N input in young lava flows was mainly driven by N fixation, although fixation rates were low. This has been confirmed in several other studies, which demonstrated a high abundance of nonheterocystous N-fixing cyanobacteria like *Microcoleus vaginatus* (Yeager *et al.*, 2004; Nemergut *et al.*, 2007; Abed *et al.*, 2010). It is obvious that in initial ecosystems, cyanobacteria play a prominent role in ecosystem engineering. They not only improve the N status of soils by N-fixation, but also secrete a polysaccharide sheath, resulting in the formation of soil crusts. This leads to a stabilization of substrates, capture of nutrients and an increase of the water-holding capacity, which paves the way for other organisms and processes (Garcia-Pichel *et al.*, 2001; Schmidt *et al.*, 2008). Therefore, at early stages of soil development heterotrophic microorganisms, which are able to mineralize the N derived from air-driven deposition (e.g. chitin) or ancient and recalcitrant materials are able to find their niches and stimulate N turnover (Bardgett *et al.*, 2007; Brankatschk *et al.*, 2011). However, this process is highly energy demanding and thus the turnover rates typically low. Obviously, as only limited competition for N resources exists at this stage (due to a lack of plants), the amount of ammonia is sufficient for the development of microbial communities involved in nitrification. This process results in the formation of nitrate, which leaves the ecosystem mainly by leaching. Therefore, N accumulation rates at initial sites are low (Tscherko *et al.*, 2004).

If the total N concentrations in soil exceed 0.2%, plant development starts and cyanobacterial soil crusts are displaced by shadowing by plant growth (Brankatschk *et al.*, 2011). Therefore, the intermediate stage of ecosystem development is characterized by increasing plant coverage and surface stabilization resulting in an increased C input

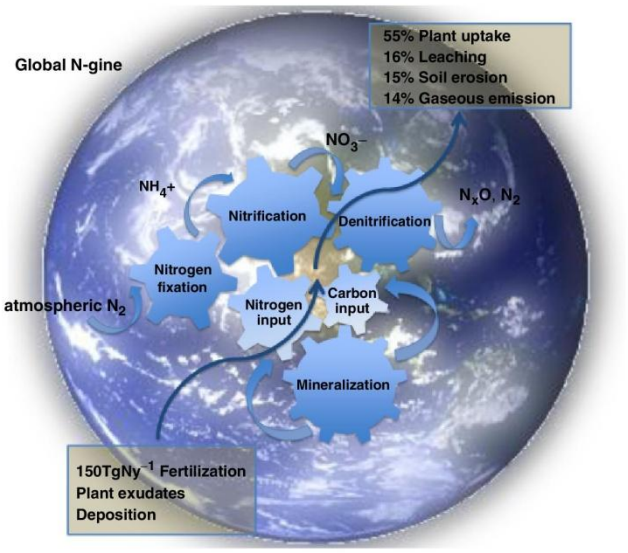

Fig. 1. Nitrogen turnover at the global scale.

via exudation and litter material. However, ammonium and nitrate contents are still much lower (Kandeler *et al.*, 2006; Brankatschk *et al.*, 2011) than in well-developed grassland sites (Chronáková *et al.*, 2009). Although it has been argued that this stage of ecosystem development is characterized by a competition between microorganisms and plants for N (Schimel & Bennett, 2004; Hämmerli *et al.*, 2007), associative or symbiotic networks between N-fixing microorganisms (mainly bacteria) and plants become a central element at this stage (Duc *et al.*, 2009). This results in an increased N-fixation activity in the rhizosphere and a highly efficient share of nutrients between plants and microorganisms. Because of the patchy distribution of C and N concentrations at those sites, many studies have revealed the highest microbial diversity at intermediate stages of ecosystem development by targeting functional genes like *nifH* (Duc *et al.*, 2009) or general microbial diversity by 16S rRNA gene (Gomez-Alvarez *et al.*, 2007). This fits with the intermediate-disturbance hypothesis, postulating that medium disturbance events cause the highest diversification (Molino & Sabatier, 2001). However, besides the development of plant–microorganism interactions, the intermediate phase of ecosystem development is also characterized by highly efficient degradation of litter and subsequent N mineralization (Esperschütz *et al.*, 2011) as well as an increase in fungal biomass (Bardgett & Walker, 2004), probably also of arbuscular mycorrhiza, which may contribute to a better distribution of the N in soil with ongoing succession. At this stage, the abundance and activity of nitrifiers (Nicol *et al.*, 2005) and denitrifiers (Smith & Ogram, 2008) is still low due to the high N demand of the plants. Whether typical plants at those sites are able to produce nitrification inhibitors to better compete for ammonium might be a highly interesting question for future research (Verhagen *et al.*, 1995).

In contrast, when total N concentrations above 0.7% are reached in soils at well-developed sites and vegetation is no longer dominated by legumes, nitrification becomes a highly significant process. Interestingly, in ecosystems of glacier forefields, nitrification activity seems to be driven by ammonia-oxidizing archaea (AOA), although being lower in abundance than their bacterial counterpart [ammonia-oxidizing bacteria (AOB)]. This might be due to the better adaptation to relative ammonium-poor environments (Di *et al.*, 2009) and low pH (Nicol *et al.*, 2008). In combination with pronounced root penetration resulting in increased exudation, enhanced water retention potential and less oxygen diffusion (Deiglmayr *et al.*, 2006), denitrification becomes a key process for the overall N budget at those sites in soil. Interestingly, Brankatschk *et al.* (2011) only found a good correlation of a part of the functional genes of the denitrification cascade, for example, *nosZ* (nitrous oxide reductase) gene abundance and potential denitrification activity, whereas *nirK* and *nirS* (nitrite reductases) gene abundance did not correlate with the rates of potential activity. Moreover, the highest relative gene abundance of *narG* was observed in early development stages of soils (Kandeler *et al.*, 2006), while the nitrate reductase activity peaked at late stages of soil development (Deiglmayr *et al.*,

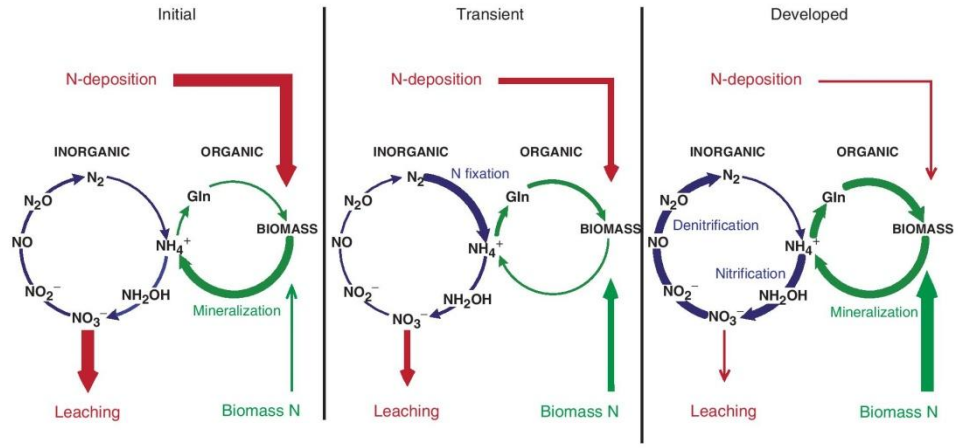

Fig. 2. Scheme of the development of the nitrogen cycle during ecosystem development (initial, transient and developed). The size of the arrows represents the impact of the corresponding process for nitrogen turnover.

2006). Similar observations were made by Smith & Ogram (2008) along a restoration chronosequence in the Everglades National Park. The mechanistic bases for these observations are still not clear. In addition to high activities of nitrifiers and denitrifiers at well-developed sites, the highest values of mineralization activity have been observed there in several studies (Tscherko *et al.*, 2004; Brankatschk *et al.*, 2011). These data are congruent with the observations of Frank *et al.* (2000), who found a positive correlation between nitrification, denitrification and N mineralization processes in Yellowstone Park grasslands.

Overall, the studies performed so far using the chronosequence approach to describe ecosystem development have revealed surprisingly similar patterns of the participation of different functional groups of microorganisms involved in N cycling at the three different phases (Fig. 2). In summary, all systems described were characterized by very low C and N concentrations in soil as well as less pronounced organismic networks of interaction at the initial stages of soil development.

Changing land-use patterns

A generalization of the results described above to other scenarios of global change related to ecosystem development, for example, in response to natural disasters (earthquakes), after manmade destructions (clear cuts of forest sites) or due to land-use changes is not possible. This is due to the different quality and amount of C and N present in soil as well as the biodiversity, mainly related to soil animals and plants at the initial stages in these disturbed systems. Whereas the consequences of natural disasters for N turnover have been rarely addressed, the impact of land-use changes on N turnover and the corresponding functional communities has been studied extensively. However, in this context, it is difficult to identify *one* main driver for shifts in the microbial population structure, as land-use changes often encompass a combination of different forms of management. For example, the use of extensively used grassland for crop production will not only change aboveground biodiversity, but will also result in changes in pesticide application, tillage and fertilizer management.

Overall, the conversion of forests or grasslands to agricultural land has an impact on almost all soil organisms (Postma-Blaauw *et al.*, 2010). Therefore, the functional diversity of microorganisms involved in N cycling is also highly influenced by land-use changes. This has been well documented for nitrifiers and denitrifiers, whereas surprisingly for N-fixing bacteria, clear response patterns have been described in only a small number of cases. In some cases, even no response of *nifH* towards land-use changes was detected (Colloff *et al.*, 2008; Hayden *et al.*, 2010), which might be related to the high concentrations of ammonium and nitrate before land-use change. In terms of nitrification, good correlations between gene abundance and land use have been described for AOB in several studies. Colloff *et al.* (2008) found higher gene abundance of the bacterial *amoA* gene in agricultural soils compared with soils from rainforests. By contrast, Berthrong *et al.* (2009) observed consistently reduced nitrification rates in soils that were converted from grassland into forest. These trends were also confirmed by Bru *et al.* (2010) comparing land-use changes between forests, grassland and agricultural soils in different

parts of the world. The authors found a strong correlation between AOB and the form of land use. Interestingly, in the same study, no differences were observed for archaeal ammonia oxidizers (AOA) in relation to the investigated land-use types. Hayden *et al.* (2010) almost consistently observed a greater abundance of AOB *amoA* genes in managed compared with remnant sites. The good correlation between AOB and land use might be related to the different ammonium concentrations in soil in response to different land-use types. AOB often colonizes habitats with high ammonium concentrations, whereas for AOA abundance, so far, no general dependency on ambient ammonium concentration has been documented. Furthermore, the results might be related to the high sensitivity of AOB towards low pH, which is often present in forest soils and leads to low availability of ammonia.

It was reported that land-use changes from forest to grassland soils are often accompanied by high N losses from soil (reviewed by Murty *et al.*, 2002). However, no clear trends are visible so far, if these losses occur in general due to increased denitrification rates or leaching of the nitrate formed during nitrification, as both observations have been described in the literature. This might be explained by the different soil types under investigation in the various studies. Whereas in loamy soils, which tend to have more anoxic microsites, denitrification might be stimulated (Rich *et al.*, 2003; Boyle *et al.*, 2006), in sandy soils, the nitrate formed may leach fast to the groundwater (Murty *et al.*, 2002). For denitrifiers, land-use changes overall influence the abundance and diversity patterns of selected functional groups. Attard *et al.* (2010) described, for example, higher potential denitrification rates in grassland soils compared with soils under cropping management. This was in accordance with a 1.5–5-fold higher abundance of denitrifiers (based on the abundance of *nirK* genes) in grassland soils than arable soils found in various studies (Baudoin *et al.*, 2009; Attard *et al.*, 2010), including shifts in the diversity patterns of *nirK*-harboring bacteria. Whereas a strong correlation between gene copy numbers of *nirK* and potential denitrification rates has been described, no correlation was found between the diversity patterns of *nirK* and turnover of nitrate. This indicates highly similar ecophysiological patterns of nitrite reducers of the *nirK* type.

Agricultural management

Not only changes in land-use patterns, but also shifts in agricultural management practice can result in alterations of functional microbial communities involved in N cycling. In general, there is consensus that an intensification of agriculture and subsequent increased fertilization regimes result in higher nitrification and denitrification rates as well as an increase of both functional groups (Le Roux *et al.*, 2003, 2008; Patra *et al.*, 2006). In the case of ammonia oxidizers, mainly AOB benefit from the increased availability of ammonium in soil (Schauss *et al.*, 2009a). For N-fixing prokaryotes, several studies have indicated a reduction based on the abundance of *nifH* and consequently also lower N-fixation activity in highly fertilized soils (Coelho *et al.*, 2009). Interestingly, the inoculation of seeds from legumes with rhizobia, which is a common practice in low-input farming to enhance N-fixation, does not only increase *nifH* abundance in the rhizosphere, but also leads to higher abundance of nitrifiers and denitrifiers (Babic *et al.*, 2008). This indicates that at least a part of the fixed N is released into soil, despite the symbiotic interaction (Babic *et al.*, 2008). As the use of monocultures and the intensification of agriculture *per se* (including the transformation of sites, which are less suited for agriculture, for the production of renewable resources) is often accompanied by a loss in nutrients (Malézieux *et al.*, 2009), which is primarily compensated by the application of inorganic fertilizers, changes in N turnover and the corresponding microbial communities might be primarily a result of changed fertilization regimes, as described by Drury *et al.* (2008). It has been confirmed in several studies that the type of fertilizer (mineral vs. organic fertilizer) has a clear influence on the N budget of soils and the corresponding functional microbial groups (Hai *et al.*, 2009; Ramirez *et al.*, 2010). As expected, the application of a mineral fertilizer based on ammonia-nitrate increases the nitrification and denitrification patterns in soil shortly after application, when the fertilizer is not taken up by the plant due to increased availability of the corresponding substrates. In contrast, the application of an organic fertilizer leads to higher abundance of microorganisms involved in mineralization and only relatively slight increases of nitrifiers and denitrifiers and their activity in the long run. Because of the overall more balanced N budget in soil when organic fertilizers are applied, N-fixing microorganisms are favored by this practice (Pariona-Llanos *et al.*, 2010). Not surprisingly, the effects observed in soils that have been used for grazing can be compared with those where manure has been applied, including clear shifts mainly in the diversity patterns of ammonia- and nitrite-oxidizing microorganisms as well as denitrifiers (Chroňáková *et al.*, 2009) Furthermore, grazing also induces shifts in root exudation patterns (Hamilton & Frank, 2001), which may further influence the abundance and activity of microorganisms involved in N turnover.

In the last decades, the influence of tillage management on N turnover has been studied in several projects, as nontillage systems have been described to be of advantage in terms of nutrient supply and are very popular in organic farming (Hansen *et al.*, 2011). Overall, changes in nitrification activity after modifying the tillage practice were well

explained by the accumulation of ammonium in the top soil due to nontillage and the corresponding changes in the abundance of nitrifiers (Attard *et al.*, 2010). In most studies, performed so far, a higher nitrification activity and subsequent higher nitrate concentrations in soil were linked to increased denitrification rates in the top soil layer in nontillage compared with tillage treatments (Petersen *et al.*, 2008; Baudoin *et al.*, 2009; Attard *et al.*, 2010). This is due to tillage-induced higher C concentrations in top soils and a stronger formation of aggregates with anoxic microsites due to a lack of tillage-induced mixing. In addition, tillage results in a merging of the surface soil layers with the lower layers, the latter being characterized by lower denitrification potential (Attard *et al.*, 2010), which causes overall lower denitrification rates and abundance of the corresponding functional genes (especially *nirK*). However, as stated above, in most cases, changes in tillage management are accompanied by changes in pest management and cropping sequences. The changes observed in long-term studies therefore cannot be linked conclusively to tillage management alone. Thus, most studies performed so far in this area were linked to short-term perturbations. They may not reflect the typical response patterns of the soil microorganisms to the new conditions after the change of the tillage management, as they do not account for microbial adaptation, in the context of the intermediate-disturbance hypothesis (Molino & Sabatier, 2001) as well as the increasing C contents in the top soils over time where nontillage practice has been performed.

Changing climatic conditions

Because of ongoing climate change, various modifications in land use and agricultural management have been implemented. Thereby, climate and land management are highly interlinked and cannot be separated. In addition, it is well accepted that climatic conditions notably influence microbial performance in soil. Thus, several studies have been performed to estimate the consequences of increased atmospheric temperature or carbon dioxide (CO_2) concentrations as well as shifts in precipitation on N turnover and the corresponding functional communities.

In general, it is difficult to simulate increased temperature scenarios in experiments, as an increase of the average temperature of 3 °C over the next 50 years would at most result in an annual increase of < 0.2 °C. Therefore, experiments comparing soils with ambient temperature with soils increased in temperature by 2–5 °C do not simulate climate change, but are more appropriate to understand the overall stress response of the soil microbial community. An air temperature increase of 3 °C for example, induced shifts in the AOB community structure, decreased AOB richness and concurrently increased potential nitrification rates in the rhizosphere of legumes. It remains open whether AOA adopted the ability to transform ammonia, while their bacterial counterparts were sensitive to the elevated temperature (Malchair *et al.*, 2010a). Besides questioning the relevance for studying climate change effects, it is unclear whether the observed shifts were a direct effect of the temperature or were rather related to changes of the plant performance, for example, increased exudation, in response to the increased temperature.

More relevant in the context of temperature-related effects are questions addressing changes in soils of permafrost regions, as here, only a slight increase of air temperature results in a prolonged period in which soils are unfrozen during the summer time. In these studies, the focus has mostly been on C turnover and methane emission, although clear effects on N transformation have been described. There is broad agreement that thawing of permafrost soils leads to a rapid increase of denitrification and hence high N_2O emissions, due to the high water saturation and the availability of easily degradable C and nitrate in those soils (Repo *et al.*, 2009; Elberling *et al.*, 2010). Measured emissions were comparable to values from peat soils (0.9–1.4 g N_2O m^{-2} and year). In contrast, nitrifying communities did not benefit from the changed environmental conditions in the short run. Metagenomic analysis and clone library studies revealed a low diversity and a relatively low abundance for ammonia oxidizers (AOA and AOB) (Liebner *et al.*, 2008; Yergeau *et al.*, 2010). Obviously, the high concentrations of available C as well as the anoxic conditions do not favor the growth of AOA and AOB. Therefore, not surprisingly, in permafrost soils, clear evidence for anaerobic ammonia oxidation has been obtained (Humbert *et al.*, 2010), in contrast to many other soil ecosystems. N-fixing microorganisms did not play a major role in the investigated sites and did not change in abundance and diversity after thawing (Yergeau *et al.*, 2010).

However, also in moderate climatic zones, small shifts in the temperature affect freezing and thawing regimes in soil during winter time and increased numbers of freezing–thawing cycles are expected. Therefore, this topic is of interest for agricultural management practice, notably when intercropping systems are used over winter. Like in permafrost regions, soil thawing is mainly accompanied by an accelerated release of nutrients, but also by the emission of greenhouse gases, such as N_2O and nitric oxide (NO), as well as CO_2 and methane. Considerable research was focused on gaseous N losses and the N_2O/N_2 ratio in the last two decades (Philippot *et al.*, 2007). A modeling study by De Bruijn *et al.* (2009) indicated that N_2O emissions resulting from freezing–thawing are not monocausal and mainly depend on the amount and quality of available C and N, the microbial biomass and the redox conditions in soil after thawing. Although N_2O emissions were reported from

soils that are generally characterized by a low temperature (< 15 °C), these values are far lower than the N_2O concentrations emitted from thawing soils (Koponen & Martikainen, 2004). Wolf *et al.* (2010) could show that up to 70% of the annual N_2O emissions from agricultural fields might occur in the winter period. Peak emissions of N_2O were reported from arable soils during or shortly after thawing (Dörsch *et al.*, 2004) and could only be attributed in part to N_2O physically trapped in soil aggregates during freezing (Teepe *et al.*, 2001). A large part of N_2O arises from the microbial denitrification process, which fits with decreased oxygen and increased C and N availabilities in soils that were subject to freezing–thawing cycles (Öquist *et al.*, 2004). Sharma *et al.* (2006) observed an increase in transcripts of the nitrate and nitrite reductase genes *napA* and *nirK*, respectively, straight after thawing began. Other studies have shown a significant increase in N mineralization compared with nonfrozen soils (De Luca *et al.*, 1992). In contrast to permafrost soils, where aerobic ammonium oxidation did not play an important role, increased nitrification rates were measured after thawing in soils from moderate climatic zones. Su *et al.* (2010) demonstrated that bacterial ammonia oxidizers were impaired by freezing and thawing, whereas their archaeal counterparts even increased in abundance. This is in accordance with the hypotheses by Schleper *et al.* (2005) and Valentine (2007), who presumed that archaea are more tolerant to stress conditions than bacteria. Therefore, archaea could be the main contributors to ammonia oxidation after freezing and thawing.

Studies on the effects of changes in precipitation on microbial N turnover are rare, notably when questions about the effects of extreme weather events are addressed, although it is well accepted that the increased variability in precipitation and the resulting soil water dynamics directly alter N cycling in terrestrial ecosystems (Corre *et al.*, 2002; Aranibar *et al.*, 2004). Not surprisingly, irrigation increased, on the one hand, nitrate leaching rates mainly in sandy soils (Olson *et al.*, 2009). On the other, increased denitrification activities were measured. For example, scenarios simulating high rainfall events resulted in 2.4–13-fold increases in ammonia, nitrate, NO and N_2O fluxes in clay loam, whereas NO and N_2O fluxes decreased in sandy soils in response to water drainage (Gu & Riley, 2010). Ruser *et al.* (2006) found maximum N_2O emission rates in differently compacted soils after rewetting of dry soil that increased with the amount of water added. Muhr *et al.* (2008) postulated that rather than the intensity of rewetting, the length of the drought period might be more important for the process patterns and the microbial communities involved in N_2O and NO emissions. Again, the effects of precipitation depend on other factors like agricultural management. For example, it could be shown that the effects of irrigation depend on the type of cover crop in soil (Kallenbach *et al.*, 2010). Overall, studies mainly focused on the effects of precipitation on denitrification rates. Other processes of the N cycle as well the corresponding communities have been rarely studied so far. It must also be assumed that these processes are also highly affected directly or indirectly by dryness and precipitation, respectively. Interestingly, Zavaleta *et al.* (2003) demonstrated changes in plant diversity patterns in different grasslands in response to different precipitation regimes, which may indicate indirect effects of different precipitation regimes on nitrifiers as well as on N-fixing microorganisms.

The same authors could show that enhanced CO_2 concentrations in the atmosphere decrease plant diversity at grassland sites. However, C input into the soil via exudation was enhanced, which resulted in an overall stimulation of most microorganisms. Mainly N-fixing bacteria benefited from the additional C input, as their abundance was increased at grassland sites with increased CO_2 (He *et al.*, 2010). As expected, enhanced CO_2 concentrations also stimulated denitrifiers in soil due to a general reduction of the redox potential in soil as a result of the increased microbial activity (Pinay *et al.*, 2007). Furthermore, a stimulation of N mineralization has been proven (Muller *et al.*, 2009). Consequently, elevated CO_2 values in the atmosphere resulted in reduced abundance of autotrophic microorganisms like ammonia oxidizers (Horz *et al.*, 2004) in combination with reduced activity patterns (Barnard *et al.*, 2006) due to competition from heterotrophs as well as lower and lower activity in grassland soils. In addition, several studies have described a positive correlation between plant species richness and AOB richness in grassland soils. Malchair *et al.* (2010b) hypothesized that this link could be due to the spatial heterogeneity of ammonia, promoted by the plant species richness. In contrast, AOB were unaffected by increased atmospheric CO_2 (Nelson *et al.*, 2010) in soils under intensive agricultural use (e.g. soybean or maize cultivation), probably as the present ecotypes in these soils are already adapted to higher C input into the soil, for example, by manuring, litter application and intensive exudation by the cultivated crop. However, when relating those results to ongoing climate change, it must be considered, as described above for temperature effects, that we are challenged with an continuous increase in CO_2 concentrations in the atmosphere and not with a doubling from 1 day to another as simulated in most experiments.

Xenobiotics

New climatic conditions and changed agricultural practice have led to an emerging pressure from weeds and phytopathogens, which complicates farming practice and has resulted in the increased use of (new) chemical substances

worldwide. Pesticides, i.e. herbicides, fungicides and insecticides, can exert collateral effects on soil microorganism and important functions such as N cycling. Some of these compounds also represent a source of N to microbial communities through mineralization. For example, the ability of microorganism to use atrazine as a sole N source has been demonstrated (Mandelbaum *et al.*, 1995; Struthers *et al.*, 1998). As bioavailability of pesticides depends on the formulation as well as on diverse crop and soil factors (e.g. percentage crop cover of the soil surface, soil type, structure, pH, N and C contents, pore volume, water-holding capacity) determining sorption, leaching and degradation of the compound, the response of the microbial biomass is expected to be linked to both the soil type and the pesticide used. Moreover, herbicides are typically applied onto bare soil while fungicides and insecticides are used on dense crops and the exposure of the soil is consequently lower (Johnsen *et al.*, 2001).

The effects of pesticides on bacterial groups involved in N transformation have been thoroughly studied using cultivation-dependent methods in the past, for example, *Rhizobium* fixing N in symbiosis with leguminous plants (Aggarwal *et al.*, 1986; Kishinevsky *et al.*, 1988; Mårtensson, 1992; Revellin *et al.*, 1992; Ramos & Ribeiro, 1993; Singh & Wright, 2002), free-living diazotrophs *Azotobacter* and *Azospirillum* (Banerjee & Banerjee, 1987; Jena *et al.*, 1987; Martinez-Toledo *et al.*, 1988) and nitrifying bacteria (Doneche *et al.*, 1983; Banerjee & Banerjee, 1987; Martinez-Toledo *et al.*, 1992a, b). On the contrary, only a few recent studies have used culture-independent approaches to better gain insight into the effects on the structure and function of soil microbial communities (Engelen *et al.*, 1998; Rousseaux *et al.*, 2003; Seghers *et al.*, 2003; Devare *et al.*, 2004; Saeki & Toyota, 2004; Bending *et al.*, 2007). In many cases, pesticides applied at the recommended field rate concentration did not have a significant impact on the structure and function of the soil microbial communities (Saeki & Toyota, 2004; Ratcliff *et al.*, 2006). Seghers *et al.* (2003) demonstrated that the community structure of AOB in bulk soil of a maize monoculture was unaltered by 20 years of atrazine and metolachlor application. Some other studies have indicated more pronounced effects. Thus, Chang *et al.* (2001) observed a severe impact of atrazine on both the abundance and the community structure of AOB. However, in this study, short-term microcosm experiments were performed with high herbicide concentrations (*c.* three orders of magnitude higher than the field rates). There is also increasing evidence that chloropicrin and methyl isothiocyanate can stimulate N_2O production (Spokas & Wang, 2003; Spokas *et al.*, 2005, 2006). For other herbicides like prosulfuron, glyphosate and propanil as well as the fungicides mancozeb and chlorothalonil, decreased N_2O emissions were observed, possibly because the compounds inhibited nitrification and denitrification (Kinney *et al.*, 2005). Cernohlávková *et al.* (2009) confirmed this hypothesis and demonstrated that mancozeb and dinocap can impair nitrification at a field rate in an arable and a grassland soil.

Besides pesticides, antibiotics are also extensively used in agricultural production systems, predominantly in livestock husbandry. As slurry and manure are usually applied as organic fertilizers in agricultural farming, a substantial fraction of the administrated compounds enters the environment (Lamshöft *et al.*, 2007). Unlike pesticides, antibiotics are explicitly designed to affect microorganisms. The impact of, for example, sulfadiazine, a broad-spectrum bacteriostatic agent, has been intensively evaluated due to its frequent use, high excretion rate and persistence in soil (Thiele-Bruhn, 2003; Lamshöft *et al.*, 2007; Schauss *et al.*, 2009a). Similar to pesticides, soil and crop characteristics are major factors influencing the response patterns of the microbial communities toward antibiotics in soil (Heuer & Smalla, 2007; Hammesfahr *et al.*, 2008; Kotzerke *et al.*, 2008; Schauss *et al.*, 2009a; Ollivier *et al.*, 2010). Potential nitrification activity remained unchanged under low sulfadiazine concentration conditions in bulk soil when applied in combination with manure (Kotzerke *et al.*, 2008). This might have been due to a substitution of the highly affected AOB by their archaeal counterparts (Schauss *et al.*, 2009b). Similar observations concerning sulfadiazine effects on the abundance patterns of AOB and AOA were made in the rhizosphere of maize and clover (Ollivier *et al.*, 2010). Also, both functionally redundant groups of nitrite reducers were negatively influenced by antibiotic addition to manure. Hence, not surprisingly, potential denitrification rates decreased in treatments where sulfadiazine was applied (Kotzerke *et al.*, 2008). While nitrite reducers harboring the *nirS* gene increased in abundance after bioavailable sulfadiazine had declined, the abundance of *nirK*-harboring nitrite reducers remained on the level of the nonmanured control treatment (Kleineidam *et al.*, 2010). Clearly, pronounced effects of sulfadiazine on the denitrifying bacteria were also observed in the rhizosphere of maize and clover, where the dominating *nirK*, but also the *nirS* nitrite reducers as well as the *nosZ*-harboring N_2O reducers were significantly impaired (Ollivier *et al.*, 2010). Furthermore, the abundance of *nifH* genes, coding for key enzyme of N fixation, was significantly impacted by sulfadiazine in the rhizosphere of both plant types, but to a greater extent in the rhizosphere of the legume.

Conclusions and outlook

The research over the last two decades linking N transformation processes in soil to the corresponding functional microbial communities has improved our knowledge

significantly about the factors driving the abundance, diversity and activity mainly of microorganisms involved in the inorganic N cycle as well as the dynamics of the corresponding turnover processes and nutrient fluxes. Overall, most studies that addressed questions linked to the consequences of land-use changes or agricultural management included data for nitrifiers, denitrifiers and N-fixing microorganisms, whereas studies in the area of climate change in most cases focused only on consequences for denitrification and N_2O emissions. This reflects well the areas of interest of the various scientific communities involved in the different research areas. However, it must be taken into account that the processes of the N-cycle are closely interlinked and thus influence each other. Thus, even if the focus is on trace gas emissions from soil, knowledge of processes like nitrification and N-fixation is of key importance too. In general, data on the diversity and abundance of N-mineralizing microorganisms are rare in microbial ecology, due to the huge variety of different biochemical pathways, which are so far mostly unknown. Therefore, not surprisingly, in most studies that are of relevance for consequences of global change on N-transformation, this functional group of microorganisms has been excluded from analyses. Nevertheless, it is generally accepted that the amount of mineralized nitrogen is one major driver for the inorganic nitrogen cycle mainly in nonfertilized natural soils.

From the recently published data, the following conclusions can be drawn generally: (1) global change-related modifications of environmental factors affect nitrifiers, denitrifiers and N-fixing microorganisms and alter the corresponding processes. (2) The abundance of the autotrophic ammonia oxidizers and nitrite oxidizers in soil is negatively correlated with additional C input by plants as a result of land-use changes towards agricultural land or a more intensive agriculture as well as enhanced CO_2 concentrations in the atmosphere. This results in soils, where no inorganic fertilizer has been applied, in reduced nitrate concentrations and consequently, despite the presence of easily degradable carbon sources, in reduced denitrification activity under anoxic conditions. Although N-fixing microorganisms benefit from the additional carbon input, their activity is only increased under low ammonia concentrations in soil, for example, conditions where most of the ammonia is taken up by the plant or by soil microorganisms for biomass production. Overall, plants might benefit from this scenario due to reduced competition for ammonium with ammonia-oxidizing microorganisms in soil. Furthermore, such conditions may reduce the amount of leached nitrate as well as emissions of N_2O. (3) By contrast, ammonia oxidizers might benefit from the application of xenobiotics as AOA in particular seems to tolerate a number of compounds that, like antibiotics, are toxic for other prokaryotes (Schauss *et al.*, 2009a, b). This may result in increased nitrification rates if enough ammonia is available and consequently in the formation of nitrate. As denitrifiers might be reduced in their activity under the given scenario, nitrate could leach to the ground water, if it is not taken up by the plants. (4) Water conditions and the oxygen content in soil highly influence nitrifiers and denitrifiers. Under anoxic conditions, however, the activity of denitrifiers again depends on the amount of available nitrate and, therefore, either on fertilization regimes or the activity of nitrifiers in non-water-logged habitats in soil.

As stated in the introduction, 'global change' encompasses *interlinked* activities of the different scenarios described above. Because each scenario results in a different response pattern of the investigated microbial communities, a prediction of what happens if two or more scenarios are mixed is almost impossible. For example, whether the addition of xenobiotics and increased carbon inputs by increased atmospheric CO_2 concentrations will lead to higher or lower concentrations of nitrate in soil cannot be predicted from currently available data. However, these types of predictions are needed to transform scientific results into concrete recommendations for practice. Another important aspect of research linked to global change is to understand the long-term consequences of changes in the environment for microbial life in soil. As yet, most studies in the past have concentrated on short-term effects using sometimes highly unrealistic predictions of future conditions. Therefore, in many cases, results represent data more relevant for disturbance ecology than for global change research. As described above, this is true for many experimental setups in the frame of climate change. Finally, the different scales of relevance must be taken into account. Microorganisms act on the μm^2 scale; however, the scales that need to be addressed in terms of political recommendations are at regional or even at a global scale. And conceptual approaches to overcome the scale problem are far from being 'on the market'. This holds true for 'upscaling' from 1 g of soil to the ha or km^2 scale, but also for 'downscaling' 1 g of soil to microsites of μm^2, where microbial life occurs. In this respect, research addressing questions about the relevant scale that must be considered for different scenarios of global change is currently absent.

Authors' contribution

J.O. and S.T. contributed equally to this work.

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Publications

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Oral presentation

Ollivier J., Kleineidam K., Thiele-Bruhn S. , Siemens J., Wilke B.-M., and Schloter M.: Effect of sulfadiazine-contaminated pig manure on microbes involved in nitrogen cycling in the rhizosphere of crops. BAGECO11, Corfu 2011