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Drivers for the performance of nitrifying organisms and their temporal and spatial interaction in grassland and forest ecosystems

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Meinen Eltern

Der Beginn aller Wissenschaften

ist das Erstaunen, dass die Dinge so sind, wie sie sind.

Aristoteles

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Zusammenfassung

Nitrifikation, die Umwandlung von Ammoniak in Nitrat, ist ein essentieller und zentraler Prozess von Nährstoffkreisläufen in terrestrischen Ökosystemen und beeinflusst dadurch nicht nur Prozesse im Boden, sondern auch die oberirdische Flora und Fauna im Hinblick auf Pflanzenertrag und darüber hinaus andere bedeutende Ökosystemdienstleistungen wie Grundwasserqualität. Um ein tiefgehendes Verständnis für das Verhalten von Schlüsselorganismen der Ammoniak- und Nitritoxidation zu entwickeln und für deren zeitliche und räumliche Interaktionen, ist es grundlegend zunächst einen Einblick in die Faktoren zu gewinnen die deren jeweiliges Verhalten und deren Zusammenspiel bestimmen. Unter anderem wurden Boden pH sowie Landnutzung als zwei der wichtigsten Faktoren identifiziert, die Substratverfügbarkeit und –zugänglichkeit für Ammoniak- und folglich auch für Nitritoxidierer bestimmen. Das Ziel der vorliegenden Arbeit war es, Muster im Hinblick auf Aktivität, Abundanz und Diversität von Mikroorganismen im zeitlichen Verlauf und verschiedenen räumlichen Auflösungen aufzudecken und im Speziellen auf deren unterschiedliche Reaktionen auf ausgewählte Umweltparameter in verschiedenen Ökosystemen einzugehen.

Das Nitrifikationspotential blieb über das Jahr hinweg stabil und selbst unter ungünstigen Umweltbedingungen wie beispielsweise in Waldböden mit niedrigem pH Wert oder unbewirtschafteten Grünlandstandorten noch detektierbar, wenn auch reduziert. Die konstant bleibenden potentiellen Nitrifikationsraten und Abundanzen von ammoniak-oxidierenden Archaeen (AOA) entlang eines pH Gradienten, gingen einher mit Diversitätsveränderungen von AOA, was darauf schließen lässt, dass spezielle Gruppen von AOA für die Aufrechterhaltung des Nitrifikationsprozesses auch unter Substratmangel sowohl in Wald als auch Grünland-Ökosystemen verantwortlich sind. AOA scheinen nicht nur in unbewirtschafteten Grünlandflächen oder sauren Böden, die durch geringen Substrateintrag gekennzeichnet sind, zu dominieren, sondern auch auf bewirtschafteten Flächen. Einhergehend mit der Intensivierung der Landnutzung stieg die potentielle Nitrifikation gleichzeitig mit der Abundanz von Ammoniak-Oxidierern an, was auf einen positiven Einfluss von Landnutzung auf die mikrobielle Gemeinschaft der Ammoniak-Oxidierer in Mineralböden auf regionaler Ebene schließen lässt. Auf einer Plot Skala im Meterbereich, konnte die Nitrifikation in unbewirtschafteten Flächen auf die Interaktion von speziellen Untergruppen von *Nitrospira* und AOA zurückgeführt werden, worauf deren gemeinsames Vorkommen auf der betrachteten Skala in einem begrenzten Zeitrahmen hindeutete. Die vorliegenden Ergebnisse legen nahe, dass Umweltparameter unterschiedliche Effekte auf das Verhalten von Nitrifizierern ausüben: während Aktivität und Abundanz eher von dynamischen Parametern, die sich im jahreszeitlichen Verlauf ändern, beeinflusst werden, bilden stabile Parameter wie beispielsweise konstant bleibender pH Wert unterschiedliche

Nischen für Nitrifizierer und beeinflussen so deren Diversität. Des Weiteren hebt die vorliegende Arbeit die Notwendigkeit hervor, den Einfluss auf mikrobielle Gemeinschaften in unterschiedlichen räumlichen Skalen zu beobachten und auch die Wechselwirkungen von verschiedenen Einflussfaktoren in Betracht zu ziehen. Die Arbeit trägt dazu bei, die Reaktion bestimmter mikrobieller Gruppen auf sich verändernde Umweltbedingungen besser einschätzen zu können und lässt dadurch Rückschlüsse auf die Anfälligkeit miteinander verbundener Prozesse auf Umweltstörungen zu.

Summary

Nitrification, the conversion of ammonia to nitrate, in terrestrial ecosystems is an essential and central process in soil nutrient cycling, thereby affecting not only belowground processes but also aboveground biota in terms of plant productivity and influences further other important ecosystems processes such as groundwater quality. To achieve a profound understanding of the performance of key players involved in ammonia- and nitrite-oxidation and their temporal and spatial interactions, it is essential to gain insights in the drivers that trigger their individual performance and influence their interplay at the same time. Amongst others, soil pH and land management have been identified as two major parameters shaping substrate availability and accessibility for ammonia-oxidizers and in consequence also nitrite-oxidizers in soils. This study aimed at observing patterns of microbial performance in terms of their abundance, activity and diversity at the temporal and spatial scale in particular focusing on their differential response to selected environmental drivers across ecosystems.

The nitrification potential was stable along the year and still detectable though reduced under adverse environmental conditions such as low pH forest soils or at unmanaged grassland sites. The nearly stable potential nitrification and abundances of archaeal ammonia-oxidizers (AOA) along a pH gradient were accompanied by changes in the diversity of AOA, implying that specialized clades of AOA were responsible for maintaining nitrification even under substrate shortage both in forest and grassland soils. But AOA seem to dominate not only in unmanaged grassland sites or acidic soils, characterized by low substrate input, but also in managed sites. Upon land-use intensification, potential nitrification increased in tandem with abundances of ammonia-oxidizers, implying that land-use positively affects the ammonia-oxidizer community in mineral grassland sites at the regional scale. At the plot scale in the metre range, nitrification could be ascribed to interacting specific *Nitrospira* sublineages and AOA, as indicated by their co-occurrence detectable during a limited period of time at the observed scale. The results present evidence for differential effects of environmental variables on the performance of nitrifiers: while activity and abundance are affected rather by dynamic seasonally changing parameters, stable variables such as constant soil pH shape distinct niches for nitrifiers, thereby influencing their diversity. The study further highlights the necessity to observe effects on microbial community at multiple scales and also take the putative interplay of various drivers into account. This study contributes to a better estimation of the responsiveness of particular groups to changing environmental conditions, which allows drawing conclusions on the robustness of intertwined processes to ecosystems perturbations.

I INTRODUCTION

1 Functional biodiversity research

Decades of intensive research have been dedicated to investigating the consequences of global change in terms of greenhouse gas emissions, elevated temperatures and nitrogen deposition, nevertheless this issue is more topical than ever. It is a challenging task to preserve ecosystem function by counteracting negative effects of anthropogenic perturbations, while at the same time increasing productivity against the background of a growing world population. This increases the need to monitor consequences of anthropogenic intrusions into ecosystems and to ensure the preservation of biodiversity (Hooper et al., 2005).

The term biodiversity, commonly used to describe species and genetic diversity in terms of richness and composition, is also associated with various organizational levels of biological diversity, including e.g., their global distribution (see Hooper et al., 2005 for details). All these levels of biodiversity fundamentally affect ecological processes. Different responses of ecosystem processes' functionality to biodiversity change have been observed, and thus, it is difficult to transfer response patterns of few taxa to others in order to predict the functional consequences of biodiversity change (Hooper et al., 2005). Intensification of anthropogenic land-use, for example, goes hand in hand with biodiversity loss for many taxa in many ecosystems (Fischer et al., 2010).

Biodiversity is important not only in terms of community composition in determining ecological function in an ecosystem, but also in the context of species richness, playing a prominent role in maintaining ecosystem processes even against the background of ecosystem change (Fischer et al., 2010; Hooper et al., 2005; Wolters et al., 2000). Stability of functionality is ensured by the diversity of functionally redundant organisms (Hooper et al., 2005). In this respect, functional stability of processes has been observed among aboveground biota and linked to productivity, despite changing nutrient-levels which induced shifts in community composition (Hooper et al., 2005). The same holds true for relationships between soil processes (e.g. decomposer community composition) and plant productivity (Hooper et al., 2005; van der Heijden et al., 1998). Still, the complex interactions of biota in the field, including above- and belowground biodiversity and the different levels of biodiversity organization (Fischer et al., 2010) essential for effective ecosystem functioning, remain elusive. Thus, to generalize the effects of biodiversity and belowground ecosystem processes connected to aboveground productivity, large scale approaches are necessary to close the gaps in our knowledge

and to contribute to a deeper understanding of different observed response patterns (Hooper et al., 2005).

To close this gap, an interdisciplinary research platform for functional biodiversity, the Biodiversity Exploratories (www.biodiversity-exploratories.de), was established. The long-term and large-scale Biodiversity Exploratories project, funded by the DFG priority program 1374, involves several research areas of biodiversity. A standardized study site design, central project coordination, and database make possible the interdisciplinary synthesis of obtained results. The major focus of the BE research project is on "the feedbacks between land use, biodiversity and ecosystem processes in real-world ecosystems" (Fischer et al., 2010), i.e. to assess the functional connection between biodiversity and ecosystem changes (Fischer et al., 2010; Hooper et al., 2005). As it is assumed that biodiversity change occurs along with ecosystem change (Fischer et al., 2010; Sala et al., 2000) caused by anthropogenic influences, different levels of human impact have been included in the concept of the Biodiversity Exploratories. In German terrestrial ecosystems, the most prominent changes have occurred as a result of a shift from deciduous into production forests and by intensification of land management to formerly unmanaged grassland ecosystems. To cover a broad spectrum of different grassland and forest ecosystems, sites with varying properties, soil-types and management intensities were selected across Germany in the regions Schwäbische Alb (ALB), Hainich-Dün (HAI) and Schorfheide-Chorin (SCH).

To predict ecological consequences for functional biodiversity in these ecosystems, it is essential not only to monitor which changes occur but also why they occur. Understanding the driving forces behind biodiversity change is a fundamental aim of biodiversity research and helps estimate the tolerance of natural ecosystems to these changes (Carney et al., 2004; Wolters et al., 2000). According to Birkhofer et al., variations in abundance and diversity patterns of different soil biota could be explained by location, land management practices or abiotic soil properties at the investigated sites (Birkhofer et al., 2012). Presumably important drivers for biodiversity change at sites of the Biodiversity Exploratories have been identified and are introduced in the following section with a particular focus on those factors which also influence soil nutrient and in particular nitrogen cycle processes such as nitrification, as this is the major focus of our study.

In terrestrial ecosystems, above- and belowground biota are strongly coupled, mediated in part via nutrient-cycling (Horz et al., 2004; Wolters et al., 2000). Nutrient availability is regulated to a large extent by microbial mobilization and immobilization processes. Thus, variations in microbial diversity and activity can consequently alter the functionality of associated processes (Hooper et al., 2005). In

this regard, processes of the nitrogen cycle are essential for ecosystem stability and are of particular importance for plant productivity and management of agricultural soils by controlling inputs and outputs of nitrogen through the ecosystem. These processes can also have negative impacts on climate change by increasing greenhouse gas emissions, and on ecosystem services, such as groundwater quality, through contamination by nitrogen species (Carney et al., 2004; Le Roux et al., 2013).

2 Ecological importance of nitrification

The fundamental importance of nitrification becomes evident when considering the indispensable role of nitrifiers in wastewater treatment. In the absence of nitrifier activity, toxic amounts of ammonia and/or nitrite would remain in wastewater and might in consequence lead to contamination of groundwater and/or eutrophication of surface waters such as lakes or rivers (Siripong and Rittmann, 2007; Smith et al., 1999). However, the nitrification process is not only crucial for the efficient removal of ammonium and urea from wastewater, but is also fundamental to soil nitrogen availability (Ward et al., 2011). This is essential for plant productivity because it affects the metabolite pools of ammonia and nitrate – both of which are required inorganic compounds along with organic nitrogen (amino acids) for plant uptake (Boudsocq et al., 2012). Increasing evidence suggests that some plant species may take up different nitrogen forms while others have been demonstrated to prefer one particular form (Boudsocq et al., 2012; Gherardi et al., 2013; Kastl et al., 2015; Warren and Adams, 2007). This affects not only plant productivity but also the availability of those compounds in soil and may consequently alter nitrogen cycling processes (Boudsocq et al., 2012). At the same time, nitrification may promote soil acidification or limit the efficiency of soil fertilization using ammonium-based compounds, additionally contributing to nitrogen loss via nitrate leaching and consequent groundwater pollution (Fierer et al., 2009; Schleper and Nicol, 2010; Schlesinger and Bernhardt, 2013; Ward et al., 2011). Transformation of nitrate by denitrification steps is also therefore crucial for the removal of nitrate from soils.

2.1 The nitrogen cycle in soils

Major processes of the nitrogen cycle are described briefly below, highlighting the molecular mechanisms and organisms able to perform these reactions. The respective reactions and genes used as molecular markers in our study, which encode the reaction-catalyzing enzymes, are listed in the

Table 1 below. A particular focus is on the nitrification process and its key players since this is the major nitrogen-transformation step investigated in this study.

The nitrogen cycle is composed of inorganic and organic partial cycles; both are linked via the central metabolite ammonium (NH_4^+), which is accessed by processes such as nitrogen-fixation, nitrification, mineralization and biomass synthesis. Ammonium is utilized for glutamine synthesis in the **organic nitrogen cycle**. This organically bound nitrogen is used for the biomass synthesis of organisms. Different mineralization processes carried out by a variety of bacteria, fungi and archaea then convert organic nitrogen into ammonium again by degrading dead biomass (Cliff et al., 2007; Xiao et al., 2005). The **inorganic nitrogen cycle** (**Figure 1**) describes the fixation of elementary atmospheric nitrogen (N_2) to ammonium (NH_4^+) which is then available for subsequent redox reactions: under aerobic conditions, nitrification produces nitrate (NO_3^-), which can then be converted by denitrifiers in sequential reduction processes by simultaneous oxidation of organic compounds under anoxic conditions. Different nitrogen oxidation states are generated whereby gaseous nitrogen compounds and finally dinitrogen gas are released again into the atmosphere (**Figure 1**, **Table 1**) (Le Roux et al., 2013; Tiedje, 1988). Additionally, ammonia can also be converted under anaerobic conditions in a process called anammox (the anaerobic oxidation of ammonium to dinitrogen). Anammox is performed by only a few bacteria, such as *Planctomycetes*, that perform ammonia oxidation (AO) for energy generation coupled to nitrite oxidation (NO) to NO_3^- whereby NO_2^- serves as e- acceptor (Jetten et al., 2009; Liu et al., 2008).

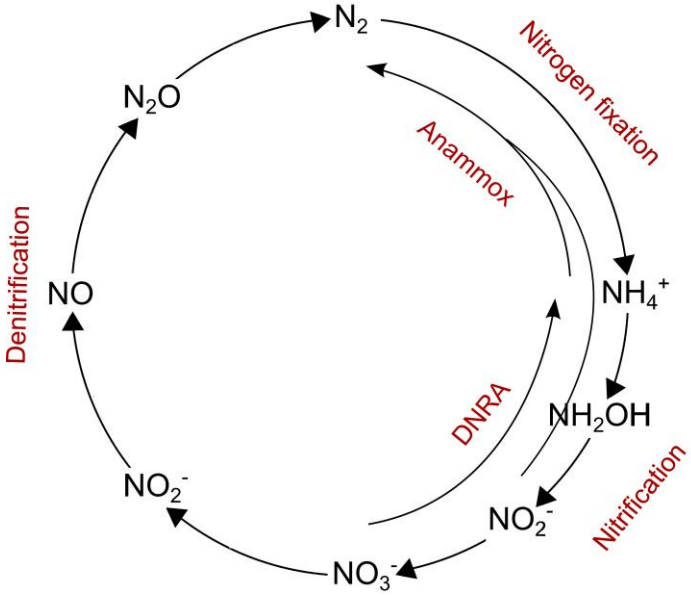

FIGURE 1: **Inorganic nitrogen cycle**.

Scheme depicts processes of the inorganic partial nitrogen cycle.

Biological **nitrogen fixation** (NF) is carried out by so called diazotrophs, comprising free-living or plant associated nitrogen-fixing microbes and symbiotic-living bacteria (Leigh, 2000; Limmer and Drake, 1998; Rusch, 2013). Symbiotic nitrogen-fixers (as *Rhizobium* and *Bradyrhizobium* species) are associated with legumes and contribute to the overall fixation activity in agricultural soils to a greater extent than can be achieved by non-symbiotic NF (Zahran, 1999). The reaction is catalyzed by components of the nitrogenase complex consisting of a dinitrogenase and a dinitrogenase-reductase. The former constitutes a Mo-Fe-protein heterodimer whose task is to bind the substrate, and the latter is responsible for ATP hydrolysis and electron transport. Since the process is very energy-intensive, associated genes such as *nifH*, which encodes the dinitrogenase reductase subunit of the nitrogenase, are only expressed under conditions of nitrogen shortage (Zehr et al., 2003).

	Process	**Reaction**			**Enzyme**	**Marker gene**
	Nitrogen fixiation	$N_2 + 8H^+ + 8e^-$	→	$2NH_3 + H_2$	Nitrogenase	*nifH* *
Nitrification	Ammonia oxidation	$NH_3 + 2H^+ + 2e^- + O_2$	→	$NH_2OH + H_2O$	ammonia monooxygenase	*amoA* *
	Hydroxylamine oxidation	$NH_2OH + H_2O$	→	$HNO_2 + 4H^+ + 4e^-$	hydroxylamine oxidoreductase	*hao*
	Nitrite oxidation	$NO_2^- + H_2O$	→	$NO_3^- + 2H^+ + 2e^-$	nitrite oxidoreductase	*nrxA* *
Denitrification	Nitrate reduction	$NO_3^- + 2H^+ + 2e^-$	→	$NO_2^- + H_2O$	nitrate reductase	*napA, narG*
	Nitrite reduction	$NO_2^- + 2H^+ + 2e^-$	→	$NO + H_2O$	nitrite reductase	*nirS, nirK* *
	Nitric oxide reduction	$2NO + 2H^+ + 2e^-$	→	$N_2O + H_2O$	nitritc oxide reductase	*qnor, cnor*
	Nitrous oxide reduction	$N_2O + 2H^+ + 2e^-$	→	$N_2 + H_2O$	nitrous oxide reductase	*nosZ* *

TABLE 1: **Transformation processes of inorganic nitrogen-cycle.**

Reactions are given to complement the transformation steps catalyzed by the respective enzymes. Encoding genes which are commonly used as marker for molecular analyses are listed according to the respective processes. Gene abundances detected in the frame of this study are marked with asterisks.

Nitrate, an ecologically important compound in the nitrogen cycle, can easily be washed out from the system due to its volatile nature. It can be assimilated by plants or used by microorganisms for transformation processes. Nitrate can be dissimilated during denitrification or DNRA (dissimilatory nitrate reduction to ammonium) (Song et al., 2013). In DNRA, nitrate is reduced to ammonium and retained in the system (Song et al., 2014), while nitrogen is released into the atmosphere in **denitrification**. The latter reaction is the stepwise reduction of NO_3^- to N_2, performed by a variety of bacteria, archaea and fungi under anaerobic conditions (Hayatsu et al., 2008; Philippot et al., 2007; Rusch, 2013; Zehr and Ward, 2002) The complete denitrification pathway comprises four consecutive reaction steps: Reduction of nitrate (NO_3^-) to nitrite (NO_2^-), to nitric oxide (NO), to nitrous oxide (N_2O) and to dinitrogen (N_2) (Giles et al., 2012). The first reaction in which NO_3^- serves as electron acceptor is catalyzed by both periplasmic (NAP) and membrane-bound nitrate reductase (NAR) (Philippot et al., 2007; Zumft, 1997). The second reduction step of NO_2^- to NO is catalyzed by either a cytochrome cd1 or a copper containing nitrite reductase (NIR) (Kandeler et al., 2006; Philippot et al., 2007). *Nir* genes of denitrifying organisms are usually expressed under anaerobic conditions and in the presence of nitrite. Interestingly, nitrite reductase encoding genes have also been detected in nitrifiers, with different expression patterns depending on nitrite (NO_2^-) availability and under low-oxygen or aerobic conditions (Lücker et al., 2010; Starkenburg et al., 2008; Urich et al., 2008). N_2O, a potent greenhouse gas, is the product of subsequent nitric oxide reduction, catalyzed by membrane-bound nitric oxide reductases (NOR). Homodimeric nitrous oxide reductase (NOS) catalyzes the last conversion of N_2O into dinitrogen gas N_2 which is the most O_2 sensitive step that can be carried out by bacteria and archaea (Philippot et al., 2007; Rusch, 2013). The complete denitrification process releases dinitrogen gas after the conversion of nitrification-derived nitrate from the system.

2.2 The nitrification process

Nitrification – the conversion of ammonia (NH_3) to nitrate - delivers the substrate for denitrification in a stepwise oxidation process: ammonia-oxidation, hydroxylamine oxidation and nitrite-oxidation. The oxidation of NH_3 to NO_2^- is commonly carried out by ammonia-oxidizers whereas the turnover to NO_3^- is performed by a distinct group, the nitrite-oxidizing bacteria (NOB) (**Figure 2**).

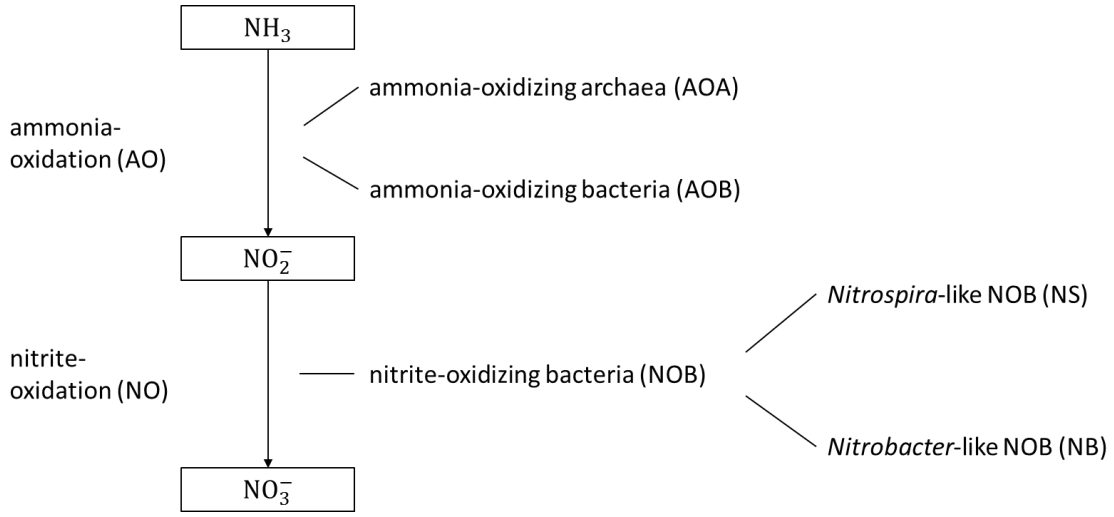

FIGURE 2: **Key players in nitrification.**

Scheme depicts simplified transformation steps of nitrification including the major functional groups performing these steps.

In the process of chemolithoautotrophic **ammonia-oxidation** (AO), ammonia serves as a reductant that is oxidized utilizing oxygen as an electron acceptor (Stein et al., 2013). The energy is used to fix carbon dioxide (CO_2), which constitutes the sole carbon source (Norman and Barrett, 2014; You et al., 2009). Ammonia, the substrate for AO, reaches the cell cytoplasm via passive diffusion across cell membranes, however, additional transporters for NH_4^+ / NH_3 have been proposed that might supplement passive uptake by an active transport system in the cell (Burton and Prosser, 2001; Schmidt et al., 2004; Suzuki et al., 1974; Ward et al., 2011). The first step, the oxidation of ammonia (NH_3) to hydroxylamine (NH_2OH), is carried out by ammonia-oxidizing bacteria (AOB) (Kowalchuk and Stephen, 2001; Ward et al., 2011) and ammonia-oxidizing archaea (AOA) (Hallam et al., 2006; Schleper et al., 2005; Treusch et al., 2005; Venter et al., 2004; Ward et al., 2011). The reaction is catalyzed by the membrane-bound enzyme ammonia monooxygenase (AMO) and is oxygen dependent (Hooper et al., 1997). The three subunits of this enzyme are encoded in the bacterial *amoCAB* operon (Arp et al., 2007); for AOA, the gene order *amoBCA* and an uncoupling of the *amoC* gene to another locus have been observed (De La Torre et al., 2008; Nicol and Schleper, 2006; Spang et al., 2012). The *amoA* gene transcription is upregulated in the presence of ammonium and its abundance is used as a proxy for the ammonia-oxidation process (Rotthauwe et al., 1997; Stein et al., 2013). Albeit for AOA one copy per cell has been proposed, this gene seems to be present in the genomes of AOB strains in up to three copy numbers which might be regulated differently (Hallam et al., 2006; Norton et al., 1996; Walker et al., 2010; Ward et al., 2011). AMO seems to be capable of oxidizing a variety of different substrates besides ammonia: e.g. alkanes, ethers or aromatic

hydrocarbons, and exhibits high sequence and structural similarity to particulate methane monooxygenase (pMMO) in methanotrophs, which enables the switch of substrates (Holmes et al., 1995; Kowalchuk and Stephen, 2001; Stahl and de la Torre, 2012; Ward et al., 2011). AMO has been shown to be inactivated by nitrite (Ward et al., 2011).

The following reaction is the oxidation of hydroxylamine (NH_2OH) to nitrite, which takes place in the periplasm of the cell. In AOB, this step is catalyzed by the heme-containing enzyme hydroxylamine oxidoreductase (HAO), encoded by the *hao* gene that is also present in multiple copies (Arp et al., 2007). This second reaction couples the oxidation of ammonia to the electron transport system. Four electrons are channeled through a cytochrome c- dependent electron transport system to the ubiquinone pool; two electrons are used for energy generation via ATP, the other two are transferred back to AMO (Vajrala et al., 2013). The two finally released protons of the whole process contribute to acidification of the surrounding environment (Klotz and Stein, 2008; Ward et al., 2011). Neither could be a homologue gene to bacterial *hao* detected in AOA so far, nor was any evidence found for an iron-based cytochrome dependent electron transfer system (Hallam et al., 2006; Walker et al., 2010). It is supposed that copper-based systems might take over this function instead (Walker et al., 2010; Ward et al., 2011). Nevertheless, studies providing evidence for the active conversion of ammonia to nitrite in AOA strongly support the assumption that the final oxidation product is indeed nitrite even though intermediate steps are still unknown (De La Torre et al., 2008; Konneke et al., 2005). The generation of an unidentified novel intermediate as e.g. nitroxyl (HNO) or NO, that could then be further oxidized to nitrite by a multi-copper protein or the presence of a yet unknown novel enzyme catalyzing the oxidation of hydroxylamine has been proposed (Vajrala et al., 2013; Walker et al., 2010). In this context, high abundances of genes encoding nitrite reductases in AOA, which are also expressed under aerobic conditions, have also gained attention (Hatzenpichler, 2012; Urich et al., 2008).

Under aerobic conditions, nitrite is oxidized to nitrate in the ATP-generating **nitrite-oxidation** (NO) process (Ehrich et al., 1995). The reaction is catalyzed by membrane-associated nitrite oxidoreductase (NXR), whose subunits are encoded by *nxrA* and *nxrB*, and which are present in multiple copies, whereby one copy may be constitutively expressed and the transcription of *nxrB* genes may be increased only in the presence of nitrite (Lücker et al., 2010; Starkenburg et al., 2006; Sundermeyer-Klinger et al., 1984). Subunit A harbors the binding site of the substrate; subunit B, an iron-sulfur containing protein, is involved in the electron transport system. The process may also be supported by the function of a putative subunit C (encoded by *nxrC* gene) connecting the NXR subunits to the membrane. In *Nitrobacter*, the location of NXR is cytoplasmic and intracytoplasmic;

Nitrospira, however, lack intracytoplasmic membranes (Bock and Wagner, 2006; Starkenburg et al., 2008; Starkenburg et al., 2006). Consequently, the localization of the NXR complex in *Nitrospira* is periplasmic, which facilitates the transport processes to and from the cytoplasm and increases the efficiency of energy generation, allowing growth at low nitrite concentrations (Bock and Wagner, 2006; Lücker et al., 2013; Spieck and Bock, 2005; Spieck et al., 1996; Starkenburg et al., 2006).

In the absence of O_2, *Nitrobacter* NXR can also catalyze the reduction of nitrate to nitrite using organic substances like pyruvate as substrate, and NADH as electron donor with NO_3^- as electron acceptor (Bock et al., 1988; Ehrich et al., 1995; Freitag et al., 1987; Lücker et al., 2013; Sundermeyer-Klinger et al., 1984). In contrast to NO, this process occurs under anaerobic conditions, similar in function to dissimilatory nitrate reduction. The presence of *nir* genes in some *Nitrobacter* strains indicates that NO_2^- could be further reduced to nitric oxide under anaerobic conditions (Lücker et al., 2010; Starkenburg et al., 2008; Zumft, 1997).

3 Ecophysiological characteristics of nitrifying organisms

Nitrification is commonly split into ammonia- and nitrite-oxidation, referring to the fact that the conversion of ammonia to nitrite and subsequent nitrite-oxidation are performed by distinct groups of organisms (Kowalchuk and Stephen, 2001). Although specialized nitrite-oxidizers isolated from biofilm and aquaculture, have been shown to possess the genetic potential to perform the complete nitrification process, this has not been demonstrated in terrestrial environments so far (Daims et al., 2015; van Kessel et al., 2015). Heterotrophic bacteria and fungi have also been shown to perform nitrification and may be of particular importance in forest ecosystems (Killham, 1990; Schimel et al., 1984; Ward et al., 2011). Heterotrophic bacteria utilize the same substrate as autotrophic nitrifiers, though this process is not necessarily coupled to growth (Ward et al., 2011). Fungi, however, rely on organic nitrogen compounds for the reaction, which is independent of energy generation purposes (De Boer and Kowalchuk, 2001). To what extent heterotrophic organisms contribute to nitrification, especially in acidic forest soils, is still a matter of debate (Koops and Pommerening-Röser, 2001; Pennington and Ellis, 1993; Ward et al., 2011). Only the functional groups whose presence has been addressed in this study are described in detail in the following section, with respect to their phylogenetic diversity and metabolism and growth strategies of representatives. Both ammonia- and nitrite-oxidizers are ubiquitous in a large variety of ecosystems, ranging from oligotrophic to nutrient-rich environments, where they differ in their preferences for certain habitats depending on respective environmental parameters.

3.1 Diversity, distribution and metabolism of ammonia-oxidizers

Since the discovery of *amoA* gene homologues in archaea from soil (Treusch et al., 2005) and the subsequent isolation and cultivation of *N. maritimus*, an archaeon able to oxidize ammonia, it has become evident that AO can be performed both by AOB and AOA in soils (De La Torre et al., 2008; Hallam et al., 2006; Hatzenpichler et al., 2008; Konneke et al., 2005; Kowalchuk and Stephen, 2001; Leininger et al., 2006; Offre et al., 2009; Schauss et al., 2009; Schleper et al., 2005). This refuted the then-prevailing dogma that *β*- and *γ-Proteobacteria* were exclusively capable of autotrophic AO in soils, and at the same time raised questions concerning the abundances, co-occurrence patterns and relative contributions from archaea and bacteria to AO potential in soils.

Four major ecotypes of **ammonia-oxidizing bacteria** can be distinguished based on their presence in different habitats: freshwater sediments, wastewater, terrestrial, and marine environments (Koops and Pommerening-Röser, 2001; Kowalchuk and Stephen, 2001; Ward et al., 2011; Zehr and Ward, 2002). Representatives of AOB can be found amongst the *β*- and *γ-Proteobacteria* comprising *Nitrosococcus, Nitrosolobus, Nitrosovibrio, Nitrosospira* and *Nitrosomonas*. The last two genera are members of *β-Proteobacteria* (Koops et al., 2006). The latter genus can be subdivided into six lineages based on phylogenetic analyses of 16S rRNA and *amoA* gene sequences, further separated by different characteristics concerning their habitats and preferences for different substrate concentrations or pH (Koops and Pommerening-Röser, 2001). Cluster 6A, for instance, contains strains that are found in freshwater and terrestrial habitats characterized by lower substrate concentrations, whereas representatives of cluster 7, with *Nitrosomonas europaea* as representative, are found in various environments including those with higher ammonia concentrations (Ward et al., 2011). Despite higher sequence similarity, *Nitrosospira* strains can also be grouped into different clusters spanning the whole range from freshwater and terrestrial habitats to marine habitats and sediments (De Boer and Kowalchuk, 2001; Koops and Pommerening-Röser, 2001; Pommerening-Röser and Koops, 2005). Lineages assigned to cluster 3 are largely derived from different soils and dominate autotrophic AO in agricultural soils, and representatives from other clusters have even been found in acidic soils (Avrahami et al., 2002; De Boer and Kowalchuk, 2001; Jiang et al., 2014; Kowalchuk and Stephen, 2001; Webster et al., 2005; Xia et al., 2011).

The vast majority of AOB are chemolithoautotrophs, meaning they use CO_2 as their sole carbon source, ammonia as their sole energy source and reductant, which is thought to be assimilated via the Calvin-Benson-Bassham cycle; all genes involved in the respective processes of this cycle have been found to be encoded in AOB genomes (Arp and Bottomley, 2006; Arp et al., 2007; Xia et al.,

2011). Not all AOB require ammonia as their source for growth; they have also been shown to hydrolyze urea as an organic source (Burton and Prosser, 2001; Koops et al., 2006; Koper et al., 2004). Recent evidence from genome studies indicate organotrophic growth capacity (Arp and Bottomley, 2006; Schmidt, 2009; Ward et al., 2011).

Like their bacterial counterparts, **ammonia-oxidizing archaea** are distributed amongst diverse environments, such as aquatic coastal, marine and freshwater (Beman et al., 2012; Francis et al., 2005; Rogers and Casciotti, 2010; Santoro et al., 2010; Wuchter et al., 2006) as well as terrestrial ecosystems (Hai et al., 2009; He et al., 2007; Lehtovirta et al., 2009; Leininger et al., 2006; Nicol et al., 2008; Ochsenreiter et al., 2003; Schauss et al., 2009; Treusch et al., 2005). They even tolerate rather unfavorable conditions characterized by energy shortages, such as oligotrophic habitats, strongly acidic soils, aquatic systems with high salt concentrations, and temperature spans from hot geothermal springs up to 97°C to rather cold environments (DeLong, 1992; Gubry-Rangin et al., 2011; Kalanetra et al., 2009; Lebedeva et al., 2013; Lebedeva et al., 2011; Ward et al., 2011). Autotrophic AOA are assigned to the phylum *Thaumarchaeota*, previously classified as *Crenarchaeota* (Brochier-Armanet et al., 2008; Pester et al., 2011; Spang et al., 2012; Stahl and de la Torre, 2012). Sequences of an *amoA*- based phylogenetic analysis cluster according to the two major habitats where AOA are present: terrestrial ecosystems (group I) and marine environments (group II) (Prosser and Nicol, 2008). This is in line with 16S rRNA gene based phylogenetic classifications assigning sequences into distinct clades (Nicol et al., 2008): group I is further distinguished into clade I group I.1a comprising sequences derived from marine environments and sediments (representative: *N. maritimus*), group I.1b dominating in terrestrial habitats (representative: *N. gargensis*) (Hatzenpichler, 2012; Konneke et al., 2005; Prosser and Nicol, 2008; Stahl and de la Torre, 2012; Ward et al., 2011) and group I.1c prevalent in acidic soils but also freshwater and sediments (Kemnitz et al., 2007; Lehtovirta et al., 2009; Nicol et al., 2004; Oline et al., 2006). Although it has been assumed from cultivation studies that all archaea harboring *amoA* gene copies are AOA, recent data suggest that not all are indeed performing active AO and should therefore be referred to as '*amoA*-encoding archaea' (AEA) (Mußmann et al., 2011).

Autotrophic growth, specifically carbon assimilation, appears to be mediated via the 3-hydroxypropionate/4-hydroxybutyrate pathway in different AOA representatives (Walker et al., 2010; Ward et al., 2011). Genome studies have proposed that AOA is capable of different growth and metabolic strategies besides chemolithoautotrophy (Prosser and Nicol, 2012; Stahl and de la Torre, 2012; Walker et al., 2010; Xia et al., 2011). This has been further confirmed by microcosm studies demonstrating the absence of active nitrification (Offre et al., 2009). Besides growth on inorganic

compounds, there is evidence that organic compounds such as pyruvate can also be used, indicating a putative mixotrophic lifestyle, where both organic compounds and CO_2 can be used as carbon sources (Hallam et al., 2006; Jia and Conrad, 2009; Rogers and Casciotti, 2010; Tourna et al., 2011). The preference for different growth strategies and lifestyles, including autotrophic, mixotrophic, or heterotrophic growth, has been shown to vary between different lineages and even within one clade (Jia and Conrad, 2009; Treusch et al., 2004; Treusch et al., 2005). Increasing evidence indicates the utilization of alternative substrates apart from ammonia for energy generation such as urea or cyanate (Hallam et al., 2006; Mußmann et al., 2011; Palatinszky et al., 2015; Spang et al., 2012; Tourna et al., 2011).

3.2 Diversity, distribution and metabolism of nitrite-oxidizers

NOB have been found in a wide range of terrestrial and aquatic ecosystems, including hot springs and permafrost soils, which constitute extreme living conditions (Abeliovich, 2006; Alawi et al., 2009; Bartosch et al., 2002; Stein et al., 2001; Watson et al., 1986; Wertz et al., 2012). Most studies on NOB have been performed in wastewater treatment plants (WWTP), however (Daims et al., 2001; Gieseke et al., 2003; Juretschko et al., 1998; Spieck et al., 2006). The phylogeny of NOB is as diverse as their distributions, consisting of representatives from five different genera affiliated to the phyla *Nitrospirae* and *Chloroflexi* as well as to *Proteobacteria* classes (α-, β-, γ- and δ-): *Nitrospira* and *Nitrolancetus* as well as *Nitrobacter*, *Nitrotoga*, *Nitrococcus* and *Nitrospina* (Alawi et al., 2009; Attard et al., 2010; Bock and Wagner, 2006; Daims et al., 2001; Sorokin et al., 2012; Spieck and Bock, 2005).

Both *Nitrococcus* and *Nitrospina* species are abundant mainly in marine ecosystems (Juretschko et al., 1998; Lücker et al., 2013; Teske et al., 1994). So far, *Nitrotoga* have been detected in permafrost soils, but there is increasing evidence of their abundance in other environments also, such as activated sludge (Alawi et al., 2009). In the majority of terrestrial habitats, *Nitrobacter* (NB) and *Nitrospira* (NS) have emerged as the dominant genera (Bartosch et al., 2002; Ke et al., 2013; Ollivier et al., 2013) and thus are described in more detail below.

Though monophyletic ***Nitrospira*-like nitrite-oxidizing bacteria** are often the predominant NOB in natural aquatic and engineered systems, they are frequently found in sediments and soils (Bartosch et al., 2002; Cébron and Garnier, 2005; Kim and Kim, 2006). Since the growth of NS species is very slow, only a few species have been cultured or enriched to date in order to gain insight into their ecophysiological characteristics. However, 16S rRNA gene-based approaches have made possible the classification of NS-like NOB sequences into sublineages derived from different environmental backgrounds (Daims et al., 2001; Maixner et al., 2006). Sublineage I (representative: '*Candidatus* N.

defluvii') is restricted to wastewater treatment plants and other nitrifying bioreactor systems (Daims et al., 2001; Lücker et al., 2010; Spieck et al., 2006), while members of sublineage II (representative: *N. moscoviensis*) are frequently found in various habitats from soils to groundwater and surface freshwater ecosystems, in heating systems, and also in bioreactors (Daims et al., 2001; Ehrich et al., 1995; Maixner et al., 2006). Sublineage III, in contrast, has been observed exclusively in cave systems and the deep sea (Daims et al., 2001; Holmes et al., 2001), and sublineage IV (representative: *N. marina*) contains sequences mainly derived from marine habitats, including sponges (Daims et al., 2001; Hentschel et al., 2002; Watson et al., 1986). In environments similar to those of representatives of sublineage II, sublineage V (representative: *Candidatus* 'N. bockiana') has been detected, while sublineage VI (representative: *Nitrospira calida*) comprises thermophilic members in hot springs (Lebedeva et al., 2008; Lebedeva et al., 2011).

The above mentioned sublineages also differ in their metabolisms and physiological characteristics, such as morphology, temperature optima, preference for aggregation with other microorganisms and their capability to use either inorganic or organic compounds for growth. Besides chemolithoautotrophic growth, heterotrophic and also mixotrophic growth capacity has been observed (Daims et al., 2001; Ward et al., 2011; Yu et al., 2014). The utilization of organic carbon sources seems to be restricted to aerobic conditions (Daims et al., 2001). For carbon fixation in '*Candidatus* N. defluvii', it is assumed that the reverse tricarboxylic acid cycle (TCA) can be used, however reverse electron transport may be an energy consuming process. For *Nitrospira* such as *Ca.* Nitrospira defluvii, the capacity to use both inorganic and organic carbon sources has been reported (Daims et al., 2001; Lücker et al., 2010). However it is still not entirely clear whether all these compounds are assimilated for energy purposes (Daims et al., 2001; Lücker et al., 2010; Spieck et al., 2006). Sublineages V and VI comprise autotrophs and obligate autotrophs respectively, such as sublineage II (Ehrich et al., 1995; Lebedeva et al., 2008; Lücker et al., 2010). Under anoxic conditions, alternative processes might take over: nitrite can be reduced and used as an electron acceptor by hydrogen oxidation (Ehrich et al., 1995).

Information on ecophysiological characteristics of NOB is mostly derived from data of the ***Nitrobacter*-like nitrite-oxidizing bacteria**, since representatives of this genus are relatively easy to culture (Sorokin et al., 1998). Based on 16S rRNA gene sequences, all *Nitrobacter* strains exhibit high similarity and are grouped together with *Bradyrhizobium sp*. and *Rhodopseudomonas*, which, however, do not belong to the functional group of NOB (Freitag et al., 2005; Orso et al., 1994; Poly et al., 2008; Regan et al., 2003). *Nitrobacter* thrive under many different environmental conditions, including both acidic and alkaline soils, aquatic ecosystems, biofilms, and even on rocks (Bock et al.,

1990; Daims et al., 2001; Gieseke et al., 2003; Hankinson and Schmidt, 1988; Sorokin et al., 1998; Ward et al., 2011).

Their metabolisms range from obligate to facultative lithoautotrophic, with mixotrophic and heterotrophic growth, respectively, utilizing organic compounds as pyruvate, lactate, acetate or glycerol (Bock et al., 1988; Ehrich et al., 1995; Freitag et al., 1987; Steinmüller and Bock, 1977). *Nitrobacter* grow "aerobically by nitrite-oxidation and anaerobically by denitrification" and use the Calvin-Benson-Bassham-cycle for carbon dioxide fixation (Freitag et al., 1987; Ward et al., 2011). The presence of diverse unique enzyme sets may enable some species as *N. hamburgensis* to change to alternative metabolic pathways with strains containing plasmids for diverse metabolic and other functions (Freitag et al., 1987; Starkenburg et al., 2008; Sundermeyer-Klinger et al., 1984; Ward et al., 2011).

4 Determinants of ecological niche separation

Nevertheless, the relative contributions of AOA and AOB or NS and NB to actual AO and NO, respectively, is still unclear as are their responses to certain environmental parameters in terms of activity or growth. Redundancy among organisms performing the same activity is a frequently observed mechanism to ensure the maintenance of a process and consequent ecosystem stability (Wolters et al., 2000). The level of redundancy determines the level of tolerance and stability of a certain process to changing environmental conditions, and is rather low between AOA and AOB and NS and NB compared to other groups due to the limited number of key players capable of performing the same process (Giles et al., 2012; Horz et al., 2004; Schauss et al., 2009; Wolters et al., 2000). Thus, it is important to identify their different characteristics vis a vis utilization of resources and adaptations to and preferences for particular environmental properties so as to draw conclusions about ecosystem resilience. Adaptations to special environmental characteristics might allow co-existence of organisms due to the presence of distinct ecological niches. These are shaped by environmental properties and contrasting requirements of redundant organisms, thereby reducing direct competition (Giles et al., 2012). For nitrifiers, factors as soil pH, lifestyle, and metabolism have been suggested as shapers of niche differentiation, while the primary ascribed factor might be their adaptation to substrate concentration (Nicol et al., 2008; Prosser and Nicol, 2012).

4.1 Ammonia-oxidizing archaea versus ammonia-oxidizing bacteria

AOA have been found to dominate in numbers over their bacterial counterparts in various environments and under different land-use conditions (He et al., 2007; Leininger et al., 2006; Martens-Habbena et al., 2009; Schauss et al., 2009). Several studies on this topic, however, mainly addressing the effects of fertilizer application, have revealed contradicting results (Di et al., 2009; Jia and Conrad, 2009; Xia et al., 2011). Offre and colleagues conducted an acetylene inhibition study demonstrating that nitrification activity in microcosms without ammonia amendment is associated exclusively with growth of AOA (Offre et al., 2009). In contrast, other groups attributed nitrification rates to the performance of AOB also associated with their growth in nitrogen-rich soils (Di et al., 2010; Di et al., 2009; Jia and Conrad, 2009; Le Roux et al., 2008). However, these different conclusions become more understandable taking into account the experimental setups, for example in terms of the amounts of ammonia applied.

The mechanism behind the different responses to fertilization can be attributed to the distinct preferences for substrate concentrations: AOA may be particularly well adapted to environments with nutrient- and / or oxygen-limitations, whereas AOB are favored in habitats with high ammonia content, such as fertilized grasslands (Hatzenpichler, 2012; Jia and Conrad, 2009; Leininger et al., 2006; Martens-Habbena et al., 2009; Norman and Barrett, 2014; Pratscher et al., 2011; Prosser and Nicol, 2012; Schleper, 2010). Despite these preferences, the presence of AOB has been detected under low substrate environments and there are strains adapted to low NH_4^+ in culture (Bollmann et al., 2013). But the minimum substrate concentrations of about 10 nM, with which AOA are able to cope, reach far beyond those of AOB (Martens-Habbena et al., 2009; Ward et al., 2011). In consequence, AOB would starve under low ammonia conditions in unfertilized soils (required: 100 µg nitrogen per g soil), while AOA would still be capable of maintaining growth below 1 µg nitrogen per g soil (Martens-Habbena et al., 2009; Offre et al., 2009; Valentine, 2007). Growth of AOA is only observable up to concentrations of 1.5 mM of ammonia, while the maximum ammonia tolerance level of AOB is around 1,000 mM (Koops et al., 2006; Prosser and Nicol, 2012). In contrast to the high tolerance levels of AOB, AOA can be inhibited by ammonia concentrations higher than 3 mM (Hatzenpichler et al., 2008; Webster et al., 2005). Nevertheless, different preferences for nitrogen availabilities have been determined amongst AOB strains and AOA phylotypes, indicating intraspecific niche separation (Carney et al., 2004; Rogers and Casciotti, 2010; Santoro et al., 2010; Schramm et al., 1998). Different requirements in terms of substrate supply can be explained by the comparatively high substrate affinity of AOA in combination with cell concentrations and specific activity determining the relative activity per cell, which seems to be higher in AOB than in AOA

(Könneke et al., 2005; Martens-Habbena et al., 2009; Prosser and Nicol, 2012; Stahl and de la Torre, 2012). The larger cell size of AOB, determining the surface to volume ratio, has also to be taken into account when relative contributions to AO are compared in the context of per cell activity rate (Könneke et al., 2005; Koops and Pommerening-Röser, 2001; Prosser and Nicol, 2012).

4.2 *Nitrospira*-like versus *Nitrobacter*-like nitrite-oxidizing bacteria

Community composition of NOB is considered to be dependent on substrate concentration and oxygen status as well as on quantity and quality of available organic compounds (Daims et al., 2001; Freitag et al., 2005). Contrasting results were obtained on the dominant NOB genera in WWTPs and reactors (Juretschko et al., 1998; Schramm et al., 1999; Schramm et al., 1998). Inter- and intraspecific ecological niche separation has been proposed, based on their co-occurrence at the same spatial scale under changing NO_2^- conditions (Bartosch et al., 2002; Daims et al., 2001; Freitag et al., 2005; Maixner et al., 2006): both genera and lineages might have different demands on and adaptions to their environment (Daims et al., 2001). Not only substrate availability but also substrate affinity is a major determinant of niche separation between NB and NS (Attard et al., 2010; Kim and Kim, 2006). A lower nitrite half-saturation constant value (K_s) for NS compared to NB revealed that substrate affinity of NB was significantly lower (Blackburne et al., 2007; Schramm et al., 1999). Thus, NS as *K*-strategists show high activity and growth even under low nitrite conditions, while NB, as *r*-strategists, would require much higher concentrations of nitrite in the environment with low substrate affinity but would exhibit higher growth rates under high substrate availability (Lebedeva et al., 2011; Schramm et al., 1999). *Nitrobacter* can tolerate concentrations up to 45 mM that would be even inhibitory for NS (Bartosch et al., 2002; Blackburne et al., 2007; Kim and Kim, 2006; Schramm et al., 1999; Ward et al., 2011). This fits well to the ecological distribution of both groups, where NS seem to have an advantage in nitrite-limited environments (Bartosch et al., 2002; Ehrich et al., 1995). Different preferences for optimum substrate concentrations also account for interspecific niche separation (Schramm et al., 1998): members of NS sublineages were found at different distances to nitrite-producing AOB in a biofilm. This indicated that NS follow a nitrite gradient dependent on their substrate concentration preferences (Maixner et al., 2006).

Besides nitrite concentration, dissolved oxygen availability is also a niche shaping factor. NS with lower K_O values for oxygen may be better able to cope with low dissolved oxygen and to out-compete most NB in oxygen-limited environments (Schramm et al., 2000; Ward et al., 2011). Remarkably, NB and some NS sublineages seem to have an advantage under anoxic conditions by using pyruvate for growth (Bock et al., 1988; Daims et al., 2001; Park and Noguera, 2008).

5 Interactions of ammonia- and nitrite-oxidizers

The low or almost undetectable amounts of nitrite in natural terrestrial habitats (Burns et al., 1995) give reason to presume that the activity and thus abundance of ammonia-oxidizers might be closely coupled to the activity of NOB in a mutualistic relationship. Nitrite, generated from ammonia-oxidation, is likely to be immediately converted as substrate into nitrate during the process of nitrite-oxidation (Gieseke et al., 2003; Teske et al., 1994). Thus the performance of ammonia-oxidizers was meant to be an essential prerequisite for substrate supply to autotrophic nitrite-oxidizers, considered to be the rate-limiting step. However, recent studies postulate putative feedback processes via ammonium, derived from cyanate transformation by NOB, which is in turn available as substrate for AO (Palatinszky et al., 2015; Stein, 2015). Ammonia- and nitrite-oxidation processes depend on each other; thus their key players are also likely to interact (Graham et al., 2007; Kim and Kim, 2006). Ammonia- and nitrite-oxidizers often are spatially associated and their distance seems to be actively controlled (Grundmann and Debouzie, 2000). This indicates a putatively strict regulation of distributions and interaction partners to meet both the requirements of substrate concentrations while at the same time preventing toxic or inhibiting concentrations of the product nitrite (Maixner et al., 2006).

Interactions in terms of the co-occurrence at the same spatial scale between representatives of ammonia- and nitrite-oxidizers are mainly assessed in nitrifying bioreactors or WWTPs and biofilms. NS-like NOB have been reported to be highly dependent on nitrite produced by spatially segregated *Nitrosospira* (AOB), while *Nitrobacter* and *Nitrosomonas* (AOB) co-occurred (Mobarry et al., 1996; Schramm et al., 1999). But the co-existence of different AOB populations and both NS- and NB-like NOB have also been observed in biofilms (Gieseke et al., 2003), and this has been attributed to different oxygen requirements of AOB, whereas different adaptations to substrate concentrations may shape ecological niches of NB and NS. This is also valid for the distribution of NS sublineages, found to co-occur with ammonia-oxidizers at different distances (Maixner et al., 2006). The choice of interaction partners might differ between ecosystems: in marine habitats, for instance, the interplay of AOA and *Nitrospina* seems likely as they exhibit congruent abundance depth profiles (Mincer et al., 2007). AOB and NB, both adapted to higher substrate concentrations, have been demonstrated to dominate nitrification in fertilized forest soils (Wertz et al., 2012). Interaction partners might differ not only between soil ecosystems but may also be related to soil compartments. In the rhizosphere of a rice paddy soil, AOA and NB co-occurred, whereas AOB and NS were abundant in the surface soil compartment (Ke et al., 2013).

However, interactions and in particular distributions of ammonia- and nitrite-oxidizers may also rely on oxygen, on substrate and carbon contents in soil, in gradients of nitrifying reactors, and in marine systems (Schramm et al., 1999). In these contexts, successful nitrification may be impeded when ammonia- and nitrite-oxidizers compete for oxygen, as AO are superior competitors (Gieseke et al., 2003; Ke et al., 2013; Schramm et al., 1999). Nitrite-oxidizers are also inhibited by too high NH_4^+ concentrations or hydroxylamine in oxygen-limited environments (Maixner et al., 2006; Monreal et al., 1986; Stüven et al., 1992). Adaptions of nitrite-oxidizers to these inhibiting substances may shape their spatial distances to ammonia-oxidizers and interaction partners, respectively. Not only similar modes of substrate utilization may require interactions but also adaption and tolerance of similar environmental conditions. For example, thermophilic sublineages of AOA and NS have been proposed to interact in volcanic grassland soils (Daebeler et al., 2014). Hence, most information on ecophysiological characteristics of key players are mainly obtained from cultivation studies, such as their optimal growth conditions and niche requirements, and thus might be valid only for the particular strain of interest (Ward et al., 2011). It remains unclear how changing environmental conditions may affect interacting groups of microorganisms catalyzing the sequential steps of transformation processes such as nitrification in natural ecosystems.

6 Drivers of microbial performance

To understand the behavior of soil microorganisms, one has to take a deeper look at the environmental conditions they are exposed to, as multiple environmental factors influence the abundance and community structure of microbial groups in soil. The complex interplay of those factors often impedes the identification of drivers and relationships of interacting soil microbes (Fierer et al., 2009). A selection of drivers that might be of relevance for the dominant community under observation at our study sites is described below and schematically depicted in **Figure 3**.

Drivers can be commonly arranged into different classification schemes. Usually, factors are classified into abiotic (climatic conditions and chemical / physical soil properties), biotic (below- and aboveground biota such as plants and microbes) and anthropogenic factors with regard to different land management regimes. Here, only individual variables are introduced although a complex network of cross-actions might exist involving the modulation of such variables by superordinate drivers. This network may then indirectly interfere with belowground biota and in particular influence important nitrogen cycle processes such as nitrification in soil (Kowalchuk and Stephen, 2001; Le Roux et al., 2013).

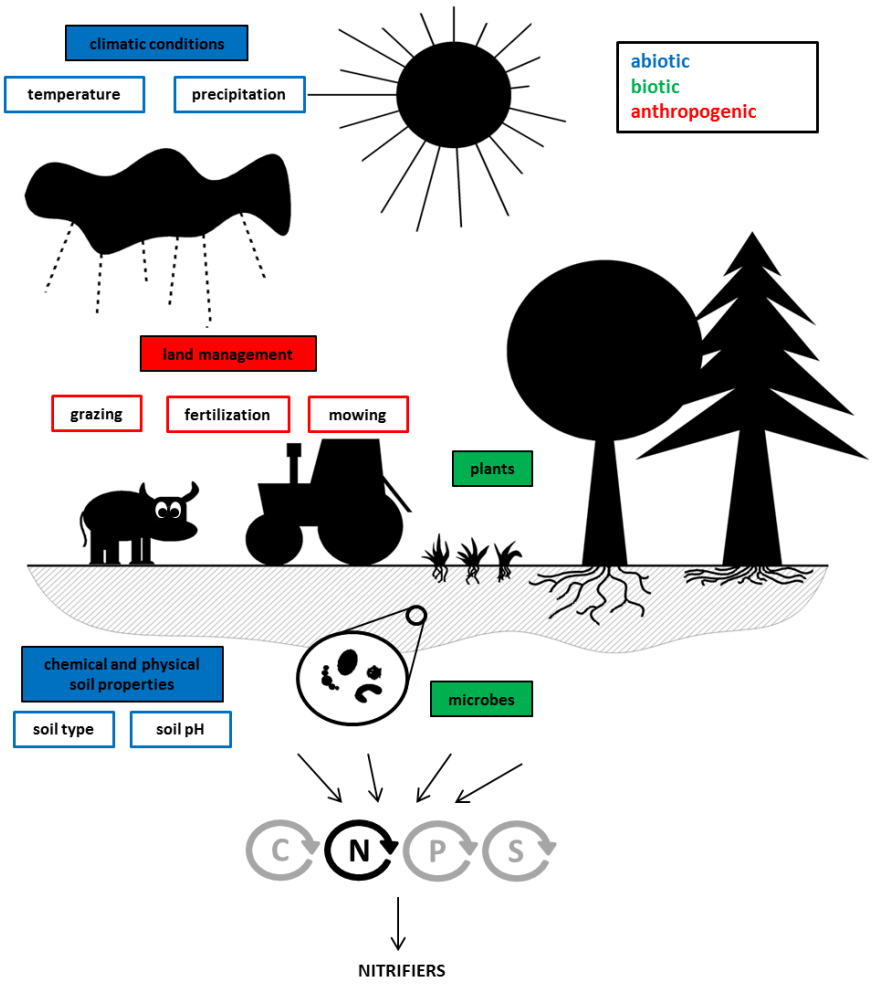

FIGURE 3: **Drivers of nitrifying communities in terrestrial habitats.**

Different levels of environmental variables affecting nutrient cycles in soil and driving microbial performance are classified into abiotic, biotic and anthropogenic factors. Selection of factors assigned to the three major classes of environmental drivers is exemplary. Classification is indicated by color code.

6.1 Abiotic site-specific factors

Soil pH

Besides other chemical and physical soil properties, mainly soil pH influences nutrient availability and associated processes in soils as well as plant productivity and organic matter turnover (Kemmitt et al., 2006). Soil pH is generally considered one of the major drivers for microbial performance and a "predictor of overall bacterial diversity" (Fierer and Jackson, 2006; Kemmitt et al., 2006; Kuramae et al., 2012; Nicol et al., 2008; Ward et al., 2011). It exerts particular influence on the growth and

ecological niche separation of the ammonia-oxidizing community, which is present in neutral, alkaline, and acidic soils (De Boer et al., 1991; He et al., 2007; Nicol et al., 2008; Shen et al., 2008; Yao et al., 2011).

The conversion of ammonia to its ionized form ammonium is pH-dependent: low pH shifts the equilibrium towards ammonium and vice versa. As ammonia availability is reduced under low pH conditions, acidic soils are considered a substrate-limited environment in particular for microbes such as ammonia-oxidizers, which rely on NH_3 for energy generation (Frijlink et al., 1992; Nicol et al., 2008; Suzuki et al., 1974; Tarre and Green, 2004). They use ammonia rather than ammonium as substrate for the reaction-catalyzing enzyme, which requires energy-dependent active transport into the cell instead of diffusion (Nicol et al., 2008). Though both pH-dependent processes such as nitrification and abundance of nitrifying organisms could be reduced under low pH conditions, some organisms are in principle capable of dealing with substrate shortage (De Boer and Kowalchuk, 2001; Nicol et al., 2008; Yao et al., 2011) in low pH environments by developing different adaptions. Mechanisms for overcoming these substrate limitations, such as shifts to alternative substrates, have been assessed in several studies and are discussed in detail later-on.

Climatic conditions and soil moisture

The impacts of climate change are multifactorial and can hardly be drawn back to a single variable such as elevated CO_2, temperature or precipitation (Fierer et al., 2009; Gschwendtner et al., 2015; Horz et al., 2004; Long et al., 2012; Tourna et al., 2008). Temperature influences not only the kinetics of biochemical processes but also affects the ammonia-oxidizing community (Avrahami et al., 2002; Fierer et al., 2009). Changes in climatic conditions are accompanied by differences in soil moisture and thus the redox potential of the soil. Both parameters fluctuate seasonally and may influence N-processes differently during the season. Balanced soil moisture is essential to many processes. Soil moisture influences nutrient availability both by facilitating solubility and transport of nutrients, and by influencing mineralization rates: stress due to water shortage should be prevented, but water content must not exceed a certain level if respective enzymatic function and activity are to be maintained (Horz et al., 2004).

Soil water content, especially at microsites, is also important for the dynamics of aerobic and anaerobic interlinked processes such as nitrification and denitrification (Adair and Schwartz, 2008; Kuramae et al., 2012; Le Roux et al., 2008; Long et al., 2012). Such transition zones are optimal for specific processes, such as methane-oxidation (Bodelier and Frenzel, 1999).

6.2 Biotic site-specific factors

Competitive interactions of biota

Aboveground and belowground diversity are tightly linked at several hierarchical levels. Direct interactions occur between populations in mutualistic (as e.g. symbiotic interaction of nitrogen-fixing bacteria in legumes) or antagonistic relationships. Indirect interactions exist between particular plant and microbial communities in terms of resources; they can either provide resources such as particular nutrients to each other, or they may also compete for the same resource. Furthermore, plant community composition alters soil properties such as soil moisture, pH, and organic matter content by litter input, which may further directly trigger other processes such as nitrification and denitrification in beneficial and adverse ways (Carney et al., 2004; Kowalchuk and Stephen, 2001; Le Roux et al., 2013; Regan et al., 2014).

Competition for organic or inorganic nitrogen compounds between plants or heterotrophic and autotrophic microbes takes place, as different soil biota all access the same nitrogen pools in soil for assimilation or microbial immobilization and nitrification transformations, especially in nitrogen-limited environments. For access to inorganic or organic nitrogen compounds, plants require mineralization by heterotrophic microorganisms or rely on the nitrification product nitrate. They compete with autotrophic nitrifiers for inorganic ammonia, and with denitrifiers for nitrate (Kaye and Hart, 1997; Le Roux et al., 2013). The preferential uptake of either nitrate or ammonium by plants can reduce competition between species by allowing niche separation, but can also induce belowground effects (Warren and Adams, 2007). While ammonium assimilation consumes less energy than nitrate assimilation, storage and accessibility of nitrate is less problematic than that of ammonium (Boudsocq et al., 2012). General principles concerning plant preferences for different nitrogen forms are still under investigation, but are likely based on plant physiological characteristics or succession stage, environmental conditions, species specific uptake and storage capacity, or their root systems (Boudsocq et al., 2012; Gherardi et al., 2013). Furthermore, preferences for nitrogen compounds might depend on the availability of particular nitrogen forms and even induce some plants to switch between inorganic nitrogen forms, hence, plant preference for particular nitrogen compounds is species specific and its extent may vary with the season (Kastl et al., 2015).

At the same time plants compete with other heterotrophic bacteria and fungi for organic carbon and nitrogen (Hankinson and Schmidt, 1988; Pennington and Ellis, 1993). However, secretion of, for example, polyphenolic compounds or monoterpenes by plants can also impede the growth or activity of nitrifiers as is the case in forest ecosystems with pine stands (Paavolainen and Smolander, 1998).

Beneficial interactions of biota

The rhizosphere compartment is of particular importance in terms of microbe – plant interactions, supported by root exudation of carbon compounds and other metabolic products (Wolters et al., 2000). This creates a specialized microenvironment for microbial groups, favoring them in an otherwise adverse habitat. This is the case for nitrifiers in paddy soils: oxygen is released into the rhizosphere where nitrifiers have been demonstrated to thrive even though they are surrounded by the anaerobic bulk soil compartment (Ke et al., 2013). Plant influence patterns are also prone to pronounced seasonal fluctuations, influencing interactions with microbes, with low root exudation at the beginning of the vegetation period, increasing during peaks of growth, and high plant litter biomass input as plants senesce. In addition to the influence that aboveground biota exert on belowground microorganisms, feedback loops from belowground to the surface can also exist, for example in nutrient-rich hot spots that may promote plant growth and influence their patterns (Ettema and Wardle, 2002). Especially in unfertilized soils, plants depend on nitrogen derived from microbial transformation processes, such as mineralization and nitrogen-fixation.

6.3 Anthropogenic land management factors

Land-use intensification has been claimed to be a major anthropogenic source of human intervention contributing to ecosystem changes. And indeed, land management activities affect physical, chemical and biological properties of the soil and thus influence not only soil structure, but also nutrient content, quality and distribution, and consequently interfere with N- and C-cycles (Herold et al., 2014; Wolters et al., 2000). Responding biota have been generally considered to increase their activity but decrease in their diversity (Birkhofer et al., 2012). “Extensive” land-use in this context means unfertilized or unmanaged soils. Practices as tillage, liming or other factors such as fertilization may play a role in the performance of nitrifying microbial communities (Attard et al., 2010; Kowalchuk and Stephen, 2001). The land-use in our study sites is determined by three major components: fertilization, grazing and mowing, which are addressed in more detail below.

The removal of aboveground plant biomass by **mowing** may have two different effects: first, plant competition for nutrient resources is impeded and second, quality and quantity of root exudates changes (Kuzyakov and Xu, 2013; Patra et al., 2006; Wolters et al., 2000). Root exudation and ammonium level increase after mowing, which leads to increasing activity and biomass changes of microorganisms (Hamilton and Frank, 2001; Herold et al., 2014; Kuzyakov and Xu, 2013; Le Roux et al., 2008). In general, mowing is associated with changes beneficial to belowground microbial communities. If plant biomass is not removed after mowing, this additional plant litter input may also

influence microbial decomposition processes as changes in plant species composition and exudation can accompany mowing (Patra et al., 2006).

Grazing exerts multiple effects on above- and belowground biota. It constitutes one source of nitrogen input by deposition of urine and excrements. Urea can be directly converted into ammonia and also increase phosphorous availability in soil (Patra et al., 2006; Ritz et al., 2004). Grazing also disturbs the vegetation, thereby triggering root exudation (Hamilton and Frank, 2001). Trampling of livestock affects soil compaction (Bardgett and Wardle, 2003; Patra et al., 2006), which in turn changes soil structure, pore space and soil moisture content. All these changes to physical and chemical properties of the soil as well as on plants may impact distribution and performance of belowground communities (Steffens et al., 2009). Several studies, often simulating grazing effects by urea or manure application, have identified correlations between grazing and the nitrifying community with regard to their abundance, activity and community composition (Avrahami et al., 2002; Le Roux et al., 2008; Patra et al., 2006).

Owing to increasing **fertilization** practices, many studies focus on the effects of fertilizer application when land-use intensity (LUI) associated changes are addressed. While most studies have related fertilization to higher microbial activity and abundance, others have observed lower bacterial abundance or no abundance shifts upon fertilization in grassland and forest soils (He et al., 2007; Ke et al., 2013; Shen et al., 2008; Zhang et al., 2010). Differences in outcomes of the studies can be explained by different levels or types of fertilizers or their application modes. It is evident that different nitrifier guilds may respond differently to particular nitrogen levels and to the type of fertilization (Leininger et al., 2006).

However, investigation of management effects on the same functional group may also deliver contradictory results at different sites or in different ecosystems (Wertz et al., 2012; Carney et al., 2004). To increase the comparability of studies dealing with the effects of LUI, a continuous measure of LUI could be useful, taking into account different management regimes. This was realized by the introduction of a compound land-use intensity index that included mowing, grazing and fertilization as major components of land-use (see Materials and Methods) (Blüthgen et al., 2012).

The described parameters can be further classified into those such as plant performance or climatic conditions which vary with the season and affect labile pools, and those comprising stable parameters; site-specific characteristics such as soil type, which are not prone to seasonal changes. Of course, these drivers do not exclusively affect nitrifier community composition and their performance but should be regarded as working in concert. These seasonally changing effects

determine the environmental conditions of a certain habitat or spatial niche (Fierer and Jackson, 2006).

7 Biogeography of microbial performance

The spatial distribution patterns of soil properties or processes and associated microbial communities are described in the field of biogeography. Microorganisms have been found to be rather heterogeneously than randomly distributed in soil (Bru et al., 2011; Franklin and Mills, 2003; Keil et al., 2011; Regan et al., 2014; Ritz et al., 2004). These spatial distributions have been shown to follow the heterogeneous spatial patterns of abiotic and biotic parameters they respond to (Fierer et al., 2009; Regan et al., 2014; Ritz et al., 2004). Thus, different functional groups present at the same scale might exhibit divergent spatial distributions. In nutrient-limited environments in particular, the patchy distribution of microorganisms follows the hotspots of nutrients (Franklin and Mills, 2003; Nunan et al., 2003).

The biogeography of microbial communities can be measured at spatial scales ranging from microscale (µm) to global scale (km) (Berner et al., 2011; Bru et al., 2011; Grundmann and Debouzie, 2000; Keil et al., 2011; Nunan et al., 2002). The scales selected for the investigation of biogeographical patterns have to account for the respective range at which effects on the target group and of investigated drivers are expected (Franklin and Mills, 2003). Different meanings are associated with the term “scale”, as there are no fixed definitions, which can be misleading without further description (Parkin, 1993). Small scales as micro- (µm to mm) and plot- (cm to m) scale allow detailed assessment of spatial distribution that can be illustrated by maps. At larger scales such as local (km), regional, continental and global scales, comparisons of the distributions based on their abundance are more difficult, due to the great number of samples that would be needed to cover the required resolution level for the construction of maps. Thus, studies of interactions of microorganisms are performed typically at smaller scales, while studies assessing edaphic effects are conducted at larger scales.

Drivers operate at different distances: for example substrate concentrations, pore structure, redox status, and root-associated effects are relevant at the µm range. Hence effects of climatic conditions on local vegetation characterize spatial variability at intermediate scales, whereas management practices or soil pH affect microbial community distribution patterns at larger scales in the range of km or even at the continental scale (Ettema and Wardle, 2002; Fierer and Jackson, 2006; Franklin and Mills, 2003; Ritz et al., 2004). An overview can be found in section III. 4.1 (**Table 4**). The size of

microbes explains why studies on their interactions and substrate-associated spatial structure are assessed at the fine scale of µm. Furthermore, spatial autocorrelation patterns change with selected drivers. Microbial community spatial patterns at the plot scale may result from factors changing at the meter range such as vegetation composition (Franklin and Mills, 2009). Additionally, microbes that have been found to be structured at the plot scale can be responding to changes in land-use intensity (Keil et al., 2011; Berner et al., 2011). Spatial distribution patterns may change according to the land-use component they reflect: grazing may lead to patchy distributions of nutrients, while fertilizer application effects might be rather homogeneous, reducing spatial heterogeneity (Berner et al., 2011).

The assessment of local differences can also be extended to the regional scale comparing different regions at ranges of several hundred km², and can be attributed to differences in topography and climatic conditions (Steffens et al., 2009; Berner et al., 2011). The relation of microbial communities and soil properties across three distinct regions located across Germany was addressed in a study by Birkhofer and colleagues (Birkhofer et al., 2012). Soil properties were correlated to soil biota abundance and diversity across regions and location influenced variability of abundances to a great extent, albeit assessed bacteria showed only weak correlations. Even at the continental scale, biogeographical patterns of bacteria can be compared across large distances. To understand the interactions of processes at small spatial scales from the µm to m range is an essential prerequisite for transferring this knowledge to the performance of the whole process at larger local or regional scales and to different ecosystems. Both spatial dependence of microbial communities and underlying associated soil properties may be subjected to seasonal variation due to changing climatic conditions and responding drivers (Ettema and Wardle, 2002; Franklin and Mills, 2009; Regan et al., 2014).

8 Open questions, objectives and hypotheses of the presented study

Most spatial interaction studies have focused either on AO or NO, demonstrating patterns from co-existing AOA and AOB at the plot scale (Keil et al., 2011) to heterogeneous distribution patterns of NOB (Gieseke et al., 2003). The co-occurrence of AO and NOB has been determined in several studies and revealed different interaction partner combinations (Daebeler et al., 2014; Xia et al., 2011; Wertz et al., 2012; Ke et al., 2013; Ollivier et al., 2013). But despite the essential interaction of both guilds for efficient nitrification in soil, it is unclear how both interact at the same spatial and temporal scales in soil. Grundmann and Debouzie demonstrated dependent spatial autocorrelations of AO and NO at

the mm range in soil (Grundmann and Debouzie, 2000). However identification of spatial interaction patterns of AO and NO as they manifest at the plot scale and how their interaction might change during the season in response to dynamic environmental variables remains elusive. As the response of functionally redundant key players to substrate preferences have been addressed primarily in wastewater treatment systems for NOB (Daims et al., 2001; Kim and Kim 2006) and studies in terrestrial systems have focused mainly on the relative abundance of AO (Di et al., 2009; Leininger et al., 2006), our study aimed to investigate spatial interaction patterns in an unfertilized habitat, to exclude additional fertilizer effects, bridging the gap of knowledge between ecophysiological characteristics determined in cultivation studies and in natural environments.

Despite the emerging attention on global change issues and the increase in research projects dealing with functional biodiversity, most experiments are performed as small laboratory scale experiments assessing biodiversity changes in a limited number of species at a single site, often focusing on the effects of one particular driver. Few studies have investigated relationships between nitrifiers and particular drivers both at smaller local scales and larger regional scales (Birkhofer et al., 2012; Gubry-Rangin et al., 2011). It remains to be clarified for the group of nitrifiers whether the respective effects of drivers of the same group of organisms observed at the local scale also apply to different regions or might be superseded by other environmental factors (e.g. Keil et al., 2011).

Various studies have identified different contributions and response patterns of AO to fertilization levels or land-use regimes (Di et al., 2009; Fischer et al., 2010; Hooper et al., 2005; Leininger et al., 2006; Patra et al., 2006). However, no study so far has attributed LUI effects at the regional scale to a continuous measure of different land-use components acting in concert, which reflects the real management situation (Blüthgen et al., 2012). It remains further to be clarified whether LUI, by shaping substrate conditions, is a general driver of the nitrifying community across regions. Soil pH has been identified as another factor affecting nitrifying communities via control of substrate accessibility (Kemmitt et al., 2006; Ward et al., 2011). Albeit studies have shown that pH in particular shapes the AO community (Nicol et al., 2004; Nicol et al., 2008), the question of whether pH shapes similar AOA communities in forest and grassland soils, including sites of ultra-acidic soil pH, remains unanswered.

In our study, we aimed to identify major factors driving the performance of microbial groups involved in the nitrogen cycle in forest and grassland ecosystems with a particular focus on the nitrification process. In this context we assessed whether putative niche shaping factors would permit the co-existence of functionally redundant organisms performing the same transformation step. We also

wanted to address their specific influence on microbes which interact in performing sequential transformation steps in terms of temporal and spatial variation. We assumed that multifactorial influences on the performance and diversity of nitrifying organisms could be assessed at different scales: effects of drivers would be observable at large spatial scales; indirect influences on microbial performance, such as substrate availability influence on niche shaping factors, would be detectable at smaller scales. Microbial abundance, diversity and activity may be dependent on both permanent stable parameters such as site-specific conditions, pH and soil-type, and more labile factors such as plant performance or climatic conditions, which vary with the season. In addition, anthropogenic effects such as land-use management affect microbial performance indirectly via substrate accessibility and availability, which in turn might directly trigger ecological niche separation.

In this context, we assume that **(i)** *stable parameters would preferably influence the community composition of nitrifiers* **(M1)**, *whereas seasonally changing drivers might influence the activity and to a lesser extent the abundance of microorganisms* **(M2, M3, M4)**. As drivers may influence nitrifiers directly by shaping substrate availability, we further hypothesize that **(ii)** *the abundance of bacterial ammonia-oxidizers might be driven to a large extent by land-use intensity* **(M2)**, since those are often found to be highly responsive to elevated ammonia levels. However, we assume that **(iii)** *archaeal ammonia-oxidizers might be active under a broader spectrum of conditions considered suboptimal for other nitrifiers and contribute to ammonia-oxidation under substrate-limited conditions as is the case in low pH environments such as forest soils* **(M1)**. However, we hypothesize that not only different preferences for substrate concentrations, but also distinct metabolism and growth strategies may affect the choice of interaction partners amongst AO and NO. We assume **(iv)** *a close interaction between* Nitrobacter *and ammonia-oxidizing bacteria, both found in high substrate environments, while* Nitrospira *can cope with lower substrate concentrations and have been found co-occurring with archaeal ammonia-oxidizers*; both are predicted to be able to switch to a mixotrophic lifestyle **(M4)**. In terms of community composition, we expected that **(v)** *the same clades of microorganisms adapted to similar conditions such as substrate-shortage would occupy similar niches across forest and grassland ecosystems* **(M1)**. To gain a representative description of community composition, the selection of primer systems is essential. With continuously growing databases of reference sequences, there is an emerging need to test primer pairs for community analysis in terms of specificity and coverage based on *in silico* analyses software tools **(M5).**

To address the research objectives and verify our hypotheses, we performed several experiments on the effects of selected putative drivers such as land-use intensity and different soil properties on the abundance, activity and diversity of nitrifiers in forest and grassland ecosystems (**Table 2**). To identify

the temporal dynamics of the biogeography of nitrogen cycle performing microbes and their interactions, we focused our studies on the small square meter scale of an extensively managed grassland site where the abundance and activity of target groups was assessed by means of qPCR and potential enzyme activity measurements **(M3, M4)**. We extended our study from the small local to large regional scales of three distinct regions in Germany comprising both forest and grassland sites to reveal putative drivers of nitrifier performance **(M1, M2)**. Based on a subset of forest plots in each region, an *amoA* (AOA) amplicon 454 high-throughput sequencing approach was conducted to reveal different ecotypes adapted to different pH ranges **(M1)**.

		M1	**M2**	**M3**	**M4**
Sampling date		05/2011	05/2011	04-11/2011	04-11/2011
Exploratory		ALB, HAI, SCH	ALB, HAI, SCH	ALB	ALB
Plot no. / -ID		9 (VIP)	50 (EP)	AEG31	AEG31
Ecosystem		forest	grassland	grassland	grassland
Abundance	*nifH* (NF)	-	✓	✓	-
	amoA (AOA)	✓	✓	✓	✓
	amoA (AOB)	✓	✓	✓	✓
	16S rRNA (NS)	-	-	-	✓
	nxrA (NB)	-	-	-	✓
	16S arch	✓	-	✓	-
	16S bact	✓	-	✓	-
Potential activity	nitrification	✓	✓	✓	-
	denitrification	-	-	✓	-
Diversity	*amoA* (AOA)	✓	-	-	-

TABLE 2: **Basic information and major aspects of conducted studies listed according to respective publications.**

Details on sampling region and dates are compared for each study. Assessed aspects of abundance (itemized to target genes), activity and diversity are listed. Basic soil properties were assessed in all studies and thus are not listed here. P5 is not addressed here as it focuses rather on methodology than ecological processes. NF = nitrogen fixation, AOA = ammonia-oxidizing archaea, AOB = ammonia-oxidizing bacteria, NS = *Nitrospira*, NB = *Nitrobacter.*

II MATERIAL and METHODS

1 Study site description

The experiments were performed in the frame of the Biodiversity Exploratories (Fischer et al., 2010). The Biodiversity Exploratories (www.biodiversity-exploratories.de) comprise three study areas located across Germany: the Biosphere Reserve Schwäbische Alb (ALB), located in the South-west of Germany, covering 422 km²; the National Park Hainich-Dün (HAI) in Central Germany, covering approx. 1300 km² and the Biosphere Reserve Schorfheide-Chorin (SCH), located in the North-east of Germany, covering approx. 1300 km². The following mean annual temperatures were determined for each region: 6.5-8.0°C (ALB), 8.0 - 8.4°C (HAI), 6.5 - 8.0°C (SCH). Mean annual precipitation values for each region are the following: 938 - 963 mm (ALB), 520 - 600 mm (HAI), 500 - 800 mm (SCH). The selection of study sites covers both grassland and forest ecosystems. Each study region consists of 50 grassland and 50 forest experimental plots (EP), i.e. in total 150 different grassland and 150 forest plots. The experimental plot size in grassland was set to 50 x 50 m, in forest to 100 x 100 m. Grassland plots of the respective study regions were termed as follows: AEG1-50 (ALB), HEG1-50 (HAI) and SEG1-50 (SCH); forest plots accordingly AEW 1-50, HEW 1-50 and SEW 1-50.

In the frame of this thesis, studies were performed at different scales: the regional, the local and the plot scale. **Table 2** depicts an overview on sampling sites and dates of sampling, listed according to the respective experiments. For our study on the **regional scale**, sites from all three study regions (exploratories) were chosen, comprising 50 grassland plots per exploratory, while region-wide studies in forest sites were restricted to 9 sites per exploratory, dedicated to intensive studies (AEW1-9, HEW1-9, SEW1-9). Analyses on the **local scale** were restricted to the experimental plots of only one exploratory. Detailed studies on the **plot scale** (selected subplot of 10 x 10 m) were performed only at one plot with plot-ID AEG31 in the ALB region.

2 Soil type and texture

The soil texture differs amongst the study regions as described below. In Hainich-Dün and the Schwäbische Alb exploratory, loamy and clayey textures are dominant. Forest and Grassland sites of the ALB region comprise both Cambisol and Leptosol soil types. In contrast, Cambisols and Stagnosols are found in HAI grassland sites, and Stagnosol as well as Luvisol in the forest sites. The grassland sites of the Schorfheide-Chorin exploratory comprise mineral soil sites as well as degraded peat soil sites. For reasons of better discrimination, sites of mineral soil were termed as SCH-MB (SCH-mineral

soil), peat soil sites were termed as SCH-NM (SCH-peatland). The dominating soil types are Histosols, Cambisols, Gleysols, Luvisols and Albeluvisols. The soil textures of both grassland and forest sites in SCH are characterized by sandy loam and sand, respectively. In forest sites of SCH, mainly Cambisols, Albeluvisols, Podsols and Regosols are found. In HAI in contrast, forest sites are restricted to Luvisols and Stagnosols. For further details, see Fischer et al. (2010).

3 Land-use intensity

The land-use intensity (LUI) level was assessed on the basis of conducted land-use surveys, providing information on frequency and intensity of the following three major components of land-use: mowing, grazing and fertilization. For our study on land-use intensity effects, we took advantage of an integrative measure for the land-use intensity, reflecting intensive and extensive land-use management, based on the above mentioned components, developed by Blüthgen and colleagues (Blüthgen et al., 2012). Briefly, the intensities of the individual components (fertilization: kg nitrogen per ha per year, mowing: events per year, grazing: livestock units per ha per year) are normalized to the prevailing forms of management in the respective regions and summed up to the compound LUI index. Further information on standardization and underlying equations for the calculation of an index (as the sum of the normalized components) can be found in Blüthgen et al. (2012). For the statistical analyses, plots were additionally grouped according to their individual LUI gradient values. The levels of LUI were termed with consecutive numbering from 1 (extensive) to 5 (intensive): level 1 = LUI indices < 1.0; level 2 = LUI indices 1.0 - 1.5; level 3 = LUI indices 1.5 - 2.0; level 4 = LUI indices 2.0 - 2.5; level 5 = LUI indices > 2.5.

For the plot scale experiment at the study site AEG31, the land-use intensity was described as follows: no fertilization, one mowing event in August (2 weeks prior to sampling), lightly grazed by sheep in October (2 weeks prior to sampling).

4 Soil sampling

All grassland and forest soil samples at the **regional** and **local** scale were collected in the frame of a soil sampling campaign in May 2011. Samples were taken from the upper 10 cm of the mineral soil at 14 predefined locations per plot, subsequently pooled and sieved to < 2 mm excluding root material. For the experiments conducted at the **plot** scale, soil samples were collected in April, May, June, August, October and November 2011 to cover the whole vegetation period. The study site was subdivided into 30 subplots, each of them containing one pair of randomly assigned sample

locations, where samples were collected from at each sampling date. Thus, in total 60 samples (2 per subplot) were taken per date (sampling scheme in Regan et al., 2014). Field work permits were issued by the responsible state environmental offices of Baden-Württemberg, Thüringen, and Brandenburg (according to § 72 BbgNatSchG). Soil samples for analyses of soil properties and potential enzymatic activity measurements were stored at 4°C; soil samples for DNA extraction were immediately frozen on dry ice at -80°C.

5 Abiotic soil properties

Basic parameters were measured in the frame of the soil sampling campaign in May 2011 at all plots by different research groups and were available for all Biodiversity Exploratories associated projects.

For nitrate and ammonium concentration determination, an extraction protocol using 1 M KCl as extractant (1:4 soil to extractant ratio w/v) was followed. The soil suspensions were shaken on a horizontal shaker for 30 min at 250 rpm followed by centrifugation (30 min at 4500 x g) (I. Schöning, personal communication). The concentrations of nitrate and ammonium in the supernatant were measured using an auto-analyzer (Bran & Luebbe, Germany). For the determination of total nitrogen and carbon, dry combustion of soil at a temperature of 1100°C followed by determination of evolving CO_2 and N_2 with a Thermal Conductivity Detector (TCD) was performed (VarioMax, Elementar, Germany) (Solly et al., 2014). The soil pH was determined using a WTW pH meter 538 (Wissenschaftlich-Technische-Werkstätten GmbH, Germany) in combination with the pH electrode SenTix61 after $CaCl_2$-extraction (0.01 M $CaCl_2$ solution, 1:2.5 soil to extractant ratio w/v) (I. Schöning, personal communication). For our study on selected forest sites, the soil pH was additionally classified according to the following classification by the Agriculture Natural Resources Conservation Service (United States Department of Agriculture, USDA): ultra acidic (< 3.5), extreme acidic (3.5 – 4.4), very strong acidic (4.5 – 5.0), strong acidic (5.1 – 5.5), moderate acidic (5.6 – 6.0), slight acidic (6.1 – 6.5) and neutral (6.6 – 7.3).

6 Potential nitrification assay

Potential nitrification rates were determined according to Hoffmann et al., 2007. 2.5 g of soil were incubated with 10 ml of 10 mM ammonium sulfate as substrate and 50 µl of 1.5 M sodium chlorate to prevent the turnover of nitrite to nitrate, then 5 h incubated at 25°C slightly shaking. Suitable incubation time was determined prior to the measurements in a pre-experiment with randomly selected samples. To stop the reaction, 2.5 ml of 2 M potassium chloride were added. After re-incubation for 20 min, the samples were centrifuged for 2 min at 2000 x g. 150 µl of supernatant

were supplemented with 90 µl ammonium chloride buffer (pH 8.5) and 60 µl of nitrite determination reagent (2 mM naphtylenediamine dihydrochloride, 2.5 M phosphoric acid and 0.06 M sulphanilamide). The following color reaction was detected spectrometrically after 15 min at an absorbance wavelength of 540 nm (SpectraMax 340, MWG BIOTECH, Germany). As control, reagents without addition of soil and soil samples where stop solution was added prior to substrate application were used.

7 Extraction of nucleic acids

DNA extraction of soil samples taken at the soil sampling campaign in May 2011 at the regional scale, comprising 150 grassland and 150 forest plots, was performed using the Power Soil DNA Isolation Kit (MO BIO Laboratories, USA) according to the manufacturer's instructions from 1g fresh weight of soil for samples. DNA was eluted in 200 µl of 10 mM Tris Buffer.

Soil samples for the plot scale experiment derived from plot AEG31 were extracted in duplicate from 0.2 g homogenized soil samples using the FastDNA® SPIN Kit for Soil (MP Biomedicals, USA). RNA extracts from the same samples were obtained using a modified extraction protocol after Lueders et al., 2004, including a chloroform-phenol-based extraction of nucleic acids followed by precipitation with polyethylene glycol. DNA content was quantified using Nanodrop 1000 Spectrophotometer (Peqlab, Germany) or via Quant-iT™Pico Green® ds DNA assay Kit (Invitrogen, USA) and measured at SpectraMax Gemini EM Fluorescence Plate Reader Spectrometer (Molecular Devices, USA).

8 Quantification of gene abundances

Real-time quantitative PCR was performed on a 7300 Real-Time PCR System (Applied Biosystems, Germany) using SyBr Green as fluorescent dye. Target genes for the respective processes, reaction mixture components, thermal cycling conditions, plasmid standard sources and corresponding primers are described in Töwe et al. (2010), Ollivier et al. (2013), Regan et al. (2014). For a summary of the qPCR assays performed, see **Table 3**. Pre-experiments were carried out to determine the optimal dilution of sample DNA to avoid a putative inhibition of the reaction by humic substances (dilution forest plots: 1:8, all grassland plots: 1:16, plot AEG31: 1:80). To prove the specificity and correct size of the amplified fragments, a melting curve analysis was carried out after each run, supplemented by gel electrophoresis on a 2% agarose gel, conducted with randomly selected samples. Serial dilutions of the respective plasmids (10^1 - 10^6 gene copies per µl) were used for standard curve calculations. Obtained efficiencies were between 80% and 92% and R^2 was determined to be above 0.99 for each qPCR assay.

Target gene	Standard source	Primer	Primer reference	Thermal profile	cycles
***amoA* (AOA)**	Fosmid clone 54d9	amo19F CrenamoA16r48x	Leininger et al., 2006 Schauss et al., 2009	94°C, 45 s / 55°C, 45 s / 72°C, 45 s	40
***amoA* (AOB)**	*Nitrosomonas sp.*	amoA1F amoA2R	Rotthauwe et al., 1997 Rotthauwe et al., 1997	94°C, 60 s / 58°C, 60 s / 72°C, 60 s	40
nifH	*Sinorhizobium meliloti*	nifHF nifHR	Rösch et al., 2002 Rösch et al., 2002	95°C, 45 s / 55°C, 45 s / 72°C, 45 s	40
chiA	*Streptomyces griseus*	chiA-2f chiA-2r	Xiao et al., 2005 Xiao et al., 2006	95°C, 30 s / 60°C, 30 s / 72°C, 60 s	40
nxrA	*Nitrobacter hamburgensis* X14 (DSMZ 10229)	F1norA R2norA	Poly et al., 2008 Wertz et al., 2008	94°C, 30 s / 55°C, 30 s / 72°C, 30 s	40
16S rRNA gene (*Nitrospira*)	*Nitrospira* 16S rRNA gene Accession No. FJ529918	Nspra 675f Nspra 746r	Graham et al., 2007 Graham et al., 2007	94°C, 30 s / 64°C, 30 s / 72°C, 60 s	40
16S rRNA gene bacteria	*Clavibacter michiganensis*	FP16S RP16S	Bach et al., 2002 Bach et al., 2002	95°C, 20 s / 62°C, 60 s / 72°C, 45 s	40
16S rRNA gene archaea	*Methanobacterium sp.*	rSAf(i) 958r	Nicol et al., 2005 Bano et al., 2004	94°C, 20 s / 55°C, 60 s / 72°C, 30 s 94°C, 20 s / 50°C, 60 s / 72°C, 30 s	5[1] 35

[1] Touchdown: -1 °C per cycle

TABLE 3: **Thermal profiles, primer and standards used for real-time PCR quantification of all genes quantified by means of qPCR in this study.** AOA = ammonia-oxidizing archaea, AOB = ammonia-oxidizing bacteria.

9 Phylogenetic analysis

9.1 454 sequencing and data processing

The amplification of the archaeal *amoA* gene was done as published in Gubry-Rangin et al., 2011, using the forward primer CrenamoA23f attached to the Roche A adapter for 454-library construction and the reverse primer CrenamoA616r (Tourna et al., 2008), attached to the Roche B adapter (10-nt barcode sequences were included for multiplexing). Amplification was performed with FastStart High Fidelity PCR System (Roche, Germany). Library purification, equimolar pooling and emPCR were conducted as recommended in the manufacturer's instructions and subsequently sequenced on a 454 GS FLX Titanium sequencer from Roche (Roche, Germany). For details see Gubry-Rangin et al. (2011).

Processing of sequences included trimming of adaptors and quality trimming (quality filtering was modified: "vfScanAllFlows" was changed from "tiOnly" to "false"). Forward reads were processed as described in Pester et al. (2012). Sequence reads in sff format underwent further quality checks using Mothur Software (Schloss et al., 2009): demultiplexing and trimming of reads, denoising (PyroNoise implemented in Mothur) and removal of reads shorter than 200 nt or homopolymers. Sequences were subjected to frameshift correction using frameD (Schiex et al., 2003) and the *amoA* sequence database published in Pester et al. (2012) was used for model building. Sequence alignment was based on the reference *amoA* database (Pester et al., 2012), using MUSCLE (Edgar, 2004) implemented in a Python script.

Data analyses of representative sequences of each OTU were done at 97% similarity level using Mothur software and were aligned to the gene reference database of archaeal *amoA* sequences (Pester et al., 2011) using ARB (Ludwig et al., 2004). The construction of a Newick-formatted, hierarchical UPGMA-dendrogram was based on distance matrix, calculated by using Yue & Clayton similarity coefficient.

9.2 ILLUMINA sequencing and data processing

For the study on the plot scale paired end Illumina sequencing was conducted on a HiSeq 2500 (Illumina, USA). First, RNA samples were reversely transcribed with GoScript (Promega, USA). The variable region 3 of the small subunit of rRNA was used as target for amplification. Primers used contained Illumina adapters and a barcode (for reverse primer) (Bartram et al., 2011). After gel purification and clean-up with NucleoSpin Extract II columns (Macherey & Nagel, Germany), amplicons were sequenced.

Raw data was subjected to different bioinformatic pipelines including the following processing steps: trimming of reads to 100 bp, removal of primer dimers, joining of the remaining reads, chimera using UCHIME (Edgar et al., 2011), and clustering with CD-HIT-OTU for Illumina (Li and Godzik, 2006; Fu et al., 2012). Sequence annotation was performed with the RDP-Classifier tool (Wang et al., 2007) (similarity threshold of 97%, confidence cutoff of 0.5).

For phylogenetic analyses and reference sequences, the following tools were used: the RDP-Classifier (with 16S rRNA training set 10), BLAST (Altschul et al., 1990), and ARB (with the SILVA 119 SSU REF NR database (Ludwig et al., 2004; Quast et al., 2013)). Subsequent processing included sequence alignment with JalView (Waterhouse et al., 2009) and the implemented MAFFT algorithm (preset G-INS-i, for maximum accuracy; Katoh et al., 2005). After using MEGA 6 for fitting the best evolutionary model (Tamura et al., 2013), the Kimura-2 parameter model with gamma distribution (K2+G) was selected for tree construction. Tree topologies were calculated taking advantage of both the Maximum Likelihood and Neighbor Joining.

10 *In silico* analyses of primer pair coverage

Several primer sets were subjected to *in silico* analyses to reveal their coverage among database annotated sequences. For this purpose, we took advantage of the Genomatix software, using the tools FastM (Klingenhoff et al., 1999) and ModelInspector (Frech et al., 1997). Prior to searching databases including sequences affiliated to bacteria / archaea or plants for matching alignments, models for the respective primer pairs were created. Settings were adjusted to one allowed mismatch and alignment with both strands. Evaluating the results was accompanied by taxonomy analyses using the NCBI taxonomy browser tool.

In silico analysis was performed for comparing the coverage of different primer sets dedicated to B-ARISA analyses as well as for the identification of OTU sequences of a 16S rRNA gene dataset that were affiliated with nitrate-oxidizing bacteria.

11 Statistical analysis

Statistical analyses were performed using the R environment (R Core Team, 2014; http://www.R-project.org). Prior to most statistical analyses, the gene abundance data were log (x+1) transformed. For multivariate analysis of gene abundance data collected from all grassland sites, a few missing values were imputed using the Singular Value Decomposition method (SVDImpute in R-package imputation, rank = 2). A permutational multivariate analysis of variance was implemented (adonis

with Euclidean distance matrix in R-package vegan). After checking the homogeneity of variance across groups (leveneTest in R-package car), ONEWAY ANOVAs were applied (lm in R). In case of a significant effect, pairwise comparisons of means (pairwise.t.test with method holm) were conducted using the multcomp package, respectively (Hothorn et al., 2008). The package ggplot2 (Wickham et al., 2009) with ggpairs was used for scatter-, jitter- and boxplots of bacterial activity data.

An integrative multivariate data analysis of sequencing data was based on the principal component analysis (correlation based, prcomp in R). The following packages for R were used for PCA: MASS (Venables and Ripley, 2010), vegan (Oksanen et al., 2013) and gplots (Warnes et al., 2009). Further analyses were based on distance matrix of dissimilarities between OTUs, calculated based on distance measure with square root-transformed relative abundance data (Hellinger Transformation). The following statistical tests were applied to a subset of samples: univariate regression for first principal component (library("pls")), permutation test for canonical correspondence analysis (library("vegan") and tests for significance (library("agricolae")). For selected datasets, also correlation networks were computed and displayed taking advantage of the qgraph package (Epskamp et al., 2012).

Mantel tests were performed to test variable correlations as a function of spatial separation (Franklin and Mills, 2009; Regan et al., 2014). Correlation significance was tested by a Monte Carlo approach with multiple permutations. For distance matrix calculation, R package vegan was used (Oksanen et al., 2013).

12 Geostatistical analysis

The spatial dependence of selected parameters at the plot scale (study site AEG31) was addressed by geostatistical methods. For underlying equations, see e.g. Steffens et al. (2009). More detailed information on geostatistical parameters that are described in brief below, are provided in Regan et al. (2014). Geostatistical semivariogram analyses were carried out in the R environment using the g-stat package (Pebesma, 2004) (R 3.0.2, RDevelopmentCoreTeam, 2008). Nugget, sill and range have been determined by semivariogram analyses. P-sill (the part of total variance that is spatially explained) and the degree of spatial dependence (dividing nugget by the sum of nugget and sill) were calculated based on the above mentioned parameters (Fortin, 2005; Steffens et al., 2011). Kriged maps were constructed by ordinary kriging by means of the ArcGIS Software (ArcMap 10.0, ESRI® 2010, Germany) wherever a spherical model could be fitted to the dataset. Step widths between 0.6 and 0.8 m were applied.

For geostatistical analyses focusing on relationships between gene abundances, activities and soil properties as well as plant functional groups (M3), settings were taken from a previous study (Regan et al., 2014) to allow comparisons. Using these settings, no spatial model could be fitted to gene abundances of marker genes in this study. Thus, the settings for the geostatistical analysis of interaction patterns of nitrifiers (M4) based on the same dataset for AO abundances, were adjusted to the weak spatial dependence of AO variables to be able to visualize their spatial patterns.

III MANUSCRIPT OVERVIEW

List of manuscripts

- Stempfhuber, B., Engel, M., Fischer, D., Neskovic-Prit, G., Wubet, T., Schöning, I., Gubry-Rangin, C., Kublik, S., Schloter-Hai, B., Rattei, T., Welzl, G., Nicol, G., Schrumpf, M., Buscot, F., Prosser, J., Schloter, M., 2015. pH as a Driver for Ammonia-Oxidizing Archaea in Forest Soils. Microbial Ecology 69, 879-883.
  (M1, first author, published)

- Stempfhuber, B., Welzl, G., Wubet, T., Schöning, I., Marhan, S., Buscot, F., Kandeler, E., Schloter, M., 2014. Drivers for ammonia-oxidation along a land-use gradient in grassland soils. Soil Biology and Biochemistry 69, 179-186.
  (M2, first author, published)

- Regan, K. M., Stempfhuber, B., Schloter, M., Rasche, F., Prati, D., Philippot, L., Boeddinghaus, R. S., Kandeler, E., Marhan, S., Spatial and temporal dynamics of a grassland nitrogen cycling microbial community at the m2 scale.
  (M3, equal contribution, submitted)

- Stempfhuber, B., Richter-Heitmann, T., Regan, K. M., Kölbl, A., Kaul, P., Marhan, S., Sikorski, J., Overmann, J., Friedrich, M. W., Kandeler, E., Schloter, M., Spatial interaction of archaeal ammonia-oxidizers and nitrite-oxidizing bacteria in an unfertilized grassland soil. Frontiers in Microbiology 6.
  (M4, first author, published)

- Purahong, W., Stempfhuber, B., Lentendu, G., Francioli, D., Reitz, T., Buscot, F., Schloter, M., Krüger, D., 2015. Influence of commonly used primer systems on automated ribosomal intergenic spacer analysis of bacterial communities in environmental samples. PLoS ONE 10, e0118967.
  (M5, equal contribution, published)

Manuscript description and contributions

Short description of papers included in this study:

M1: Influence of pH on abundance & diversity of AOA in forest soils

M2: Influence of LUI on abundance & activity of ammonia-oxidizers in grassland soils

M3: Temporal and spatial analysis of abundance & activity of nitrifiers and denitrifiers

M4: Temporal and spatial analysis of abundance of key players in nitrification

M5: Methodological approach for the assessment of different primer systems

MANUSCRIPT 1

pH as a Driver for Ammonia-Oxidizing Archaea in Forest Soils

Barbara Stempfhuber*, Marion Engel*, Doreen Fischer*, Ganna Neskovic-Prit, Tesfaye Wubet, Ingo Schöning, Cécile Gubry-Rangin, Susanne Kublik, Brigitte Schloter-Hai, Thomas Rattei, Gerhard Welzl, Graeme W. Nicol, Marion Schrumpf, Francois Buscot, James I. Prosser, Michael Schloter

* these authors contributed equally

Short description:

Since soil pH is affecting ammonia availability and accessibility, its effects on ammonia-oxidizers were under investigation at different forest sites across Germany, constituting a pH gradient from ultra-acidic to acido-neutral pH range. The abundance of AOA was not significantly influenced by soil pH, while AOB were less abundant in acidic soils. The diversity of archaeal *amoA* genes was assessed by a 454-based sequencing approach which identified distinct AOA community compositions according to the pH range. OTUs exclusively detected under ultra-acidic conditions were affiliated to *Nitrosotalea* group 1.1 and *Nitrososphaera* subcluster 7.2. A comparison with grassland sites from other regions revealed similarities in their OTU composition of ultra-acidic soils, indicating that pH indeed shaped the community composition across different ecosystems.

Contributions:

contributed to planning and design of the experiments

performed the qPCR experiments

conducted subsequent analyses of qPCR data and data interpretation of sequencing data

wrote the manuscript

Microbial Ecology 69, 879-883, 2015. doi: 10.1007/s00248-014-0548-5

MANUSCRIPT 2

Drivers for ammonia-oxidation along a land-use gradient in grassland soils

Barbara Stempfhuber, Gerhard Welzl, Tesfaye Wubet, Ingo Schöning, Sven Marhan, François Buscot, Ellen Kandeler, Michael Schloter

Short description:

Land management regimes have been identified to be a major driver of ammonia-oxidizers at the small spatial scale. Thus, this study aimed at investigating the combined effects of different land-use components such as mowing, grazing and fertilization at the local and regional scale comparing 150 grassland sites. The nitrification potential was increasing with increasing land-use intensity, accompanied by increasing abundances of AOA and AOB, using *amoA* gene copy numbers as a proxy. Although land-use intensity could be revealed as driver of ammonia-oxidizer performance at mineral sites across regions, it was demonstrated that also site-specific parameters have to be taken into account, such as soil-type associated properties. At histosolic sites, other processes such as methane-oxidation might play a more prominent role, attributed to elevated ground-water tables and peat matrix degradation.

Contributions:

contributed to planning and design of the experiments

performed qPCR experiments

conducted subsequent statistical analyses and data interpretation

wrote the manuscript

Soil Biology and Biochemistry 69, 179-186, 2014. doi: 10.1016/j.soilbio.2013.11.007

MANUSCRIPT 3

Spatial and temporal dynamics of a grassland nitrogen cycling microbial community at the m^2 scale

Kathleen Regan*, Barbara Stempfhuber*, Michael Schloter, Frank Rasche, Daniel Prati, Laurent Philippot, Runa S. Boeddinghaus, Ellen Kandeler, Sven Marhan

* these authors contributed equally

Short description:
Soil processes exhibit both temporal and spatial variations. Since this heterogeneity may affect the outcome of studies on microbial interactions, it is essential to assess temporal dynamics of microbial performance at spatial scales. This study followed spatial and temporal patterns of microbial communities involved in nitrogen cycling, both their abundances and potential activities, at the meter scale in an unfertilized grassland plot. While potential nitrification and denitrification activities were relatively stable over the sampling period, genes involved in the respective steps varied more strongly over time, suggesting functional redundancy among them. The observed short duration of their variability could well be linked to aboveground processes, although this link has proved elusive at multiple scales. Overall, investigated microbial groups rather exhibited temporal than spatial variability at the investigated scale.

Contributions:
contributed to planning of the experiments
performed parts of the qPCR measurements
conducted subsequent statistical analyses and data interpretation
critically revised the manuscript

submitted

MANUSCRIPT 4

Spatial interaction of archaeal ammonia-oxidizers and nitrite-oxidizing bacteria in an unfertilized grassland soil

Barbara Stempfhuber*, Tim Richter-Heitmann*, Kathleen M. Regan*, Angelika Kölbl, Pia Kaul, Sven Marhan, Johannes Sikorski, Jörg Overmann, Michael W. Friedrich, Ellen Kandeler, Michael Schloter

* these authors contributed equally

Short description:

The temporal and spatial interaction patterns of nitrifying guilds of ammonia- and nitrite-oxidizers were examined at an unfertilized grassland plot. The abundances of key players of the respective processes, AOA and AOB as well as *Nitrospira*-like and *Nitrobacter*-like NOB were assessed by quantification of marker genes. Diverse and seasonally varying patterns of spatial distribution were revealed for NOB implying both niche separation and co-occurrence patterns, likely dependent on spatial heterogeneity of environmental variables such as substrate or oxygen levels. Congruent spatial patterns of AOA and *Nitrospira* however, were only demonstrated at the investigated scale in August and October and could be also linked to the respective nitrogen-pools in October, indicating their functional interaction to perform the complete nitrification process. A phylogenetic analysis additionally led to the assumption that in particular sublineage V affiliated *Nitrospira*-like NOB would contribute to NO from August onwards, since the relative abundance of assigned OTU sequences was increased.

Contributions:

contributed to planning and design of the experiments

performed qPCR measurements

conducted subsequent analyses and data interpretation

wrote the manuscript

Frontiers in Microbiology 6, 2016. doi: 10.3389/fmicb.2015.01567

MANUSCRIPT 5

Influence of commonly used primer systems on automated ribosomal intergenic spacer analysis of bacterial communities in environmental samples

Witoon Purahong*, Barbara Stempfhuber*, Guillaume Lentendu*, Davide Francioli, Thomas Reitz, François Buscot, Michael Schloter, Dirk Krüger

* these authors contributed equally

Short description:

Due to severe limitations of isolating bacteria from diverse environmental samples, cultivation-independent approaches are essential to assess the microbial community composition of ecosystems. However, commonly used fingerprinting methods are often biased due to the selection of the respective primer sets. In this respect, the study aimed at the comparison of different primer sets used in B-ARISA fingerprinting approaches. *In silico* testing of the primer coverage revealed differences in the coverage of bacterial taxa and in the exclusion of plant sequences. The results were further confirmed by testing the primer pairs in environmental samples. The observed discrepancies in the investigated primer pairs highlight the necessity to check the primer pair coverage prior to the analysis.

Contributions:

performed the *in silico* analyses with Genomatix software

conducted data analysis of *in silico* data and data interpretation

critically revised the manuscript

PLoS ONE 10, e0118967, 2015. doi: 10.1371/journal.pone.0118967

IV DISCUSSION

1 Nitrification in grassland and forest ecosystems

Nitrification plays a crucial role not only in the preservation of ecosystem stability and in linking above- and belowground processes, but also connects important nitrogen pools in soils. It is therefore essential to monitor the performance of key players in nitrification, both their activity and abundance, and their relative contributions to the process itself, to draw conclusions about their function in an ecological context as affected by environmental parameters. One of the central aims of this study was to address the effects of major drivers of the nitrifying community: LUI in grassland and pH in forest ecosystems, which we expected to exert the largest influences on the nitrifying community in the respective ecosystems. In addition to demonstrating reduced abundances, we also showed that pH may exert basic limitations that shape the AOA community independent of the ecosystem investigated, thereby favoring particular AOA clades adapted to extremely low pH (M1). Land-use intensity, in contrast, positively affected abundance and activity of both AOA and AOB at higher pH levels in mineral grassland soils (M2). Furthermore, our study revealed that those drivers which exhibited dynamic seasonal behavior affected activity and abundances of members of the nitrifying community (M2, M3, M4), while more stable edaphic factors shaped community composition (M1).

To assess major drivers of soil nutrient cycles and estimate the consequences of disturbances on the stability of ecological processes, it is essential to know the underlying principles of major processes. We attributed the effects of both kinds of drivers to the ways they influence substrate availability and accessibility, thereby shaping ecological niches of key players. We hypothesized that these dynamics induce shifts in the spatial and temporal patterns of nitrifiers and affect the selection of interaction partners. By investigating seasonal variation in spatial distribution patterns, we indeed found co-occurrence of AOA and NS, both of which are particularly well adapted to low substrate conditions, at the same spatial scale at unmanaged sites (M4), corresponding to the spatial distribution of ammonium.

1.1 Abundance and activity patterns in relation to nitrogen pools

One major aspect of this study was the determination of abundance and activity patterns of nitrifiers. AOA were found to dominate in numbers over their bacterial counterpart irrespective of the ecosystem or land management investigated. Nevertheless, the abundance of archaeal *amoA* genes

was reduced in forest soils (in the range of 10^4 to 10^7) compared to grassland soils (in the range of 10^7 to 10^8) (M1, M2). Potential nitrification rates in grassland mineral sites largely followed the dynamics of gene abundances, implying their functional contribution to ammonia-oxidation (M2). Several studies have identified a positive relationship between the abundance of ammonia-oxidizers and nitrification activity (Di et al., 2009; Leininger et al., 2006; Prosser and Nicol, 2008; Wuchter et al., 2006). In line with those studies, PNR were correlated to archaeal (in ALB: r=0.471, in HAI: r=0.725) and bacterial *amoA* gene abundances (in ALB: r=0.455, in HAI: r=0.598) in mineral soils under different land-use regimes (M2), albeit weaker correlations were detected in SCH-MB sites (for AOA: r=0.386, for AOB: r=0.402). In contrast, over the whole season in an unmanaged grassland plot of the ALB region correlations between activity and abundance were not seen (M3). However, nitrification activity measurement in the presented study was restricted to the determination of potential activity. Absence of correlations have been reported for denitrification as well (Le Roux et al., 2013), and this lack of relatedness may be due to a discrepancy between highly stable activity rates in contrast to more dynamic abundances of key players (M3).

Functionality of nitrification is often confirmed by linking the sequential transformation steps of ammonia- and nitrite-oxidation to substrate and product nitrogen pools. One would expect that the activity and abundance of nitrifiers would be positively correlated to the end-product nitrate (NO_3^-) and negatively correlated to the substrate of the nitrification process - ammonia (NH_3) (measured in the form of ammonium (NH_4^+)). Nitrate as the end-product of nitrification is available as substrate for denitrification in natural environments. A positive correlation of nitrate to potential nitrification rates (PNR) was investigated in mineral soils of grassland sites (PNR – NO_3^-: $r > 0.5$ in each region), indicating complete nitrification and additionally implying that the nitrification process dominated over low denitrification in these soils (M2). This may not have been the case in forest soils where no positive correlation of PNR and nitrate could be observed ($r = -0.4$), implying that other processes could have interfered (M1). PNR and ammonium concentrations were not significantly correlated in grassland (M2, M3) or in forest sites (M1) which may be a result of the role of NH_4^+ as central metabolite of the soil nitrogen cycle, involved in a variety of associated processes including assimilation by plants or heterotrophic organisms. However, decreasing ammonium concentrations along with increasing PNR, a possible indicator of ongoing nitrification, was observed, albeit this was not significant (M2). Consistent inverse spatial distribution patterns of AOA abundances and NH_4^+ could be visualized (M4), indicating a high turnover of substrate co-localized at sites where AOA were highly abundant (Prosser and Nicol, 2012). These congruent patterns of AOA and NH_4^+ distribution and the additional co-occurrence of NO at the same spatial scale in October may indicate that

complete nitrification was temporally limited under optimal conditions. This was further supported by a positive correlation of NS-like NOB and AO abundances to nitrate (M4), which points to a minimal influence by competing organisms or side processes.

1.2 Inter- and intraspecific ecological niche separation

These observations were striking evidence of functional redundancy in the investigated soils. The abundance patterns of AOA and AOB, followed over a year in an unfertilized grassland site, revealed inverse seasonal dynamics: highest abundance of archaeal *amoA* gene was reached in May and November (in the range of 10^8), when lowest copy numbers of bacterial *amoA* gene (in the range of 10^6) were determined, and vice versa (M3, M4). Inverse dynamics of AOA and AOB abundances were also observed for spatial patterns (Wessen et al., 2011). Despite measured seasonally changing abundances, nitrification rates remained at the same level, and this has been previously demonstrated for extensively used sites of the ALB region (Meyer et al., 2013). The stability of the process supports the assumption that functionally redundant microorganisms could, in principle, maintain ecologically important processes over the whole year (Giles et al., 2012). Redundancy of ecological processes is of particular importance for the sustainability of ecosystem services as it ensures ecosystem stability. In other words, functional redundancy might buffer the impacts of environmental change and determine ecological niche separation, thereby allowing the co-existence of functionally redundant species by minimizing the competition for resources (Giles et al., 2012; Wolters et al., 2000). Despite increasing knowledge of the general mechanisms of niche differentiation, central questions still remain, such as: what factors influence the contribution of functionally redundant organisms to nitrification activity in different ecosystems?

In terrestrial environments, nitrification efficiency has been ascribed to the performance of ammonia-oxidizers, as this was considered to be the rate limiting step. Several studies addressing the relative contributions of key players in nitrification, when comparing, for example, different habitats, depth profiles or soil compartments, yielded contradictory results concerning the dominance of either AOA or AOB (e.g. Boyle-Yarwood et al., 2008; Di et al., 2009; Kim and Kim, 2006; Leininger et al., 2006; Rogers and Casciotti, 2010; Schauss et al., 2009). Independent of the investigated ecosystem (forest vs. grassland), geographic region or soil type, we found AOA to dominate in numbers over their bacterial counterpart (M1, M2), which is in line with other studies (Adair and Schwartz, 2008; He et al., 2007; Ke et al., 2013; Leininger et al., 2006; Schleper 2010). AOA abundances estimated by the detection of archaeal *amoA* gene copy numbers ranged from 10^5 to 10^7 in forest soils, and from 10^7 to 10^8 and 10^5 to 10^6 in extensively and intensively used grassland soils,

respectively (M1, M2, M3, M4). NS-like NOB were more abundant in an extensively used grassland soil than NB-like NOB, with 16S rRNA gene copy numbers ranging from 10^7 to 10^8 and 10^5 to 10^6, respectively (M4). Numerical dominance, however, is not necessarily linked to higher contributions or efficiencies. Specific activity per cell must be taken into account as well, and this has been proven to be higher in AOB than in AOA. The latter, however, may compensate for this disadvantage by its higher abundance. Nevertheless, other studies have detected opposing trends for AO and NO key players in soils (Di et al., 2009; Grundmann and Debouzie, 2000). These contradictory observations are largely attributable to differences in underlying substrate concentrations, highlighting the importance of taking site-specific factors into account. Gradients of nitrite concentrations, for example, determine the relative contributions and distributions of NS and NB (Kim and Kim, 2006). In turn, data on abundance and activity of AO in nutrient-rich vs. nutrient-limited soils provide contrasting results, perhaps attributable to different preferences of AOA and AOB concerning substrate quantity and quality. Requirements for substrate availability may also differ depending on whether it is needed for activity or growth (Prosser and Nicol, 2012). However, niche differentiation may be due not only to **(i) substrate concentrations** but also **(ii) growth strategies** or the capacity to switch to alternative metabolic strategies, which will be discussed in the following passages. Meanwhile, it must be kept in mind that those characteristics are largely derived from laboratory cultures or metagenomics studies and therefore may not be transferrable one-to-one to the behavior and properties of microorganisms in natural ecosystems.

The presence of the substrates ammonia and nitrite as well as oxygen availability serving as a terminal electron acceptor are essential for nitrification (Martens-Habbena et al., 2009; Ward et al., 2011). It is not surprising, therefore, that these factors act as niche determining factors. In this context **(i) substrate or oxygen affinity** plays a major role, particularly when limitation occurs. Affinity is defined as the ratio between the maximum uptake rate and the half-saturation constant (Laanbroek et al., 1994) and controls the optimal substrate range needed to satisfy the cell's energy demand at non-inhibiting concentrations. Enzyme affinities to the respective substrates of AOA and NS-like NOB are higher than those of their counterparts AOB and NB (Lücker et al., 2013; Martens-Habbena et al., 2009; Schramm et al., 1999). This implies that AOB and NB would require higher substrate levels for growth (Abeliovich, 2006; Martens-Habbena et al., 2009; Stahl and de la Torre, 2012). And indeed, autotrophic nitrification activity has been correlated to active AOB performance in a variety of nitrogen-rich or fertilized grassland soils (Di et al., 2009). Our study showed that intensively managed sites harbored higher abundances of AOB than extensively managed ones (M2): measured gene copy numbers of marker genes ranged from 2.8×10^6 (ALB) and 4.6×10^6 (HAI) under

intensive management to $9x10^4$ (ALB) and $6x10^5$ (HAI) under extensive management (low LUI). Even at an unfertilized site, AOB dynamics followed those of ammonium through the season: observed abundance of AOB was highest at most dates characterized by high ammonium content (M3). Furthermore, our observed increase in the number of bacterial *amoA* gene abundances with increasing soil pH (M1) supports the hypothesis that AOB are indeed adapted to higher nitrogen sources and concentrations. While we also found AOA to be responsive to increasing land-use intensity levels associated with higher NH_4^+ loads, AOA abundance was reduced at the highest levels (M2) likely due to inhibition of ammonia-oxidation by high ammonia concentrations (Hatzenpichler et al., 2008; Stahl and de la Torre, 2012). Also in acidic soils, the archaeal *amoA* gene copy numbers fell in the range of $7x10^6$ compared to bacterial *amoA* in the range of $5x10^5$ -$1x10^6$ (M1). The dominance of AOA vs. AOB in low-ammonia environments such as extensively used soils (M3) or acidic soils (M1), where pH might play a crucial role in shaping NH_3 availability by controlling the NH_3:NH_4^+ equilibrium, further supports the assumption that AOA may play an important role in nutrient-limited sites (Chen et al., 2014; Offre et al., 2009; Schleper, 2010; Verhamme et al., 2011; Zhang et al., 2010), based on their tolerance of low ammonia levels and high substrate affinity (Prosser and Nicol, 2012).

No evidence for spatial niche differentiation of functionally redundant ammonia-oxidizers could be found because spatial distributions for both AOA and AOB could not be visualized by kriged maps at the same date (M4). Nevertheless, spatial niche distribution patterns could be characterized for NS and NB; spatial niche separation with minimal overlap of patches of high gene abundances of NB and NS was visible at the plot scale in April, whereas in August maps depicted congruent spatial patterns. This demonstrated their co-occurrence when ammonia-oxidizers were observable at the same spatial scale, leading to the assumption that NOB may have had access to AO-derived nitrite (M4). Specific adaptions could therefore make possible the co-existence of functionally redundant NOBs. Nitrite availability, for example, has been demonstrated to induce shifts between NS and NB (Attard et al., 2010): whereas NS could deal with lower concentrations (Lücker et al., 2013; Schramm et al., 1999), NB-like NOB were associated with high potential nitrite-oxidation activity under high-nitrogen levels (Wertz et al., 2012). Unfortunately, nitrite concentrations could not be determined, but have been shown to be below the detection limit in similar studies (Attard et al., 2010; Spieck et al., 2006).

Oxygen status can also act as a niche determining factor, e.g., in paddy soils (Ke et al., 2013), and this may have been the case in April at our study site due to the relatively high soil moisture content (58%) at that date (Regan et al., 2014). Simultaneously, substrate-limited conditions could have also prevailed at this date due to the absence of AO at the same spatial scale (M4). Low oxygen conditions

and the presence of organic carbon derived from mineralization processes in spring and autumn might additionally trigger NOB to switch to nitrate reduction (Ehrich et al., 1995; Freitag et al., 1987). The fact that nitrite concentrations above a certain level would inhibit this process fits with our assumption that nitrite-limited conditions might favor the above mentioned processes (Freitag et al., 1987). Although NXR in general has been proven to catalyze the reversible process of NO – nitrate reduction, NB might be particularly well adapted to low-oxygen environments by their potential to completely or partially denitrify (Lücker et al., 2010; Starkenburg et al., 2008). This is supported as well by the presence of *nir* genes in NB (Ehrich et al., 1995; Freitag et al., 1987). These specific ecological adaptions trigger intraspecific niche differentiation (Daims et al., 2001; Gieseke et al., 2003; Placella and Firestone, 2013; Schramm et al., 2000).

Interspecific niche differentiation may occur amongst both AO and NO. The existence of different ecotypes adapted to particular substrate concentrations and even "overlapping ecological niches", have been proposed for NS-like NOB (Lebedeva et al., 2008; Lebedeva et al., 2011; Lücker et al., 2010; Maixner et al., 2006), further reducing competition and facilitating co-existence even under very similar nitrite concentrations (Bartosch et al., 2002; Cébron and Garnier, 2005). In a more detailed subgroup analysis of NS sublineages, the co-occurrence of several sublineages was detected. Operational taxonomic units (OTUs) 01 and 02 were affiliated with representatives of NS sublineages I, II and VI, while OTU03 was associated with *Ca.* NS bockiana as cultured representative of lineage V (M4). The results further indicated changes in the relative abundances of sublineage-associated OTUs with OTU03 increasing in its relative abundance from August on (M4). Although widely congruent spatial distributions of AOA and NS in October were visualized using abundance data of the whole NS community, NS-like NOB sublineages may have relied on optimal substrate concentrations coupled with AOA-driven release of NO_2^- (Tourna et al., 2011). Slight shifts might be explainable by NS residing at an optimal distance to the NO_2^- source (Grundmann and Debouzie, 2000). This suggests that the abundance of a particular sublineage may be coupled to a particular nitrite concentration, determined by its particular growth requirements and tolerance levels (Bartosch et al., 2002). Adaptations to different substrate levels also seem to be the case within ammonia-oxidizers with *Nitrosomonas* (AOB) and *Ca.* Nitrosoarchaeum or *Nitrosospharea* (AOA) being associated with higher, *Nitrosospira* (AOB) and *Ca.* Nitrososphaera or *Nitrosotalea* (AOA) with lower ammonia levels (Daebeler et al., 2014; Hatzenpichler et al., 2008; Jung et al., 2011; Schramm et al., 1998; Tourna et al., 2011).

Distinct substrate affinities alone would not necessarily account for niche separation; growth limiting conditions may also play a role (Prosser and Nicol, 2012). **(ii)** Different **growth strategies,** including

the capacity to utilize organic compounds or alternative metabolic strategies would give a competitive edge to organisms that can to switch to other energy generating modes (Hallam et al., 2006; Prosser 2012). Maintaining energy generation for growth under nutrient-limited conditions may be of particular importance for organisms with low substrate affinity. Thus, the switch to heterotrophic growth, albeit coupled to lower generation time than that of bona-fide heterotrophs, might constitute an advantage in competition under conditions of limited resources, but would not likely be preferred to the still more efficient autotrophic mode (Bock et al., 1988; Lücker et al., 2010; Norman and Barrett, 2014; Verstraete and Focht, 1977). Since we did not assess heterotrophic nitrification and fungal communities in forest soils, the discussion will focus primarily on autotrophic nitrifiers, their metabolic diversity, and their capacity to switch to other growth strategies. Responses of AO to organic carbon content might be indifferent: both positive correlations and inhibition by high organic substance concentration have been reported (Chen et al., 2008; Erguder et al., 2009; Könneke et al., 2005; Wessen et al., 2011). AOB abundances in SCH-NM soils were positively correlated to total organic carbon content (r=0.511) (M2) which we consider is due to indirect effects of carbon, which would increase the nutrient supply by triggering the performance of microbes that mineralize organic N, delivering ammonium as substrate for AO, not due to a switch to heterotrophic growth (Gubry-Rangin et al., 2010; Hallin et al., 2009; Lehtovirta-Morley et al., 2011; Pratscher et al., 2011; Wessen et al., 2011). Similar to AOB, high AOA abundance was detected in soils characterized by high organic matter content (Stopnišek et al., 2010; Zhalnina et al., 2012). Adair and Schwartz reported increased AOA abundance in May (Adair and Schwartz, 2008), concomitant with the major plant growth stage leading to increased root exudation, which is in line with this study, where highest abundance of AOA was also detected in May and November (M3, M4). Increases in autumn may be related to increased organic carbon content, favoring lineages capable of a heterotrophic or mixotrophic livestyle (Brown et al., 2013). However, no significant correlation could be found in our study, perhaps due to other soil microbes accessing carbon pools at this time. The discovery that some AOA did not contribute to autotrophic nitrification, the *amoA*-encoding archaea (Mußmann et al., 2011), supports the hypothesis that archaea may be able to switch to other strategies for energy generation, metabolizing alternative substrates to ammonia (Lebedeva et al., 2013; Spang et al., 2012). This would give AOA a survival advantage under a wide range of growth conditions, varying amongst AOA phylogenetic lineages (Offre et al., 2009; Treusch et al., 2004; Treusch et al., 2005; Ward et al., 2011), giving those AOA an advantage over autotrophic AOB and increasing their competitiveness in substrate limited sites (Rogers and Casciotti, 2010).

Amongst NOB, growth strategies differ inter- and intraspecific. NB strains have been shown to use a variety of organic substances for mixotrophic and heterotrophic growth (Bock et al., 1983; Daims et al., 2001; Steinmüller and Bock, 1977). Especially under oxygen limitation, NB exhibit higher growth rates and faster turnover by switching to a heterotrophic lifestyle and denitrification. Thus, their competitiveness is likely to increase under high soil moisture conditions, which we expected to see in April when pronounced changes in NB abundance with respect to spatially restricted patches of very high NB abundance were detected concomitant with the highest measured soil moisture content of the year (M4). This may further have contributed to niche differentiation based on adaptation to different oxygen conditions. But amongst NS sublineages, different lifestyles and growth strategies are also likely to vary from obligate autotrophic growth to heterotrophic or mixotrophic growth strategies, utilizing organic compounds such as pyruvate under nitrite limited conditions (Daims et al., 2001; Lücker et al., 2010). Distinct contributions of metabolically diverse NOB sublineages are reflected by different ^{13}C based activity patterns (Wang et al., 2015), and could explain the patterns and shifts of the NS-like NOB-assigned relative abundances observed in this study - the co-occurrence of different sublineages with divergent growth strategies at the same spatial scale (M4). Nevertheless, other niche determining factors, such as varying tolerances or preferences for different substrate sources, may also account for niche separation (Prosser and Nicol, 2012).

2 Interactions of key players in the nitrification process

In terrestrial environments, the successful interaction of AO and NO at the same temporal and spatial scales is essential to sustain nitrification efficiency and prevent negative environmental consequences, such as toxic levels of nitrite accumulation through failure of the conversion of AO-derived nitrite by NO (Grundmann et al., 2001). Lack of nitrate would also have crucial impacts on plants and other microbes that assimilate nitrate as major nitrogen source. At the local plot scale of our study site, co-existence of AO and NO was demonstrated both at temporal and spatial scales (M4). NS-like NOB showed a continuous spatial autocorrelation during the year, while NB-like NOB patterns could be visualized in April, August and November. AOA and AOB spatial patterns varied between May and November: AOA were spatially structured at the investigated scale in August and October, while AOB exhibited spatial autocorrelation in the remaining months (M4). A study of Grundmann and colleagues attributed nitrification efficiency in soils to the detected spatial co-occurrence of AO and NO (Grundmann et al., 2001). Since nitrite rarely accumulates in terrestrial environments, a tight mutualistic interaction of ammonia- and nitrite-oxidizers has been proposed (Ke et al., 2013). AOA species in particular have been shown to exhibit a pronounced sensitivity to

elevated nitrite levels (Lehtovirta-Morley et al., 2014). Therefore, a close interaction with nitrite-oxidizers would be of particular importance to AOA for removal of toxic amounts of nitrite (Lehtovirta-Morley et al., 2011; Prosser and Nicol, 2012). NOB may profit as well from a close interaction with AO, both in terms of substrate supply for energy generation and to avoid too-high ammonium concentrations, as nitrite oxidoreductase has been demonstrated to be both nitrite- and ammonium-sensitive (Abeliovich 2006; Monreal et al., 1986).

AOB and NOB have been shown to form microcolonies exhibiting patchy distributions (Gieseke et al., 2003; Grundmann and Debouzie, 2000; Grundmann et al., 2001; Mobarry et al., 1996; Schramm et al., 1999). Zones of AO and NO activity could not be clearly separated, indicating both the coexistence of different groups of ammonia- and nitrite-oxidizer populations (Maixner et al., 2006) and their pronounced scale heterogeneity as a response to changing NH_4^+ and O_2 levels at distances in the µm range (Gieseke et al., 2003). Changes in the abundance and / or community composition of AO have been related to changes in NO, pointing to shifts in interaction partners (Knapp and Graham, 2007). This is congruent with our observations of changing correlations of key players in nitrification: AOA and NS were positively correlated in October (r=0.574), AOA and NB in April (r=0.576) and October (r=0.561), but no spatial dependence could be visualized by kriged maps in the latter case; AOB was only positively correlated with NB in April (r=0.506) (M4). Thus, our data imply shifts in the co-occurrence patterns of AO and NO at the investigated spatial scale. Nevertheless, these correlations could not be visualized as kriged maps. Changes in the selection of interaction partners might be ascribed to one of the following mechanisms: **(i)** similar preferences for substrate concentrations, **(ii)** capacity to adopt a mixotrophic or heterotrophic lifestyle, **(iii)** similar adaptations to adverse environmental conditions.

(i) Spatial autocorrelations of nitrifiers have been determined at ranges from cm to several meters (Keil et al., 2011). In our study, spatial dependencies of nitrifiers were investigated at the meter scale (M4). Since nitrite concentrations were not determined in the present study, we took the presence of AO as proxy for nitrite concentration: the release of nitrite into the surrounding environment of AO might generate a concentration gradient constituting different NO_2^- levels for NO (Maixner et al., 2006). Our geostatistical approach confirms the interaction of AOA and NS through observed congruent spatial distribution patterns under the nutrient-limited conditions we assume would prevail at the investigated unfertilized plot (M4). Both AOA and NS have higher substrate affinity than their counterparts and are thus extremely well adapted to low substrate environments (Daebeler et al., 2014). Therefore, NS might have an advantage over NB in areas of low nitrite supply, which we attributed to the putatively low nitrite-production rates by AOA at the same scale. AOA in

turn were likely to respond as well to low substrate concentrations at the unmanaged site under investigation. The interactions between NS and AOA seemed to be temporally and spatially restricted: temporal dynamics of both abundances increased from April to May, followed by a decline until August / October. Highest abundances of both archaeal *amoA* gene abundance and 16S rRNA gene for NS were obtained in November (M4). Interactions of AOA and NS have been found mainly under substrate-limited conditions (Daebeler et al., 2014; Ke et al., 2013), while those of AOB and NB have been found in nitrogen-rich environments (Ke et al., 2013; Wertz et al., 2012). We did not assess spatial autocorrelation patterns under high nutrient availability, as would be found in fertilized soils, but we assume that NB and AOB might interact due to their preference for nitrogen-rich soils. Although NOB abundances were not determined at our other experimental sites, those monitoring different land-use intensities, it can be assumed that interactions with AOB as key players in fertilized soils would gain importance because AOA abundance decreased under very intensive land-use level 5 (M2). Support for this assumption comes from a study showing that AOB reduction leads to a decline in NB-like NOB abundances and a simultaneous increase of NS-like NOB; this is attributed to NS preferences for lower NO_2^- concentrations (Ollivier et al., 2013).

(ii) Another similarity of AOA and NS, apart from their preferences for low-substrate environments, is their capacity for mixotrophic or heterotrophic lifestyles. Evidence for mixotrophic growth comes in part from the absence of labelled CO_2 incorporation by AOA in particular soils (Daebeler et al., 2014; Jia and Conrad, 2009; Wang et al., 2015). In this context, Wertz and colleagues attributed the missing interaction patterns and the absence of nitrification-associated growth of AOA and NS in acidic forest soils to alternative growth strategies (Wertz et al., 2012). We speculate that nitrification during the second half of the year of our study can be attributed to autotrophic growth when substrate availability increases slightly, and additionally triggered by a mowing event in August, impeding plant competition for ammonia (M4) (Chen et al., 2014). In contrast to our assumption, reduced AOA abundance after mowing, attributed to reduced root exudation, was suggested in other studies as indicative of the presence of mixotrophic or heterotrophic AOA (Chen et al., 2014). Nevertheless, we assume that non-autotrophic AOA and NS could also exhibit interactions apart from those which are strictly autotrophic (Daebeler et al., 2014). Although no spatial correlations could be determined at the beginning and end of the year, we speculate that alternative growth strategies would increase the competitiveness of AOA and NS towards their counterparts and give them a growth advantage in soils with more organic material, as might be the case at these dates (M4), enabling them to cope with suboptimal environmental conditions. Nevertheless, we ascribed nitrification in October to the interaction of AOA and the autotrophic NS-like NOB associated with *Ca.* Nitrospira bockiana

sublineage, additionally supported by the results of a Pearson-coefficient-based network correlation analysis (M4), confirming significant correlations of AOA and NS as well as OTUs of NS. Furthermore, NS OTU03, which had increased in its relative abundance, was correlated to nitrate.

(iii) Not only alternative growth strategies but also shared adaptions to adverse growth conditions, characterized by energy or nutrient shortage or extreme climatic conditions, might determine the choice of interaction partners. The identification of dominant AOA phylotypes in ultra-acidic soils further supports the proposed persistence of AOA in suboptimal surroundings (M1). NO at forest sites have not been assessed in this study, but results from other studies lead to the speculation that here too NS would be the respective interaction partners, since they are known to deal particularly well with acidic pH environments through the formation of microcolonies (Larsen et al., 2008; Lebedeva et al., 2011). In thermal springs under elevated temperatures and also in volcanic grassland soils autotrophic NS have been exclusively found interacting with AOA (Daebeler et al., 2014; Lebedeva et al., 2011; Lebedeva et al., 2013). NS were found serving as a seeding population in a starvation mode of inactivity (Gieseke et al., 2003; Schramm et al., 1999). The expression of *nxr* genes has been shown to be not only substrate induced but also constitutive, allowing these organisms to respond immediately to changes in environmental conditions after starvation (Lücker et al., 2010). As high nitrite concentration has been demonstrated to inhibit growth of *Nitrosotalea*, which were present in the forest soils of this study mainly under low pH ranges (M1), a close interaction with NO in acidic sites to counteract growth inhibition would be likely (Lehtovirta-Morley et al., 2011). NB could also be isolated from an acidic soil (pH 5.5) (Hankinson and Schmidt, 1988; Spieck et al., 2006), implying that NB are also in principle capable of living under acidic conditions. Furthermore, AOB and NB show rapid rewetting-induced upregulation after a starvation period, while AOA and NS respond later, although maintenance of transcription level or the stability of transcripts would also affect their responsiveness to changing conditions (Placella and Firestone, 2013; Stein et al., 2013). The flexibility to react to energy stress or substrate shortage under energy-limited or adverse environmental conditions might influence the selection of interaction partners (Lehtovirta-Morley et al., 2014; Rogers and Casciotti, 2010; Valentine 2007).

3 Effects of selected environmental parameters on the nitrifying community

Because drivers influence microorganisms either directly or indirectly, via substrate concentrations for example, our aim was not only to assess the direct correlation of substrate concentrations to microbial performance but also to identify super-ordinated drivers. For our detailed study on drivers

of nitrification, we selected LUI and pH as the major factors shaping substrate concentration and substrate accessibility in grassland and forest soils. One has to consider that other non-assessed factors might be important for nitrification as well. Environmental parameters are linked to microbial behavior in soils and may be classified as abiotic, biotic and anthropogenic variables (**Figure 1**). These factors can be additionally distinguished as labile and stable parameters (**Figure 4**). Labile factors, such as temporally varying climatic conditions, are unlikely to change the spatial location of microbes but may influence their activities, while preserving stable community composition (Felske and Akkermans, 1998). While abundance patterns are considered to be influenced mainly by labile parameters, community composition may be shaped by stable parameters such as soil type or soil pH, factors exerting long-term effects. These could be considered as indirect effects on substrate availability or concentration, and could additionally directly affect community composition by shaping specific lineage adaptions (Attard et al., 2010; Hallin et al., 2009; Martens-Habbena et al., 2009). Both environmental factor classes can have different effects on the performance of AO and NO communities and thereby change their interaction patterns.

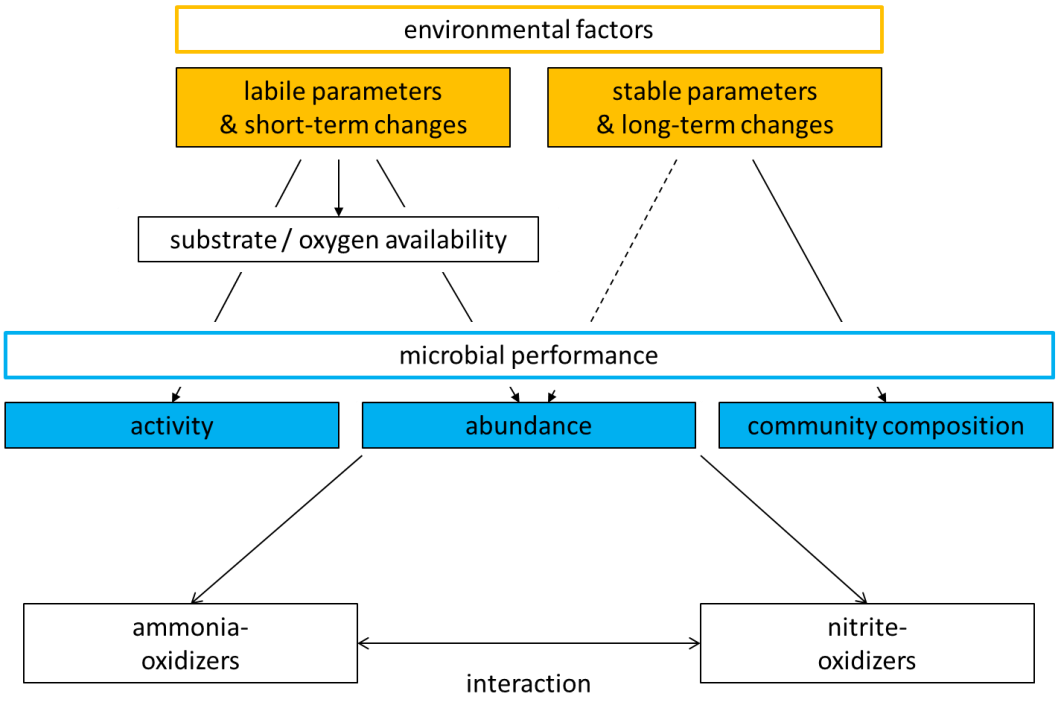

FIGURE 4: **Hierarchical levels of environmental factors.**

Variables are classified into stable and labile parameters influencing different aspects of microbial performance such as activity, abundance and community composition (indicated by arrows).

3.1 Effects of stable soil parameters on abundance and diversity of nitrifiers

Although site-specific soil properties have been identified as major drivers of microbial community composition, activity and abundance (Herold et al., 2014; Zhalnina et al., 2012), site-dependent characteristics cannot easily be related to individual variables (Horz et al., 2004) due to the heterogeneity and complexity of the soil matrix, which provides a mixture of stable (e.g. soil texture, pH) and labile parameters (e.g. soil moisture, organic carbon content). Nevertheless, a clear relationship between biomass, activity and diversity of microbial communities to soil properties was shown in a study conducted on the same experimental sites that were investigated in this study (Herold et al., 2014). Focusing on the effects on nitrifiers, no correlation of AOA or AOB abundance to soil parameters could be determined either at the extensively used, unfertilized site in the ALB region (M3) or at the mineral soil sites of ALB and HAI regions (M2). For a detailed analysis of stable parameters, soil pH and soil type were chosen; their effects will be discussed in the following section.

Soil pH, postulated to be the best predictor of microbial community structure, is considered to "affect bacterial community structure, growth and activity of all microbes" (Fierer and Jackson, 2006; Kuramae et al., 2012; Ward et al., 2011). Besides the general effects on soil microbes, pH seems to impose restrictions on nitrifiers, particularly to ammonia-oxidizers (Gubry-Rangin et al., 2011; Hallin et al., 2009; He et al., 2007; Nicol et al., 2004; Nicol et al., 2008; Yao et al., 2011), albeit nitrification has been found to occur even in acidic soils with pH values below 3.5 (De Boer and Kowalchuk, 2001; Burton and Prosser, 2001; Erguder et al., 2009). The major problems AO and NO have to face in acidic environments are to keep internal pH constant and to deal with NH_3 limitation (Gubry-Rangin et al., 2011). At low pH, ammonia is ionized to ammonium, which leads to reduced ammonia availability (Frijlink et al., 1992; Tarre and Green, 2004). Low but still detectable PNR levels have been found in acidic environments such as forest soils. PNR levels were below 50 ng NO_2^--N g^{-1} h^{-1} in acidic forest sites in ALB and HAI regions, while values were slightly higher in SCH forest sites (M1), compared to mineral grassland soils of neutral pH range in ALB and HAI regions in the range of 276-1121 ng NO_2^--N g^{-1} h^{-1} (M1, M2). Neither abundance nor activity of AO was correlated to pH in the majority of the investigated grassland sites across three different regions in Germany (M2). The mean values of soil pH were comparable, in the neutral pH range of 6.3 (ALB) and 6.9 (HAI and SCH-NM). At sites with lower pH of about 5.8 in SCH-MB, positive correlations of AOA and pH were detected (r=0.606). Also in acidic forest soils, AOA exceeded their bacterial counterpart in numbers (M1). In contrast to AOA, which are able to cope with low pH, growth of AOB is severely inhibited in acidic pH (Nicol et al., 2008; Stopnišek et al., 2010; Suzuki et al., 1974; Zhang et al., 2012). Gene and transcript abundances of AOB increased with increasing pH but were almost absent in different acidic terrestrial habitats;

those of AOA decreased (Isobe et al., 2012; Gubry-Rangin et al., 2010; Nicol et al., 2008; Stopnišek et al., 2010; Yao et al., 2011). Highest AOA abundances in forest sites were detected in the range of soil pH 3 – 4.5, and ratios of AOA to AOB increased with decreasing soil pH, again highlighting the important role of AOA in acidic environments. This fits well to observations demonstrating a positive correlation of AOA abundances to activity in acidic soils (He et al., 2007; Yao et al., 2011). Sustained high growth rates even under acidic conditions would rely on adaption mechanisms to prevent NH_3 shortage (Nicol et al., 2008). Active substrate transport mechanisms, aggregation, storage mechanisms of NH_3 and the utilization of alternative substrates have been proposed (Stahl and de la Torre, 2012). Apart from passive diffusion, active energy-consuming ammonia uptake might occur via transporters encoded by *amt* genes, which have been identified in AOA (Booth et al., 2005; Burton and Prosser, 2001; Hallam et al., 2006; Martens-Habbena et al., 2009; Schmidt et al., 2004; Stahl and de la Torre, 2012; Ward et al., 2011; Weidinger et al., 2007).

Despite indeterminate results on the effect of pH on *amoA* transcript and gene abundances (Bru et al., 2011; Hallin et al., 2009; He et al., 2007; Jia and Conrad, 2009; Nicol et al., 2008; Yao et al., 2011), soil pH had no effect on the abundance of AOA at the forest sites of this study (M1), which is not surprising given that the number of all *amoA* encoding organisms was included, disregarding the fact that only few phylotypes might be active in the respective pH range. Thus, pH-dependent shifts in the abundances would not have been observable unless relative abundances of phylotypes were considered (Wessen et al., 2011; Yao et al., 2011).

Since AOA dominated under acidic soil pH, the community structure of AOA was assessed in more detail: Soils with higher pH ranges harbored microbial communities with higher OTU richness than those found in low pH environments, and evenness was reduced compared to AOA communities in acidic sites (M1). These findings imply that a less diverse AOA community composition seems to be present in low pH soils, indicating that only a few AOA phylotypes are adapted to the conditions prevailing under acidic sites and are exclusively associated with the respective pH ranges (Fierer and Jackson, 2006; Nicol et al., 2008). The same clades (*Nitrososphaera* subcluster 7.2 and *Nitrosotalea* cluster) adapted to low pH ranges were found both in forest and grassland soils (M1). Soil pH indeed shaped the community structure of AOA in terms of a pH-induced niche separation (Nicol et al., 2008; Yao et al., 2011): Sequences exclusively found in acidic (pH <5) soils were assigned to archaeal soil lineages 1.1a and 1.1a-associated, while 1.1b related sequences were detected only under higher pH ranges at the observed site (Gubry-Rangin et al., 2011; Hatzenpichler 2012). The putatively heterotrophic lineage 1.1c was dominant mainly in forest sites with acidic pH range 3.8 – 4.3 (Kemnitz et al., 2007; Nicol et al., 2005; Oline et al., 2006), but was also present in grassland soils of

pH range 4.5 – 6.0 (Lehtovirta et al., 2009; Ochsenreiter et al., 2003) indicating that pH does not shape community composition exclusively; the ecological site-specific context has to be taken into account. However, other studies point to pH-independent nitrification in different soils (Booth et al., 2005). This highlights the importance of considering combined effects of other factors also, for instance the source of ammonia.

In our study, sequences clustered into two groups clearly separated by pH 4.6. The community composition of AOA in acidic soils revealed high similarity with those detected under ultra-acidic (pH < 3.5) grassland soils from another study (Gubry-Rangin et al., 2011), while AOA community composition of forest and grassland soils in the acido-neutral pH range were more dissimilar in terms of shared OTUs (pH > 4.5) (M1). OTUs identified in the ultra-acidic pH range, OTU4 and OTU1, have been assigned to *Nitrososphaera* subcluster 7.2 (Gubry-Rangin et al., 2011) and *Nitrosotalea* (He et al., 2007; Lehtovirta-Morley et al., 2011) sequences, respectively (M1). As previously mentioned, communities in low pH environments may require particular adaptation mechanisms. Assigned cultivated representatives such as *Nitrososphaera viennensis* seem to prefer low NH_4^+ concentrations, and this archaeon was further characterized by its capability of mixotrophic growth, which was observed to be much faster upon application of pyruvate than under autotrophic conditions alone (Daebeler et al., 2014; Tourna et al., 2011). One might speculate that there is a critical pH range, optimal for autotrophic growth, identified as pH 4-5 for *Nitrosotalea* in culture. Interestingly, this coincides with the pH range we identified as critical for the separation of acidophilic communities in our soils (Lehtovirta-Morley et al., 2011) (M1).

Furthermore, the ability to switch to alternative substrates such as urea would be a beneficial characteristic for growth under ultra-acidic suboptimal conditions (Gubry-Rangin et al., 2011). As the process of ureolysis is considered to be functional in a pH-independent manner, it could constitute a promising advantage of an alternative metabolism for AO in acidic soils (De Boer and Kowalchuk, 2001). NH_3 from hydrolyzed urea remains in the cell and serves as substrate for AO in AOA and AOB; both have been proposed capable of ureolysis, since growth of AO on urea has been observed in cultures (Burton and Prosser, 2001; Prosser and Nicol, 2001) and was further confirmed by the discovery of urease genes (Koper et al., 2004; Lu and Jia, 2013; Tourna et al., 2011). There is evidence that a few AOB might also be able to deal with acidic sites, probably also having recourse to further adaptations such as aggregate formation (De Boer et al., 1991) and those AOB lineages might co-occupy similar niches at low pH environments (Schleper and Nicol, 2010). Given that similar results have been obtained regardless of the investigated scale (at the global and regional scale), the

adaptation mechanisms to soil pH might be universal. We conclude that AOA dominates ammonia-oxidation in acidic soils independent of the ecosystem investigated, and that decreasing AOB abundance and growth might be attributed to low NH_3 content and / or to negative effects with regard to their cell physiology (Nicol et al., 2008; Norman and Barrett, 2014; Suzuki et al., 1974; Yao et al., 2011; Zhang et al., 2012).

A comparison of different **soil types** at sites situated in similar geographical regions (and thus subjected to the same climatic conditions) revealed the importance of taking site-specific soil type into account. The site specific properties of the three investigated regions have been thoroughly described in Fischer et al. (2010). In brief, mineral sites (ALB, HAI, SCH-MB) are characterized by loamy and clayey texture and histosolic sites (SCH-NM), which are groundwater dependent and periodically water saturated (water content at our site 101-122%), are characterized by high water holding capacity and a comparatively high soil pH. Peat degradation, the increased availability of organic carbon and mineralization processes might fuel nitrogen-fixation, a high energy consuming process. This assumption is supported by the extremely high *nifH* gene abundances in SCH-NM plots, in the range of 2.5 – 9.7 x 10^7, compared to the mineral soil sites under investigation (M2). Interestingly, organic carbon and AOB abundance were correlated exclusively at histosolic sites, which is surprising because AOB are considered autotrophs. Thus, this effect might be attributed to soil type specific high organic carbon content, which drives mineralization and nitrogen-fixation processes. Both in turn increase NH_4^+ content, which is then available for ammonia-oxidizers (Brankatschk et al., 2011; Wessen et al., 2011), and which might favor AOA through mineralization-derived ammonia (Prosser and Nicol, 2012). A study by Lethovirta-Morley and colleagues underlined this assumption by demonstrating co-enrichment of AOA with nitrogen-fixers and heterotrophs that may deliver ammonium by mineralization processes (Lehtovirta-Morley et al., 2011). Owing to high groundwater tables and periodical flooding, the high water content in histosolic sites would also hamper oxygen availability and accessibility for aerobic processes, favoring anaerobic organisms and others specially adapted to conditions in transition zones (Bodelier and Frenzel, 1999; Herold et al., 2014). Since the described effects are mediated by primary soil moisture effects rather than to direct impacts of soil type, affected processes are discussed in the section dealing with soil moisture. Soil texture and labile factors such as soil moisture content collectively affect nutrient accessibility (Franklin and Mills, 2009; Kuramae et al., 2012). Thus, it can be concluded that histosols, which are characterized by extremely high water holding capacity or exposed to long-term periods of flooding, are likely to inherit their own communities in transition zones, such as methane-oxidizing bacteria (MOB), which have indeed been detected at the investigated sites (M2). In contrast, short-term soil

moisture content in temperate mineral soils seemed instead to affect microbial activities and abundances as will be discussed below.

3.2 Effects of dynamic soil parameters on abundance and activity of nitrifiers

Dynamic or labile parameters that change over the season are often subjected to climatic conditions such as temperature or precipitation. Attention to climatic change is increasing, including elevated temperatures due to global warming, and experiments assessing the response of microbial communities to drought and subsequent rewetting and to estimating the resilience of ecosystems in their response to water content fluctuations have gained prominence.

Soil moisture content may be a primary influence on the activity of aerobic and anaerobic processes and thus also induce shifts between nitrification and denitrification dynamics. The aerobic process of nitrification would be impaired if water content was too high, which was proven by the strong negative correlation of water content to potential nitrification activity ($r = -0.7069$) measured at different sites at one sampling date in May (M1). While PNR was positively correlated to nitrate in mineral soils under rather low water content (42-22% WC) (M2), denitrification played a more prominent role in histosolic sites: high water content in addition to low oxygen availability (101-122%); additionally, high levels of organic carbon favoring denitrification impeded the correlation of nitrification potential to the nitrate pool (M2). Followed over an entire season, neither PNR nor AO abundances were correlated to soil moisture, dynamics of marker gene abundances associated with denitrification transformation processes followed the seasonal soil moisture dynamics, decreasing from April to May and subsequently increasing again (M3). Nevertheless, at the investigated spatial scale, congruent distribution patterns of potential denitrification and nitrification activity could be detected at some dates (M3), indicating that the prevailing soil moisture conditions allowed both processes to co-occur.

In turn, drought has also been shown to counteract nitrification by limiting access to their substrate due to lower diffusion of NH_4^+ (Placella and Firestone, 2013; Stark and Firestone, 1995). Ammonia-oxidizers seem to react with different dynamics to changing soil moisture content: after a short lag phase, first AOB increase their transcript levels, followed by AOA, while a NB-like NOB response is delayed due to the retarded production of nitrite (Placella and Firestone, 2013). Adaptions to microaerophilic redox conditions, not least due to the high oxygen affinity of AOA, might partially explain their presence in high numbers in SCH histosolic soil (gene copy numbers of archaeal *amoA* in the range of 10^8 at an average soil water content of >100 %) and paddy soils (M2) (Bannert et al., 2011; Shen et al., 2008; Wang et al., 2015). Furthermore, high soil moisture content can increase

mineralization rates (Stark and Firestone, 1995), in turn constituting the preferred substrate source for AOA. Nevertheless, taking into account that nitrification is an aerobic process and that the abundance of AOA is often reduced under oxygen limitation in soils characterized by high moisture levels (Szukics et al., 2012), the relatively high presence of AOA at intermediate LUI levels in SCH-NM may not be necessarily coupled to AO activity (AOA-PNR: r = 0.41 weakly correlated, AOB-PNR: r = -0.31 no correlation in SCH-NM soils) (M2). Transition zones with special redox conditions might constitute the ideal habitat for closely related and putatively intertwined processes such as ammonia- and methane-oxidation, albeit both processes are connected to completely different metabolisms (Bodelier and Frenzel, 1999; Daebeler et al., 2014; Holmes et al., 1995). Methanotrophs, carrying a pMMO which catalyzes methane oxidation (MO), might well perform AO due to the structural similarity of pMMO to AMO, which allows both groups to switch substrates (Alam and Jia, 2012; Holmes et al., 1995; Kowalchuk and Stephen, 2001; Stahl and de la Torre, 2012). Genes encoding pMMO were found to be present exclusively in histosolic soils under investigation, (M2) which reinforces our assumptions.

It can be further speculated that soil moisture might fundamentally influence the distribution of microorganisms also, based on their adaptions and preferences to different redox status. The spatially separated distribution patterns at the plot scale in April were likely attributed to high soil moisture content, albeit no spatial correlation could be detected: NB dominated in numbers and they are known to be capable of dealing with lower oxygen levels than NS (Bock et al., 1988; Bock and Wagner, 2006; Sundermeyer-Klinger et al., 1984) (M4). Organisms such as NB might adapt to lower oxygen content e.g. by increasing specific oxygen affinity, though it may take some time to react and switch to denitrification and heterotrophic growth (Freitag et al., 1987; Laanbroek et al., 1994).

Seasonal effects exert a major influence on **vegetation**, which in turn affects different functional microbial groups (Hallin et al., 2009; Patra et al., 2006). Interactions with plants may have both negative and positive outcomes for microbes. The preference of particular plant species for either nitrate or ammonium and immobilization during plant growth stages control inorganic nitrogen compound pools and thus belowground processes such as nitrification or denitrification, as well as the competition between both guilds and vice versa – microbes influence the availability of inorganic nitrogen forms for plant uptake (Boudsocq et al., 2012). Plants might also inhibit or promote nitrification (Boudsocq et al., 2012), which in turn affects the belowground community favoring either nitrifiers or denitrifiers by additionally changing the accessibility of the respective substrate pools, thereby creating a competitive situation with microorganisms which rely on ammonia or nitrate as substrate (Hawkes et al., 2005; Ward et al., 2011; Woldendorp and Laanbroek, 1989).

Ammonia-oxidizers might be at a disadvantage in competing for resources with plants or heterotrophs due to lower generation times, which is further supported by the finding that nitrifier abundance is negatively affected by plant composition (Bock et al., 1988; Kaye and Hart, 1997; Le Roux et al., 2003). Albeit a general strong coupling of above- and belowground performance has been proposed (Ritz et al., 2004), no relationship of the observed microbial communities to any plant parameter measured could be detected at the small spatial scale in our study (Regan et al., 2014). This was the case for both combined plant parameters and plant species specific parameters integrated into the analyses (M3). However, indirect plant effects via nutrient distributions or competition patterns remained unassessed in our study. AOB spatial distribution in the summer months exhibited a patchy heterogeneous structure as illustrated by kriged maps that may follow nutrient distributions predetermined by plants (M4). Interestingly, the temporally limited occurrence of nitrification in autumn coincides with a mowing event in August which removed aboveground biomass and led to a likely sudden drop in plant nitrogen uptake (M4). Unfortunately, exudation patterns that could have provided information on the release of nitrification inhibiting substances or carbon compounds that may either trigger or impede microbial performance (Subbarao et al., 2007), were not assessed. Inhibiting substances derived from competing biota might be present as well in forests. In coniferous forests, substances as monoterpenes or polyphenols might be secreted that lead to allelopathic inhibition of nitrification (Paavolainen and Smolander, 1998). AOA seem to be more prone to nitrification inhibitors in coniferous forests than AOB as abundances of AOB in beech and spruce forests did not differ across regions, while AOA seem to be slightly reduced in the ALB region, for example (M1).

Land-use intensity effects were addressed only in grassland sites where we took advantage of a compound index of LUI measures such as grazing, mowing and fertilization, which are the dominant land-use management regimes at the study sites (Blüthgen et al., 2012). Detailed information on the calculation of the LUI index can be found in the material and method section. In mineral soil sites of ALB and HAI regions, both potential nitrification activity and ammonia-oxidizer abundances were significantly positively correlated with LUI (M2). The responsiveness of ammonia-oxidizers to inorganic and organic **fertilization** has been previously reported (Di et al., 2009; Jia and Conrad, 2009; Nicol et al., 2004; Schauss et al., 2009), supporting the findings in this study (M2) and clearly identifying LUI as a major driver of nitrifier performance in mineral grassland soils. We attributed the increases in abundance and potential activity at intensively used sites mainly to the combined effects of grazing and fertilization in terms of high fertilizer amounts concomitant with elevated nitrogen levels derived from urine and dung deposition, respectively.

The response of AOA to fertilization seems to be indifferent: AOA are frequently found in nitrogen limited and unfertilized environments, but also after organic manure input (Schauss et al., 2009). In contrast, high abundance of AOB was mainly restricted to soils with high nitrogen content such as fertilized soils (Di et al., 2009; Kastl et al., 2015; Wertz et al., 2012). The ratio of AOA to AOB decreased with decreasing land-use intensity, which additionally highlights the particular role of AOB in nitrogen-rich soils (Chen et al., 2014; Xia et al., 2011). Simultaneously, the abundance of AOA was reduced at high LUI levels (M2), which refers back to the sensitivity of AOA towards high ammonium concentrations that might be connected to high LUI (Hatzenpichler et al., 2008; Tourna et al., 2008). Shifts become even more evident when comparing the ratios of AOA to AOB upon mineral and organic fertilization (250), mineral fertilizer (<500) to even higher ratios without fertilizer addition (Leininger et al., 2006). In general, fertilization has been postulated to trigger microbial activity mainly via increasing nutrient cycling (Enwall et al., 2007; Kuramae et al., 2012). Nevertheless, in a more detailed view, fertilization itself might act either as trigger or inhibitor - dependent on the amount and type of fertilizer as well as the tolerance levels of key players to fertilization levels (Hallin et al., 2009; Oved et al., 2001). Thus, it is no surprise that, at the first glance, contradictory LUI effects were found in different studies (Freitag et al., 2005; He et al., 2007; Long et al., 2012; Yao et al., 2011). The application of ammonia-based fertilizers might primarily affect AO (Ke et al., 2013), having a secondary effect on NOB: Studies indicate that NB might thrive under high N input in both agricultural and forest soils and that fertilization might change community composition of NS (Freitag et al., 2005; Ke et al., 2013; Wertz et al., 2012). Fertilization in forest soils led to an increase in AOB and NB abundances accompanied by community shifts, while AOA and NS remained unaffected (Wertz et al., 2012). Albeit the abundance of NOB was only determined at one study site of low LUI in our experiments, one might conclude that AOA and NS interact under low-substrate environments, which corresponds to their preference for low substrate concentrations (M4). AOB (M2, M4) and NB in turn were lower in numbers compared to their counterparts at the extensively used sites, but responded positively to fertilization (Ke et al., 2013; Wertz et al., 2012).

Mowing or grazing events may also exert indirect effects on microbial communities apart from direct ammonium or nitrate input as is the case under fertilization: Changing plant species composition might change nutrient competition or exudation patterns, soil compaction might alter soil pore structure shift redox conditions in soil, while urine or dung deposition could have direct effects in terms of elevated nutrient levels (Patra et al., 2006; Schauss et al., 2009). Upon mowing and grazing, AOA could gain importance in the nitrification process (M4), which was observed after a mowing event in August. Mowing excludes plants from the competition for nutrients in soil, which might then

be available to microorganisms. Increased nitrification has also been demonstrated in the absence of plants in this context (Norton et al., 1996). Furthermore, the release of organic compounds by root exudation is increased, thereby favoring mixotrophic lineages (Ke et al., 2013), albeit another study showed contrasting observations (Chen et al., 2014), which might be explained by the presence of distinct predominant phylotypes. A moderate increase in NH_4^+ availability due to mowing, instead of a sudden peak of NH_4^+ as in the case of fertilization, might further favor AOA instead of inhibiting them which seems to be the case at high ammonia levels (Chen et al., 2014; Hatzenpichler 2012) (M2).

The influence of **grazing** is often simulated in studies by urine or dung application (Hallam et al., 2006; Webster et al., 2005). For example Di and colleagues related potential nitrification rates induced by high loads of urine deposition to AOB performance, which increased at the expense of AOA (Di et al., 2009). Nevertheless, it is likely that AOA might also respond to urine input as they have been demonstrated to harbor urease genes (Tourna et al., 2011). Heterotrophic or mixotrophic lineages of NOB might also be favored at grazed sites due to changed availability of carbon compounds (Patra et al., 2006; Wertz et al., 2008). Although the compound LUI index allows the estimation of the combined effects of different land-use components, the reaction to individual components cannot be predicted for individual key players, which might respond differently to nitrogen input due to grazing / fertilization (M2). One pitfall of the attempt to assess LUI via coupled LUI index is the equal weighting of all included components in this context (Blüthgen et al., 2012), which might not reflect the real effects of each single component on microbial performance.

The above mentioned data indicate that LUI affects activity and abundance of nitrifiers. However, to understand simultaneously observed changes in community composition, it might be valuable to distinguish between the effects of short- and long-term LU management. Long-term fertilization of 19 years was demonstrated to influence community structure but neither abundance nor activity of AO (Long et al., 2012). This leads to the question of how LUI should be classified in our study. Our data might reflect both long-term effects (compound LUI index, calculated for 5 years) and the actual measured status at the sampling date (M2). Our study design does not allow us to clearly distinguish both effects. However, a more detailed view on long- and short-term LUI changes might give insights into how to classify LUI into the proposed scheme of distinction between labile and stable parameters. Long-term fertilization might preferentially change soil pH and other basic soil properties as well as root exudation patterns (Long et al., 2012), which would be classified as stable. Against this background it is not surprising that long-term fertilization is considered to shape the community composition of nitrifiers: *Nitrosospira* and *Nitrosomonas* cluster dominated the AOB

community in fertilized soils (Webster et al., 2002). The study of Meyer and colleagues gave first hints to changing AOA patterns under different land-use management regimes in the ALB region (Meyer et al., 2013) and it has also been shown that NB-like NOB sequences varied between intensively and extensively used sites (Poly et al., 2008). The presence of highly abundant OTUs detected in fertilized vs. unfertilized soils was due to an increase in relative abundances of particular genotypes that were in principle present both in fertilized and unfertilized soils (Ke et al., 2013; Long et al., 2012). This was further confirmed by Hallin and colleagues, who assumed that fertilization affects community size rather than structure, albeit shifts in the ratio of AOB to AOA were detected (Hallin et al., 2009). Long-term fertilization-induced community shifts have persisted several years after fertilizer application and the level of resilience seems to be dependent on regime and quality of fertilization (Shen et al., 2008; Wertz et al., 2012) (see also section 4.2).

4 Multifactorial impacts on the nitrification process

To reveal individual microbial responses to environmental changes is a challenging task because multifunctional effects on above- and belowground interactions have to be assessed simultaneously (Wolters et al., 2000).

4.1 Driver effects at multiple scales

These interactions additionally vary on both spatial and temporal scales (Fierer, 2008). Since the terminology of “scale” is different in various publications, it is often difficult to compare scale effects. To circumvent this limitation, the term “scale” in this thesis is defined as follows: scale describes the range at which observations have been made, independent from geostatistical maps, which are commonly constructed only for small spatial scales.

“Microbial communities respond simultaneously to multiple factors operating at different spatial scales” (Franklin and Mills, 2003). This statement means that the spatial distribution of soil microorganisms has to be considered as a result of interactions between microbial autocorrelations determined by microorganisms’ preferences or tolerances and the heterogeneity of soil properties they respond to (Ettema and Wardle, 2002). We propose that spatial heterogeneity in soils is achieved by interacting organization levels of stable and labile parameters (Berner et al., 2011; Ettema and Wardle, 2002; Franklin and Mills, 2003) whereby the spatial distribution of microbial communities and soil properties exhibit temporal flexibility under the impact of dynamic parameters.

Drivers controlling spatial distributions and seasonal variations can be assigned to different hierarchical levels and visualized at different scales which we also assessed in our studies (M1, M2, M3, M4, see **Table 4**) (Ettema and Wardle, 2002).

	scale	**distance class**	**environmental factors**		**this study**
small scale	micro scale	µm / mm	pore space & soil structure, root exudates, nutrients		
	plot scale	cm / m	**vegetation**, land-use management**,** **soil moisture, nutrients**		M3 M4
large scale	local / field landscape	sev. m / km	**land-use management** soil moisture vegetation climatic factors	**soil-type** **soil pH** organic matter content topography	M1 M2
	regional	sev. Km			
	continental	across countries			M1

TABLE 4: **Scales for the detection of associated variables.**

Distance classes are given for different small and large scale classes. Environmental variables detectable at the respective scales that were addressed in this study are highlighted in bold. Variables assessed at large scales are further distinguished in labile (left) and stable (right) parameters. Selection of variables and associated scales are exemplary from different publications and thus no claims to be completed can be made.

Drivers operating at higher levels might also affect others influencing a variety of subordinate single environmental characteristics and properties that in turn shape the microbial group under investigation e.g. via substrate availability (M1, M2) (Kowalchuk and Stephen, 2001). Despite homogeneous, stable, large scale variables, organisms respond to small scale variation (Ettema and Wardle, 2002). Thus, it is obvious that scales for the observation of chosen variables have to be selected according to the range of action of the driver investigated. Processes occurring at microsites, such as microbial spatial organization and interactions, have to be assessed at the µm to mm distance classes for instance, determined mainly by substrate availability or by microbial activity functional groups carrying out subsequent transformations (Grundmann and Debouzie, 2000; Nunan et al., 2003). Soil chemical and physical properties such as soil moisture or nutrient status have been spatially auto-correlated at the plot to local scales over distances of several meters (Fierer and Jackson, 2006; Ritz et al., 2004). Plant species effects, in contrast, might operate at scales in the range of cm to m where vegetation changes could be expected, while land-use management might

affect soil properties at larger scales which in turn influence the spatial structure of microbes (Ettema and Wardle, 2002; Lauber et al., 2008). Soil pH might even operate at multiple scales: at the regional, landscape and continental scales (Bru et al., 2011; Fierer and Jackson, 2006; Wessen et al., 2011).

This indicates that the influence of drivers on spatial autocorrelation of soil properties and soil microorganisms can be assessed at multiple scales (Berner et al., 2011; Bru et al., 2011; Franklin and Mills, 2003; Nunan et al., 2003; Steffens et al., 2009; Wessen et al., 2011). Microbial communities in the focus in this study were found to be auto-correlated at highly dynamic scales from 2-4mm (Grundmann and Debouzie, 2000), 1.4-7.6m (Keil et al., 2011) and 1-20m (M3, M4), varying with the season and comparable with other studies assessing the same range (Franklin and Mills, 2009; Nunan et al., 2003). The detected co-localization of NO with AO at ranges within a few mm (Grundmann and Debouzie, 2000) could be also assumed in the presented study (M4). Partially high nugget values indicated unassessed small scale heterogeneity, which might reflect interaction patterns at the mm range. Association of microbial patterns to spatial distributions of the substrate could also be observed at the plot scale, confirming the assumption that microbial spatial patterns often follow patchy substrate distributions under nutrient-limitation (M4) (Nunan et al., 2003). Both niche separation and co-occurrence patterns could be visualized for NOB in April, August and November (M4) implying that the spatial dependence of functional guilds changed over the year mainly due to responses to fluctuations in seasonally dynamic environmental parameters. Similar spatial niche separation could be demonstrated for nitrite reducers (Keil et al., 2011). We would assume that spatial separation occurs under limiting environmental conditions in order to reduce competition for the same resources such as oxygen (M4). In this context, soil heterogeneity makes possible the co-existence of functionally redundant organisms (Ettema and Wardle, 2002).

A high level of spatial dependence was also demonstrated for plants at the meter range, regulating belowground communities mainly via root exudation (Griffiths et al., 1998; Ritz et al., 2004). However, at the plot scale, no spatial relationships were found between abundances of ammonia-oxidizers and plants or abiotic properties (M3).

Correlations of drivers and soil biota were observed at the large scale across all regions of the Biodiversity Exploratories. Soil biota were affected to a different extent: clear relationships could be associated with land-use management, while soil properties and location accounted to a lesser extent for changes in the abundances and diversity of soil biota (Birkhofer et al., 2012). LUI has been demonstrated to drive spatial heterogeneity and shape niches at the plot scale in previous studies on sites of the ALB region (Berner et al., 2011; Keil et al., 2011). This study investigated the effects of LUI

as a major driver both at the local (each individual exploratory) and regional scale (all exploratories across Germany) and could prove the influence of LUI on microbial nitrifier performance operating at both scales (M2). In another study, LUI explained spatial variance for AOB even at the landscape scale (Bru et al., 2011).

At the continental scale, pH was the best predictor of changes in bacterial diversity and richness, explaining 73% of variation (Fierer and Jackson, 2006). This fits well to our observations on the regional scale (M1), indicating that pH is a major driver of community composition, which we assume to be attributed to its effects on substrate concentrations (see **Figure 4**). While a comprehensive study by Bru and colleagues at the landscape scale could not identify any of the measured soil properties as spatial niche separation factors for AOA and AOB distribution, they confirmed an effect of pH on the ratio of both ammonia-oxidizers (Bru et al., 2011).

It is important to keep in mind that the same functional group can exhibit relationships to different drivers at different ranges and across ecosystems (Fierer et al., 2009). To this end, a multiscale approach, conducted at nested scales to assess all organizational levels would be advantageous (Franklin and Mills, 2003). Our results again highlight the need to select appropriate scales for biogeographical approaches carefully, according to the drivers under investigation and the objectives of the study (Franklin and Mills, 2009; Grundmann and Debouzie, 2000; Nunan et al., 2003; Steffens et al., 2009).

4.2 Long-term effects and interactions of multiple drivers

In addition to the classification of drivers into labile and stable parameters, it may also be possible to distinguish the long- and short-term effects of drivers, the latter resembling labile parameters in their operation mode (**Figure 4**). Long-term studies in this context commonly refer to a multiannual period of time between 19 years (Long et al., 2012) and up to 50 years of fertilization (Hallin et al., 2009). Long-term effects play an important role as prerequisites for the response patterns of microbial communities to short-term changes, in particular with regard to land management. In this respect, microbial communities in soils that had been fertilized for longer periods have been shown to react differently to fertilizer application than communities under extensive land-use history (Aronson and Helliker, 2010). Long-term fertilization studies have identified similar AOB community composition under fertilized and unfertilized conditions. Albeit clades adapted to high and low substrate concentrations, respectively, could be detected under both high and low LUI environments, fertilization induced an increase in the abundance of only one particularly well adapted genotype. Also for the AOA community, increasing OTU abundance of specific strains responsive to fertilization

could be demonstrated (Long et al., 2012). Thus, there is increasing evidence that long-term fertilization induces shifts mainly in the relative abundances of high-nitrogen adapted lineages rather than shaping two different communities (Hallin et al., 2009; Long et al., 2012). This might represent an advantage for lineages that are able to survive under suboptimal conditions, prepared to respond to changing substrate concentrations in combination and regaining growth under conditions optimal for their respective preferences and tolerances.

Thus, not only stable parameters but also variables inducing long-term effects are likely to shape microbial communities which then might react immediately to dynamic changes. Labile factors directly affect transformation processes e.g. via shaping redox conditions for enzymatic activity. As discussed previously, stable parameters such as soil type or pH affect activity and abundance to a lesser extent than labile factors (M1, M2). Reinforcing these presumptions, OTU composition of the AOA community in forest soils was influenced by pH (43% variance in principal component analysis) rather than by soil moisture content (19.6%). Labile nitrogen compounds had minor effects on OTU composition (0% NH_4^+, 13.8% NO_3^-), and thus played a negligible role in terms of shaping diversity (M1). Nevertheless, relative abundances of different lineages might exhibit seasonal dynamics (M4). In contrast, potential nitrification activity was correlated to soil water content as labile factor rather than to pH (M1), again highlighting the different modes of action of labile and stable parameters. Our data support the objective that community structure is shaped by stable or long-term parameters (Hallin et al., 2009), while labile factors influence abundance and activity of the observed functional groups. Changes in substrate availability, whether triggered by pH or fertilization, would first change the community size by increasing the abundance of adapted lineages, preceding changes in their activity (Le Roux et al., 2008; Webster et al., 2005; Wertz et al., 2012). Thus, a community shift towards species adapted to high substrate conditions occurs prior to a detectable increase in activity.

The large proportions of variance in the spatial abundance of key players could be explained by the major drivers pH and LUI (Bru et al., 2011). Long and colleagues correlated OTU composition of the AOA community with soil pH and fertilization, accounting for approximately 30% of variance (Long et al., 2012). Other studies, investigating effects of both LUI and pH on soil biota, revealed that pH might shape bacterial community composition independent of different land-use types (Kuramae et al., 2012; Yao et al., 2011) in terms of a clear phylogenetic niche separation, based on adaptions to particular pH ranges. Especially in ultra-acidic environments, not only substrate limitation but also specific adaptions to deal with low pH might restrict the community composition to few lineages (M1). In this respect we could demonstrate reduced richness in acidic sites. Clades of AOA and AOB have been found exclusively at high or low pH ranges, implying clear separation of pH-adapted

communities (Fierer and Jackson, 2006; Nicol et al., 2008; Yao et al., 2011). Yao and colleagues, for example, found T-RFs (terminal restriction fragments) that were present exclusively either in soils pH < 4.4 or pH > 4.4 (Yao et al., 2011). This is in line with our findings revealing pH 4.5 as a critical pH value for community separation, operating as a threshold value for cluster separation of lineages (M1). In ultra-acidic soils, OTU1 and OTU4 were identified that could be preferentially related to *Nitrososphaera* cluster 7.2 and *Nitrosotalea*, while OTU2 was associated with higher pH ranges (M1). The similarity of communities in forest soils below pH 4.6 to communities from grassland soils with pH < 3.5 could be demonstrated, which suggests that pH shapes AOA communities in ultra-acidic pH ranges across ecosystems independent of soil type or climatic conditions. However, we could not pin the "threshold effect" to either critical substrate concentrations restricting growth below pH 3.5 to very few selected lineages or to the requirement of specific cell adaptions at ultra-acidic pH levels. In this context, pH might be the underlying "limiting" factor shaping community structure. One might speculate that other "secondary" factors such as LUI or labile parameters would in turn have limiting effects on the relative abundances of key players and their activity within the community shaped by different pH ranges. Some T-RFs responded to LUI across different pH ranges (Yao et al., 2011), which would point to LUI as a secondary driver. Although abundances of both AOA and AOB changed under fertilization regimes in acidic and alkaline soils, fertilization under low pH shapes AOA community structure (He et al., 2007), while only in alkaline fertilized soils AOB community composition is affected (Shen et al., 2008). Additionally, both processes might also be interlinked as fertilization has been shown to influence community composition indirectly also, by inducing changes in soil pH (Philippot et al., 2007).

Taking the above mentioned observations into account, one might assume that (i) pH shapes primary community composition and (ii) fertilization induces shifts in relative abundances and affects activity, with both major drivers working in concert. Despite the above mentioned data elucidating an overall context of major drivers in nitrification, we must not leave out of consideration that, depending on the prevailing environmental conditions of study sites, other factors apart from pH or LUI might also shape nitrifying communities (Fierer et al., 2009). This implies that the effects of LUI have to be interpreted against the background of pH and soil-type (Chen et al., 2014).

5 Methodological caveats

5.1 Limitations in assessing activity and abundance of nitrifiers

Field studies often have to face the question of to what extent "functional gene expression (would) reflect ecosystem function?" (Prosser and Nicol, 2008). In this regard, activity and growth have often been associated with abundances of genes or transcripts of genes encoding the investigated function (Brown et al., 2013; Prosser and Nicol, 2008). However, this does not necessarily imply activity of the respective function nor does it necessarily have to be accompanied by growth (indicated by 16S rRNA content) (Placella and Firestone, 2013; Prosser and Nicol, 2008; Prosser and Nicol, 2012). Hence, abundance data reflect the potential of nitrification processes in soils. In the case of DNA based qPCR measurements, the transcription level of genes cannot be estimated, and this could be an obstacle to taking the data as proxy for activity or starvation levels (M1, M2, M3, M4) (Blazewicz et al., 2013). Furthermore, RNA-based studies also do not necessarily reflect activity of organisms (Ke et al., 2013) against the background of high stability of transcription (M4). In this context it has also to be considered that *amoA* bearing organisms might not be active AOA (Agogue et al., 2008), and also *Thaumarchaeota* bearing *amoA* genes without autotrophic AO have been found (Mußmann et al., 2011). Furthermore, one has to be careful when inferring the relative contributions of key players from gene abundances. Taking gene abundance numbers as proxy for cell numbers requires knowledge of gene copy numbers per cell. In the case of AOA, *amoA* gene abundances could be directly linked to cell numbers, as only one copy per cell has been observed so far. However, multiple copies per cell for AOB and NB (strain dependent) do not allow an exact estimation of cell numbers (Poly et al., 2008). In addition, cell size also has to be taken into account as activity levels might be different: the activity per cell is higher in AOB, thus they could compensate for their lower numerical abundance by increased rates (Schauss et al., 2009).

Potential activity under standardized conditions does not represent natural environmental conditions and might thus differ from activity in situ, although comparable rates have been determined in some studies (Erguder et al., 2009). Furthermore, the respective contributions of key players to potential nitrification cannot be assessed by our experimental approach, hence it has to be taken into account that high ammonia levels might inhibit AOA, while at the same time AOB could be tolerant of higher levels of accumulating nitrite (Cua and Stein, 2011; Tourna et al., 2011). Nevertheless, although PNR does not reflect real activity, measurements confirm that the present ammonia-oxidizers indeed perform active nitrification (M1, M2, M3). The utilization of alternative substrates or growth strategies further challenge the attempt to link gene abundances to the functionality of regarded processes such as autotrophic ammonia-oxidation (Prosser and Nicol, 2008).

5.2 Limitations in assessing community composition of nitrifiers

The culture-based investigation of microbes provides essential information on their ecophysiological characteristics in terms of their metabolisms, substrate ranges and tolerances that would remain unassessed without cultivation studies, and would severely obstruct the investigation of their ecological functions; this was the case for AOA because cultured representatives were lacking for decades. Since artificial culture conditions might select for particular organisms and exclude others, one has to keep in mind that most characteristics are predicted from few cultured or enriched representatives and thus might not be transferrable to all community members. This impeded, for example, the identification of a clear relationship between nitrifier performance and different soil parameters (M2) or the identification of lineages capable of alternative growth strategies throughout the year (M4). Cultivation studies provide insights into ecophysiology, albeit assessing communities in natural environments would be more relevant for estimating their ecological function. In their natural habitats, microbes are embedded in a network of ecological functions and factors influencing their performance. Thus, assessing their diversity and community structure by sequencing approaches or fingerprinting methods might deliver a more realistic description of their network in soil.

Cultivation-independent sequencing approaches however, are limited not only by extraction method associated biases, but also to a large extent by the selection of the respective primer set chosen to describe the whole community of interest (DeLong and Pace, 2001). Thus, a comparison and careful selection of primer pairs is essential to assess the community of interest; primer selection is essential to the outcome of the study. Addressing the diversity of functional guilds based on 16S rRNA genes might counteract the identification of different sublineages as sequences reveal a very high similarity (98%) (M4). This has been demonstrated for some lineages of AO (Fierer et al., 2009), but was also seen in our study on NOB, with NB-like NOB exhibiting low phylogenetic resolution (M4). To circumvent this issue, several other methods have been applied to facilitate the identification of NB lineages from the utilization of primers targeting *nxrA* gene sequences (Poly et al., 2008; Wertz et al., 2008), to labeled antibodies targeting different NB strains in biofilms (Grundmann and Debouzie, 2000) or intergenic spacer analysis of 16S and 23S rRNA gene sequences (Navarro et al., 1992).

To this end, comparing primer sets *in silico* and *in vitro* is helpful to reduce this bias and to perform analyses representative of the soil microbial community structure of interest. Not only diversity should be largely covered, but also the bias of amplifying of undesired species should be kept as low as possible. Furthermore, primer sets should apply to different soils under investigation (Fierer et al.,

2009). Primer sets have been evaluated for the use in B-ARISA (automated rRNA intergenic spacer analysis) in terms of their specificity and the coverage of the community under investigation, tested in soil and wood samples (M5). For the culture-independent B-ARISA method, different primer sets were tested that should fulfill the desired criteria: (i) broad coverage of taxa at the (ii) exclusion of undesired sequences as those from chloroplasts or mitochondria. Primer sets varied in their capacity to amplify sequences from different phyla. Some phyla were not covered by one of the primer sets at all in *in silico* analyses of database sequences. Importantly, a comparative study on the effects of e.g. fertilization on bacterial community structure revealed similar results for all primer sets. Similarities in primers *in silico* could also be confirmed by similar results obtained in vitro. The study (M5) highlights the necessity to use software tools repeatedly to test and select different primer sets prior to the analyses as databases are rapidly changing and expanding. Nevertheless, one has to consider that "no technique is without bias, but bias in molecular techniques applies equally to bacteria and archaea and can apply in both directions" (Prosser and Nicol, 2008).

5.3 Limitations in relating phylogeny to ecophysiology

Two highly abundant OTUs assigned to *Nitrososphaera* subcluster 7.2 and *Nitrosotalea* were identified, dominating in both grassland and forest soils at extremely acidic sites (pH < 3.5), implying that those representatives might be responsible for the detected nitrification rates in low pH environments. The data obtained from sequences of *amoA* genes derived from DNA samples (M1), provided evidence for the relative abundance of AOA clades harboring the respective gene and thereby putatively inheriting the function of interest. Our RNA-based sequencing approach targeting the NOB community aimed at identifying metabolically active key players, taking the presence of 16S rRNA genes on RNA level as proxy (M4). We cannot conclude from the presence of 16S rRNA genes on RNA level that organisms assigned to NOB lineages would indeed contribute to NO activity based on their sequence similarity, as functions such as cell maintenance, starvation mode or simply the presence of multiple gene copies have to be taken into account as well (Blazewicz et al., 2013).

Albeit the abundance of marker genes or the presence of OTU sequences is commonly used as proxy for the performance of the process as such and as indicator for the identification of process performing key players, respectively, it is still a matter of debate to what extent this would reflect the functionality of the investigated process; in other words: is phylogeny indicative of ecophysiology of the assessed microbial community?

For a long period of time, predictions of AOA physiology resulted solely from environmental genomic studies due to a lack of cultivated representatives that might give some indication of their

metabolism and functional role (Schleper et al., 2005). However, drawing inferences from phylogenic data on the function might be critical. This might become more evident against the background of the discovery of *amoA*-encoding archaea lacking ammonia-oxidation activity (Mußmann et al., 2011). This highlights the need to complement environmental studies delivering experimental data on diversity with ecophysiological characteristics from cultivation studies to get final confirmation of the ecological role of investigated organisms. In this respect, not only the presence of *amo* genes (Treusch et al., 2005), but also the isolation and cultivation studies have confirmed AO activity (De la Torre et al., 2008; Hallam et al., 2006; Hatzenpichler et al., 2008; Konneke et al, 2005; Kowalchuk and Stephen, 2001; Leininger et al., 2006; Offre et al., 2009; Schauss et al., 2009; Schleper et al., 2005). Gene expression patterns upon substrate application, autotrophic growth by labelling experiments, the presence of transport systems for the substrate, all can be taken as serious evidence for an active role of the respective clade in the process of interest (Hallam et al., 2006; Treusch et al., 2005). Phylogeny cannot provide information about the ecological role - it only gives indications of the ecophysiological potential in soils. Thus, to be able to draw reliable conclusions from phylogenetic information on the function of AOA, it is essential to consider the metabolism of respective lineages investigated in culture.

To conclude, our data on the phylogeny of AOA under different pH values do not necessarily imply their functional activity in nitrification, but rather indicate their potential to perform nitrification. To reveal the presence of active ammonia- and nitrite-oxidizers, transcriptomic information for the respective lineages is required (Schleper et al., 2005). Additionally, studies with isotope-labelled substrates would allow tracking of the community actively performing the process of interest. Assessing the active fraction of the nitrifying community would also complement our study on the abundance, potential activity and phylogeny of ammonia-oxidizers under diverse environmental influences.

6 Outlook and perspectives

An increasing number of studies are dealing with the consequences of anthropogenic influences on biodiversity and the stability of ecosystem services. To generalize effects of anthropogenic change on the microbial communities maintaining ecosystem functions, it may be necessary to address the interrelatedness of processes performed by distinct key players. When individual drivers are investigated at a single scale, assessment of drivers' effects on the nitrifying community may be hampered: responses of key players that are influenced by multiple factors operating at other

distinct scales can be misinterpreted and thus, dynamics may remain undetected at the chosen scale. This highlights the necessity of multifactorial multiscale approaches to identify drivers at distinct spatial scales. Though awareness of this limitation has increased, interaction patterns of nitrifiers remain largely unassessed at different scales. Thus, experiments supplementing our local scale geostatistical approach to plots under intensive land-use at nested scales could lend support to our assumptions of interaction partner shifts under nutrient-rich environmental conditions.

Although the effects of major drivers of microbial performance such as pH or LUI are well examined with respect to induced changes in activity and community composition of particular taxa or overall bacterial community (Erguder et al., 2009; Kuramae et al., 2012), the effects on interacting groups of microorganisms catalyzing the sequential steps of transformation processes such as nitrification are rarely addressed. Recent studies have surprisingly proposed a novel key player in nitrification. *Ca.* NS inopinata, an autotrophic representative of *Nitrospira* sublineage II, may combine the major steps in nitrification that have to date been ascribed to two distinct functional guilds in one organism: ammonia- and nitrite-oxidation. Metagenomic sequencing revealed the genetic potential for conducting the complete nitrification process by identifying *amo* (dissimilar in sequence to known *amoA* marker genes), *hao* and *nxr* genes. This was complemented by incubation experiments revealing the capacity to transform ammonia into nitrate coupled to growth (Daims et al., 2015; van Kessel et al., 2015). It would be interesting, therefore, to investigate the differential response patterns of AO and NO clades and sublineages to substrate concentrations.

This study highlights not only the need to gain basic knowledge of the metabolisms of putative key players, revealing mutual dependencies and alternative metabolic pathways, to gain a complete picture of the nitrification process and its drivers, but also introduces new insights into an exciting research area.

7 Conclusion

Considering the spatial and temporal variability of microbial abundance and diversity, the questions arise of which factors drive these temporally changing biogeographical patterns and whether functionally redundant microbes and their interaction partners might respond differently to these changes. The assessment of redundancy is important to determine the tolerance of ecosystems to changes and perturbations and to predict their responses and resilience capacity (Prosser and Nicol, 2012). Changes in the abundance and diversity of key players performing the examined processes do

not necessarily result in pronounced alterations in ecosystem function (Wolters et al., 2000) since we observed rather stable potential nitrification rates over the year (M3,M4). We attributed the relative niche adaptions to both mechanistic requirements of organisms involved in nitrogen cycling and their tolerance levels, as well as to distinct concentration preferences for substrate.

Although AOA dominated in numbers regardless of the ecosystem and land management (M1-4), their contribution to the maintenance of nitrification potential especially in substrate-limited (low pH) and unmanaged (low LUI) soils may be especially important (M1, M4) (**Figure 5**). Nevertheless, AOA abundance also increased with LUI levels, inhibited only at very intensive sites, indicating their ability to deal with a broad range of environmental conditions. Increasing dominance of AOB was determined in nitrogen-rich environments, which is in line with our hypothesis (M1, M2).

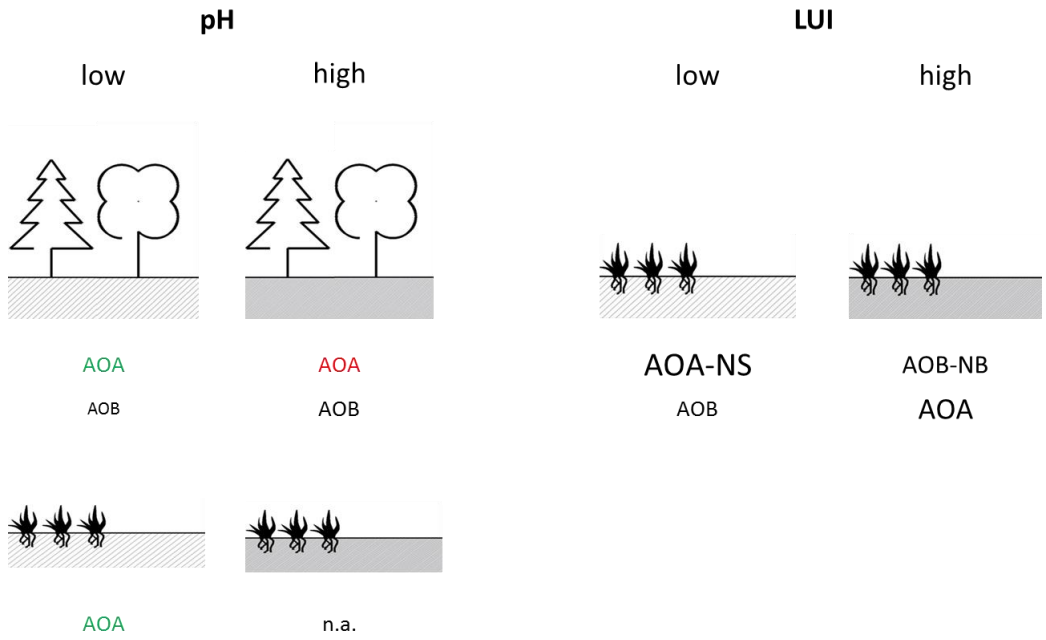

FIGURE 5: **Comparison of the effects of major drivers of nitrifiers abundance and community composition across ecosystems.**

Bright grey indicate low soil pH and LUI, dark grey high pH and LUI levels. Size of letters gives an estimation on the dominance of the respective group in soil, mainly based on abundance data. Differences in community structure are indicated by different color code and have only been assessed in M1.

Different substrate preferences, among other factors, also shaped the choice of interaction partners in soil: AOA and NS have been shown to interact at unmanaged sites at a spatially and temporally restricted scale (M4). It is tempting to speculate that AOB and NB may in turn interact at higher LUI levels. Shifts in interaction partners in response to changing environmental conditions might occur

inter- and intraspecifically (M4). In this context, specific AOA lineages seem to be particularly well adapted to environments under energy-shortage, such as acidic soils, where specialized lineages of AOA were found exclusively under the ultra-acidic pH range below pH 3.5, differing from the community composition at higher pH, and independent of the ecosystem under investigation (**Figure 5**) (M1). This is in agreement with our assumption that the same clades adapted to similar environmental conditions would occupy similar niches. Confirming the central hypotheses of this study, stable parameters such as soil pH shaped AOA community composition both in forest and grassland ecosystems (M1, M2), while dynamic variables such as soil moisture content or LUI affected activity and abundance of nitrifiers in grassland sites (M2, M3) (**Figure 4**).

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List of abbreviations

AEA	amoA-encoding archaea
ALB	Schwäbische Alb exploratory
AMO	ammonia monooxygenase
amoA	gene encoding subunit A of the ammonia monooxygenase
amoCAB	operon encoding the ammonia monooxygenase
amoC	gene encoding subunit C of the ammonia monooxygenase
AO	ammonia-oxidation
AOA	ammonia-oxidizing archaea
AOB	ammonia-oxidizing bacteria
ATP	adenosine triphosphate
B-ARISA	bacterial automated ribosomal intergenic spacer analysis
BE	Biodiversity Exploratories
Ca.	Candidatus
cd1	dimer of cytochrome c and d1 heme group
chiA	gene encoding the bacterial chitinase group A
CO_2	carbon dioxide
DFG	Deutsche Forschungsgemeinschaft
DNA	deoxyribonucleic acid
DNRA	dissimilatory nitrate reduction to ammonium
e^-	Electron
e.g.	for example
EP	experimental plot
Fe	Iron
HAI	Hainich-Dün exploratory
HAO	hydroxylamine oxidoreductase
hao	gene encoding the hydroxylamine oxidoreductase
HNO	nitroxyl
i.e.	this means
K_O	half-saturation constant for oxygen
K_S	half-saturation constant
LU	land-use
LUI	land-use intensity
MOB	methane-oxidizing bacteria
Mo	molybdenum
N^-	nitrogen
N_2	dinitrogen
N_2O	nitrous oxid
NADH	dihydronicotinamide adenine dinucleotide

NAP	nitrate reductase
NB	*Nitrobacter*-like nitrite-oxidizing bacteria
NF	nitrogen-fixation
NH_2OH	Hydroxylamine
NH_4^+	Ammonium
nifH	gene encoding the dinitrogenase reductase subunit of the nitrogenase
NIR	nitrite reductase
nirK	enzyme encoding the copper containing nitrite reductase
nirS	enzyme encoding the cytochrome cd1-nitrite reductase
NO_2^-	Nitrite
NO_3^-	Nitrate
NOB	nitrite-oxidizing bacteria
NOR	nitric oxide reductase
NOS	nitrous oxide reductase
nosZ	gene encoding the nitrous oxide reductase
NS	*Nitrospira*-like nitrite-oxidizing bacteria
NXR	nitrite oxidoreductase
nxrA	gene encoding subunit A of the nitrite oxidoreductase
nxrB	gene encoding subunit B of the nitrite oxidoreductase
nxrC	gene encoding subunit C of the nitrite oxidoreductase
O_2	Dioxygen
OTU	operational taxonomic unit
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
pMMO	particulate methane monooxygenase
PNR	potential nitrification rate
rRNA	ribosomal ribonucleic acid
SCH	Schorfheide-Chorin Exploratory
SCH-NM	histosolic soil sites at Schorfheide-Chorin exploratory
SCH-MB	mineral soil sites at Schorfheide-Chorin exploratory
TCA	tricarboxylic acid cycle
T-RF	terminal restriction fragment
VIP	very intensive plot
vs.	Versus
WWTP	wastewater treatment plant

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APPENDIX

A Manuscript 1

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SOIL MICROBIOLOGY

pH as a Driver for Ammonia-Oxidizing Archaea in Forest Soils

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Abstract In this study, we investigated the impact of soil pH on the diversity and abundance of archaeal ammonia oxidizers in 27 different forest soils across Germany. DNA was extracted from topsoil samples, the *amoA* gene, encoding ammonia monooxygenase, was amplified; and the amplicons were sequenced using a 454-based pyrosequencing approach. As expected, the ratio of archaeal (AOA) to bacterial (AOB) ammonia oxidizers' *amoA* genes increased sharply with decreasing soil pH. The diversity of AOA differed significantly between sites with ultra-acidic soil pH (<3.5) and sites with higher pH values. The major OTUs from soil samples with low pH could be detected at each site with a soil pH <3.5 but not at sites with pH >4.5, regardless of geographic position and vegetation. These OTUs could be related to the *Nitrosotalea* group 1.1 and the *Nitrososphaera* subcluster 7.2, respectively, and showed significant similarities to OTUs described from other acidic environments. Conversely, none of the major OTUs typical of sites with a soil pH >4.6 could be found in the ultra- and extreme acidic soils. Based on a comparison with the *amoA* gene sequence data from a previous study performed on agricultural soils, we could clearly show that the development of AOA communities in soils with ultra-acidic pH (<3.5) is mainly triggered by soil pH and is not influenced significantly by the type of land use, the soil type, or the geographic position of the site, which was observed for sites with acido-neutral soil pH.

Keywords Ammonia-oxidizing archaea · *amoA* · Soil pH · Forest soil · 454 pyrosequencing

Barbara Stempfhuber, Marion Engel and Doreen Fischer have contributed equally to this work.

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Introduction

The amount and quality of available nitrogen determine both productivity and biodiversity in most natural and semi-natural ecosystems [1]. In forest ecosystems, for example, the distribution patterns of ammonia and nitrate in soil are strongly influenced by the plant community composition and the growth kinetics of trees [2]. In this respect both the subsequent delivery of inorganic nitrogen via nitrogen fixation or mineralization and the conversion of ammonia to nitrate via nitrification are of interest. Previously, many studies have investigated agricultural soils to identify environmental characteristics that influence the

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abundance and activity of ammonia oxidizers and nitrite oxidizers, the two major groups of microorganisms that perform nitrification [3]. However, despite the importance of nitrification in the productivity of woodlands, datasets from these ecosystems are rare [4], though they could be useful in improving our understanding of the role of abiotic and biotic parameters that influence nitrifiers in forest soils. In addition, the role of heterotrophic nitrification in such ecosystems cannot be ruled out, but has been reported in relatively few studies [5, 6].

We recently identified soil pH as a key driver of the composition of ammonia-oxidizing microbes in soils, potentially through its influence on the ammonia:ammonium ratio and described a highly specialized group of ammonia-oxidizing archaea (AOA) that occurs mainly in extremely acidic soils (pH 3.5–4.5), with consequent low availability of ammonia [7]. However, as the aim of the underlying study (UK Countryside Survey) was to characterize a large number of different soils with different land use types, other co-variables may have influenced AOA communities, and most of the acidic soils were from moorlands. Furthermore, the behavior of AOA in ultra-acidic soils, defined here as those with pH <3.5, where ammonia availability is even more reduced than in extreme acidic soils, is not clear. Therefore, in the present study, we focused on one land use type (forest) and included sites with soil pH values from 6.8 to 3.2 to (i) evaluate the role of pH as a driver for AOA in forest soil, (ii) evaluate the occurrence of the AOA clade in ultra-acidic forest soils that has been identified as a major contributor to ammonia oxidation in moorland soils with extreme acidic soil pH, and (iii) assess potential relationships between the ammonia oxidizer community structure and the potential nitrification rate.

Material and Methods

Topsoil was sampled from 27 experimental forest plots in Germany, which are part of the three sites of the Biodiversity Exploratories (www.biodiversity-exploratories.de/): Schwäbische Alb (ALB, sites AEW1-9; Southern Germany), Hainich-Dün (HAI, sites HEW1-9, Central Germany), and Schorfheide-Chorin (SCH, sites SEW1-9, Northern Germany). Major study site characteristics are given in the Supplemental Material and Table S1. In brief, study sites of regions ALB and SCH were composed of age-class forests and natural forests; whereas in HAI besides age-class forests, single-tree selection systems were the predominant forest types. Spruce and beech tree species are prevalent in ALB and HAI; and at SCH sites, the vegetation is composed of beech and pine stands.

The soil pH was measured using 10 g sieved air-dried soil in 25 ml of 0.01 M $CaCl_2$ solution. Based on these results, soils were grouped according to the following classification by the Agriculture Natural Resources Conservation Service (United States Department of Agriculture, USDA): ultra acidic (<3.5), extreme acidic (3.5–4.4), very strong acidic (4.5–5.0), strong acidic (5.1–5.5), moderate acidic (5.6–6.0), slight acidic (6.1–6.5), and neutral (6.6–7.3). Thus, the experimental design included 9 sites from each of the three explorations, representing a pH gradient from ultra acidic (<3.5) to acido-neutral (>6.0). As the pH of the litter layer did not always follow the trend of the underlying mineral soil layers, and the pH of the litter layer is highly dynamic temporally, in response to the degradation status of the litter material, only the mineral soils were used for further analysis.

Ammonium and nitrate concentrations were determined colorimetrically [8]. The abundances of AOA and AOB were determined from DNA extracts based on the ammonia monooxygenase subunit A gene (*amoA)* using quantitative PCR (qPCR) [7, 8], and the diversity of AOA was assessed by amplicon-based pyrosequencing of *amoA* genes [7]; for details, see (Supplemental Material).

Results and Discussion

Archaeal *amoA* genes were detected in all DNA extracts derived from forest soils, even in those from ultra-acidic soils. The abundance of archaeal *amoA* genes ranged from 1×10^4 to 3.4×10^7 g^{-1} soil dry weight (Figure S1 A), with the highest abundance at the sites with a soil pH ranging from 3 to 4.5. The abundance of bacterial *amoA* genes was in the range of 2×10^4 to 4×10^6 g^{-1} soil dry weight (Figure S1 B), and AOA were more abundant than AOB in the majority of the sites, as assessed by the *amoA* gene abundance. This is consistent with the previous studies performed in some agricultural soils [8, 9], although AOB are often more abundant particularly in well-fertilized soils [10]. The highest AOA:AOB ratios were found at sites with the lowest pH values (SEW3,4,9; data not shown), confirming the previous data published by [11]. In the observed forest sites, archaeal abundance (determined via 16S rRNA gene abundance, data not shown) ranged from 10^6 to 10^7 g^{-1} dw soil in ALB and slightly higher values in HAI. The abundance was higher in SCH with values up to 2×10^8 g^{-1} dw soil. The ratios of archaeal 16S rRNA:archaeal *amoA* genes were similar in ALB and HAI sites (0.8–4.3 for ALB and 0.3–5.3 for HAI), with a few plots with ratios as high as 24. In the SCH region, the mean ratio was higher (mean value 24) with only two sites with ratios above 10 (data not shown). Bacterial 16S rRNA gene abundance was in the range of 10^8 for all sites. The ratios of 16S rRNA:bacterial *amoA* gene abundance were similar in range to HAI (mean value ca. 450) but varied strongly in ALB and SCH (80–4011 for ALB and 360–49,077 for SCH). A number of factors, in addition to pH, have been suggested as determining niche differentiation between AOA and AOB. These include ammonia concentration and mixotrophy, but both are based largely on the analysis of a very small number of laboratory cultures, with little evidence

for either in soil [12]. A further factor with potential to influence relative abundance of AOA and AOB is the allelopathic inhibition of nitrification, and the allelopathic inhibition by monoterpenes or polyphenolic compounds on nitrification has been reported in coniferous forest soils [13]. This may explain the lower abundance for *amoA* genes in SCH sites with pine stands, but there is currently no evidence of the differential inhibition of AOA and AOB by such compounds. As pH influences the equilibrium of ammonium to ammonia, the latter being substrate for ammonia oxidation, an effect of different pH levels on PNR is expected. Interestingly, potential nitrification rate was weak, negatively correlated to soil pH (r=−0.518) as expected, but was influenced more by site-specific conditions, such as soil water content (r=−0.706), see table (S2). Thus, the highest potential nitrification rates were found in soils from the Schorfheide region, where soil water content was lowest (Table S1). Ammonium and nitrate concentrations at the time of sampling were in the range of 0.1–4.4 mg ammonium kg^{-1} dw soil and 0.5–7.7 mg nitrate kg^{-1} dw soil, and neither were correlated with AOA abundance (AOA—NO_3 r=−0.063 and AOA—NH_4 r=−0.221) or potential nitrification rate (PNR—NO_3 r=−0.403 and PNR—NH_4 r=−0.177), see table (S2). However, if AOB are favored by higher ammonia concentrations, the relatively high concentration of ammonia used in PNR assays will lead to greater rates in soils with high AOB:AOA ratios, while use of chlorate to inhibit nitrite oxidation may also favor AOB, which may be less sensitive to nitrite toxicity.

To investigate potential relationships between pH and AOA community structure, an *amoA* gene-targeted OTU-based approach was performed. To characterize the diversity of archaeal *amoA* gene-harboring communities for each site, indices for richness (Chao1) and evenness (Shannon) were calculated (Figure S3). AOA communities of sites with higher soil pH comprised remarkably higher OTU richness than sites with a more acidic soil pH. The distribution of OTUs was more even at sites with low pH, while a lower number of highly abundant OTUs dominated AOA communities at higher pH values. These observations indicate that pH indeed shapes the evenness and richness of AOA communities in forest soils as they seem to be more different under high pH levels, whereas fewer AOA seem to be adapted to low pH.

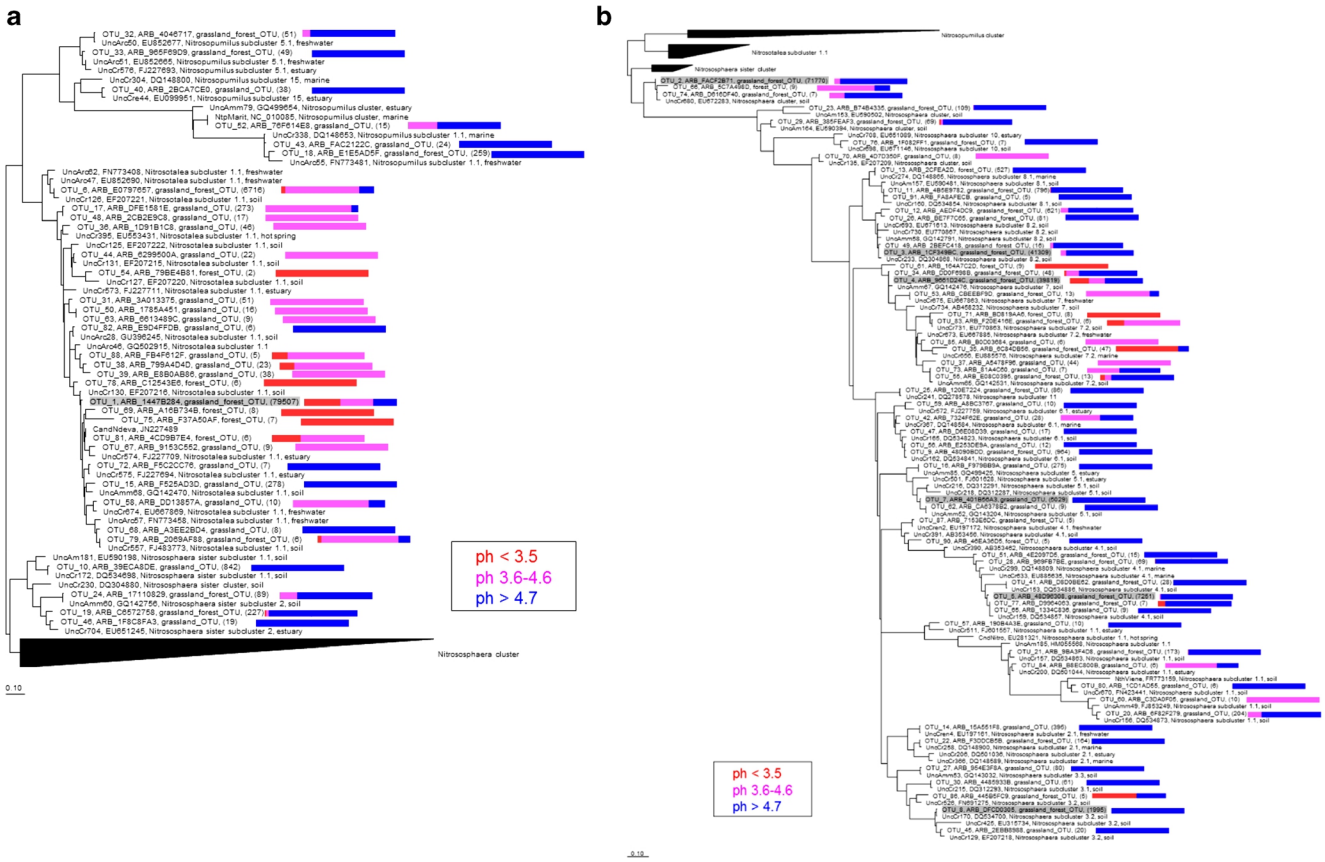

Fig. 1 Maximum likelihood phylogenetic analysis of partial archaeal *amoA* gene sequences detected in soil samples of grassland and forest (clustered at 97 % sequence identity, OTU1-99). *Color code* indicates the range of soil pH values where sequences were derived from (*red*, pH <3.5; *pink*, pH 3.6–4.6; *blue*, pH >4.7). *Bar plots* show relative abundance of each OTU in the different samples. Numbers in *brackets* show absolute abundance of the OTU. Number of OTUs derived from grassland: 45, forest; 10 and shared, 36. **a** OTUs related to the *Nitrosopumilus*/*Nitrosotalea*/*Nitrososphaera* sister cluster. **b** OTUs showing similarity to the species of the *Nitrososphaera* cluster. The *grey bars* indicate the most abundant sequences

This interpretation must, however, be treated with caution as saturation of rarefaction curves was not approached in the majority of the sites, indicating that full coverage had not been achieved (Figure S2), and comparisons between sites are complicated by analysis of different numbers of sequences. Two clusters dominated the archaeal *amoA* gene-harboring communities (Figure S4), on the basis of OTU-based dissimilarity, which clearly followed the pH values of the particular sites at all three study regions. The dominant clusters were separated at a pH of 4.6. The main clusters were confirmed by unifrac and parsimony tests (p <0.001). Interestingly, cluster A-included sites showed a pH <4.7 with generally reduced *amoA* gene diversity and more heterogeneous composition than cluster B.

Overall, 10 major OTUs (representing >1 % in any sample) could be distinguished and were shared between the two clusters (Figure S5). OTUs 1 and 4 were highly abundant in ultra-acidic soils (<pH 3.5); whereas OTU 6 was highly abundant in one sample with pH 4.1, and OTU 2 was mainly representative of the sites with neutral-acidic soil pH (>pH 4.7). The phylogenetic analysis (Fig. 1) revealed OTU 4 to be closely related to the *Nitrososphaera* subcluster 7.2, confirming the data of [7] who also described a major group of AOA from agricultural soils with an ultra-acidic pH belonging to the same group. In contrast, OTU 1 showed large similarities to *Nitrosotalea*; this OTU has also been observed in an ultra-acidic upland soil in China [14].

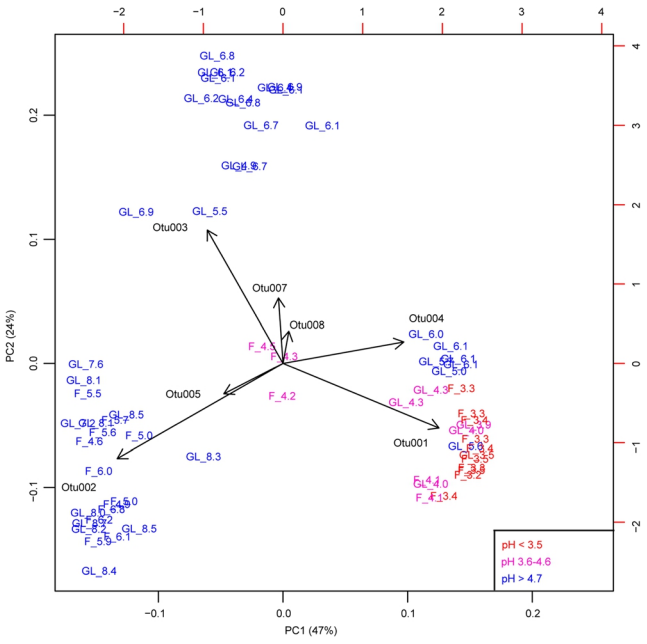

Fig. 2 Principal component analysis (PCA) of *amoA* gene-defined community structure in soils sampled from grassland (prefixed with GL) and forest (prefixed with F) with the influence of OTUs: *length of the arrows* show the strength of influence, the *direction* shows loading to samples investigated. R software was used for calculations. Datasets from the study of [7] and this study were compared to assess the relevance of pH as a general driver of AOA community structure

The dataset of this experiment was compared with the sequences obtained in a previous study of agricultural soils [7], and the importance of soil pH for the AOA community structure was assessed using the same pipeline, including PCR and sequence data processing. The principal component analysis (PCA) (Fig. 2) indicated that agricultural and forest sites with a soil pH >4.5 showed little similarity in terms of shared AOA OTUs, whereas communities were similar in soils with pH<3.5, regardless of vegetation type (agricultural vs. forest) or location and soil type. The PCA on grassland and forest sites was supplemented by further statistical analyses based on a PCA of the forest sites of the study (data not shown) to test the influence of selected environmental parameters, including soil moisture content, pH, ammonium, and nitrate, on the distribution of OTUs. Univariate regression of the first principal component revealed that pH value explained 43.8 % of variance, whereas soil moisture content accounted for only 19.6 % (data not shown). The percentages of explained variance for ammonium and nitrate concentration were negligibly low (0 % and 13.8 %, respectively). This supports our assumption that indeed, pH and not soil moisture content affects OTU composition in our forest sites.

The importance of pH as driver of AOA composition in forest soils was further confirmed by a permutation test conducted for canonical correspondence analysis (p value <0.05, data not shown). This again clearly indicates that soil pH is a major driver of AOA community distribution in forest soils.

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Supplemental Information Manuscript 1

Material and Methods

Sampling sites

The experiment was performed in the frame of the Biodiversity Exploratories (Fischer et al 2010). The Biodiversity Exploratories comprise three regions within Germany: the Biosphere Reserve Schwäbische Alb (ALB), located in the South-west; the National Park Hainich-Dün (HAI) in Central Germany and the Biosphere Reserve Schorfheide-Chorin (SCH), located in the North-east of Germany. Mean annual temperatures for each region are: 6.5 - 8.0°C (ALB), 8.0 - 8.4°C (HAI), 6.5 - 8.0°C (SCH) and mean annual precipitation values are: 938 – 963 mm (ALB), 520 – 600 mm (HAI), 500 – 800 mm (SCH). Each exploratory consists of 50 grassland and 50 forest experimental plots (EP). Of these, 9 sites per exploratory were selected for intensive studies, abbreviated as very intensive plots (VIP). These plots are termed AEW1 to AEW9 (Schwäbische Alb), HEW1 to HEW9 (Hainich-Dün), SEW1 to SEW9 (Schorfheide-Chorin). Soil texture in the Schorfheide-Chorin exploratory is sandy loam and sand, mainly loam and clay in the Hainich-Dün exploratory, and clay in Schwäbische Alb. For further details see Fischer et al. (2010) and attached supplementary table 1, providing information on forest type, tree species and respective soil types. Data on general properties of exploratories and plots were obtained from the Biodiversity Exploratories database, respectively. Fieldwork permits were issued by the responsible state environmental offices of Baden-Württemberg, Thüringen, and Brandenburg (according to § 72 BbgNatSchG).

Soil sampling

Soil samples were obtained in the frame of a coordinated soil sampling campaign in May 2011. At 14 predefined locations within each plot, samples were taken from the upper 10 cm of the mineral soil and sieved to ≤2mm without root material. Mineral soil was sampled using a splittube auger (diameter ca. 50 mm). The samples from 14 different sampling points were mixed and homogenized directly after sampling. Soil samples for DNA extraction were immediately frozen on dry ice at -80°C.

Extraction of nucleic acids and DNA quantification

DNA was extracted from 1 g fresh weight soil. DNA extraction was carried out using a Power Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, USA) according to the manufacturer's instructions. Elution was done in 200 µl of 10 mM Tris. DNA content was quantified using a Quant-iT™Pico Green® ds DNA assay Kit (Invitrogen, Carlsbad, USA) according to manufacturer's instructions. Pico Green was diluted 1:200 in 1x TE buffer. A lambda DNA standard was used for standard dilution series.

Measurements were performed with a SpectraMax Gemini EM Fluorescence Plate Reader Spectrometer (Molecular Devices, Sunnyvale, California, USA).

Quantification of *amoA* and 16S rRNA genes

Genes were quantified by quantitative PCR on a 7300 Real-Time PCR System (Applied Biosystems, Germany) with SyBr Green as fluorescent dye. Reaction mixture, cycling conditions and components such as plasmids used for standard dilutions and primers for *amoA* gene abundance measurements were used as described by Töwe et al (2010). For archaeal 16S rRNA gene amplification, primers rSAf(i) from Nicol et al (2005) and 958r from Bano et al (2004) were used, and DNA extracted from *Methanobacterium* sp. served as a source for standard generation. The thermal profile was as follows: 94 °C, 20 s / 55 °C, 60 s / 72 °C, 30 s (touchdown -1 °C per cycle for 5 cycles, followed by 35 cycles). 16S rRNA bacterial genes were quantified as described in Gschwendtner et al (2010). Serial plasmid dilutions (10^1-10^6 gene μl^{-1}) were used for standard curve calculations. Samples were diluted 1:8, which was determined as the optimal dilution in a previous experiment revealing putative reaction inhibiting effects. Dissociation stage for melting curve analysis was performed after each run in order to assess specificity of amplified qPCR products. Electrophoresis was conducted on a 2% agarose gel with randomly selected samples for confirmation of correct amplicon size. Calculated efficiencies of qPCR assays were 86% and 83% for bacterial and archaeal a*moA* genes, respectively, >80% for 16S rRNA gene assays, with R^2>0.99 for all assays.

Potential nitrification assay

A potential nitrification assay was performed according to Hoffmann et al. (2007) as described in detail in Stempfhuber et al. (2014). In brief, 2.5g of soil (fresh weight) were supplemented with 10mM ammonium sulfate solution serving as substrate and 1.5M sodium chlorate inhibiting the nitrite oxidation step. After 5h incubation at 25 °C, the reaction was stopped by adding 2M potassium chloride, incubated for additional 20 min, followed by a centrifugation step. Ammonium chloride buffer and a reagent for nitrite determination (2mM naphtylenediamine dihydrochloride, 2.5M phosphoric acid, 0.06M sulphanilamide) were added to the supernatant. Spectrophotometric measurement of the color reaction was carried out at a Spectra Max 340 device (MWG BIOTECH, Germany). Samples where stop solution was applied prior to incubation with substrate and reagents without soil served as controls.

454 sequencing

The amplification of the *amoA* gene fragment was performed according to Gubry-Rangin et al (2011). For *amoA* gene targeting PCR, the forward primer CrenamoA23f (5'-ATGGTCTGGCTWAGACG-3`) attached to the Roche A adapter for 454-library construction and the reverse primer CrenamoA616r (5'-GCCATCCATCTGTATGTCCA-3') (Tourna et al 2008), attached to the Roche B adapter for bidirectional sequencing were used. For multiplexing, a 10-nt barcode sequence was included for each primer as recommended by Roche. Three independent PCRs were performed for each sample with FastStart High Fidelity PCR System (Roche, Mannheim, Germany) and pooled for purification. Library purification, equimolar pooling and emPCR were performed according to the manufacturer's recommendations (Roche). Bidirectional sequencing was performed on a 454 GS FLX Titanium sequencer from Roche as described previously in Gubry-Rangin et al (2011).

Data processing

The resulting sequences were processed using the amplicon signal processing pipeline of the Roche software for base calling, trimming of adaptors and quality trimming with one modification in the quality filtering section, where "vfScanAllFlows" was changed from "tiOnly" to "false". Rawdata contained 427000 reads after the first quality check (referring to more than 15,000 reads per sample). Further processing was carried out only for forward reads. According to Pester et al (2012), similar results would be expected for a reverse reads dataset. Sequence reads in sff format were subjected to further quality checking using Mothur Software (Schloss et al 2009). Briefly, after demultiplexing the reads and trimming them to 720 flows, sequences where subjected to denoising (PyroNoise implemented in Mothur). After removal of reads shorter than 200 nt or containing homopolymers more than seven nucleotides long, sequences were frameshift corrected using frameD (Schiex et al 2003) using the curated *amoA* sequence database published in Pester et al (2012) for model building. Sequences were then aligned against the reference *amoA* database (Pester et al 2012) using MUSCLE (Edgar 2004) implemented in a Python script. 155,000 sequences were obtained with an average amplicon length of 359 bp.

Phylogenetic analysis of *amoA* gene sequences

Representative sequences of each OTU, clustered in Mothur at 97% similarity level to prevent overestimation of *amoA* gene diversity due to 454 sequencing errors (Kunin et al. 2009), were aligned to sequences of the archaeal *amoA gene* reference dataset published in Pester et al (2011) using ARB (Ludwig et al 2004). OTUs represented by less than 5 reads were omitted. Representative sequences for each OTU from forest samples and from grassland sites with different pH from the study of Gubry-Rangin et al. (2011) were then added to the Pester et al. archaeal *amoA* consensus

tree using the Parsimony algorithm implemented in ARB, to produce a phylogenetic affiliation of detected sequences for different sites with variable pH. Partial *amoA* gene sequences (293 nt) were added to a maximum likelihood based consensus tree with reference sequences (586 nt long) using the parsimony algorithm.

Statistical analyses

Data analyses were performed using Mothur Software and carried out on 85% and 97% sequence similarity levels. Sequence analysis of *amoA* genes was based on 85% homology level, approximately equivalent to the species level according to Pester et al (2012). A Newick-formatted, hierarchical UPGMA-dendrogram was based on distance matrix, calculated by using Yue & Clayton similarity coefficient. Differences between the gene abundances were tested using Tukey-test and HSD-Test after performing ANOVA. Multivariate statistics for Principal Component Analysis were carried out using R software. Analysis was based on distance matrix of dissimilarities between OTUs, calculated based on distance measure with square root-transformed relative abundance data (Hellinger Transformation).

The following packages for R were used for PCA (input number of samples: 27 forest sites, 40 grassland sites): MASS (Venables & Ripley, 2002, http://www.stats.ox.ac.uk/pub/MASS4), vegan (Oksanen et al., 2012, http://CRAN.R-project.org/package=vegan) and gplots (Warnes, 2012, http://CRAN.R-project.org/package=gplots). The following additional statistical tests were applied to a subset of samples (only forest plots, input number of samples: 27) - the respective packages for R are given in brackets: univariate regression for first principal component (library("pls")), permutation test for canonical correspondence analysis (library("vegan") and tests for significance (library("agricolae")).

Availability of sequences:

Forward sequences from the 454 pyrosequencing have been deposited in the Sequence Read Archive of the National Center for Biotechnology Information (accession no. SRX331496, SRX331831-SRX331852 and SRX331512-SRX331515).

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Legends:

FIGURE S1 Abundance of archaeal *amoA* (A) and bacterial *amoA* (B) genes (g^{-1} soil dw) for each site was plotted against pH. The sites were grouped according to their soil pH range. Different letters indicate significant differences.

FIGURE S2 Rarefaction curves for archaeal *amoA* gene sequences. Numbers of detected OTUs per site are plotted against total numbers of sequences obtained. Analyses were conducted separately for each region. A) Rarefaction curves for Schwäbische Alb region; A1-A9 = sites AEW1-AEW9 B) Rarefaction curves for Hainich-Dün region; H1-H9 = sites HEW1-HEW9 C) Rarefaction curves for Schorfheide-Chorin region; S1-S9 = sites SEW1-SEW9.

FIGURE S3 Indices for richness and evenness of archaeal ammonia oxidizing communities as a function of soil pH in forest ecosystems. Indices were calculated based on 85% similarity level using Mothur software. Regression coefficients and *p*-values are given below. 1A) Chao1 Index estimates of richness within the sites along the pH gradient. *p*-value indicates significant positive correlation. $p = 0.003$, $r^2 = 0.29$. 1B) Shannon Index estimates of evenness within the sites along the pH gradient. *p*-value indicates significant negative correlation. $p = 0.01$, $r^2 = 0.22$.

FIGURE S4 β-diversity analyses for AOA community composition amongst different sites.
Main clusters are referred to as cluster A and cluster B. Corresponding color codes for respective pH ranges are depicted in the graph.

FIGURE S5 Heat-map of the most abundant OTUs with associated dendrograms, showing the clustering of the samples according to pH, based of relative abundance of the OTUs with relative abundance >0.01%.

TABLE S1 Characteristics of forest sites.

For each site, corresponding plot-ID and region is given (AEW1-9, HEW1-9, SEW1-9) and related forest types, soil types, water content and pH values are listed according to the Biodiversity Exploratories database. ALB = Schwäbische Alb, HAI = Hainich-Dün, SCH = Schorfheide-Chorin.

TABLE S2 Correlation matrix across all regions.

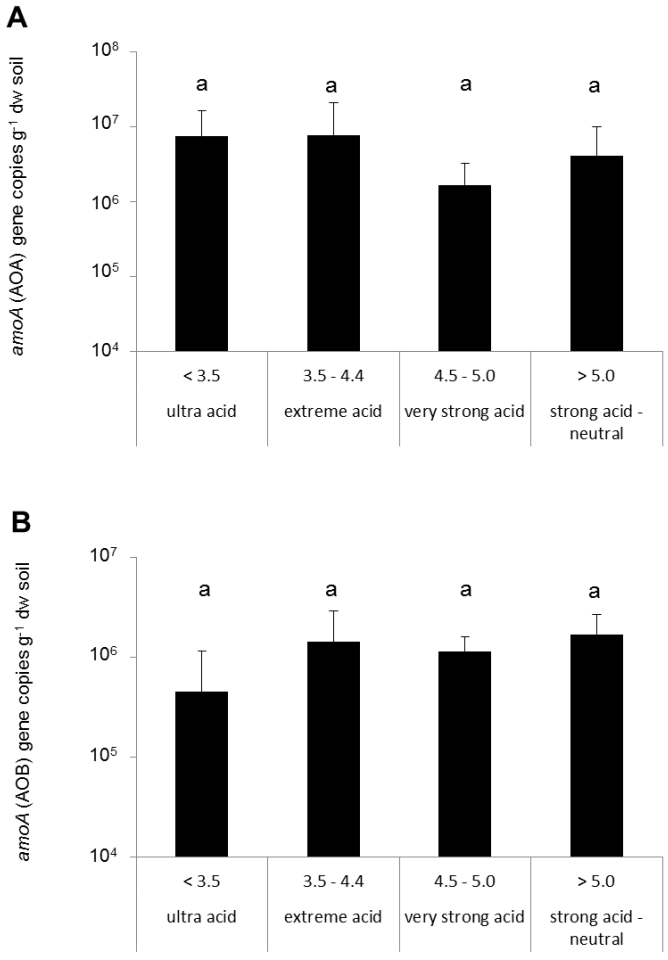
Correlation coefficients are given for *amoA* gene abundances for ammonia-oxidizing archaea (AOA) and bacteria (AOB), potential nitrification rates (PNR) and soil pH to test their linear correlation to soil nitrate and ammonium concentrations as well as soil water content.

region	plot-ID	forest type	tree species	soil type	pH	water content	ammonium concentration (mg kg^{-1} of dw soil)	nitrate concentration (mg kg^{-1} of dw soil)	potential nitrification rate (ng NO_2^--N g^{-1} h^{-1})
ALB	AEW1	age-class forest	spruce	cambisol	3.4	41%	1.74 ± 0.82	1.24 ± 0.65	23.76
	AEW2	age-class forest	spruce	leptosol	4.3	39%	2.19 ± 0.68	2.48 ± 3.07	19.67
	AEW3	age-class forest	spruce	cambisol	5.7	38%	0.78 ± 0.78	2.93 ± 2.44	45.26
	AEW4	age-class forest	beech	cambisol	6.2	67%	0.02 ± 0.05	3.02 ± 2.07	47.87
	AEW5	age-class forest	beech	cambisol	4.5	54%	1.35 ± 0.30	3.82 ± 1.89	26.69
	AEW6	age-class forest	beech	cambisol	4.9	50%	0.37 ± 0.33	1.15 ± 0.78	27.01
	AEW7	age-class forest	beech	leptosol	4.6	59%	0.82 ± 0.50	7.62 ± 1.37	47.43
	AEW8	natural forest	beech	cambisol	6.1	51%	2.30 ± 1.44	4.09 ± 2.89	14.34
	AEW9	natural forest	beech	leptosol	5.9	46%	0.10 ± 0.15	4.81 ± 1.56	35.87
HAI	HEW1	age-class forest	spruce	stagnosol	6.8	36%	1.20 ± 0.81	4.48 ± 0.55	78.41
	HEW2	age-class forest	spruce	stagnosol	5.5	23%	1.49 ± 0.87	0.37 ± 0.20	58.13
	HEW3	age-class forest	spruce	luvisol	5.0	31%	1.82 ± 1.86	0.71 ± 0.53	38.44
	HEW4	age-class forest	beech	luvisol	6.0	45%	1.52 ± 0.98	1.46 ± 0.51	39.65
	HEW5	age-class forest	beech	luvisol	5.0	35%	2.68 ± 1.44	7.71 ± 4.55	27.46
	HEW6	age-class forest	beech	luvisol	4.2	30%	4.34 ± 3.26	1.98 ± 0.68	32.74
	HEW7	single tree selection system	beech	luvisol	4.1	30%	2.92 ± 0.82	0.60 ± 0.55	51.17
	HEW8	single tree selection system	beech	luvisol	5.6	35%	1.96 ± 1.06	3.70 ± 0.43	27.91
	HEW9	single tree selection system	beech	luvisol	4.1	33%	0.95 ± 0.67	2.53 ± 1.18	33.60
SCH	SEW1	age-class forest	pine	cambisol	3.5	9%	0.14 ± 0.17	0.36 ± 0.12	80.19
	SEW2	age-class forest	pine	cambisol	3.4	11%	0.32 ± 0.15	0.52 ± 0.49	76.01
	SEW3	age-class forest	pine	cambisol	3.3	7%	0.77 ± 1.29	0.93 ± 0.94	62.31
	SEW4	age-class forest	pine	cambisol	3.3	11%	0.45 ± 0.24	0.36 ± 0.26	97.50
	SEW5	age-class forest	beech	cambisol	3.2	14%	3.72 ± 1.97	1.52 ± 0.60	115.85
	SEW6	age-class forest	beech	cambisol	3.4	15%	4.38 ± 1.88	2.36 ± 1.72	90.47
	SEW7	natural forest	beech	cambisol	3.5	16%	1.07 ± 0.99	2.48 ± 1.36	76.36
	SEW8	natural forest	beech	albeluvisol	3.3	23%	1.44 ± 1.63	2.93 ± 1.07	91.32
	SEW9	natural forest	beech	cambisol	3.3	14%	1.25 ± 0.67	0.50 ± 0.69	88.71

Table S2:

	Ammonium	Nitrate	Water content
Soil pH	- 0.085	0.530	0.729
AOA	- 0.221	- 0.063	- 0.261
AOB	0.170	0.344	0.414
PNR	- 0.177	- 0.403	- 0.706

Figure S1:



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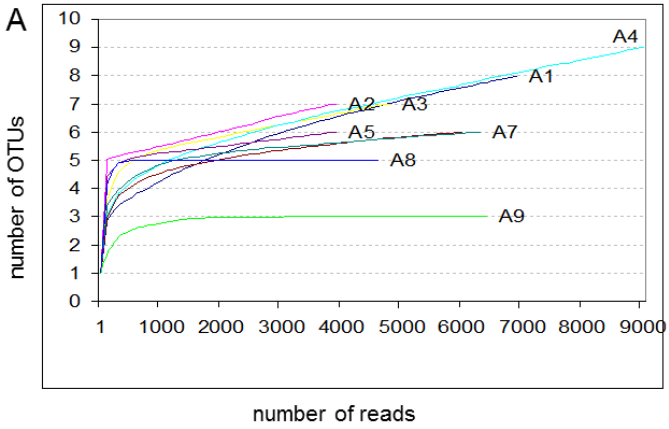
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number of OTUs
number of reads
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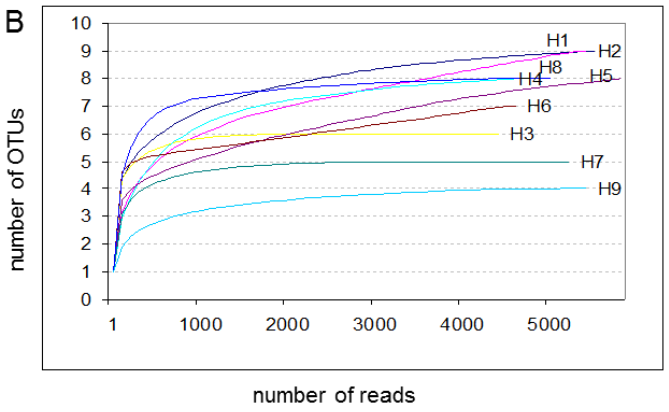
A2
A3
A4
A5
A7
A8
A9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number of OTUs
number of reads
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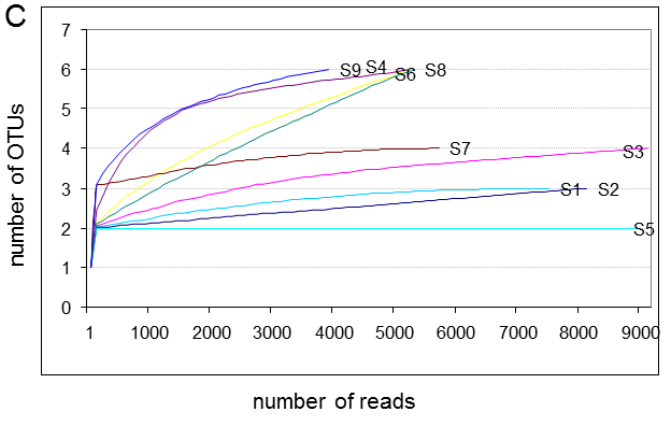
H2
H3
H4
H5
H6
H7
H8
H9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number of OTUs
number of reads
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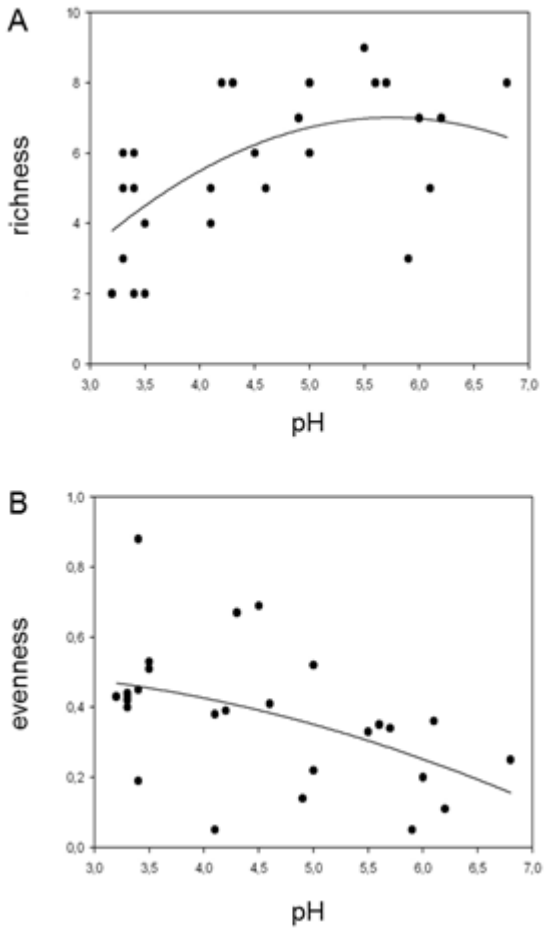
S3
S4
S5
S6
S7
S8
S9

Figure S3:



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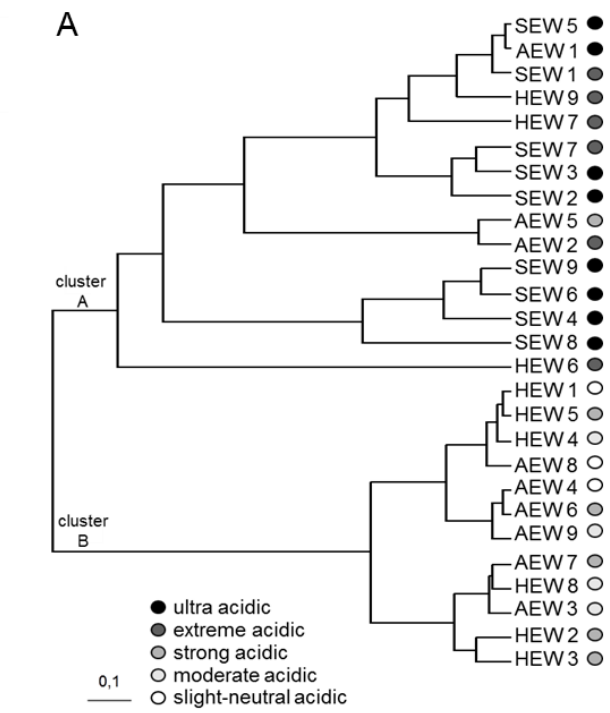

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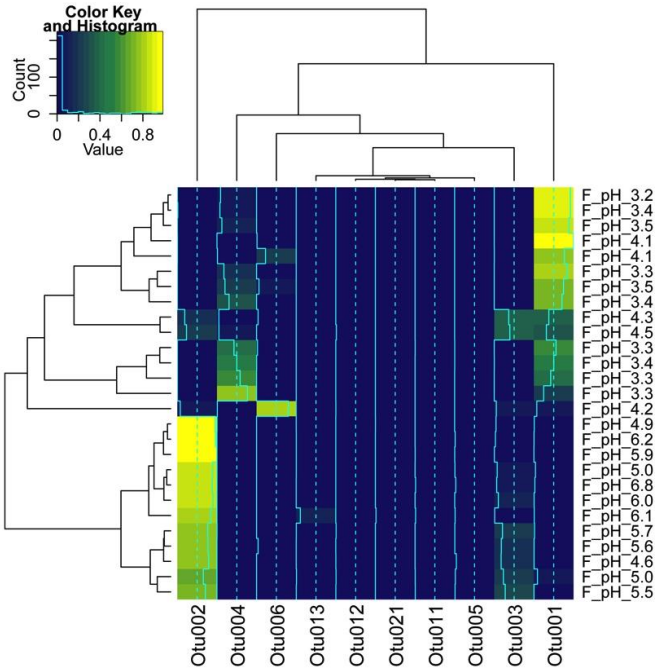

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Drivers for ammonia-oxidation along a land-use gradient in grassland soils

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ABSTRACT

In this study, drivers for ammonia-oxidation and the related microbial communities (ammonia-oxidizing bacteria and archaea) were investigated in grassland soils on the local as well as on the regional scale focusing on the role of land-use intensity (LUI). To this end, 150 sites from three distinct regions across Germany were selected, covering the whole range of LUI levels (from natural grasslands up to intensive managed meadows). Furthermore, the role of contrasting soil types was analyzed in one of the regions (high vs low organic matter content) for ammonia-oxidation. We revealed a significant increase in potential nitrification rates and abundance of ammonia-oxidizing microbes at two sites on the local level from extensively to intensively managed sites, which indicates that the response pattern of ammonia-oxidizing microbes in grassland soils is likely triggered to a large extent by LUI. However at a third site, where two different soil types were investigated, no correlation between LUI and potential nitrification rates was observed, and only a site-specific effect was apparent. At this site, on the one hand the specific soil type (Histosol) and the related continuous nutrient mobilization from the former peat matrix, as well as the high groundwater level, which could induce a high abundance of methane- oxidizing microbes in the top soil, may be of greater importance as a driver for potential nitrification rates and abundance of ammonia- oxidizing microbes than LUI. On the other hand, the mineral soils of this site were characterized by extreme water shortage, which may also explain the lack of potential nitrification and the abundance of ammonia-oxidizing bacteria and archaea. Thus any extrapolation of local data to regional predictions must be made with care, as factors other than LUI may be of importance if the nitrification potential of a soil is to be described.

1. Introduction

Ammonium is the central metabolite of the nitrogen cycle as it links the inorganic to organic phases of nitrogen species. Most organisms (including plants and animals) are able to uptake ammonium and use it for the synthesis of amino acids. Therefore, ammonium availability is strongly linked to the productivity of ecosystems, and can be regarded as an indicator for sustainable use, for example in unfertilized soils (Kowalchuk and Stephen, 2001; Ollivier et al., 2011). Under aerobic conditions in non-fertilized soils the availability of ammonium is mainly controlled by the transformation rates of nitrogen into ammonium via nitrogen fixation and the activity of nitrifiers, which use ammonia as the principal electron donor for autotrophic growth (Stahl and de la Torre, 2012), besides uptake of ammonium for biomass generation and mineralization (the stepwise degradation of dead biomass proteins and polypeptides into ammonium). Thus microbes capable of oxidizing ammonia, which is the first step of nitrification, determine the productivity of ecosystems to a large extent.

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The oxidation of ammonia to nitrite can be performed both by ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) (Treusch et al., 2005), which have been detected in a large variety of habitats ranging from aquatic freshwater ecosystems (reviewed in Kowalchuk and Stephen (2001)) to terrestrial habitats (Fierer et al., 2009; Ochsenreiter et al., 2003; Treusch et al., 2005). Several studies have focused on the respective contributions of AOA and AOB to the ammonia-oxidation in these habitats (e.g. Adair and Schwartz, 2008; Beman et al., 2012; Di et al., 2009; Jia and Conrad, 2009; Leininger et al., 2006; Martens-Habbena et al., 2009; Santoro et al., 2008; Zhang et al., 2010) with partially contradicting observations, which indicate the presence of different drivers of ammonia-oxidizer performance in the investigated environments. Various factors influencing the soil microbial performance of ammonia-oxidizers have been identified so far, amongst the most intense studied are climatic conditions (Tourna et al., 2008), soil chemical and physical properties (He et al., 2007; Nicol et al., 2008; Zhalnina et al., 2012), plant diversity (Kowalchuk et al., 2002) and land-use management. The term land-use intensity (LUI) comprises different management regimes such as fertilization, mowing or grazing which have been demonstrated to affect microbial communities in soils (Nicol et al., 2004; Patra et al., 2006), mainly due to nitrogen-input via fertilizer and manure application or soil compaction by grazing livestock or usage of heavy machinery.

It can be assumed that the land-use intensity (LUI) strongly also drives the abundance, diversity and activity of AOB and AOA in soils. However, as LUI integrates a large variety of factors that influence ammonia-oxidizers to a different degree, such as different fertilization rates, soil compaction or plant species composition, it is not clear if a general link between LUI and ammonia-oxidation can be found, mainly taking into account that site specific conditions like climate or soil type also drive AOA and AOB performance. Overall studies of AOA and AOB dynamics in the response to different LUI levels are rare and mainly cover small scales (Di et al., 2009; Keil et al., 2011; Nicol et al., 2004; Schauss et al., 2009).

In this study we present results from a systematic study on the influence of LUI on AOA and AOB in grassland soils on the local as well as on the regional scale. In this respect, our main focus was to determine overall underlying principles across a large number of different study sites. The selected regions representing the local scale had an area of 300 km^2 each and were located in three areas of Germany (southwest; central; northeast). In each of these regions 50 plots were selected and classified in different LUI levels according to an index of LUI developed by Blüthgen et al. (2012). This index includes the main determining factors of land-use intensity dominating at the study sites, in particular fertilization intensity, mowing frequency and grazing level. At each plot, the abundance of ammonia-oxidizing microbes was measured based on the quantification of the gene coding for the subunit A of the ammonia monooxygenase gene (*amoA*). In addition, potential nitrification rates as well as in situ ammonium levels were determined. To quantify the ammonium input via nitrogen fixation also the abundance of nitrogen fixing microbes was measured based on the nitrogenase gene *nifH*.

We hypothesized an overall positive correlation of LUI and abundance of ammonia-oxidizing microbes and according potential nitrification rates on the local as well as on the regional scale, independent from the soil type and climatic conditions, due to increased ammonium availabilities by fertilizer input or nitrogen fixation in combination with increased soil carbon contents as well as increased oxygen availability in mineral soils. However, very high LUIs could lead to a decrease due to soil compaction by heavy machinery and grazing livestock and less oxygen availability as well as higher competition with the heterotrophic microflora in response to higher nutrient contents. In soils with natural high carbon content, like Histosols, the ammonia-oxidation rates are assumed to be lower independent from the land-use due to the natural high water levels and they do not follow the described trend of mineral soils due to low redox potentials.

2. Materials and methods

2.1. Site description

The experiment was performed in the frame of the 'German Biodiversity Exploratories' (www.biodiversity-exploratories.de; Fischer et al., 2010). The Biodiversity Exploratories are based in three different areas located across Germany: the Biosphere Reserve Schwäbische Alb (ALB) in the South-west of Germany; the National Park Hainich-Dün (HAI) in Central Germany, and the Biosphere Reserve Schorfheide-Chorin (SCH), in the North-east of Germany. Mean annual temperatures have been determined as follows: 6.5–8.0°C (ALB), 8.0–8.4°C (HAI), 6.5–8.0°C (SCH). Mean annual precipitation levels, detected for each region, are the following: 938–963 mm (ALB), 520–600 mm (HAI), 500–800 mm (SCH). Each region comprises 50 grassland study sites, so called experimental plots (EP), so in total our study focused on 150 different grassland plots. For reasons of clarity, the grassland plots were termed as follows: AEG1-50 (ALB region), HEG1-50 (HAI region) and SEG1-50 (SCH region). The Schorfheide-Chorin exploratory comprises mineral soil sites as well as degraded peat soil sites. For reasons of better discrimination, sites of mineral soil were termed as SCH-MB (SCH-mineral soil), peat soil sites were termed as SCH-NM (SCH-peatland). The dominating soil types are Histosols, Cambisols, Glysols, Luvisols and Albeluvisols. The experimental sites of the Schwäbische Alb comprised Cambisols and Leptosols, whereas in Hainich-Dün mainly Cambisols and Stagnosols were found. The respective soil textures are characterized by predominantly sandy textures in the Schorfheide-Chorin exploratory. In Hainich-Dün and Schwäbische Alb exploratory, loamy and clayey textures are found. For further details see Fischer et al. (2010).

2.2. Land-use intensity

On the basis of conducted land-use surveys, Blüthgen et al. (2012) developed an integrative measure for the land-use intensity based on the following components: fertilization, including organic and inorganic fertilizers (in kg nitrogen per ha per year), mowing frequency per year and grazing intensity by cattle, horses or sheep, measured via livestock density (in livestock units per ha per year). The measured intensities of the three components were normalized to the typical forms of management for the respective regions. The calculated LUI index is the sum of all three normalized components and reflects a numerical gradient, reflecting extensive respectively intensive land-use management. Underlying equations for the calculation of the index as well as further information can be found in Blüthgen et al. (2012). The originally proposed LUI index has been based on a three year index, whereas for our studies the recently calculated five-year-index was used based on the same equations (Blüthgen et al., unpublished). To monitor changes across different regions along the land-use intensity gradient, the results were either plotted against corresponding LUI – indices or grouped according to their individual LUI values to meet the demands of a rather detailed gradation on the one hand and the availability of a sufficient number of independent replicates, required for subsequent comprehensive statistical analyses at the same time on the other hand. The levels of LUI were termed with consecutive numbering from 1 (extensive) to 5 (intensive) for reasons of clarity (see also Table 1): Level 1 = LUI indices below 1.0; Level 2 = LUI

Table 1
Major abiotic and biotic soil properties. LUI levels are depicted for each exploratory (SCH is separated into SCH-NM and SCH-MB) with respective numbers of sites within each level. Ammonium, nitrate and total nitrogen concentrations as well as total organic carbon content in soil are listed, related to g of dry soil for reasons of comparability. Abundance data of *nifH* gene is included. Values represent mean values and corresponding standard deviations for each level. Levels with site numbers comprising less than three sites contain no standard deviation values. ALB = Schwäbische Alb, HAI = Hainich-Dün, SCH-NM = Schorfheide-Chorin, peat soil, SCH-MB = Schorfheide-Chorin, mineral soil.

Exploratory	LUI-Index level	LUI-Index range	No. of sites	Soil water content %	Ammonium μg N g^{-1} dw	Nitrate μg N g^{-1} dw	Total nitrogen mg N g^{-1} dw	Total organic carbon mg C g^{-1} dw	*nifH* gene abundances copies g^{-1} dw
ALB	1	<1.0	8	45.5 ± 16.1	27.1 ± 9.3	3.9 ± 3.2	5.6 ± 1.9	62.1 ± 18.5	$8.87 \cdot 10^6 \pm 1.72 \cdot 10^7$
	2	1.0–1.5	13	41.8 ± 9.2	24.0 ± 9.8	9.4 ± 9.2	6.0 ± 1.3	62.0 ± 14.9	$9.08 \cdot 10^6 \pm 1.16 \cdot 10^7$
	3	1.5–2.0	17	44.0 ± 12.0	20.4 ± 7.4	17.8 ± 13.8	6.7 ± 1.3	68.0 ± 13.3	$1.46 \cdot 10^7 \pm 1.76 \cdot 10^7$
	4	2.0–2.5	9	44.2 ± 8.1	18.2 ± 8.1	22.2 ± 10.1	6.7 ± 1.4	66.0 ± 12.1	$1.30 \cdot 10^7 \pm 1.91 \cdot 10^7$
	5	>2.5	3	35.7 ± 6.1	15.3 ± 10.5	30.6 ± 13.7	6.9 ± 0.5	67.4 ± 3.7	$1.28 \cdot 10^7 \pm 9.14 \cdot 10^6$
HAI	1	<1.0	12	22.3 ± 6.0	16.6 ± 5.3	5.1 ± 4.7	4.1 ± 1.5	45.0 ± 15.0	$6.13 \cdot 10^6 \pm 6.95 \cdot 10^6$
	2	1.0–1.5	9	22.2 ± 4.2	8.0 ± 3.0	11.7 ± 5.2	4.8 ± 0.8	48.6 ± 7.5	$1.56 \cdot 10^7 \pm 2.52 \cdot 10^7$
	3	1.5–2.0	13	22.2 ± 5.0	9.4 ± 4.1	13.9 ± 5.9	4.7 ± 1.1	46.6 ± 10.7	$4.59 \cdot 10^6 \pm 3.67 \cdot 10^6$
	4	2.0–2.5	12	19.7 ± 6.6	8.8 ± 3.7	14.7 ± 7.6	4.1 ± 1.1	41.7 ± 11.4	$9.13 \cdot 10^6 \pm 1.16 \cdot 10^7$
	5	>2.5	4	23.5 ± 2.9	5.0 ± 2.9	14.7 ± 9.4	5.1 ± 2.1	49.3 ± 19.5	$5.72 \cdot 10^6 \pm 3.24 \cdot 10^6$
SCH-NM	1	<1.0	2	101.0	15.0	14.0	10.2	103.7	$2.50 \cdot 10^7$
	2	1.0–1.5	17	113.4 ± 49.0	29.6 ± 9.4	21.3 ± 13.8	16.2 ± 6.1	188.9 ± 92.3	$9.70 \cdot 10^7 \pm 1.47 \cdot 10^8$
	3	1.5–2.0	2	122.0	24.5	16.4	22.4	295.0	$4.64 \cdot 10^7$
	4	2.0–2.5	–	–	–	–	–	–	–
	5	>2.5	6	105.7 ± 20.3	17.7 ± 8.1	34.5 ± 7.6	12.8 ± 3.6	131.2 ± 40.5	$5.02 \cdot 10^7 \pm 3.69 \cdot 10^7$
SCH-MB	1	<1.0	1	7.0	7.7	1.5	1.9	18.9	$1.49 \cdot 10^7$
	2	1.0–1.5	11	8.2 ± 3.1	7.6 ± 5.0	3.1 ± 1.9	2.1 ± 0.5	22.5 ± 4.6	$7.30 \cdot 10^6 \pm 8.20 \cdot 10^6$
	3	1.5–2.0	6	7.5 ± 3.4	8.6 ± 5.7	3.1 ± 2.9	1.8 ± 0.3	18.8 ± 3.4	$1.16 \cdot 10^7 \pm 7.94 \cdot 10^6$
	4	2.0–2.5	5	7.2 ± 2.3	7.5 ± 5.5	5.1 ± 5.5	2.3 ± 0.6	23.2 ± 6.0	$6.47 \cdot 10^6 \pm 7.56 \cdot 10^6$
	5	>2.5	–	–	–	–	–	–	–

indices between 1.0 and 1.5; Level 3 = LUI indices between 1.5 and 2.0; Level 4 = LUI indices between 2.0 and 2.5; Level 5 = LUI indices above 2.5. The number of sites per LUI index is given in Table 1.

2.3. Soil sampling

All soil samples were taken in May 2011 in the frame of a coordinated soil sampling campaign. At 14 predefined locations within each plot, individual samples were taken from the upper 10 cm of the mineral soil and subsequently pooled and sieved to <2 mm without root material. Soil samples for potential enzymatic activity measurements were stored at 4°C until usage; samples for DNA extraction were immediately frozen on dry ice at −80°C.

2.4. Abiotic soil properties

Nitrate and ammonium concentrations were measured after extraction using 1 M KCl (1:4 soil to extractant ratio w/v). The soil suspensions were shaken on a horizontal shaker for 30 min at 250 rpm followed by a centrifugation step (30 min at 4500 × *g*). The concentrations of nitrate and ammonium in the supernatant were measured using an auto-analyzer (Bran & Luebbe, Norderstedt, Germany).

For the determination of total nitrogen and carbon, dry combustion of soil at a temperature of 1100°C followed by determination of evolving CO_2 and N_2 with a Thermal Conductivity Detector (TCD) was used (VarioMax, Elementar, Hanau, Germany).

Soil pH was determined by a WTW pH meter 538 (Wissenschaftlich-Technische-Werkstätten GmbH, Weilheim, Germany) in combination with the pH electrode SenTix61 after adding 0.01 M $CaCl_2$ solution to sieved, air-dried soil (1:2.5 soil to extractant ratio w/v).

2.5. Extraction of nucleic acids

DNA was extracted from 1 g fresh weight of soil samples. DNA extraction was carried out using Power Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, USA) according to manufacturer's instructions. Elution was done in 200 μl 10 mM Tris Buffer. DNA content was quantified using Nanodrop 1000 Spectrophotometer (Peqlab, Germany) and purity validated via determination of A260/A280 ratios.

2.6. Quantification of amoA and nifH genes

Real-time quantitative PCR was performed on a 7300 Real-Time PCR System (Applied Biosystems, Germany) using SyBr Green as fluorescent dye. For the quantification of *nifH* and *amoA* gene abundances, thermal profiles, plasmids for standard dilutions and reaction mixture components including primers were used according to Töwe et al. (2010) (Table S1). Serial plasmid dilutions (10^1–10^6 gene copies per μl) were used for standard curve calculations. In a pre-experiment, performed to avoid reaction inhibition effects, the optimal sample dilution was determined as 1:16 (data not shown). A melting curve analysis was performed by adding a dissociation stage step after each run in order to prove the specificity of the amplified qPCR products. To further confirm the correct size of the amplified fragments, gel electrophoresis for randomly selected samples was conducted on a 2% agarose gel. Efficiencies obtained were as follows: 86–87% for *amoA* AOB, 88% for *amoA* AOA and 86–92% for *nifH*. R^2 was determined to be above 0.99 for each qPCR assay.

2.7. Potential nitrification assay

The measurement of the potential nitrification rate was carried out according to Hoffmann et al. (2007). For potential nitrification measurements, 2.5 g of soil were mixed with 10 ml of 10 mM ammonium sulfate as a substrate and 50 μl of 1.5 M sodium chlorate to prevent the turnover of nitrite to nitrate. The samples were incubated at 25°C for 5 h slightly shaking. After the addition of 2.5 ml of 2 M potassium chloride to stop the reaction and subsequent incubation for 20 min, the samples were centrifuged 2 min at 2000 × *g*. 90 μl ammonium chloride buffer (pH 8.5) and 60 μl of regent for nitrite determination (2 mM naphtylenediamine dihydrochloride, 2.5 M phosphoric acid and 0.06 M sulphanilamide)

were added to 150 μl of supernatant. The color reaction was detected spectrometrically at an absorbance wavelength of 540 nm (SpectraMax 340, MWG BIOTECH, Germany). The samples with added stop solution prior to incubation and reagents without soil served as controls.

2.8. Statistical analyses

For statistical computing, the R environment (http://www.R-project.org) was used. To prepare data for the statistical analysis, the variables pN (potential nitrification), AOB, AOA, and *nifH* were $\log(x + 1)$ transformed. For multivariate analysis, a few missing values ($n = 3$) were imputed using the Singular Value Decomposition method (SVDImpute in R-package imputation, rank = 2). To test the effect of the LUI levels and of the different regions on all variables, a permutational multivariate analysis of variance was implemented (adonis with Euclidean distance matrix in R-package vegan). After checking the homogeneity of variance across groups (leveneTest in R-package car), ONEWAY ANOVAs with LUI levels as factor were applied (lm in R). In case of a significant effect, pairwise *t*-tests with adjusted *p*-values (pairwise.*t*.test with method holm) were added. An integrative multivariate data analysis was based on the principal component analysis (correlation based, prcomp in R).

3. Results

3.1. Abiotic soil properties

Total nitrogen content in ALB and HAI regions increased in response to higher LUI levels with slightly lower values for HAI (6.4 mg g^{-1} in average at ALB region, 4.6 mg g^{-1} in average at HAI region). In SCH, however, the nitrogen content was remarkably higher in the Histosols (15.2 mg g^{-1} in average), whereas the nitrogen content was lowest at mineral soil sites of SCH (2.0 mg g^{-1} in average). Evenly distributed total organic carbon content amongst different LUI levels was found in ALB (65.1 mg g^{-1}) and HAI (46.0 mg g^{-1}) with being slightly lower in HAI. Thus overall the C/N ratio at HAI and ALB decreased with increasing LUI levels. Within SCH a high variation in the total organic carbon content was detected with the highest content of total organic carbon amongst all regions in the peat soils (179.7 mg g^{-1}), whereas mineral soils of SCH were characterized by very low contents of total organic carbon (20.0 mg g^{-1}).

Nitrate and ammonium concentrations showed at all sites of HAI and ALB a clear influence of the corresponding LUI level (Table 1): nitrate concentrations increased from extensively to intensively managed sites in ALB and HAI (3.9–30.6 μg g^{-1} for ALB, 5.1–14.7 μg g^{-1} for HAI), whereas ammonium concentrations decreased (27.1–15.3 μg g^{-1} for ALB, 16.6–5.0 μg g^{-1} for HAI). Nitrate concentrations were lower at SCH-MB sites (1.5–5.1 μg g^{-1}) and showed the highest variation in SCH-NM with the highest value of 34.5 μg g^{-1}. Also for ammonium concentrations in SCH, no clear trend was visible: concentrations were comparably low for each land-use category in SCH-MB (7.9 μg g^{-1}) and were subject to strong fluctuations across LUI levels in SCH-NM.

3.2. Nitrogen input via nitrogen fixation

As a proxy for biological nitrogen input via nitrogen fixation, *nifH* gene copy numbers were determined. Copy numbers of the *nifH* gene ranged from 4.6×10^6 to 1.6×10^7 per g of soil in the study sites of ALB, HAI and SCH-MB, whereas remarkably higher abundances of 2.5×10^7 to 9.7×10^7 per g of soil were detected in sites of SCH-NM. The gene abundance was highest at intermediate LUI levels (levels 2–3) in most regions (Table 1).

3.3. Potential nitrification activity and abundance of ammonia-oxidizers

The rates of potential nitrification ranged from 276.2 ng NO_2–N g^{-1} h^{-1} to 1121.1 ng NO_2–N g^{-1} h^{-1} in soils from the ALB region and increased with increasing LUI levels (1–5). For HAI region, the same dynamics were detected with comparable rates (Fig. S1). The distribution amongst LUI levels within the mineral soil sites in SCH-MB revealed similar but not significant trends as observed in ALB and HAI: a slightly increasing potential nitrification rate with higher LUI levels. However the rates were generally lower (92.2 ng NO_2–N g^{-1} h^{-1}–346.7 ng NO_2–N g^{-1} h^{-1}) compared to the other regions. Within SCH-NM sites, no clear trend was notable, although highest rates were detectable for LUI level 5. Overall SCH-NM sites (level 5) had the highest potential nitrification rates amongst all investigated (1483.6 ng NO_2–N g^{-1} h^{-1}).

Copy numbers of *amoA* genes for AOA ranged from 1.8×10^7 to 1.3×10^8 copies per g of soil in the ALB region and from 2.8×10^7 to 1.3×10^8 copies per g of soil in HAI (Fig. S2). The gene copy numbers in ALB increased from lower to higher LUI levels with highest copy numbers observed at LUI level 4. When comparing gene copy numbers of extensively and most intensively used sites in HAI, a significant increase in the abundance of AOA was visible. For SCH-NM and SCH-MB sites, the highest gene copy numbers were found at intermediate LUI levels 2 and 3 with declining abundance at the highest land-use intensity independent from the respective soil type. Gene copy numbers within SCH-NM sites were comparable to ALB and HAI, whereas the lowest copy numbers were detected for SCH-MB (4.7×10^6 to 5.6×10^7 copies per g of soil).

In contrast to AOA, *amoA* gene copy numbers for AOB were generally lower across all sites. The copy numbers showed a significant dependency to LUI and ranged from 9.0×10^4 at extensively used sites to 2.8×10^6 copies per g of soil at intensively used sites in the ALB region. Also in HAI sites, the copy numbers increased with increasing LUI (6.0×10^5 to 4.6×10^6 copies per g of soil). The highest *amoA* copy numbers for AOB were associated with intermediate LUI for both soil types in SCH. However, AOB were found to be more abundant in mineral soils than in peat soils. Overall, the observed response pattern did not change, if the obtained gene copy numbers were related to ng extracted DNA (which is a good proxy for microbial biomass) neither for AOA nor for AOB (data not shown).

4. Discussion

4.1. The influence of land-use intensity on potential nitrification rates and ammonium/nitrate dynamics on the local and regional scale

Several studies have reported an impact of the land-use management on ammonia-oxidation in grasslands or forest soils at the plot scale (e.g. Patra et al., 2006). Here, we could determine that the same effect could be observed at larger local and regional scales by comparing different grassland sites located across Germany. We demonstrated an increase in the potential nitrification rates correlated to increasing land-use intensities mainly within ALB and HAI region (Fig. 1). The impact of LUI on the potential nitrification rate was further confirmed by one-way ANOVA for ALB and HAI ($p = 0.030$ for ALB, $p < 0.001$ for HAI). In contrast, no correlation was found for both SCH-NM and SCH-MB ($p = 0.570$ for SCH-NM, $p = 0.858$ for SCH-MB; see Table 2); similar results were obtained for correlation coefficients ($r = 0.494$ for ALB, $r = 0.517$ for HAI, $r = 0.078$ for SCH; see Table 3). The observed effects in ALB and HAI were most pronounced when comparing LUI levels 1 and 5. Despite the fact that LUI-indices do not discriminate against different

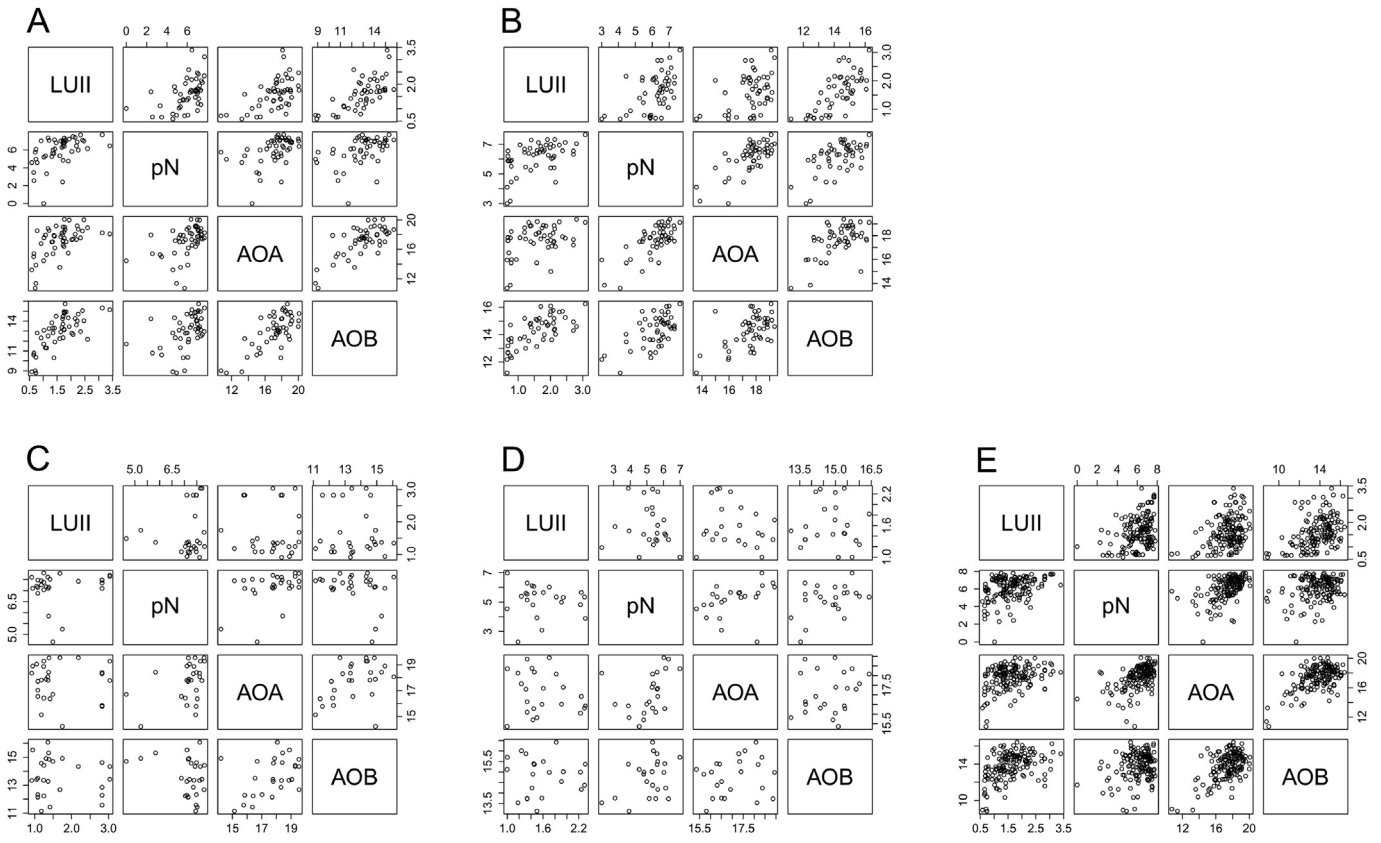

Fig. 1. Scatterplots for potential nitrification rate and gene abundances across land-use intensities. Scatterplots depict data obtained for potential nitrification rate (pN), *amoA* gene abundances for AOA (AOA) and AOB (AOB) as well as the land-use intensity index values for each site plotted against each other. Corresponding correlation coefficients were calculated. Analysis was conducted separate for each region, i.e. ALB (1A), HAI (1B), SCH-NM (1C), SCH-MB (1D) and for all three exploratories combined (1E). ALB = Schwäbische Alb, HAI = Hainich-Dün, SCH-NM = Schorfheide-Chorin, peat soil, SCH-MB = Schorfheide-Chorin, mineral soil.

compounds (Blüthgen et al., 2012), we assume that the increase in potential nitrification rates at intensively used sites is largely attributed to higher nitrogen input by fertilizer application or animal grazing. Thus, it would be interesting to reveal the particular influence of each land-use intensity component in subsequent studies.

Nitrate levels in ALB, HAI and SCH-MB were strongly positively correlated to the potential nitrification rate ($r = 0.58$ for ALB, $r = 0.68$ for HAI, $r = 0.56$ for SCH-MB, see Table 3) and increased with increasing LUI. This indicates that at the time point of sampling, the denitrification activity was obviously low at those sites. This is in contrast to SCH-NM, where no correlation between nitrate concentrations and potential ammonia-oxidizing activity was observed, which may serve as an indication for denitrification in the Histosol under investigation, as a result of lower oxygen availability induced by higher water content. ALB and HAI regions displayed average water contents of 42% and 22%, respectively, evenly distributed amongst the LUI levels. The soil water content in peat soil sites of SCH-NM ranged from 101% to 122% in average as a result of the high groundwater table, whereas mineral soil sites in SCH-MB had an extremely low average water content of 7.5% (Table 1), but showed in addition a low maximal water holding capacity in contrast to the relatively high water holding capacity of Histosols.

Table 2
Significance levels of the measured parameters in response to LUI. ANOVA *p*-values are given for each region to test impacts of land-use intensity on potential nitrification, *amoA* gene abundances for AOA and AOB. Significant impacts are marked in bold ($p < 0.05$). ALB = Schwäbische Alb, HAI = Hainich-Dün, SCH-NM = Schorfheide-Chorin, peat soil, SCH-MB = Schorfheide-Chorin, mineral soil.

	Potential nitrification	*amoA* AOA	*amoA* AOB
ALB	**0.030**	<**0.001**	<**0.001**
HAI	<**0.001**	**0.002**	<**0.001**
SCH-NM	0.570	0.560	0.818
SCH-MB	0.858	0.142	0.808

Although correlation coefficients revealed that ammonium concentrations were not correlated to potential nitrification rates ($r = -0.25$ for ALB, $r = 0.22$ for SCH-NM, $r = -0.08$ for SCH-MB, see Table 3), probably due to the rapid turnover of available ammonium in soil by heterotrophic microorganisms and plant uptake, the concentrations decreased from extensively to intensively managed sites in ALB and HAI ($r = -0.48$). The contribution of nitrogen fixation to the present ammonium pool in soil is low as indicated by the low *nifH* gene copy numbers found at all sites, with the only exception of SCH-NM. Here, the highest concentrations of total organic carbon were determined providing the required sources for energy which is in accordance to observations in Brankatschk et al. (2011). Moreover the highest water contents were found in these soils, which could favor the nitrogenase activity of free living diazotrophs due to low redox conditions. At the other sites (mainly LUI levels 4 and 5) obviously mainly the presence of ammonium and the lack of easy degradable carbon inhibit the nitrogen fixation (Gadkari and Stolp, 1974; Limmer and Drake, 1998).

4.2. Linking potential nitrification rates to the abundance of ammonia-oxidizing microbes on the local and regional scale in relation to land-use intensities

There is still a strong debate in the literature to which extent AOA and AOB contribute to the oxidation of ammonia in soils.

Table 3
Correlation matrix for potential nitrification rates. Correlation coefficients are given for each region and across all regions to test linear correlation of potential nitrification rates to land-use intensity, gene abundances of AOA and AOB, as well as to nitrate and ammonium concentrations. ALB = Schwäbische Alb, HAI = Hainich-Dün, SCH-NM = Schorfheide-Chorin, peat soil, SCH-MB = Schorfheide-Chorin, mineral soil.

	LUI-index	*amoA* AOA	*amoA* AOB	Nitrate	Ammonium
ALB	0.494	0.471	0.455	0.583	−0.246
HAI	0.517	0.725	0.598	0.677	−0.481
SCH-NM	0.114	0.410	−0.318	0.253	0.218
SCH-MB	0.037	0.386	0.402	0.564	−0.083
All regions	0.355	0.509	0.246	0.607	0.065

Whereas Jia and Conrad (2009) and Di et al. (2009) postulated that ammonia-oxidation is driven mainly by AOB, especially in soils with high nitrogen loads, other authors could prove a high contribution of AOA to the process, mainly under nutrient limiting conditions in soil (Schleper, 2010) or if abiotic stressors were applied to soil (Schauss et al., 2009). Thus, it is still not clear to what extent gene copy numbers of AOA and AOB might reflect active contribution to the nitrification process hence different studies revealed a positive correlation (Leininger et al., 2006; Wuchter et al., 2006; Prosser and Nicol, 2008; Di et al., 2009). Besides the different cell size of the AOA and AOB also the ecophysiology of the different groups of ammonia-oxidizers might trigger ammonia-oxidation rates. Whereas AOB are considered to depend on the substrate availability of ammonium (Martens-Habbena et al., 2009), AOA have been shown to be able to switch to urea as alternative substrate due to their mixotrophic lifestyle (Tourna et al., 2011), which might affect differential contributions of AOA and AOB to the oxidation of ammonia, especially at sites with intensive livestock grazing.

We found higher gene copy numbers for *amoA*-AOA than *amoA*-AOB (AOA:AOB ratio 10), independent from the region and soil under investigation, which is in line with previous findings (Leininger et al., 2006; He et al., 2007; Adair and Schwartz, 2008). ANOVA values (Table 2) indicate significant interactions of AOB abundance and LUI in ALB ($p < 0.001$) and HAI ($p < 0.001$) regions, which is in agreement to Nicol et al. (2004) and Di et al. (2009), who obtained similar data on the plot scale. However, these observations could not be confirmed either for the mineral soil or for the Histosols of SCH, indicating the importance of the particular soil type present at the respective site. Overall the AOA:AOB ratio decreased with decreasing LUI index, which again pinpoints the importance of added ammonia for the performance of AOB. A significant effect of LUI on AOA could be determined by ANOVA for ALB ($p < 0.001$) as well as for HAI sites ($p = 0.002$). However, for ALB, an increase in gene abundance with increased land-use intensities could be demonstrated only for LUI levels 1–4. A declining trend of ammonia-oxidizer abundances was visible at high LUI levels for both soil types of SCH. Different studies indicated that AOA could be inhibited at intensively managed sites due to high ammonia concentration there, although controversially discussed (Tourna et al., 2008; Verhamme et al., 2011). Thus again our data pinpoint the importance of the soil type and the site conditions if the response of AOA to different LUI levels is discussed. However it also has to be considered that fertilizer impacts might vary according to the type of fertilizer used (Schauss et al., 2009) independent from the LUI level, thus mainly at LUI level 5, the different response pattern of AOA in the different exploratories may also be a result of different fertilizer types used in the respective regions. For example intensive grazing was demonstrated to affect microbial communities in soil by excrement deposition (Nicol et al., 2004; Patra et al., 2006) as well as via soil compaction and consequently reduced the oxygen concentrations in soil pores possibly favoring other processes besides nitrification. In contrast, manure application often leads to a fast increase in gene copy numbers of AOA (Schauss et al., 2009).

4.3. Influence of the soil type and soil properties on the local scale for ammonia-oxidation

Besides the strong correlation of AOA and AOB abundance, as well as potential nitrification rates to land-use intensity in ALB and HAI regions, we found neither significant correlation to specific soil parameters as total organic carbon or pH in these regions (Table S3) nor correlations between LUI and soil properties (Table S2). However as SCH region comprises Histosols in addition to mineral soils, we could not only compare different regions in terms of response of ammonia-oxidizers toward different land-use intensities looking at comparable soil types, but also include questions on the influence of different soil types on the local scale on the performance of AOA and AOB. In SCH-NM, total organic carbon content was positively correlated to AOB abundance ($r = 0.511$), which could be due to the fact that higher carbon and nitrogen contents trigger mineralization rate, which subsequently improves nutrient supply that would in turn favor AOB in these nutrient-rich environments as suggested by Wessen et al. (2011). According to our hypothesis, carbon content may be also considered to affect ammonium levels by serving as energy donor for nitrogen fixation.

Besides carbon availability differences in soil pH values (which trigger the ratio of ammonium : ammonia) may play an important role as driver for AOA and AOB (Gubry Rangin et al., 2011). Mean values of soil pH of the investigated regions ALB (pH 6.3), HAI (6.9) and SCH-NM (pH 6.9) were in a comparable range, thus, an additional influence of the pH has been considered as unlikely. However, for SCH-MB sites, where lower pH values were measured (pH 5.8), a clear influence of soil pH on the community structure of ammonia-oxidizers was visible. This is confirmed by the calculated correlation coefficients indicating no significant relationship between pH and AOA or AOB abundances for ALB, HAI and SCH-NM, whereas a positive correlation to pH (Table S3) was found at SCH-MB sites for AOA. These findings are in accordance to previous studies at both regional and landscape scale (Bru et al., 2011; Wessen et al., 2011) where soil pH has been identified as a major driver for ammonia-oxidizers, although often contradicting positive and negative correlations of AOA and AOB abundances with soil pH were determined (Hallin et al., 2009; He et al., 2007; Nicol et al., 2004; Yao et al., 2011). However, the high dominance of AOA in SCH-NM might be a result of changing environmental conditions at groundwater dependent, periodically flooded peat-rich soils in SCH-NM and the higher persistence of AOA to changes in abiotic soil properties (Chen et al., 2008; Schleper et al., 2005; Valentine, 2007).

We assumed that potential nitrification rates would be lower at Histosol sites due to high water levels there, but surprisingly, we detected the highest potential nitrification activity at these sites. Also at extensively used sites of SCH-NM, a relatively high potential nitrification activity was detected may be as a result of the nutrient mobilization during the history of these soils (degradation of peat). Another possible explanation for the lack of linear correlation between abundances and potential nitrification rates (corresponding *r* values, see Table 3) might be the presence of methane-oxidizing microbes, as it has been stated that ammonia monooxygenase is closely related to particulate methane monooxygenase (pMMO) in methanotrophs (Holmes et al., 1995; Kowalchuk and Stephen, 2001; Stahl and de la Torre, 2012), enabling both methane- and ammonia-oxidizing microbes to switch substrates (Alam and Jia, 2012). Therefore, we speculated that periodically groundwater-

dependent degraded peat soils would constitute a habitat triggering both processes (Horz et al., 2002), as postulated by Bodelier and Frenzel (1999), providing the optimal environment with "oxic and anoxic conditions in close proximity". To verify our assumptions, we tested the presence or absence of a pMMO-coding gene, *pmoA*, in our soils. Indeed, in contrast to ALB and HAI sites, we could detect *pmoA* at SCH-NM sites (data not shown), indicating that methane-oxidation might occur at these sites, leading to the speculation that methanotrophic microbes could contribute to or be responsible for surprisingly high levels of potential nitrification rate in these soils. Alam and Jia (2012), reported an inhibiting effect of fertilization on methane-oxidation in paddy soil, leading us to the assumption that methane-oxidizers would switch to ammonia-oxidation under high fertilizer input as present at intensively used sites at LUI level 5 in SCH-NM.

4.4. Integration of variables influencing ammonia dynamics

Despite the high heterogeneity of sites attributed to different soil types and other site-specific conditions, we were able to identify some underlying region-independent principles across large spatial scales as depicted by principal component analysis (PCA), integrating all investigated variables of each region, including SCH-NM sites as well. Principal components 1 and 2 explained 42%, respectively 25% of variance and provided strong evidence that the management intensity, represented by the different LUI levels mainly affected potential nitrification rates and abundance of AOB and AOA at intermediate and high LUI (Levels 3–5), in ALB and HAI sites, which is additionally indicated by the arrows A and B in Fig. 2. The different LUI levels of SCH sites in contrast show no linear distribution, instead a separate clustering of SCH-MB and SCH-NM sites (Fig. 2, circles C and D) was observed.

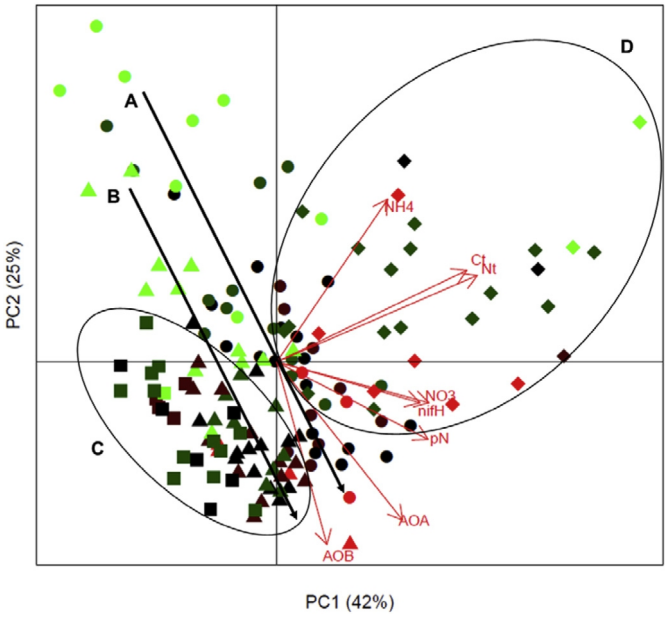

Fig. 2. Principal component analysis integrating variables measured for all regions across different land-use intensities. Variables for regions ALB, HAI and SCH were included in the analysis. Colors represent different land-use intensity levels: level 1 = light green, level 2 = dark green, level 3 = black, level 4 = dark red, level 5 = light red. Arrows A and B indicate distribution of upward LUI levels for ALB (line A) and HAI (line B) sites. Circles indicate clustering of SCH-MB (circle C) and of SCH-NM (circle D) sites. ALB = Schwäbische Alb (circle), HAI = Hainich-Dün (triangle), SCH-NM = Schorfheide-Chorin, peat soil (rhomb), SCH-MB = Schorfheide-Chorin, mineral soil (square).

This points out that regional effects may play a larger role than originally assumed.

These observations might be of relevance for the development of more site specific management techniques including mainly soil type and carbon contents of soils. To understand the dynamics of ammonium in soil in a better way however, data on the organic nitrogen turnover is also needed, which obviously triggers ammonium dynamics to a far larger extent than the abundance and activity of ammonia-oxidizers.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2013.11.007.

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Supplemental Information Manuscript 2

Table S1: Thermal profiles, primer used and standards used for real-time PCR quantification

Target gene	Standard source	Primer	Primer reference	Thermal profile	No. of cycles	Primer
amoA AOA	Fosmid clone 54d9	amo19F	Leininger et al., 2006	94°C, 45 s / 55°C, 45 s / 72°C, 45 s	40	0.5
		CrenamoA16r48x	Schauss et al., 2009			
amoA AOB	*Nitrosomonas sp.*	amoA1F	Rotthauwe et al., 1997	94°C, 60 s / 58°C, 60 s / 72°C, 60 s	40	0.5
		amoA2R	Rotthauwe et al., 1997			
nifH	*Sinorhizobium meliloti*	nifHF	Rösch et al., 2002	95°C, 45 s / 55°C, 45 s / 72°C, 45 s	40	0.5
		nifHR	Rösch et al., 2002			

Table S2: Correlation matrix for land-use intensity. Correlation coefficients are given for each region and across all regions to test linear correlation of LUI-index total nitrogen and organic carbon, soil pH, nitrate and ammonium concentrations. ALB = Schwäbische Alb, HAI = Hainich-Dün, SCH-NM = Schorfheide-Chorin, peat soil, SCH-MB = Schorfheide-Chorin, mineral soil.

	total nitrogen	total organic carbon	soil pH	nitrate	ammonium
ALB	0.371	0.213	- 0.055	0.626	- 0.342
HAI	0.096	- 0.025	- 0.021	0.487	- 0.564
SCH-NM	- 0.228	- 0.284	0.323	0.614	- 0.371
SCH-MB	- 0.258	- 0.269	- 0.323	- 0.225	0.124
all regions	0.018	- 0.038	0.033	0.482	- 0.252

Table S3: Correlation matrix for total organic carbon and soil pH. Correlation coefficients are given for each region and across all regions to test linear correlation of total organic carbon and pH to AOA and AOB abundances. ALB = Schwäbische Alb, HAI = Hainich-Dün, SCH-NM = Schorfheide-Chorin, peat soil, SCH-MB = Schorfheide-Chorin, mineral soil.

	total organic carbon		soil pH	
	AOA	AOB	AOA	AOB
ALB	0.208	0.259	- 0.278	- 0.260
HAI	0.219	0.110	0.216	0.066
SCH-NM	0.291	0.511	0.026	- 0.460
SCH-MB	0.069	0.039	0.606	0.129
all regions	0.165	- 0.009	0.101	- 0.116

Figure S1: Potential nitrification rates across different land-use intensities. Land-use intensities are grouped into consecutive levels: level 1 corresponds to extensive, level 5 to intensive land-use. Potential nitrification rates are depicted for ALB, HAI, SCH-NM and SCH-MB. ALB = Schwäbische Alb, HAI = Hainich-Dün, SCH-NM = Schorfheide-Chorin, peat soil, SCH-MB = Schorfheide-Chorin, mineral soil.

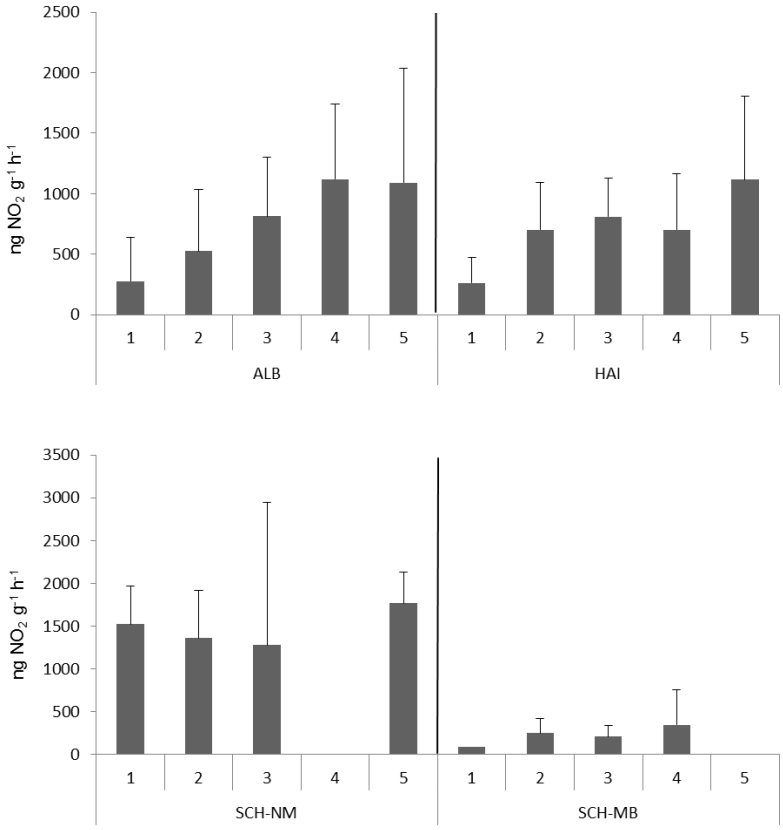
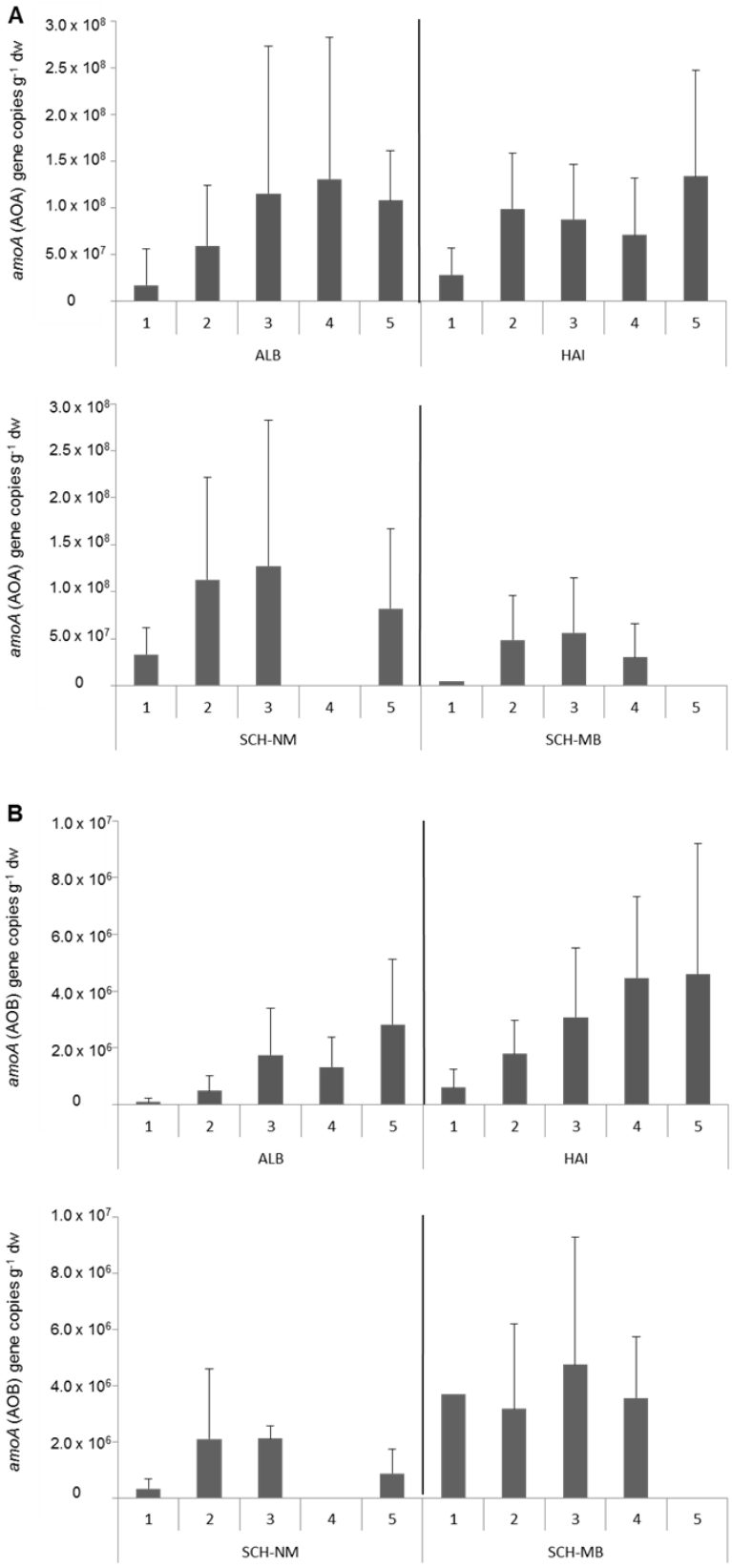


Figure S2: Gene copy numbers for *amoA* across different land-use intensities. Land-use intensities are grouped into consecutive levels: level 1 corresponds to extensive, level 5 to intensive land-use. Abundances for *amoA* gene for AOA (Figure 2A) and for AOB (Figure 2B) are depiced for ALB, HAI, SCH-NM and SCH-MB. ALB = Schwäbische Alb, HAI = Hainich-Dün, SCH-NM = Schorfheide-Chorin, peat soil, SCH-MB = Schorfheide-Chorin, mineral soil.


A
amoA (AOA) gene copies g-1 dw
3.0 x 10^8
2.5 x 10^8
2.0 x 10^8
1.5 x 10^8
1.0 x 10^8
5.0 x 10^7
0
1 2 3 4 5 1 2 3 4 5
ALB
HAI
amoA (AOA) gene copies g-1 dw
3.0 x 10^8
2.5 x 10^8
2.0 x 10^8
1.5 x 10^8
1.0 x 10^8
5.0 x 10^7
0
1 2 3 4 5 1 2 3 4 5
SCH-NM
SCH-MB
B
amoA (AOB) gene copies g-1 dw
1.0 x 10^7
8.0 x 10^6
6.0 x 10^6
4.0 x 10^6
2.0 x 10^6
0
1 2 3 4 5 1 2 3 4 5
ALB
HAI
amoA (AOB) gene copies g-1 dw
1.0 x 10^7
8.0 x 10^6
6.0 x 10^6
4.0 x 10^6
2.0 x 10^6
0
1 2 3 4 5 1 2 3 4 5
SCH-NM
SCH-MB

C Manuscript 3

Spatial and temporal dynamics of a grassland nitrogen cycling microbial community at the m^2 scale

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Running Head: Temporal and spatial variability of nitrogen cycling microorganisms

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ABSTRACT

The heterogeneity of soil presents challenges of scale, both spatial and temporal, to the understanding of soil microbial community interactions. To characterize the spatial and temporal dynamics of the nitrogen cycling microbial community at the meter scale, we sampled an unfertilized temperate grassland plot (10 m x 10 m) in southwest Germany six times over one growing season. Our objectives were to identify spatial and temporal niche differentiation patterns in the nitrogen-cycling microbial community members and potential enzyme activities, as well as their association with stages of plant growth. We tested potential linkages among abundances of marker genes responsible for nitrogen fixation, ammonia-oxidation, denitrification, and potential nitrification and denitrification enzyme activities. Gene abundances exhibited significant temporal variability but little spatial variability and no discernible spatial structure at this scale. Both potential activity measurements, however, were spatially structured at dates corresponding to periods of rapid plant growth. Differences in temporal and spatial variability between marker genes and their respective potential activity measurements suggest both short-lived temporal niche partitioning and a degree of microbial functional redundancy. Our results indicate that temporal variability is more discernible than spatial variability among nitrogen cycling microorganisms at the studied scale.

Keywords: ammonia-oxidation, denitrification, potential enzyme activity, biogeography, grassland

INTRODUCTION

Soils are considered hotspots of microbial diversity. Recent estimates have indicated that in addition to a large number of fungi, protists and other micro-eukaryotes, one gram of soil may harbor more than 1 million bacterial and archaeal species (Paul 2014). This enormous biodiversity is a result of multiple interfaces with differing biogeochemical properties that are formed in soil as a result of interactions between microbes and their abiotic environment (Totsche *et al*., 2011). Not surprisingly, the issue of "scale" has become a topic of interest in microbial soil ecology in recent years. Studies at different scales have identified different influences on the soil microbiome and its functions. For example, Grundmann *et al*. (2001), using a modelling approach, demonstrated significant differences in microbial communities which catalyze processes of nitrification and denitrification in different soil compartments at the sub-millimeter scale. At this scale, individual substrates or physicochemical properties have been identified as drivers for microbial community development, while at field scales cultivation regimes, landscape gradients, edaphic factors, and topography have been shown to drive the spatial distribution of microbes (Ettema & Wardle 2002). At the landscape scale, factors such as soil type, climate, and rainfall regimes influence microbial communities and their functional traits (Lauber *et al*., 2008; Bru *et al*., 2011; Parker & Schimel 2011; Hartmann *et al*., 2013). Most spatial studies of the nitrogen cycling microbial community have to date been done at field (Hallin *et al*., 2009; Enwall *et al*., 2010) and regional scales (Philippot *et al*., 2009 (a); Petersen *et al*., 2012).

The importance of temporal variation in microbial communities has also become clearer, and should therefore be included as a component of a study scale in addition to spatial heterogeneity. For example, the relationship of natural vegetation cycles to temporal heterogeneity in soil has been demonstrated (Herron *et al*., 2013; Lauber *et al*., 2013). Temporal patterns are now also known to exhibit hierarchies of scale, with those temporal differences providing different answers to questions of controls on processes (Bardgett *et al*., 2005). Temporal studies capture transient changes in individual variables over time, providing insight into processes that may be important but ephemeral (Burke *et al*., 1997). In contrast, spatial analyses provide insight into biogeographical distributions and links between microorganisms and their soil environment, but can mask temporal variability.

Studies in which both temporal and spatial aspects are addressed are rare, although there is clear evidence that both factors interact strongly at multiple scales (Chapin *et al*., 2002). Over short time periods, such as days, weeks, or plant growth periods, temporal variation in factors such as root exudates and ephemeral changes in substrate availability may be more influential at the plot scale

than edaphic factors, such as pH, CN ratios, or soil texture (Burke *et al*., 1997; Bardgett *et al*., 2005). Edaphic factors may in turn interact with spatial and temporal abiotic factors such as micro-climatic conditions, resulting in temporal or spatial niche differentiation among, for example, those members of the microbial communities which perform the same steps in inorganic N-cycling. As a consequence, the concept of hotspots (Parkin 1983; Nunan *et al.*, 2003) has been expanded to include hot moments (Groffman *et al*., 2009; Kuzyakov & Blagodatskaya 2015). The duration of hot moments, however, is highly variable, and observed interactions among members of the nitrogen cycling microbial community vary depending on the choice of time scale.

In this study we investigated the dynamics of soil microbial communities in a small grassland plot over a season to gain understanding of temporal and biogeographical patterns of microorganisms involved in soil inorganic nitrogen cycling. One 10 m x 10 m plot was intensively sampled both temporally and spatially; six times over a complete growing season from early April, before plants had begun to actively grow, until November of the same year when plant growth had ceased after a hard frost. Sampling times were selected to coincide with plant growth stages. We selected an unfertilized grassland because it provided high plant biodiversity and low agricultural management (Regan *et al*., 2014). As plants depend solely on soil-available nitrogen, most of which is derived from fixation of atmospheric nitrogen and nitrogen release from dead biomass through mineralization, there is a strong link between microbial performance and plant growth in unfertilized grasslands. Resource availability for microbes in soil is modified as well by the degree of plant diversity, and this can influence niche differentiation among members of the nitrogen cycling microbial community (Zak *et al*., 2003; Eisenhauer *et al*., 2010; Lange *et al*., 2014).

We used a molecular approach based on quantitative PCR amplification of marker genes to quantify microbial populations involved in nitrogen fixation, ammonia-oxidation, and denitrification. We also determined potential enzyme activity patterns for both nitrification and denitrification to provide an additional evaluation of the activity potential of both nitrifiers and denitrifiers in the field under the more controlled conditions of the laboratory. Our objectives were to characterize the temporal and spatial variability of microorganisms involved in inorganic N-turnover and to gain insight into the relationship between stages of plant growth and the nitrogen cycling microbial community at the meter scale. The study is descriptive; its purpose is to observe and understand these processes in the complexity of a field setting in order to refine further experimental studies, which are lacking in unfertilized grasslands at the meter scale.

MATERIALS AND METHODS

Site description

The present study is part of a larger, interdisciplinary project of the German Biodiversity Exploratories (Fischer *et al*., 2010). The study site is located in the Swabian Alps, a limestone middle mountain range in southwest Germany, near the village of Wittlingen, Baden-Württemberg (48°25´0.01´´ N, 9°30´0.00´´ E)**.** One 10 m x 10 m plot was established within a larger grassland site that is managed at low intensity – no fertilizer added, mown once per year and grazed briefly by sheep for 1 - 2 weeks in late summer or early autumn. Annual precipitation in 2011, the year in which samples for this study were collected, was 810 mm and average temperature was 8.1°C. FAO classification of the soil type is Rendic Leptosol, a calcareous, shallow A-C soil (typically 10 cm deep), with average pH of 6.7, organic carbon (C) of 66.0 mg g^{-1}, and total nitrogen (N) of 7.0 mg g^{-1}. Soil texture is silt of mean 84%, clay of mean 15%, and sand less than 2%. C, N, and pH values were uniform over the sampling period. The site has been managed without added fertilizers since at least 1994.

Sample design

A 10 m x 10 m plot was established within the grassland site and divided into 30 subplots (each 2 m x 1.67 m). Within each subplot six pairs of sampling locations were randomly assigned, with one pair sampled at each of six dates over one growing season. Sample pairs were separated by 50 cm to provide appropriate lag distances for later geostatistical analyses. A detailed description of the sample design can be found in Regan *et al.,* (2014). Over the season, 360 total samples were collected (60 per date x 6 dates). Sampling dates were chosen to correspond to stages of plant growth on the plot: April 5th, the beginning of the vegetation period; May 17th, during the main growth phase; June 27th, at peak plant biomass; August 16th, two weeks after mowing; October 5th, nine weeks after mowing during a second period of plant growth; and November 21st, after the first frost, when plants had senesced.

Sample collection

Soil samples were collected with core augers (58 mm diameter) to 10 cm depth. The top one cm, consisting entirely of undecomposed plant residues, was removed from each core to avoid introduction of plant material into the soil samples. Cryovials for DNA extraction were filled with homogenized soil (sieved at 5 mm), frozen in liquid nitrogen in the field, and stored afterwards at -20°C. Physical (soil texture, pH, soil moisture, and bulk density), chemical (ammonium, nitrate, phosphate, C, N, extractable organic carbon – EOC, and extractable organic nitrogen – EON), biological soil properties (C_{mic}, N_{mic}, bacterial and fungal phospholipid fatty acids – PFLAs), and roots,

litter, as well as aboveground biomass of grasses, legumes, and forbs were analyzed as described in Regan *et al.,* (2014).

DNA extraction and quantification of marker genes

DNA was extracted from duplicate homogenized soil subsamples (300 mg each) using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) following the manufacturer's protocol. Concentrations of DNA extracted from both sample replicates were measured independently on a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA), then pooled and re-measured to confirm the final DNA concentration of each sample. Samples for quantitative real time PCR (qPCR) measurement were diluted with ultra-pure water to a target concentration of 5 ng DNA μl^{-1} The following microbial groups were measured by qPCR: abundance of bacterial16S rRNA as proxy for the total bacterial community; abundance of archaeal 16S rRNA to assess the total archaeal community; *nifH* gene to assess the nitrogen fixing community; genes encoding the catalytic subunits ammonia monooxygenase enzymes of archaea (*amoA* AOA) and bacteria (*amoA* AOB) to evaluate the ammonia-oxidizing community; and *nirS*-, *nirK*- and *nosZ*-type denitrifier genes, which encode cytochrome cd_1 heme nitrite reductase, copper-nitrite-reductase, and nitrous oxide reductase, respectively, for the denitrifying community. Amplification of the qPCR products for nitrogen fixers and ammonia-oxidizers was conducted on a 7300 Real-Time PCR System, (Applied Biosystems, Foster City, CA, USA). The 25 µl reaction mixture was composed of Power SYBR Green master mix (12.5 µl), BSA (3%, 0.5 µl), respective primers (10pmol, 0.5 µl), DEPC and template DNA (2 µl). Bacterial 16S rRNA, *nirS* and *nirK* were analyzed on a Fast Real-Time 7500 PCR (Applied Biosystems, Foster City, CA, USA). The 15 µl reaction mixture was composed of 4.125 µl ultra-pure water, 0.75 µl each of forward and reverse primers, 7.5 µl SYBR Green master mix, 0.375 µl T4, and 1.5 µl template DNA. Amplification of *nosZ* was done on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with a 25 µl reaction mixture consisting of 8.25 µl ultra-pure water, 12.5 µl SyBr Green master mix, 1.0 µl each of forward and reverse primers, 0.25 µl T4, and 2.0 µl template DNA. To control the specificity of qPCR products and their correct fragment size, a melt curve analysis (dissociation stage) and/or a gel electrophoresis on a 2% agarose gel were performed after each run. Standard curves were obtained with serial plasmid dilutions of the respective genes. Thermal profiles, primers, the standards used and references for all analyses are given in Table S1.

Potential activity measurements

The potential nitrification assay (PNA) was performed according to the procedures described in Hoffmann *et al.* (2007). For determination of potential nitrification rates, 10 ml of ammonium sulfate solution (10 mM) were added as substrate to 2.5 g fresh weight of soil. The transformation of

nitrite to nitrate was inhibited by addition of 50 µl of sodium chlorate (1.5 M). After an incubation of 5 h with shaking at 25°C, the reaction was stopped by applying 2.5 ml potassium chloride (2 M), followed by an additional incubation period of 20 min. Samples were centrifuged for 2 min at 2000 x g and 150 µl of supernatant was transferred to each of 96 well plates. After the addition of 90 µl of ammonium chloride buffer and 60 µl of nitrite determination reagent (naphtylenediamine dihydrochloride (2 mM), sulphanilamide (0.06 M), phosphoric acid (2.5 M) to each sample, the subsequent color reaction was measured at a wavelength of 540 nm on a spectrometer (SpectraMax 340, MWG BIOTECH, Germany) and used for subsequent calculations of nitrite produced and expressed as µg NO_2^--N g^{-1} dry soil h^{-1}. For controls, the potassium chloride solution was added prior to incubation. Additionally, reagents without soil samples served as negative controls.

Denitrifying enzyme activity (DEA) was determined using a modified assay of Smith & Tiedje (1979), without the addition of chloramphenicol as preliminary assays showed that no de novo synthesis of denitrifying enzyme occurred within a 2-h incubation period. In brief, 2 g field-moist soil was incubated in air-tight bottles (inner volume 118 ml) with 5 ml solution containing 1.1 mM KNO_3 and 1 mM glucose. Anaerobic conditions were established by evacuating and flushing the headspace with N_2 gas three times. From each bottle 10 ml N_2 was removed and replaced by 10 ml acetylene (C_2H_2) to inhibit nitrous oxide reductase activity. Bottles were incubated at 25°C on a horizontal shaker (150 rpm). Headspace samples (1 ml) were taken after 30, 60, 90 and 120 min from each bottle and transferred into evacuated 5.9 ml septum-capped exetainers (Labco Ltd., UK). These samples were diluted with 10 ml N_2 before gas chromatographic analysis (Agilent 7890 gas chromatograph equipped with an ECD detector, Agilent, Santa Clara, CA, USA). Potential N_2O release (µg N_2O-N g^{-1} dry soil h^{-1}) from soil was calculated from the linear regression of N_2O concentration against time.

Statistical analyses

All statistical analyses were carried out in the R environment (R Development Core Team 2012). To test whether functional gene abundances and enzyme activities exhibited significant changes by sampling date, one-way ANOVAs with sampling date as a factor were performed, followed by Tukey's HSD post-hoc test at the significance level of $P< 0.05$.

To test whether spatial autocorrelation could be determined in gene abundances or in potential enzyme activities at any given date, empirical semivariograms were assessed using the gstat 2.4.0 Package (Pebesma 2004). Empirical semivariograms were calculated for all functional genes and for potential enzyme activities to a maximum distance of 8 m for each date. Where spatial structure was evident, a spherical, exponential, or linear model was then fitted based on RSME and

visual control. When a model could be fitted, an interpolated (kriged) map of the distribution of that property on the plot could be constructed. Maps were constructed using ArcGIS (ESRI 2010, Environmental Research Institute, Redlands, CA, USA).

Mantel tests were employed to determine whether the measured marker genes and potential enzyme activities were correlated to other aspects of the community as a function of spatial separation, and of how the measured genes, as representing the nitrogen cycling microbial community, were related to multivariate descriptors of the environment. A Mantel test is a regression in which the variables are similarity or distance matrices of all pairwise comparisons among samples; the Mantel statistic (rM) is computed by determining the sum of the cross-products of the corresponding values in each of these matrices (Franklin & Mills 2009). The significance of the resulting correlation is determined using a Monte Carlo approach with multiple permutations. Distance matrices, using Euclidean distance, were calculated between all pair-wise combinations of samples for marker genes, potential enzyme activities, plant functional groups, soil environmental conditions, and geographic location, using the R package 'vegan' (Oksanen *et al*., 2013). Matrices were constructed as follows: 1) spatial distances among pairs of sampling points using their unique x and y coordinates, 2) distances in abiotic and soil chemical properties as measures of the environment (soil moisture, bulk density, texture, pH, soil organic C, soil total N, EOC, EON, NH_4^+, NO_3^-, and PO_4^{-3}); 3) distances in biomass of different plant functional groups (grasses, forbs, and legumes); 4) distances in the marker gene abundances; and 5) distances in both PNA and DEA. Environmental variables were scaled to unit variance and zero mean to account for different units of measurement. Environmental matrices were then tested against measured marker genes, plant functional groups and potential enzyme activity measurements with soil abiotic properties and soil chemical properties analyzed both separately and together. Functional genes were standardized with respect to one another for each sample. Mantel statistics were then calculated for all pairs of distance matrices using the default settings of 999 permutations in the R package 'vegan' (Oksanen *et al*., 2013).

RESULTS

Temporal changes in abundance of measured marker genes and potential enzyme activities

One way-ANOVA indicated significant differences in the abundances of both the archaeal and bacterial 16S rRNA genes ($P < 0.05$) over the sampling season. Archaeal 16S increased from April to May, decreased from May to October, and increased again at the last sampling time point in November (Table 1). Changes in abundance of the bacterial 16S rRNA gene contrasted with that of

the archaeal 16S rRNA gene. Bacterial 16S rRNA gene abundance decreased at the first two sampling dates, and increased in June, after which its abundance remained high for the duration of the season (Table 1). Over the entire season, abundance of the archaeal 16S rRNA gene was two orders of magnitude lower than that of its bacterial counterpart.

The abundance of the *nifH* gene, which was used as a marker for nitrogen fixing microbes, was highest in May and lowest in August and October; at the other sampling times values did not significantly differ from one another (Fig. 1 a). Abundance of ammonia-oxidizing archaea (AOA), based on the quantification of the archaeal *amoA* gene, was three to four orders of magnitude higher than that of its bacterial counterpart AOB, and did not vary significantly at the first three sampling dates (April-June) (Fig. 1 a). It was lowest in August and highest in November, while the abundance of AOB changed in the opposite direction from that of AOA over most of the sampling period (Fig. 1 a).

Denitrifiers harboring the *nirK* gene declined from April to June sampling dates with lowest abundance in June, significantly different from all other sampling dates, and significantly highest in November (Fig. 2 a). Abundance of *nirS* harboring denitrifiers, in contrast, was highest in April and lowest in May and October, different from *nirK* harboring denitrifiers (Fig. 2 a). Abundance of *nosZ* harboring denitrifiers was also significantly highest in April (Fig. 2 a). In October, *nosZ* gene copy numbers had dropped by an order of magnitude from earlier measured values. In November *nosZ* gene copy numbers increased, but never reached the high values of April and August (Fig. 2 a).

Activity measurements of PNA varied over the sampling period, with significantly highest activities in April and November, while from May through October differences were lower but not significantly different from one another (Fig. 1 b). Although DEA appeared more variable than PNA, both its highest (June and October) and its lowest activities (August) shared significance with other sampling dates (Fig. 2 b). No significant correlations between PNA and abundance patterns of AOA or AOB either separately or summed could be determined (data not shown). There were also no correlations between *nirK* and DEA, *nirS* and DEA, or the sum *of nirK* and *nirS* with DEA (data not shown).

Relationships between abundances of marker genes and potential enzyme activities with soil properties over time

Soil abiotic properties at this studied plot have previously been described in detail (Regan, *et al*., 2014). Briefly, bulk density, pH, soil C and N, and C/N ratios were stable over the sampling period. Soil moisture, however, varied over the same period; lowest in May and October (Fig. S1). Only *nirS* and *nosZ* exhibited a correlation with soil moisture (Fig. 3 a, b). Neither 16S rRNA genes (archaeal and bacterial) nor AOA, AOB, *nifH*, or *nirK* showed a relationship to soil moisture. Potential enzyme

activity measurements were also not correlated with soil moisture (data not shown). The strength of the correlations of soil chemical properties NH_4^+, NO_3^-, and EOC to changes in soil moisture varied. NH_4^+ exhibited a strong positive relationship to soil moisture, while NO_3^- and EOC exhibited weaker positive correlations with soil moisture (Fig. 3 c - e).

Correlations of the different marker genes and potential enzyme activities with their putative substrates were also examined. No significant correlations could be determined for AOA or AOB with respect to NH_4^+. Among the denitrifiers, only *nirS* and *nosZ* exhibited weak positive relationships to NO_3^-, and *nosZ* was also positively correlated with EOC (Fig. 4 a - c). Neither PNA nor DEA were influenced by their putative substrates NH_4^+ and NO_3^- respectively (data not shown), but both were positively, albeit weakly, related to total N (Fig. 4 d, e).

Spatial variability and spatial structure

Spatial variability of both the measured marker genes and potential activities at every sampling date was low, as indicated by the small standard errors for each time point (Figs. 1 and 2). Geostatistical analyses of abundances of all marker genes, whereby empirical variograms were determined from measured data, also indicated no spatial structure at our sampling scale using the given settings for geostatistical analyses (data not shown). Potential activity measurements, by contrast, were spatially structured and could be spatially modeled at this scale at several sampling dates, although they also exhibited very low spatial variability at all dates. The resulting empirical variograms made it possible to fit model variograms to PNA in June, August and October; and to DEA in June, October, and November. For the two dates at which both PNA and DEA distributions could be fitted with spatial models, June and October, kriged maps were generated in order to visualize and compare their spatial distributions on the plot (Fig. 5 a - d).

Multivariate spatial relationships

Mantel tests were used to identify spatial correlations at the community level; between soil abiotic and chemical properties, plant functional groups, the measured marker genes and their potential enzyme activities. Soil abiotic and chemical properties were strongly spatially correlated from April through August, but not in October or November. The measured marker genes were not spatially correlated at our sampling scale at any date. Potential enzyme activities were correlated with space only in November (Table 2).

Mantel tests of the measured marker genes with plant functional groups were not significant at any date. Plants and potential enzyme activities were spatially correlated only in May, and genes and enzyme activities were spatially correlated only in October. No spatial correlations between soil abiotic properties and plants or soil abiotic properties and the analyzed genes could be determined.

Soil abiotic properties and potential enzyme activities, however, were spatially correlated at all dates (Table 2).

DISCUSSION

Reviews by Groffman *et al*. (2009) and Kuzyakov & Blagodatskaya (2015) emphasized the importance of both temporal and spatial dynamics in soil function, expanding on the concepts of "hot spots and hot moments". In this study we investigated spatial and temporal dynamics of microbial community patterns involved in inorganic N-turnover as well as their potential activities at the scale of meters over an entire vegetation period, taking into account the influence of plant growth stage on nitrogen availability and the resulting competition with the microbial community for substrate. Potential enzyme activities associated with both nitrification and denitrification exhibited clear spatial structure at the dates of rapid plant growth on the plot. PNA changed in the same direction as AOB abundance early in the season, but changed in direction similar to AOA and *nifH* later in the year, while DEA was highest at the dates corresponding to the appearance of legumes, June and October (Regan *et al* 2014) on the plot. Le Roux *et al* (2013) demonstrated similar associations of DEA with legumes. Overall, the spatial response patterns of the investigated groups of marker genes did not vary significantly at our sampling scale, but clear temporal differences were detected.

Spatial heterogeneity

PNA and DEA were spatially structured at the scale of our plot at some dates although neither exhibited high spatial variability at any given time point. Interestingly, the dates with the strongest spatial structure corresponded to periods of rapid plant growth and high plant biomass on the plot. Kriged maps of this spatial distribution showed areas of similar minima and maxima for both PNA and DEA (Fig. 5 a - d). Spatial structure at these dates was also observed in PLFA patterns and soil abiotic properties from previous analyses at this plot (Regan *et al*., 2014), suggesting that while it is difficult to directly connect above- and below-ground processes in grasslands, those links exist, but may be very short-lived. This structure weakened in November, perhaps as a result of plant senescence. The finding of Stempfhuber *et al*. (2014) that PNA was correlated to NO_3^- rather than to NH_4^+ could help explain the similarity in spatial distributions of PNA and DEA in June and October, suggesting that both were influenced by changes in availability of the same substrate, as both were correlated, albeit weakly, with total N (Fig. 4 d, e). The lack of spatial heterogeneity at the investigated scale with respect to measured abundance patterns of marker genes may be linked to the dense rooting system that is typical for grassland soils (Huyghe *et al*., 2014), resulting in

effectively homogeneous conditions at the scale of our measurements. However, soil mineral nitrogen is more completely utilized in high than in low plant diversity grasslands (Palmborg *et al*., 2005). Our inability to discern spatial heterogeneity in the measured marker genes at our sampling scale may have been compensated for by robust nitrogen mineralization activity over the season, making subtle temporal changes easier to detect.

Plant relationships to temporal heterogeneity of microbes responsible for N turnover in grassland soils

It is well accepted that plants can strongly influence microbial communities and their functional traits in the rhizosphere by the excretion of exudates during the growing season (Philippot *et al*., 2009 (b); Berg *et al*., 2014). However exudation is not constant; clear shifts in root exudation patterns, both in amount and quality, occur at different stages of plant growth (Ziegler *et al*., 2013; Huang *et al*., 2014). In addition to exudates, competition with plants for nitrogen is another factor driving the development of microbial communities, as the uptake of NH_4^+ during plant growth also often results in depletion of NH_4^+ in soils (Boudsocq *et al*., 2012; Kuzyakov & Xu 2013). This in turn can reduce the abundance of ammonia-oxidizers, especially at the plant-soil interface during periods of most rapid plant growth, typically in May. However, whereas in May AOA benefited from reduced ammonia availability, increasing slightly in abundance, in October AOA abundance was low, as was NH_4^+ (Regan *et al*., 2014). At this date, a second, smaller pulse of plant growth was observed, and the abundance of AOB remained high, possibly a result of additional N input due to N-fixation by rhizobia associated with legumes, which had re-appeared on the plot in October. AOB has been shown to increase in the presence of legumes (Malchair *et al*., 2010, Le Roux *et al*., 2013). While legumes were present on the plot in October, in May, by contrast, no legumes had yet appeared (Regan *et al*., 2014), so in May no nitrogen would have been available as a result of rhizobial activity. However, it has to be considered as well that the dynamics of *nifH* gene abundance were similar to archaeal *amoA* abundances from April to June. It remains elusive whether different key players could be associated with different groups of nitrogen-fixers: *NifH* gene abundances could also well be associated with free-living nitrogen-fixers providing lower fixation rates which might be in favor of AOA preferences (Meyer et al., 2013), while AOB are rather associated with nitrogen-fixing microbes in legumes, i.e. symbiotic nitrogen-fixation (Le Roux et al., 2013). Observed differences in the dynamics of AOA and AOB may also be attributable to differences in their ecophysiologies. AOA are capable of mixotrophic metabolic strategies; for example, the metabolization of organic carbon, while AOB are considered obligate autotrophs, solely dependent on NH_4^+ availability (Kowalchuk & Stephen 2001; Schleper *et al*., 2005; Daebeler *et al*., 2012; Zhalnina *et al*., 2012). AOB abundance was

generally highest at dates with high NH_4^+ concentrations (Regan *et al*., 2014), declining with decreasing NH_4^+, though this correspondence did not persist into October. At that date NH_4^+ was lowest, but AOB did not decrease. This suggests that AOA were able to out-compete AOB when NH_4^+ availability was low in May, which is in accord with previous studies (Di *et al*., 2010), but that the competitive environment may have shifted in October, making it possible for AOB to compete more effectively for limited NH_4^+ at this date. In November AOA increased, possibly due to the decrease in soil temperature, which has been associated with increased AOA (Rasche *et al*., 2011). Wessén & Hallin (2011) also identified patterns in AOA and AOB distributions similar to ours, with higher abundance and greater variability in AOA as compared to AOB over time. Our measured higher abundances of AOA compared to AOB in an unfertilized grassland are also consistent with data reported by others (Phillips *et al*., 2000; Okano *et al*., 2004; Leininger *et al*., 2006). Over time, the dynamics of AOA were most similar to those of total archaea, indicating that a large proportion of soil-borne archaea in the investigated grassland plot were capable of oxidizing ammonia. Similar to ammonia-oxidizers, denitrifiers, specifically those harboring the *nirS* and *nosZ* genes, were also influenced by substrate availability, nitrate and EOC respectively.

In addition to substrate availability, soil moisture dynamics contributed to changes in the abundances of denitrifiers. Lange et al (2014) demonstrated that soil moisture plays a key role in the interactions between plants, abiotic soil properties and microbial biomass. An increase in soil moisture triggers both microbial activity and reduced transport of oxygen, thus a decrease of redox potential is likely, in turn promoting the growth of microbes which can use alternative electron acceptors such as nitrate. There is some evidence that both *nirS* and *nos*Z harboring denitrifies are more sensitive to soil moisture than those microbes harboring *nirK* (Regan *et al*., 2011; Petersen *et al*., 2012). All quantified marker genes for denitrification declined from April to May, corresponding to decreasing soil moisture, while *nirS* increased and *nosZ* increased in May, June and August, corresponding to increasing soil moisture. However, in October, a steep decline in *nosZ* gene abundance was observed. Recent work has identified heretofore unknown *nosZ*-carrying microorganisms (Sanford *et al*., 2012; Graf *et al*., 2014; Jones *et al*., 2014), some of which do not harbor known genes for other reductase-driven denitrification steps. The measured *nosZ* values in our study represent the sum of *nirS* and *nirK* denitrifiers plus their associated *nosZ* as well as additional microorganisms that carry only *nosZ*. It is possible that this last group of microorganisms were influenced by a transitory condition in October that we did not identify, however, our study did not take this group into account.

In this study, only free living N-fixing microbes were analyzed, not those which live in nodules; therefore the increase in legume density in October is not reflected in our *nifH* data. The high abundance of N-fixing microbes in May could have resulted from increased exudation levels of the plants, but in October their abundance declined during the second period of plant growth. At this time, carbon could have been allocated directly to the symbionts of the legumes living in the nodules (Simms & Taylor 2002; Bever *et al*., 2009).

Notably, *nirK* decreased in June to its lowest point of the sampling period. This could have been due to competition with plants for copper, as the denitrifying gene *nirK* is a member of the multi-copper oxidase metalloprotein family, requiring copper to produce the nitrite reductase enzyme (Zumft 1997). Although we did not measure copper availability in our plot, copper deficiencies have been identified in up to 25% of German soils, especially calcareous soils with relatively high organic matter (Alloway 2005), a description which fits the soil on our plot. The steep decline in *nirK* in June corresponded to the period when plant demand for copper was highest; that of flowering, pollen formation, and seed setting (Alloway 1995; Marschner 1995). Enwall et al (2010) also saw a transitory relationship between soil copper levels and *nirK* abundances, lending support to our speculation.

Conclusions

Transient soil abiotic and chemical properties clearly impacted abundances of the measured marker genes even though spatial shifts in microbial abundances could not be discerned at this sampling scale. A spatially structured response of potential enzyme activities to soil abiotic properties was detected at the dates of most rapid plant growth, however. Our study demonstrated that while small and ephemeral changes in soil environmental conditions can result in changes in gene abundances, these changes may not necessarily reflect process rates. Potential activity measurements may integrate the results of inorganic N-cycling by different members of the respective microbial communities over time; the observed stability of our measurements over the season may have reflected such temporal integration (Le Roux *et al*., 2013).

A more nuanced understanding of nitrogen cycling at multiple scales, both temporal and spatial, is critical given the rapid increase in nitrogen inputs to the biosphere. The observational nature of this study provides an overview at the meter scale which can be used to develop targeted experimental designs to resolve these processes at scales suitable for management. It also suggests some heretofore unidentified ephemeral soil conditions that merit further study.

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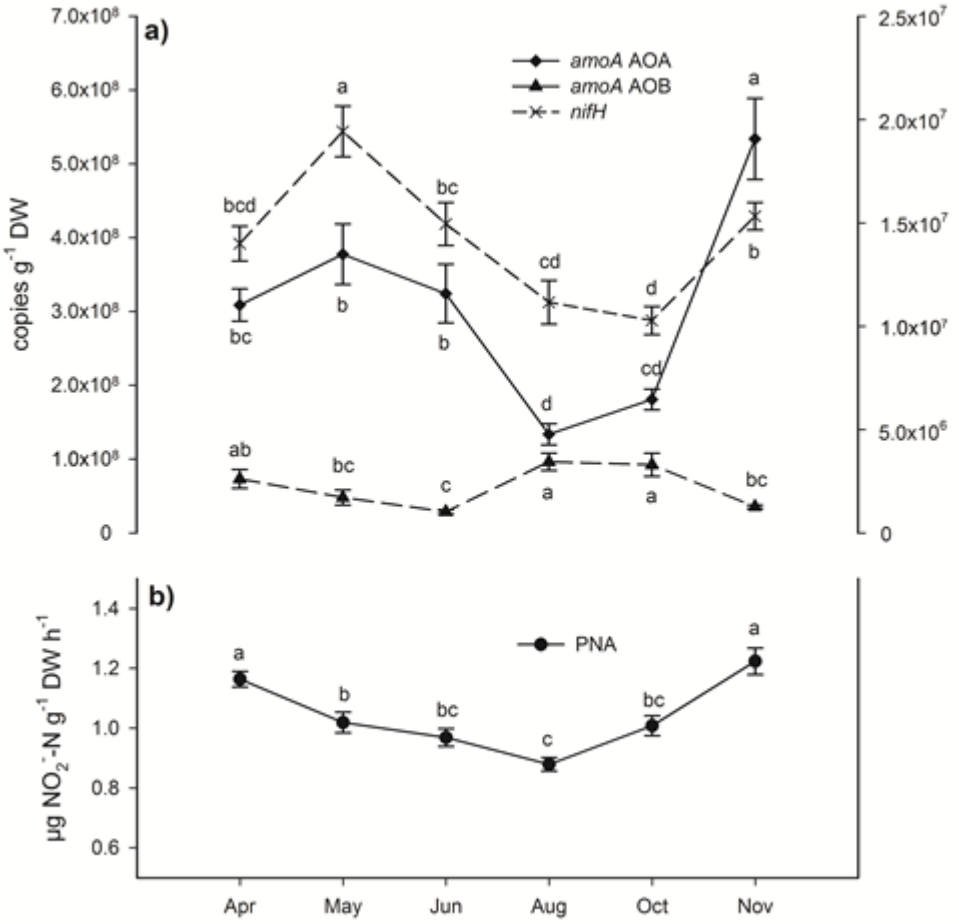

Figure 1:

a) Nitrogen fixing marker gene nifH and ammonia-oxidizing marker genes amoA of archaea (AOA) and bacteria (AOB), expressed as copies g-1 dry soil (DW). Solid line indicates that values are plotted on the left hand Y axis (AOA only). Broken lines indicate values are plotted on the right hand Y axis (both AOB and nifH). Different letters close to the points indicate significant differences between sampling dates for each measured gene. Differences were determined by one-way ANOVA followed by Tukey's HSD test (P< 0.05).

b) Potential nitrification enzyme activity (PNA). Units are µg NO2--N g-1 dry soil (DW) h-1. Different letters close to the points indicate significant differences between sampling dates for each measured gene. Differences were determined by one-way ANOVA followed by Tukey's HSD test (P< 0.05).

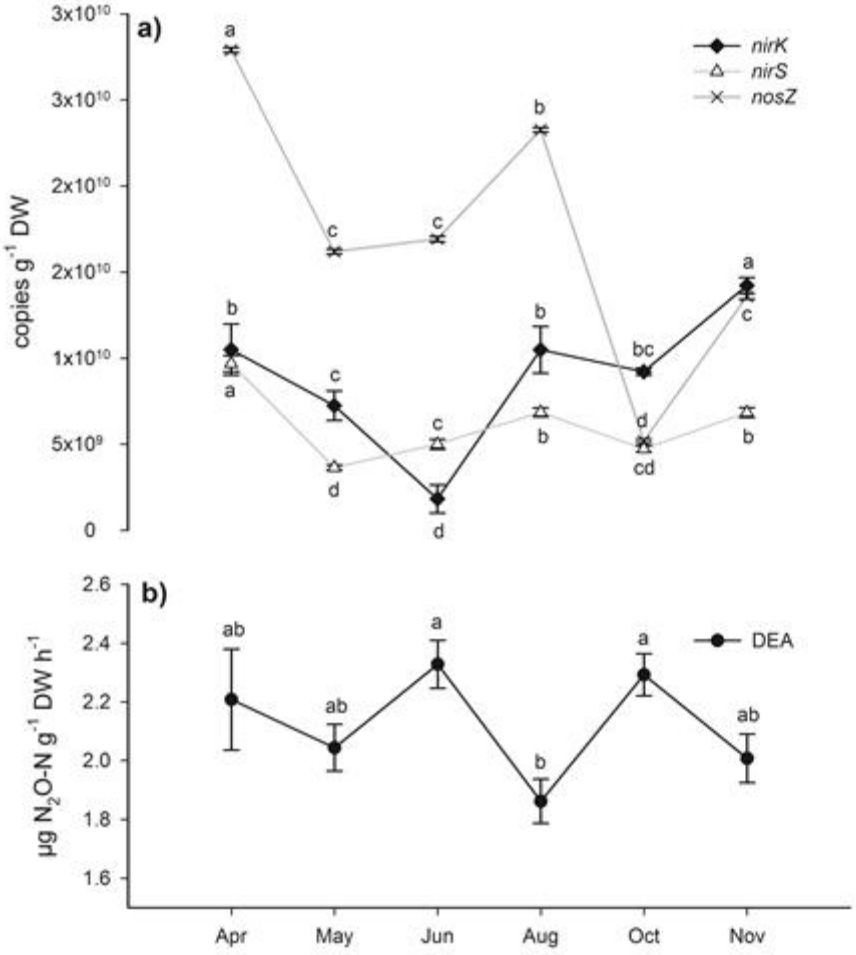

Figure 2:

a) Denitrifying marker genes nirK, nirS and nosZ expressed as copies g-1 dry soil (DW). Different letters close to the points indicate significant differences between sampling dates for each measured gene. Differences were determined by one-way ANOVA followed by Tukey's HSD test (P< 0.05).

b) Potential denitrifying enzyme activity (DEA). Units are µg N2O-N g-1 dry soil (DW) h-1. Different letters close to the points indicate significant differences between sampling dates for each measured gene. Differences were determined by one-way ANOVA followed by Tukey's HSD test (P< 0.05).

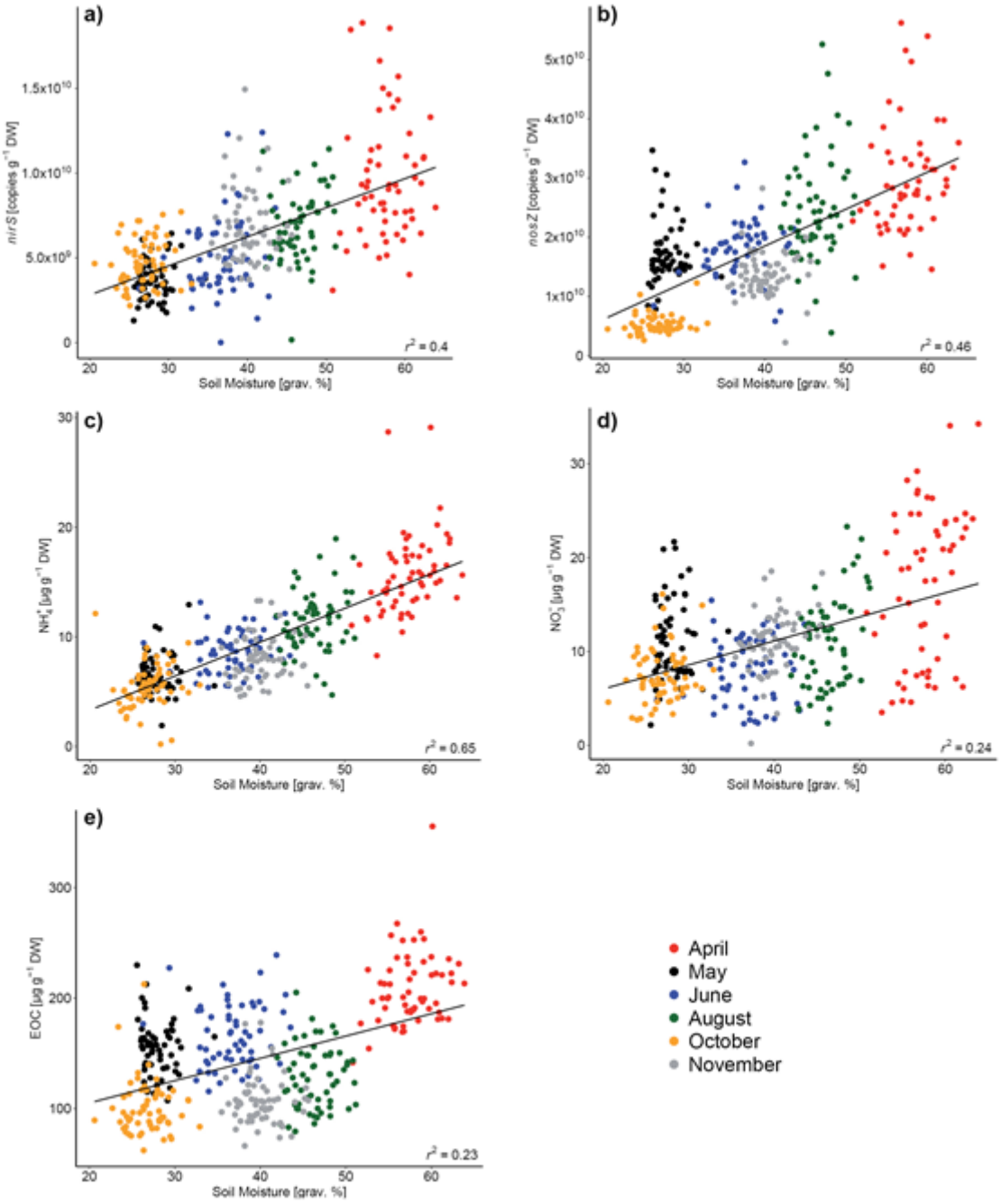

Figure 3: **Scatterplots of Pearson correlations between denitrifying marker genes** a) nirS and b) nosZ and of putative substrates c) NH4+, d) NO3- and e) EOC with soil moisture over the entire season. Sample dates are indicated by symbol color.

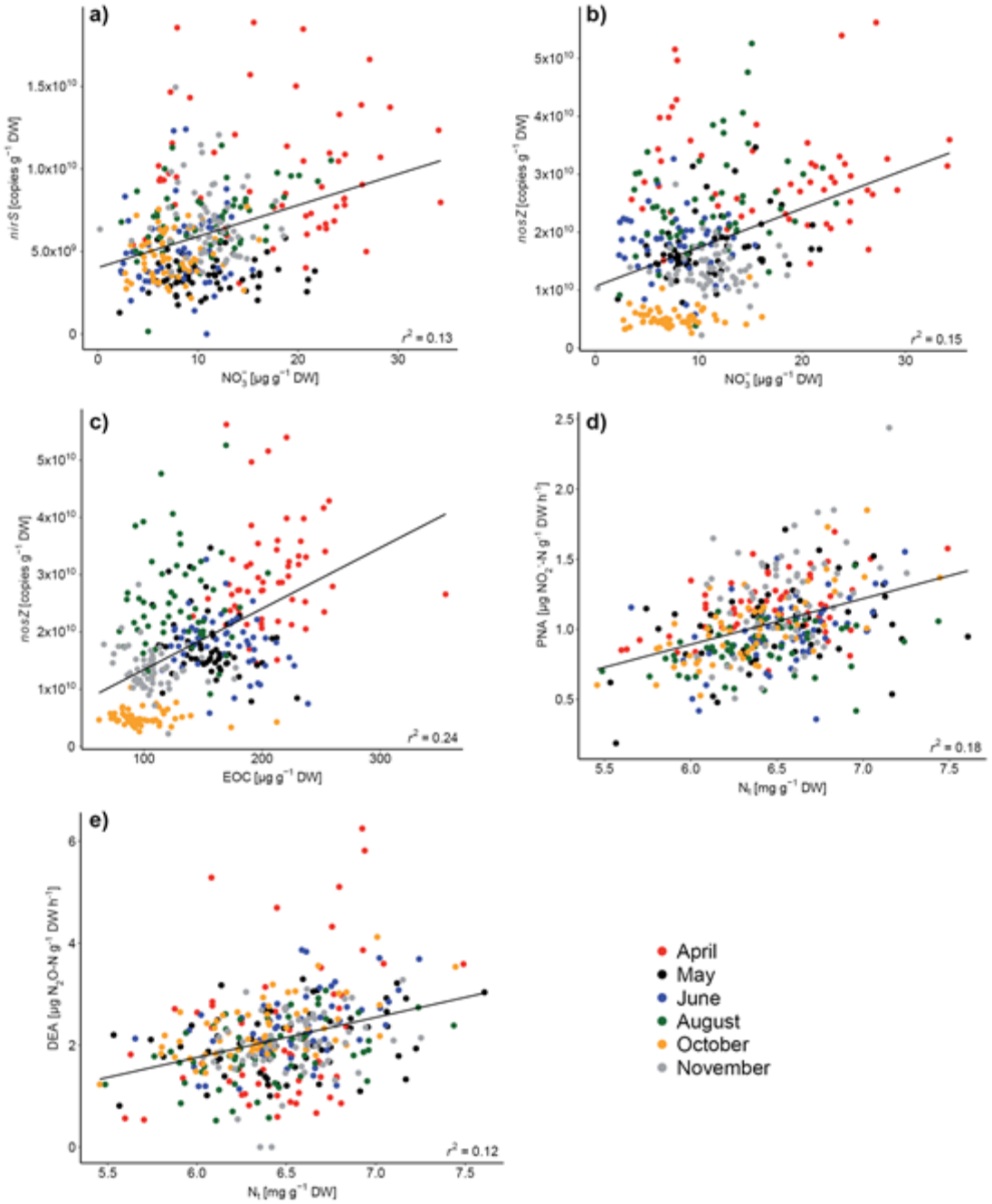

Figure 4: **Scatterplots of Pearson correlations between denitrifying marker genes** a) nirS and b) nosZ with NO3-, c) of nosZ with EOC, and correlations of potential activity measurements d) PNA and e) DEA with total soil nitrogen. Sample dates are indicated by symbol color.

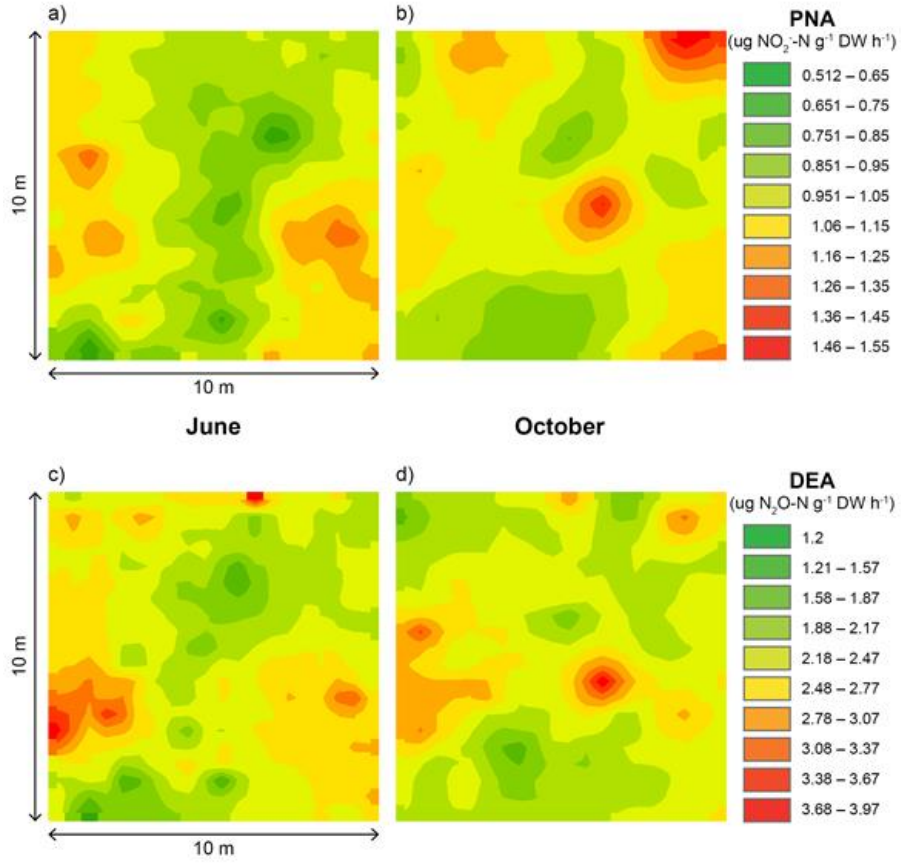

Figure 5: **Kriged maps** of potential nitrification enzyme activity (PNA) in a) June and b) October, and of denitrifying enzyme activity (DEA) also in c) June and d) October.

Date	Archaeal 16S rRNA (copies g^{-1} DW)	SE		Bacterial 16S rRNA (copies g^{-1} DW)	SE	
Apr	$2.1x10^{9}$	$1.4x10^{8}$	cd	$3.4x10^{11}$	$2.0x10^{10}$	b
May	$2.8x10^{9}$	$1.4x10^{8}$	ab	$1.9x10^{11}$	$7.3x10^{9}$	c
Jun	$2.4x10^{9}$	$1.5x10^{8}$	bc	$1.8x10^{11}$	$9.5x10^{9}$	c
Aug	$1.6x10^{9}$	$1.3x10^{8}$	de	$3.9x10^{11}$	$2.2x10^{10}$	ab
Oct	$1.5x10^{9}$	$1.1x10^{8}$	e	$3.6x10^{11}$	$2.7x10^{10}$	ab
Nov	$3.2x10^{9}$	$1.7x10^{8}$	a	$4.4x10^{11}$	$2.4x10^{10}$	a

Table 1: **Abundances of archaeal 16S rRNA and bacterial 16S rRNA genes** (copies g-1 soil dry weight (DW)). Values are expressed as mean with standard error (SE). Letters indicate significant differences by date based on one-way ANOVA followed by Tukey HSD with $P<0.05$.

Date	Space/ Abiotic	Space/ Plants	Space/ Genes	Space/ Enzymes	Abiot/ Plants	Abiot/ Genes	Abiot/ Enzymes	Plants/ Genes	Plants/ Enzymes	Genes/ Enzymes
APR	**0.193*** **	0.014	0.003	0.071	0.101	0.025	**0.229** **	0.029	-0.022	0.003
MAY	**0.146** **	-0.023	0.002	-0.056	0.050	-0.115	**0.266*** **	0.053	**0.115***	-0.082
JUN	**0.198** **	0.037	-0.022	0.052	0.106	-0.073	**0.287** **	-0.127	-0.090	-0.122
AUG	**0.213*** **	0.016	-0.036	0.012	-0.098	0.019	**0.206** **	0.081	-0.000	0.092
OCT	0.059	-0.018	-0.030	-0.040	0.030	-0.117	**0.375*** **	-0.088	0.045	**0.171***
NOV	0.042	**0.125** **	0.094	**0.089***	-0.018	0.061	**0.152***	0.073	-0.035	-0.093

Table 2: **Results of the Mantel tests including spatial structure (Space), soil abiotic properties (Abiotic), plant functional groups (Plants), enzyme-encoding genes (Genes), potential enzyme activities (Enzymes)** for the six sampling dates of this study in 2011. Pearson correlations (r-values) with significance assessed by permutation test. *P < 0.05, **P < 0.01, ***P < 0.001. Only values shown in bold are significant.

Supplemental Information Manuscript 3

Target/Primers	Sequence(5'-3')	Thermal Conditions
16S rRNA[1]	*Pseudomonas aeruginosa*	
341f	CCT ACG GGA GGC AGC AG	95 °C / 15 s, 60 °C / 30 s, 72 °C / 30 s, 75 °C / 15 s, 35 cycles
534r	ATT ACC GCG GCT GCT GGC A	
Arch 16S rRNA	*Methanobacterium sp.**	
rSAf(i)[2]	CCT AYG GGG CGC AGC AG	94 °C / 20 s, 55 °C / 60 s, 72 °C / 30 s, 5 cycles; 94 °C / 20 s, 50 °C / 60 s, 72 °C / 30 s, 35 cycles
958r[3]	YCC GGC GTT GAM TCC AAT T	
***amoA*(AOA)**	*Fosmid clone 54D9*	
19f[4]	ATG GTC TGG CTW AGA CG	94 °C / 45 s, 55 °C / 45 s, 72 °C / 45 s, 40 cycles
CrenamoA616r48x[5]	GCC ATC CAB CKR TAN GTC CA	
***amoA*(AOB)**[6]	*Nitrosomonas sp.*	
amoA-1f	GGG GTT TCT ACT GGT GGT	94 °C / 60 s, 58 °C / 60 s, 72 °C / 60 s, 40 cycles
amoA-2r	CCC CTC KGS AAA GCC TTC TTC	
nifH[7]	*Sinorhizobium meliloti*	
nifHf	AAA GGY GGW ATC GGY AAR TCC ACC	95 °C / 45 s, 55 °C / 45 s, 72 °C / 45 s, 40 cycles
nifHr	TTG TTS GCS GCR TAC ATS GCC ATC AT	
nirK[8]	*Sinorhizobium meliloti*	
nirK876f	ATY GGC GGV CAY GGC GA	95 °C / 15 s, 63 ° C / 30 s, 72 °C / 30 s, 80 °C / 30s, 35 cycles
nirK1040r	GCC TCG ATC AGR TTR TGG TT	
nirS[9]	*Pseudomonas fluorescens* C7R12	
nirS4Qf	AAC GYS AAG GAR ACS GG	95 °C / 15 s, 63 °C / 30 s, 72 °C / 30 s, 80 °C / 15 s, 35 cycles
nirS6Qr	GAS TTC GGR TGS GTC TTS AYG AA	
nosZ[10]	*Bradyrhizobium japonicum* USDA 110	
nosZ2f	CGC RAC GGC AAS AAG GTS MSS GT	95 °C /15 s, 65 °C / 30 s, 72 °C / 30 s, 6 cycles; 95 °C / 15 s, 60 °C / 30 s, 72 °C / 30 s, 40 cycles
nosZ2r	CAK RTG CAK SGC RTG GCA GAA	

[1]Lopez-Gutierrez *et al.*, 2004*;* [2]Nicol *et al., 2005* f*;* [3]Bano *et al*., 2004 r; [4]Leininger *et al*.,2006; [5]Schauss *et al*., 2009; [6]Rotthauwe *et al*., 1997; [7]Rösch *et al*., 2002; [8]Henry *et al*., 2004; [9]Throbäck *et al*., 2004; [10]Henry *et al*., 2006

*Primer covers various representatives of Thaumarachaeota, Euryarchaeota and Crenarchaeota according to Genomatix software suite search (Nov 2012).

Table S1: **Primer and thermal details for quantification by qPCR of measured marker genes.**

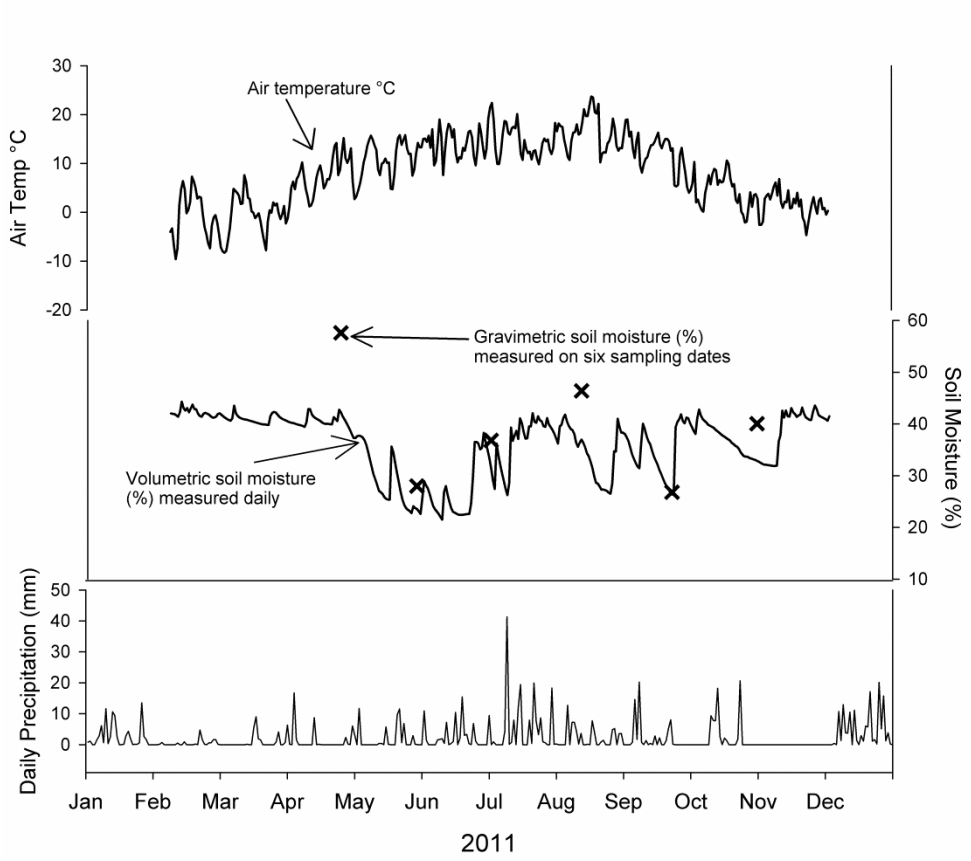

Figure S1: **Daily precipitation, volumetric soil moisture and daily air temperatures provided by the German Weather Service from station located at Münsingen, the station nearest the plot.**

Soil moisture measurements for the six sampling dates are indicated by asterisks. Discrepancies between the weather station and our data are likely due to the slightly different locations of the weather station and the sampled plot.

D Manuscript 4

frontiers in Microbiology

ORIGINAL RESEARCH
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Spatial Interaction of Archaeal Ammonia-Oxidizers and Nitrite-Oxidizing Bacteria in an Unfertilized Grassland Soil

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Interrelated successive transformation steps of nitrification are performed by distinct microbial groups – the ammonia-oxidizers, comprising ammonia-oxidizing archaea (AOA) and bacteria (AOB), and nitrite-oxidizers such as *Nitrobacter* and *Nitrospira*, which are the dominant genera in the investigated soils. Hence, not only their presence and activity in the investigated habitat is required for nitrification, but also their temporal and spatial interactions. To demonstrate the interdependence of both groups and to address factors promoting putative niche differentiation within each group, temporal and spatial changes in nitrifying organisms were monitored in an unfertilized grassland site over an entire vegetation period at the plot scale of 10 m^2. Nitrifying organisms were assessed by measuring the abundance of marker genes (*amoA* for AOA and AOB, *nxrA* for *Nitrobacter*, 16S rRNA gene for *Nitrospira*) selected for the respective sub-processes. A positive correlation between numerically dominant AOA and *Nitrospira*, and their co-occurrence at the same spatial scale in August and October, suggests that the nitrification process is predominantly performed by these groups and is restricted to a limited timeframe. Amongst nitrite-oxidizers, niche differentiation was evident in observed seasonally varying patterns of co-occurrence and spatial separation. While their distributions were most likely driven by substrate concentrations, oxygen availability may also have played a role under substrate-limited conditions. Phylogenetic analysis revealed temporal shifts in *Nitrospira* community composition with an increasing relative abundance of OTU03 assigned to sublineage V from August onward, indicating its important role in nitrite oxidation.

Keywords: nitrification, ammonia oxidation, nitrite oxidation, niche separation, spatial analysis, grassland

INTRODUCTION

Nitrification has been the focus of many studies over decades due to the ecological importance of this process, especially for agricultural ecosystems. Nitrification determines, to a great extent, whether applied fertilizers will function either as plant growth supporting components or as environmental pollutants. Nitrate leaching into water causes eutrophication, and the emission of

N_2O, a highly potent greenhouse gas, contributes to climate change (Ollivier et al., 2011). However, results of the relative contributions of key players have been contradictory – supportive either of archaeal (Leininger et al., 2006; Adair and Schwartz, 2008; Zhang et al., 2012) or bacterial ammonia-oxidizer (Di et al., 2009; Jia and Conrad, 2009) dominance – or have suffered from missing links between abundances of nitrifiers and nitrification activities (Di et al., 2009). These discrepancies can be explained in part by the designs of those studies, which have focused mainly on detailed analyses of key players involved in one or another sub-process, thereby neglecting to account for the fact that nitrification requires a strong interaction among phylogenetically differing microbes with different ecophysiologies.

The first steps, the oxidation of ammonia to hydroxylamine and nitrite, can be catalyzed by ammonia-oxidizers. The last step of the transformation process, the oxidation of nitrite to nitrate, is performed by a distinct group of organisms, the nitrite-oxidizers (Konneke et al., 2005).

Ammonia-oxidizers comprise both ammonia-oxidizing bacteria (AOB) and archaea (AOA) (Kowalchuk and Stephen, 2001; Treusch et al., 2005). Their abundances have been monitored in a wide range of ecosystems (Ochsenreiter et al., 2003; Francis et al., 2005; Treusch et al., 2005; Stahl and de la Torre, 2012). The discovery of archaeal involvement in ammonia-oxidation (AO), the frequent numerical dominance of AOA over AOB, and their active participation in AO (Leininger et al., 2006; De La Torre et al., 2008; Hatzenpichler et al., 2008; Offre et al., 2009; Schauss et al., 2009), have thrust the relative contributions of AOA and AOB into the research spotlight. Several studies have indicated that AOA and AOB colonize different niches in soil (Keil et al., 2011; Ollivier et al., 2013; Regan et al., 2014; Stempfhuber et al., 2014) and differ in their ecophysiologies (Hatzenpichler, 2012); however, their putative interaction partners have remained largely unaddressed (Prosser and Nicol, 2008).

The ability to oxidize nitrite is found in only six bacterial genera: *Nitrobacter*, *Nitrotoga*, *Nitrococcus*, *Nitrospina*, *Nitrospira*, and *Nitrolancetus*; affiliated to the alpha-, beta-, gamma-, and delta-classes of *Proteobacteria* and the phyla *Nitrospirae* and *Chloroflexi*, respectively (Daims et al., 2001; Bock and Wagner, 2006; Alawi et al., 2009; Attard et al., 2010; Sorokin et al., 2012). Nitrite-oxidizing bacteria (NOB) can be found in a variety of habitats (Abeliovich, 2006), from marine and freshwater aquatic systems (Watson et al., 1986; Stein et al., 2001), to wastewater treatment plants (WWTPs) (Juretschko et al., 1998; Daims et al., 2001; Gieseke et al., 2003; Spieck et al., 2006) and terrestrial ecosystems (Bartosch et al., 2002; Wertz et al., 2012). In terrestrial environments *Nitrobacter* (NB) and *Nitrospira* (NS) have been identified as the dominant genera (Bartosch et al., 2002; Cébron and Garnier, 2005; Kim and Kim, 2006; Ke et al., 2013). Niche differentiation amongst NOB has been proposed in several studies in both aquatic and terrestrial habitats (Schramm et al., 1999; Cébron and Garnier, 2005; Ke et al., 2013; Ollivier et al., 2013; Placella and Firestone, 2013). Shifts between NB and NS have been shown to be a consequence of different strategies related to substrate affinity (Attard et al., 2010). It has been suggested that NB are *r*-strategists, favored under high substrate concentrations owing to lower substrate affinity of their respective catalyzing enzyme. NS, however, as *K*-strategists, are capable of tolerating lower nitrite and oxygen concentrations (Schramm et al., 1999; Daims et al., 2001; Kim and Kim, 2006).

It is commonly assumed that the two transformation steps for complete nitrification are dependent on the interaction of two distinct microbial guilds in terrestrial ecosystems (Kowalchuk and Stephen, 2001). As autotrophic ammonia-oxidizers gain their energy from the conversion of ammonia to nitrite, AOB and NOB are thought to be dependent on each other in a mutualistic relationship. Nitrite, the product of ammonia-oxidation (AO) is available for nitrite-oxidizers as substrate, which, under aerobic conditions, in turn assures the consumption and the removal of the toxic nitrite in the environment by nitrite oxidation (Juretschko et al., 1998; Maixner et al., 2006). Thus, the processes of ammonia- and nitrite-oxidation are considered to be spatially dependent (Grundmann et al., 2001). Studies on the interactions and spatial structure of AOB and NOB have been performed mainly in aquatic systems or biofilm- and activated sludge-based WWTPs (Gieseke et al., 2003; Ke et al., 2013). In soils, the number of studies on interactions between ammonia- and nitrite-oxidizers is limited, suggesting an interaction of AOB with both NS- and NB-like NOB, and co-occurrence of AOA with NS (Xia et al., 2011; Wertz et al., 2012; Ke et al., 2013; Ollivier et al., 2013; Daebeler et al., 2014). Studies which take spatial and temporal dynamics of these nitrification networks into account, are, however, missing.

Hence, the focus of this study was to investigate the formation of networks of ammonia- and nitrite-oxidizers as influenced by season in a grassland soil. We postulated that the dominant forms of nitrifying networks are AOB – NB under high substrate concentrations in spring and summer and AOA – NS under lower substrate concentrations in autumn. As AOA (Jia and Conrad, 2009; Tourna et al., 2011; Daebeler et al., 2014) and NS (Daims et al., 2001; Lücker et al., 2010; Lebedeva et al., 2013) are considered to be mixotrophs, both groups may act also independently, mainly at locations with high carbon availability. To test our hypotheses, we followed the seasonal dynamics and spatial distribution patterns of AOA, AOB, NB, and NS using qPCR-based approaches to assess the abundance of marker genes for each group. We then linked these data to ammonia and nitrate availability. The dynamics of metabolically active NOB were further analyzed by screening the 16S rRNA inventory (obtained by barcoded Ilumina sequencing) both to gain a deeper insight into the active community structure of NOB as affected by time and space, and to link these to the presence of AOA and AOB.

MATERIALS AND METHODS

Study Site Description and Sampling Design

The experiment was performed in the frame of the 'German Biodiversity Exploratories'[1] (Fischer et al., 2010), a large interdisciplinary study aimed at improving our understanding

[1]http://www.biodiversity-exploratories.de/startseite/

of the effects of land use intensity on diversity at different scales. A low land-use intensity grassland site (48°25′0.01″ N, 9°30′0.00″ E), which did not receive additional fertilizer input and was subjected only to short-term grazing in the Biosphere Reserve Schwäbische Alb in the South-west of Germany, was selected for this study (Regan et al., 2014). Mean annual temperature in the year of sampling was 8.1°C; mean annual precipitation was 810 mm. The experimental site (plot ID: AEG31) was classified as Rendzic Leptosol (according to the FAO classification system). Abiotic soil parameters such as pH, carbon and nitrogen content, bulk density and soil texture were stable during the season.

In an unfertilized grassland site, a 10 m × 10 m plot was divided into 30 subplots (each 2 m × 1.67 m). Six pairs of sampling locations were randomly assigned within each subplot, each pair separated by 50 cm to provide appropriate lag distances for later geostatistical analyses. One pair from each subplot was sampled at each of six dates over one growing season. In total, 360 samples were collected in April, May, June, August, October, and November 2011 (60 per date × 6 dates). Dates were chosen to correspond to stages of plant growth on the plot. Per date, two samples were collected from the upper 10 cm soil horizon from each of the 30 subplots within the 10 m × 10 m plot (i.e., 60 samples per date in total). Soil samples were collected with a soil auger (58 mm diameter) to 10 cm depth. Soil was sieved (5 mm) and homogenized in the field. Samples for DNA extraction were frozen in liquid nitrogen in the field, and stored at −20°C. Detailed information on soil properties and sampling details can be found in the supplemental material or obtained from Regan et al. (2014).

Extraction of Nucleic Acids

A total of 360 samples were collected at six sampling dates, 60 samples per date, over one growing season, from April to November 2011. All samples were extracted in duplicate from homogenized soil subsamples (0.3 g) using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA). Concentrations of the extracts from both sample replicates were measured independently on a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA), then pooled and re-measured to confirm the final DNA concentration. For qPCR measurements, samples were diluted to a target concentration of 5 ng DNA μl^{-1} with ultra-pure water. This concentration has been determined as not inhibiting PCR in pre-experiments (data not shown). Extractions of rRNA from homogenized soil samples were conducted following a protocol modified after Lueders et al. (2004), in which the centrifugation step after addition of PEG was extended to 90 min. The nucleic acids were resuspended in 30 μl EB buffer, and the precipitation of the RNA after DNA digestion was carried out with isopropanol in the presence of sodium acetate.

Quantification of Marker Genes

Real-time quantitative PCR was performed on a 7300 Real-Time PCR System (Applied Biosystems, Germany) using SyBr Green as fluorescent dye. To quantify abundances of AOA and AOB the respective *amoA* genes were used as target. NS-like and NB-like NOBs were targeted by primer sets for 16S rRNA genes for NS and *nxrA* genes specific for NB. As primers for NS-like *nxrA* genes have been tested and shown to be non-specific (Ke et al., 2013), we chose specific 16S rRNA gene primers to target NS-like NOB. PCRs were performed according to Ollivier et al. (2013), major PCR parameters are listed in Supplementary Table S1. Serial dilutions of the plasmids containing fragments of the marker genes (Supplementary Table S1) were used for standard curve calculations. To determine the specificity and correct fragment size of the amplified qPCR products, a melting curve analysis was conducted after qPCR for each sample, followed by gel electrophoresis on a 2% agarose gel for randomly selected samples. Efficiencies obtained were above 80% and R^2 was determined to be above 0.99 for each qPCR assay.

Sequencing of 16S rRNA and Phylogenetic Analysis

We used universal primers targeting the 16S rRNA gene, and conducted paired end Illumina sequencing on a HiSeq 2500 (Illumina, San Diego, CA, USA). Besides the specific binding site 341f (Muyzer et al., 1993) and 515R (Lane, 1991), the primers contained the Illumina adapter sequence as well as the binding site for sequencing primers. Additionally, the reverse primer included a barcode region of six nucleotides. Briefly, RNA extracts from soils were reversely transcribed with GoScript (Promega, Madison, WI, USA), and PCR amplification was carried out targeting the V3 region, using primers containing Illumina adapters and a barcode (reverse primer only) (Bartram et al., 2011). Amplicons were purified from agarose gels and cleaned with NucleoSpin Extract II columns (Macherey & Nagel, Düren, Germany) prior to sequencing at the Helmholtz Center for Infectious Diseases, Braunschweig, Germany. Two samples (one in April, one in June) were lost during the process. Sequence raw data were analyzed using a bioinformatic pipeline: downstream processing included the trimming to 100 base pairs for each direction, the removal of contaminating primer dimers, and the joining of the remaining reads. Joined reads were checked for chimeric sequences with UCHIME (Edgar et al., 2011), and then clustered with CD-HIT-OTU for Illumina (Li and Godzik, 2006; Fu et al., 2012). Obtained representative sequences were finally annotated with the RDP-Classifier (Wang et al., 2007), with a similarity threshold of 97% for OTU clustering and a confidence cutoff of 0.5. After the removal of single- and doubletons, the final dataset was created.

For the identification of NOB in the dataset, suitable genera covered by the respective qPCR primer pairs for NS and NB were identified with the Genomatix software suite using the FastM and ModelInspector tool (Klingenhoff et al., 1999). OTUs affiliated exclusively with those genera were then extracted from the 16S rRNA dataset. For reference sequences, the RDP-Classifier (with 16S rRNA training set 10), BLAST (vs. the Nucleotide collection (nr/nt)) (Altschul et al., 1990), and ARB (with the SILVA 119 SSU REF NR database) (Ludwig et al., 2004; Quast et al., 2013) were used to extract type strain sequences and close relatives for phylogenetic analysis. *Nitrospina gracilis*, a marine NOB, was chosen as an outgroup (Luecker et al., 2013). The

obtained set of sequences was aligned with JalView (Waterhouse et al., 2009) and the implemented MAFFT algorithm (preset G-INS-i, for maximum accuracy) (Katoh et al., 2005). We first checked the alignment for the best fitting evolutionary model with MEGA 6 (Tamura et al., 2013). The model with the least Bayesian Information Criterion was considered to best describe the substitution pattern, and was subsequently used for tree construction, in this case the Kimura-2 parameter model with gamma distribution (K2+G). Tree topologies were then calculated with the Maximum Likelihood and Neighbor Joining algorithms as implemented in MEGA 6.

The sequence reads analyzed for this manuscript have been uploaded to the Short Read Archive under the project ID "PRJEB10957." The full study can be accessed under the following link: http://www.ebi.ac.uk/ena/data/view/PRJEB10957.

Statistics

Statistical analyses were performed using the R environment[2]. To prepare data for statistical analyses, qPCR abundance data were log (x+1) transformed. We conducted pairwise Pearson and Spearman rank correlation analyses between all variables and observations for initial data screening. Selected highly correlated pairs were corrected for autocorrelation by using functions available in the nlme package. First we formulated a null model between two variables with function lme(), then updated this model by using one of five correction procedures for spatial autocorrelation (exponential, spherical, linear, Gaussian, rational quadratic). The best fitting corrections according to the Akaike Information Criterion (AIC) were chosen for the final regression model. For pairwise comparisons of group means between the six sampling dates, we used the function glht() of the package multcomp with method "Tukey" on generalized linear models with the appropriate distribution families for each group of variables (Hothorn et al., 2008; Herberich et al., 2010). Non-random spatial dependence, i.e., the relation of data points in dependency of their distance, was analyzed using the geostatistical approach published by Steffens et al. (2009). A semi-variogram describes the degree of variability as a function of spatial separation of samples (Grundmann and Debouzie, 2000). Spherical models were fitted to each experimental semivariogram using the gstat fitting routine of R. Furthermore, exponential models were tested if no spherical model could be fitted. For underlying equations, see e.g., Steffens et al. (2009). In case no model could be fitted, either the parameter under investigation was homogeneously distributed or the spatial distribution was independent of the scale chosen (see Supplementary Table S3) and thus could not be visualized by kriged maps. More detailed information on our geostatistical approach is provided in the supplemental material. The variogram model was used in order to interpolate the measured data to non-sampled sites within the investigated plot (Steffens et al., 2011) and kriged maps were constructed to visualize the spatial structure of gene abundances at the plot scale. Maps were constructed by ordinary kriging taking advantage of the ArcGIS Software (ArcMap 10.0, ESRI® 2010, Germany) wherever a model could be fitted to the dataset.

[2]http://www.R-project.org

RESULTS

Temporal Dynamics of Ammonia- and Nitrite-Oxidizers

To assess putative temporal changes in the abundances of ammonia- and nitrite-oxidizers, we determined the gene copy numbers of the 16S rRNA gene (NS), *nxrA* (NB) and *amoA* (AOA and AOB) (Supplementary Table S2; **Figure 1**). Numbers of 16S rRNA genes for NS were in the range of 10^7 to 10^8 gene copies per g soil dry weight, whereas NB were lower in abundance with 10^5 to 10^6 *nxrA* gene copy numbers. Exceptions were a

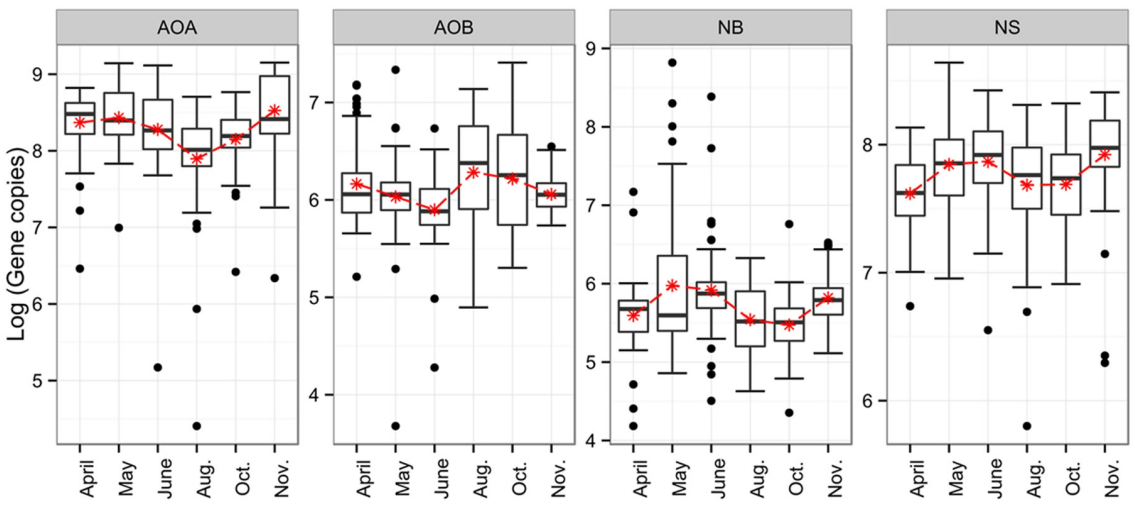

FIGURE 1 | Boxplots for seasonal dynamics of ammonia- and nitrite-oxidizers. Depicted are gene copy numbers. AOA, ammonia-oxidizing archaea; AOB, ammonia-oxidizing bacteria; NB, *Nitrobacter*-like; NS, *Nitrospira*-like.

few sampling sites with very high gene copy numbers exceeding 10^7. Gene copy numbers indicative for NS increased from April to May, and declined slightly in June and August/October when lowest values were detected. In November the abundance of NS-like NOB increased to its maximum. Interestingly, the seasonal dynamics of AOA abundance closely resembled the trend of the NS gene abundance pattern with a decline in August and October and highest values in May and November. AOB abundance, in contrast, exhibited highest gene copy numbers in August and October, coinciding with the lowest gene abundances for AOA and NS; lowest gene copy numbers were detected in May/June and November. Throughout the entire season, AOB copy numbers (in the range of 10^6) were generally lower than AOA (in the range of 10^8). In terms of statistical significance, changes in abundance for NS were not significant after the tested model was corrected for spatial autocorrelation. For AOA, AOB, and NB, however, significant changes were found for the June–August transition ($p < 0.01$), as well as for the decrease in AOA ($p < 0.001$) and NB ($p < 0.05$) between October and November, and for NB in early spring ($p > 0.01$).

Spatial Analysis of Gene Abundances of Ammonia- and Nitrite-Oxidizers

In order to detect spatial structures of the investigated groups at the plot scale of 10 m^2, geostatistical semivariogram analyses were conducted. Supplementary Table S3 shows semivariogram parameters of gene abundance data for the respective sampling dates. Spherical models could be fitted for all sampling dates for NS-like NOB, whereas spatial dependence was found at only few dates for the other genes.

Range, nugget and sill were determined to assess the spatial behavior of variables (Supplementary Table S3). For most gene abundance data, spatial dependence was captured within the sampling area with seasonally varying ranges of autocorrelations (4.9–12.8 m for AOA, 2.3–9.1 m for AOB, 1.2–21.2 m for NS, 4.5–12.3 m for NB). For some parameters, a far-reaching spatial autocorrelation would be expected when the determined range exceeds the boundaries of the plot as, e.g., for NS-like NOB with a range of 21 m in October, which did not represent a reliable range, because it exceeded the maximum distance between sampling points. Gene abundances of NB in November and NS in April and October exhibited an extremely high spatial dependency (above 87%). For NB, the degree of spatial dependence increased during the year. However, the seasonal dynamics of NS-like NOB first revealed a decline in spatial dependence visible until June, followed by an increase in August and again in November. In October, the highest spatial dependency of about 93% was reached for NS-like NOB. The degree of spatial dependence was rather low for AOA and AOB (between 2.4 and 36.5%) and the data sometimes exhibited a large nugget effect, implying high non-measured small-scale variability.

Kriged maps, used to visualize the spatial distribution of the investigated variables, revealed highly variable spatial distributions over the sampling period for both NB and NS-like NOB (**Figure 2**). In case no map could be constructed, the spatial distribution of the parameter of interest was too homogeneously distributed to be visualized by a spherical model or could not be resolved at our sampling scale. On the sampling dates for which kriged maps could be generated for NB, varying distribution patterns were detected, ranging from medium-sized patches in November (**Figure 2A6**), to large patches with hotspots in April (**Figure 2A1**), and finally more homogeneous structures in August (**Figure 2A4**) with higher abundances in the upper part of the plot interspersed by a few smaller nested patches. Spatial autocorrelation patterns of NS, observed at each sampling date, varied extensively with the season (**Figures 2B1–6**). NS abundance was spatially structured in larger patches with rather smooth transitions from areas of low to high abundance in April and May, the latter even harboring a pronounced hot spot of high abundance. This rather homogeneous distribution changed to more small-scale patchiness with a heterogeneous structure in June. In August, a continuous decline in abundances located at the upper border of the plot was evident, again becoming more homogeneous, with larger patches in October and lowest values in the right half of the plot. Pronounced small-scale heterogeneity with a relatively high number of small sharply zoned patches could be demonstrated for NS-like NOB in November; AOA distributions could be displayed in August and October (**Figures 2C4,5**) revealing larger homogeneous patchiness with gradient-like structures of gene abundances. AOB gene abundance was more heterogeneously distributed in May than in the other months with smaller patches and a more pronounced gradient-like structure in the upper right corner of the plot (**Figures 2D2,3**). Spatial variability was more homogeneous in November. **Figure 2E5** shows the spatial distribution of NH_4^+ with a pronounced large patch of high concentration on the right side of the plot, corresponding to the lowest abundances for AOA and NS gene copy numbers measured at this sampling date.

Phylogenetic Analysis of Active Nitrite-Oxidizing Bacterial Community Composition

To further differentiate the various groups of active NOB, a 16S rRNA based barcoding approach was performed and OTUs affiliated with selected NOB groups (NS and NB) were further analyzed. In the 16S rRNA dataset, we detected 40 OTUs assigned to genus *Nitrobacter* based on 97% sequence similarity of the variable region 3, but a single OTU accounted for more than 99% of reads associated with this genus. This particular OTU also was the second most abundant signal in the entire dataset and was represented by 5.4 million reads (∼1.1% of the entire bacterial dataset). For the phylum *Nitrospira*, 285,000 reads (0.063% of all bacterial reads) could be assigned to 36 OTUs. However, 33 of these OTUs were found to be spurious, hence, we focused on the remaining three generalist OTUs in this phylum, which accounted 99.3% of all NS assigned reads and appeared in all samples. The three representative sequences for these OTUs exhibited sequence similarities between 92% (01 vs. 03), 93% (02 vs. 03), and 97% (01 vs. 02), respectively.

The relative abundance of the NB OTU strongly increased from April to May ($p < 0.001$) and from August to October

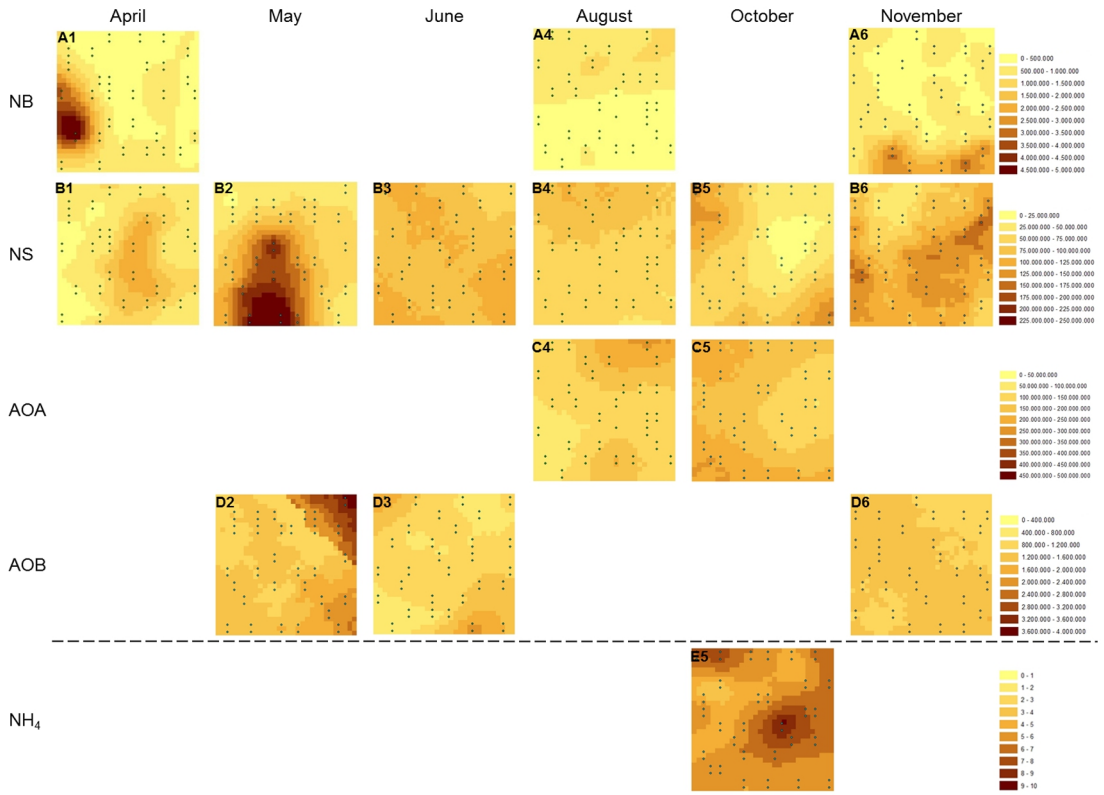

FIGURE 2 | Spatial distribution of selected variables. Kriged maps were constructed for gene abundances of **(A)** *nxrA* gene (NB), **(B)** 16S rRNA genes (NS), **(C)** *amoA* gene (AOA), **(D)** *amoA* gene (AOB), and for soil ammonium content **(E)** at different sampling dates (1–6). Gene abundances are given in gene copy numbers per g soil (dry weight), ammonium concentration is given in μg N per g soil (dry weight). AOA, ammonia-oxidizing archaea; AOB, ammonia-oxidizing bacteria; NB, *Nitrobacter*-like nitrite-oxidizing bacteria; NS, *Nitrospira*-like nitrite-oxidizing bacteria.

($p < 0.01$), when this OTU reached its annual maximum, decreasing significantly again between October and November ($p < 0.05$), maintaining relatively constant levels between May and August (Supplementary Figure S1). This NB-OTU at some dates exhibited very high correlation to the NS-OTUs (especially in April and August). Relative abundances of the three NS-OTUs were stable during the first three sampling dates of the year. For all three OTUs, the abundances increased from June to August ($p < 0.05$), except OTU01, which was not significant ($p = 0.06$). Interestingly, the activities of OTUs 01 and 02 both declined during the late season sampling dates, whereas OTU03 remained stable, thus increasing its abundance compared to the other *Nitrospira* OTUs (Supplementary Figure S1).

Nitrospira OTUs showed overall positive correlations with each other (OTU01-02: $r = 0.683$, OTU01-03: $r = 0.530$, OTU02-03: $r = 0.512$), with varying strengths of correlations if the sampling dates were analyzed separately (Supplementary Figure S2). In accordance with their sequence-based similarity of 97%, OTU01 and 02 were highly correlated at most of the sampling dates ($r > 0.650$). Correlations with NS OTU03 were generally weaker, but still significant. NS OTUs did not show any correlation to ammonium (Supplementary Figure S2). At the beginning and toward the end of the year, significant correlations of NS OTUs with nitrate content were found, especially for OTU 02 (up to $r = 0.42$ in November). A weak correlation between nitrate and the *Nitrobacter*-OTU was also found in October.

A phylogenetic tree was constructed based on the Neighbor Joining algorithm (**Figure 3**) and detailed examinations were performed on the affiliation of the NS OTU-sequences to sublineages of NS-like NOB, as designated in Daims et al. (2001) and Lebedeva et al. (2011) (Supplementary Table S4). The topology of the neighbor joining tree was further confirmed by the maximum likelihood method (data not shown). NS OTU01 and OTU02 were located in proximity to sublineages I, II and VI. It is of note that for some taxa, the variable region 3 of the 16S rRNA cannot clearly resolve the sequence affiliation beyond the genus level, which seemed to happen in the case of some of the sublineages. Both conducted methods, however, place NS OTU03 with a similarity level of 94% in the sublineage V of *Nitrospira* with *Ca. Nitrospira bockiana* as cultured representative. To determine whether only gene abundances or also the composition of the contributing NS sublineages exhibited seasonal dynamics, we followed the changes in one selected subplot over time. We chose one of the 30 available subplots (see sampling scheme in Regan et al., 2014) that exhibited the most pronounced dynamics in 16S rRNA gene abundances for NS-like NOB (**Figure 4C**). We compared shifts in the relative activity of OTUs by plotting

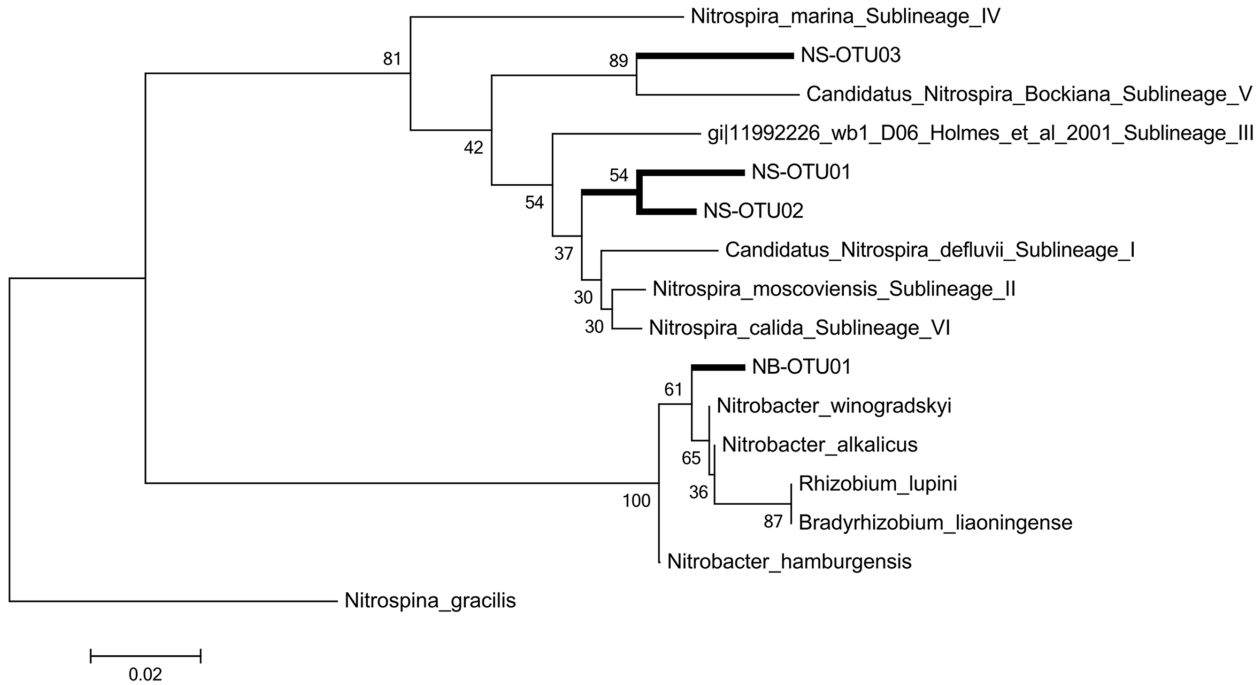

FIGURE 3 | Phylogenetic tree. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.39985022 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) and are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method with gamma distribution (K2+G) and are in the units of the number of base substitutions per site. The analysis involved 10 nucleotide sequences. All positions with less than 10% site coverage were eliminated. That is, fewer than 90% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 182 positions in the final dataset. Evolutionary analyses were conducted in MEGA6. Sequences contain sublineage designations as given in Daims et al. (2001).

their relative abundances against each other, setting the total abundance to 1 (**Figure 4A**). The proportions of the NS OTU abundances did not change during the first half of the year. From August on, the relative abundance of OTU03 in particular increased at each subsequent sampling date until the end of the year. While this effect was observed for the whole dataset (**Figure 4B**), it was especially pronounced in this location, suggesting spatial heterogeneity of species distribution.

DISCUSSION

Temporal Dynamics and Metabolic Activity of NOB

To provide insight into the temporal dynamics of active organisms and to help identify different sublineages of dominant NS-like NOB, the abundance of 16S rRNA as a proxy for metabolic activity was assessed by an Illumina sequencing approach. Discrepancies in the direct comparison of gene abundances on a DNA level to metabolic activity at an rRNA level are attributable to the fact that gene abundances do not necessarily indicate growth or reflect activity at the RNA level (Chen et al., 2008; Offre et al., 2009; Blazewicz et al., 2013; Placella and Firestone, 2013; Daebeler et al., 2014). Marginally higher abundances of NS-assigned 16S rRNA sequences on the RNA level (Supplementary Figure S1), compared with lower *Nitrospira* rRNA 16S gene abundances on the DNA level during autumn (Supplementary Table S2) may be explained by high activity of a few organisms in cell-maintenance or in the investigated processes (Blazewicz et al., 2013). In the first half of the year, the reverse was observed. This may indicate that large numbers of NS-like NOB were inactive under suboptimal growth conditions, in a state of starvation and dormancy (Ettema and Wardle, 2002). Enzyme stability (Chen et al., 2007; Ke et al., 2013) or the constitutive expression of multiple gene copies (Poly et al., 2008; Lücker et al., 2010) could be important prerequisites for an immediate reaction to changing environmental conditions such as the sporadic availability of substrate (Blazewicz et al., 2013).

Temporal analysis demonstrated pronounced seasonal dynamics of AO and NO both with respect to their abundances and to the numerical dominance of AOA within the AOs and NS within the NOs at all measured dates (Supplementary Table S2), corresponding to previous studies (Leininger et al., 2006; Adair and Schwartz, 2008; Meyer et al., 2013; Ollivier et al., 2013; Stempfhuber et al., 2014). The higher abundance of genes involved in particular transformation processes may result not only from ammonia- or nitrite-oxidation, but also from potential mixotrophic growth, as proposed for NS and AOA (Prosser and Nicol, 2008; Jia and Conrad, 2009). The high standard deviations in gene

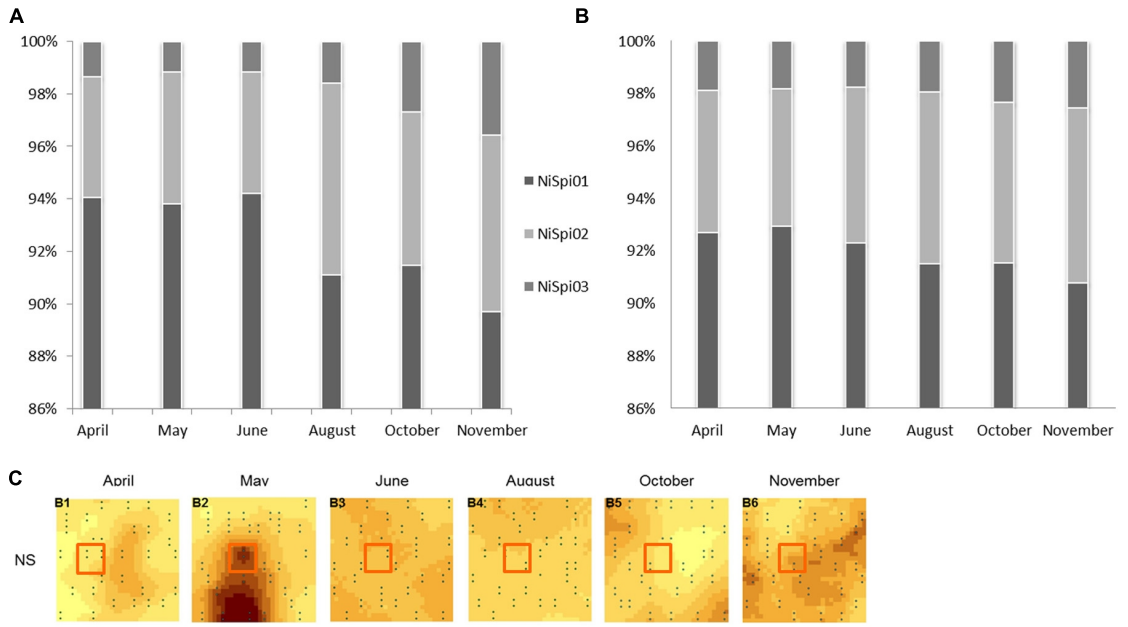

FIGURE 4 | Relative abundances of NS-assigned OTUs. Columns display the relative abundances of *Nitrospira*-like NOB OTUs 01-03 over the season. The total abundance of NS-assigned OTUs was set to 100%. NS = *Nitrospira*-like nitrite-oxidizing bacteria. Barcharts depict either relative abundances within one selected subplot **(A)** or represent the complete dataset **(B)**. The location of the selected subplot is indicated by the red square **(C)**.

copy numbers at one sampling date therefore highlight the importance of supplementing temporal analysis with spatial structure analysis in the field by the identification of local hotspots.

Temporal Dynamics of Spatial Niche Differentiation Amongst NOB

Functionally complementary microbial groups often differ in their responses to environmental changes, shaping functional niches (Maixner et al., 2006). Studies have addressed spatial niche differentiation patterns of functionally redundant organisms often co-existing at the same spatial scale (Schauss et al., 2009; Schleper, 2010; Wertz et al., 2012; Ollivier et al., 2013) or differing in their spatial distribution (Krause et al., 2010, 2013). Our data showed seasonally varying patterns of niche differentiation: spatial niche separation between NS and NB was most evident at our study site in April, as large patches of high gene abundance were clearly spatially discriminated (**Figures 2A1,B1**), whereas homogeneous and congruent abundance patterns for both NS and NB were found in August, indicating co-occurrence at the same spatial scale (**Figures 2A4,B4**). We attribute these co-occurrence patterns to different adaptations to substrate concentrations, making possible the co-existence of NB and NS by reduced "interspecific" competition (Hibbing et al., 2010): it has been suggested that NB as *r*-strategists exhibit high growth rates and activity and may therefore out-compete NS under high nitrite levels (Schramm et al., 1999; Maixner et al., 2006), while NS may have a competitive advantage over NB under nitrite-limitation (Lücker et al., 2010). In November, rather undifferentiated and very patchy patterns were detected for NS and NB, without areas of clear spatial separation or congruence (**Figures 2A6,B6**).

Nitrite concentration is usually below the detection limit in natural terrestrial systems, transformed rapidly to prevent its toxic accumulation (Burns et al., 1995; Attard et al., 2010; Xia et al., 2011; Ke et al., 2013). One can infer, however, from the absence or presence of AO spatial distribution patterns at the same investigated scale, information about the nitrite content in soil, assuming that substrate availability shapes the niche differentiation patterns of NOB. Unfortunately, we could not visualize environmental variables for April and November that could explain the spatial distribution of NOB phyla. Nevertheless, we may speculate that the absence of ammonia-oxidizers at the observed spatial scale in April (**Figure 2**) suggests that nitrite formation derived from AO was low. Under such nitrite substrate-limited conditions, other niche determining factors operating at the investigated scale may have been more important. For example, the measured high soil moisture content in April (Regan et al., 2014) suggests that oxygen status could have influenced spatial niche separation. NB are presumed to prefer high oxygen conditions and thus compete with heterotrophic organisms or AO for oxygen (Kim and Kim, 2006), while NS could occupy spatial niches with extremely low oxygen content (Gieseke et al., 2003; Lücker et al., 2010). However, especially under low nitrite/nitrate conditions, NOB can switch to nitrite reduction, i.e., the reduction of nitrate to nitrite, which can be catalyzed by NXR (Sundermeyer-Klinger et al., 1984; Bock et al., 1988; Bock and Wagner, 2006). Under

anoxic conditions, some NB may also perform the complete denitrification process (Freitag et al., 1987). The ability of NB to also exhibit heterotrophic growth could then provide a competitive advantage over NS (Freitag et al., 1987; Lücker et al., 2010).

Temporal Dynamics of Spatial Niche Differentiation Amongst Sublineages of NOB

Niche differentiation has been demonstrated within genera and species of NOB. Putative shifts within NB-like NOBs, however, would not have been captured by our approach, since the V3 region of the 16S rRNA gene might not be sufficient to distinguish between the phylogenetically highly similar NB species (Freitag et al., 2005; Alawi et al., 2009), closely related to *Bradyrhizobia* (Orso et al., 1994). Thus we restricted our subsequent phylogenetic analyses to *Nitrospira* community composition for which the co-existence of up to three distinct sublineages has been reported (Freitag et al., 2005; Maixner et al., 2006; Lebedeva et al., 2008), in line with our results. NS OTU01 and OTU02 were phylogenetically placed in close proximity to cultured or enriched representatives of different sublineages (**Figure 3**, see Supplementary Table S4 for details): sublineage VI (Lebedeva et al., 2011), sublineage II (Ehrich et al., 1995; Daims et al., 2001) and sublineage I (Lücker et al., 2010). Sublineages I (Spieck et al., 2006) and II, correlated to the presence of AOA in volcanic grassland soils (Daebeler et al., 2014), are adapted to low substrate and oxygen concentrations (Maixner et al., 2006; Wertz et al., 2012; Ke et al., 2013). OTU03 of NS was affiliated to *Ca. Nitrospira bockiana* with 94% similarity (**Figure 3**), and similar substrate preferences that hold true for *Ca. Nitrospira bockiana* as cultured representative may also apply to other members of sublineage V (Lebedeva et al., 2008), such as the inability to be stimulated by organic substrates or to take up pyruvate. NS OTU03 may exhibit similar characteristics. However, transferring knowledge on habitat preferences attained from cultivated species or enrichment studies to pathways and metabolism of microorganisms in their natural habitats has to be handled with care (Regan et al., 2003; Prosser and Nicol, 2012).

We therefore addressed the question of whether or not the microbial structure at sampling sites with high gene abundances is fundamentally different from that at sites of low abundance with regard to their NS OTU composition (**Figure 4**). We selected the subplot with the most pronounced changes in NS abundance. Despite varying gene abundances, the community composition and its relative metabolic activity did not change during the first half of the year, implying the co-existence of sublineages under substrate-limitation. In the second half of the year, the relative proportion of OTU03 in particular, affiliated with sublineage V (Lebedeva et al., 2008), increased. We speculate that nitrite operates as a niche determining factor in "intraspecific" competition and may have caused shifts in the relative abundances of OTUs and affiliated sublineages from August on (Maixner et al., 2006), as even sublineages of the genus NS have been proposed to exhibit different preferences for nitrite concentrations (Grundmann and Debouzie, 2000; Maixner et al., 2006).

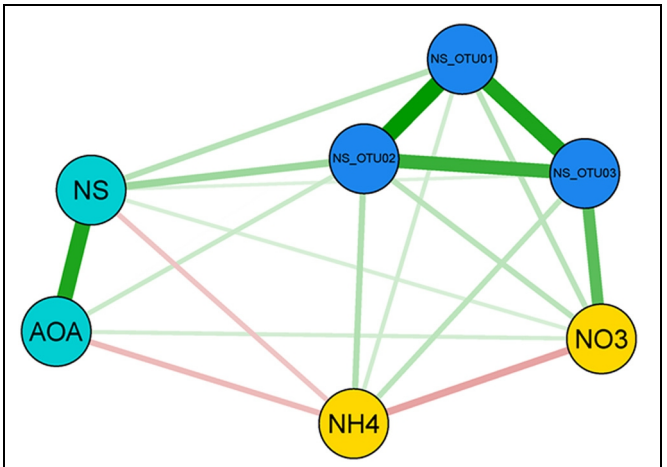

FIGURE 5 | Network analysis of interactions between NS-assigned OTUs, gene abundances and nitrification-associated nitrogen-pools in October. Depicted are Pearson correlations between three parameter groups for sampling date October: gene abundances (light blue circles), *Nitrospira* OTUs 01-03 (dark blue circles) and nitrate and ammonium concentrations (yellow circles), respectively. Edges between the nodes are weighted according to the correlation strength. Positive coefficients are colored in green, negatives are displayed in red. AOA, Ammonia-oxidizing archaea; NS, *Nitrospira*-like nitrite-oxidizing bacteria (NOB).

Spatial Interactions of Nitrifying Organisms

Studies on nitrifiers at spatial ranges from μm (Maixner et al., 2006) to the landscape scale (Grundmann and Debouzie, 2000; Bru et al., 2011) have demonstrated that the factors influencing spatial dependency operate at different scales: soil texture or land management practices operate at larger spatial scales while, for example, vegetation, can operate at smaller scales (Ettema and Wardle, 2002; Ritz et al., 2004). Nitrification at some sampling dates may have occurred at nested scales which were not characterized. High nugget effects for AOA and AOB abundances at some dates imply the presence of unmeasured variance at smaller scales (Supplementary Table S3) (Steffens et al., 2009). The ranges of spatial dependence of the abundance data in this study (Supplementary Table S3) were, however, similar to spatial autocorrelations ranging from 1.4 to 7.6 m for AOA and AOB in a previous study in the same region (Keil et al., 2011), and corresponded also to those found in studies at mm to m scales (Nunan et al., 2003; Franklin and Mills, 2009).

Surprisingly, our spatial analysis at the plot scale did not confirm the hypothesis that nitrification could be attributed mainly to a close functional interaction reflected by the spatial dependence of AOB and NOB, although many studies have reported their functional interaction (Mobarry et al., 1996; Schramm et al., 1999; Abeliovich, 2006; Xia et al., 2011; Wertz et al., 2012). AOB and NB have been shown to dominate nitrification under high substrate-conditions (Shen et al., 2008; Jia and Conrad, 2009; Di et al., 2010; Wertz et al., 2012; Ke

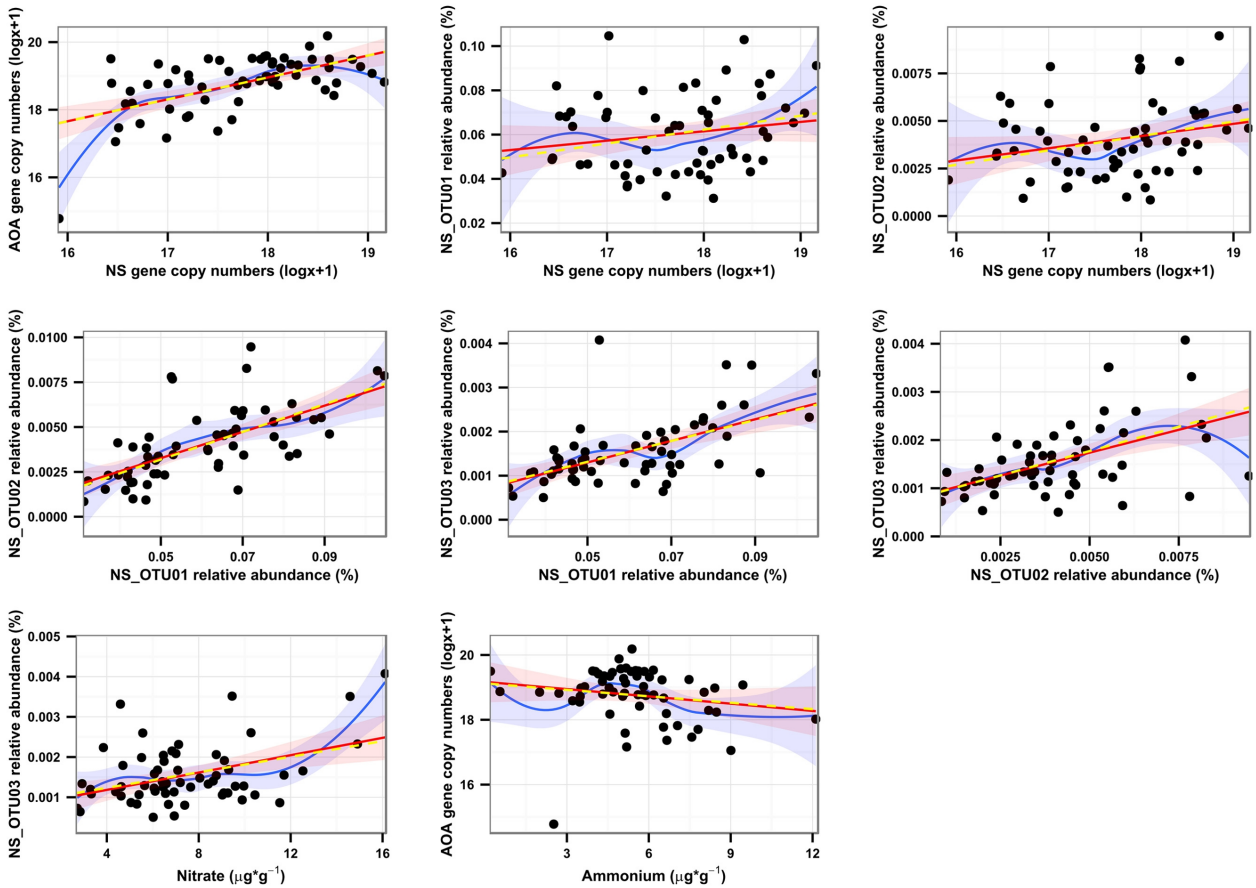

FIGURE 6 | Univariate linear models between pairs of variables in October. Pairs of variables were selected from the network analysis (**Figure 4**) to show additional support for our conclusions after accounting for spatial autocorrelation. Red lines indicate the uncorrected, Gaussian regression models, whereas yellow, dashed lines represent the same models after correction for spatial autocorrelation. Blue lines are derived from Loess fits. All models are significant at $p < 0.05$, except for AOA/Ammonium ($p > 0.1$) and NS_OTU02/NS ($p = 0.0565$), which, however, show significant spearman rank correlations, possibly pointing at significant, non-parametric models. Although the model improvements for all variables were very small according to AIC shifts, NS and the NS OTUs 01 and 02 were best described with exponential variograms and NS OTU03 with the spherical variogram. For nitrate and AOA, no spatial model led to model improvements.

et al., 2013). In contrast, the congruent spatial distributions of AOA and NS and their positively correlated abundances in autumn ($r = 0.574$ for Oct.; **Figure 2**; Supplementary Table S5), strongly suggest an interaction of AOA and NS in performing the sequential transformation steps of nitrification. This is further supported by reports on the co-occurrence of AOA and NS in the same soil compartments (Lebedeva et al., 2011; Ke et al., 2013; Daebeler et al., 2014). Since a sensitivity of AOA to nitrite accumulation was demonstrated recently for *Nitrosotalea* isolates, a close mutualistic relationship between AOA and NOB seems reasonable (Lehtovirta-Morley et al., 2014). Although the exact mechanisms are still under investigation, it has been demonstrated that both AOB and AOA are able to catalyze the transformation of ammonia to nitrite (e.g. Tourna et al., 2011). Efficiency and kinetics of ammonia-oxidation and consequently the release of nitrite might, however, vary between distinct phyla and environmental conditions (Ward, 2011). Thus it can be speculated that NOB respond to different levels of nitrite that are either determined by kinetics of ammonia-oxidation or by the relative distance of NOB to the source of their substrate (Maixner et al., 2006), according to their distinct preferences for nitrite concentrations. The temporal and spatial interaction of AOA and NS and their linkage to ammonium- and nitrate-pools were further supported by a Pearson-coefficient-based network analysis for October (**Figure 5**), when congruent spatial patterns of AOA and NS were most pronounced (**Figures 2** and **6**; Supplementary Table S5) and all investigated molecular markers were highly correlated with each other, which was observed only in October (Supplementary Figure S3). Several significant, positive pairwise correlations were detected in October. Correlations between nitrate and NS OTU03, AOA and NS, as well as NS and NS OTUs 01 and 02, respectively, were all found to be significant at $p_{\text{adjusted}} < 0.05$, and remained significant after correction for spatial autocorrelation. Furthermore, strongly positive correlations of AOA and NB were observed as well (April: $r = 0.576$, October: $r = 0.561$), but their interaction at the spatial scale could not be identified by our geostatistical analyses (Supplementary Table S5).

Nitrate concentration was positively connected most clearly with OTU03 in October ($r = 0.42$; Supplementary Figure S2), which hints at the active participation of sublineage V (**Figure 3**) in the production of nitrate and for subsequent nitrite oxidation from August on (**Figure 4**). The ability of most NOB to simultaneously convert nitrate to nitrite implies that their performance can influence the nitrate pool in different directions, impeding determination of clear positive or negative correlations (Supplementary Figure S2). The positive correlation of AOA and nitrate (**Figure 5**) was likely due to the direct connection of AO and NO processes, the former delivering the product for the latter transformation step. AOA abundance was strongly negatively correlated to ammonium content, which corresponds to their spatial distribution patterns, which varied inversely (**Figures 2C5,E5**), indicating consumption of ammonia as substrate by AOA (Schleper and Nicol, 2010; Ke et al., 2013). The negative correlation of nitrate and ammonium ($r = 0.233$; **Figure 5**; Supplementary Figure S2) could be due to a decline in the ammonia pool by AO, resulting in an increase in nitrate content due to NO. This confirms that the complete nitrification process based on interactions between ammonia- and nitrite-oxidizers can be followed at the investigated scale only at very limited periods during the year. It must be considered, however, that nitrification at other dates may be performed by organisms that catalyze complete nitrification (commamox) that have not been assessed by our study of spatial interaction patterns (Daims et al., 2015; van Kessel et al., 2015).

Different growth strategies such as potential mixotrophy or heterotrophy may obscure the interactions between AOA and NS. Consequently, the utilization of alternative substrates (Prosser and Nicol, 2008, 2012; Tourna et al., 2011) for energy production and assimilation of different carbon sources (Lehtovirta-Morley et al., 2013) must also be taken into account. The potential for mixotrophic growth (Rogers and Casciotti, 2010; Lehtovirta-Morley et al., 2014) could increase the competitiveness of AOA and NS over their counterparts by providing a growth advantage and assuring their greater flexibility in reacting to suboptimal substrate-limited conditions. An increase of organic material, as observed in autumn due to plant litter, may further support the growth of mixotrophic organisms (Brown et al., 2013). Differences in preferences for, e.g., organic compounds or other characteristics have been reported even within particular AOA species in soils (Offre et al., 2009; Hatzenpichler, 2012; Lehtovirta-Morley et al., 2014) and for ecotypes of *Nitrospira* (Maixner et al., 2006). This heterogeneity could affect patterns of spatial distribution and inhibit correlation of abundances to environmental parameters. Given this, it becomes necessary to identify drivers which may influence nitrifiers directly or indirectly via changing substrate availability or ammonia sources (Prosser and Nicol, 2012). AOA, for example, prefer mineralized nitrogen, derived from decaying plant material, which is the main source of inorganic nitrogen at the end and before the start of the vegetation period, rather than ammonium directly applied by fertilization (Offre et al., 2009; Levičnik-Höfferle et al., 2012).

Even occasional mowing or grazing may influence nitrogen availability and consequently the microbes performing nitrification (Patra et al., 2005, 2006). We assumed, therefore, that the a mowing event in August (2 weeks before sampling) affected the observed nitrification activity in autumn (Both et al., 1992), uncoupling the plants' competition for substrate, thereby enabling AO to better access the ammonium pools in soil (Wolters et al., 2000; Hamilton and Frank, 2001; Patra et al., 2006; Le Roux et al., 2008; Kuzyakov and Xu, 2013). The heterogeneous ammonium distribution may also be linked to plant diversity, as a strong spatial distribution pattern of legumes was observed mainly in October at the site (Regan et al., 2014).

This study presents evidence for both temporal and spatial correlation of AOA and *Nitrospira* in an unfertilized grassland site, indicating their interrelationship in performing the nitrification process over one growing season. The obtained results, however, are based on a 1-year study. Thus, it would be important to assess spatial interaction patterns at larger temporal scales to confirm stability of the observed patterns. However, *Nitrobacter* and ammonia-oxidizers might interact at scales not covered by our study, below the m^2 range, and may require subsequent studies using microscopic techniques.

We demonstrated an interaction of AOA and NS under unfertilized conditions, and it would be interesting to extend this approach to sites under high land-use intensity with different fertilization practices to compare both the major actors and their interactions (Keil et al., 2011). Recently, alternative possibilities have been described for nitrifiers to gain ammonia using cyanate as substrate (Stein, 2015). It has been demonstrated that ammonium derived from cyanate transformation by NS can be used by ammonia-oxidizing microbes (Palatinszky et al., 2015); such alternative feedback processes may exist between functional guilds of nitrification and play an important role for the stabilization of nitrifier networks mainly in fertilized soils.

AUTHOR CONTRIBUTIONS

BS and TR-H conducted and interpreted the experiments and wrote the manuscript. KR performed the soil sampling and sample preparation as well as critical revision of the draft. AK contributed to geostatistical analyses and data interpretation. PK and JS contributed to phylogenetic and statistical analyses. SM performed soil sampling and was responsible for the conception of the experiment. JO, MF, EK, and MS were involved in the conception of the experiments and final approval of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01567

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Supplemental Information Manuscript 4

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Supplementary Material

Spatial interaction of archaeal ammonia-oxidizers and nitrite-oxidizing bacteria in an unfertilized grassland soil

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1 Supplementary Experimental Procedures

Study site description and sampling design

In the frame of the Scalemic project, the following abiotic soil parameters were determined and did not change over the season: pH (6.7), carbon (66.0 mg g^{-1}) and nitrogen (7.0 mg g^{-1}) content, bulk density and soil texture. Extractable organic carbon was in the range between 208.25 µg g^{-1} and 100.19 µg g^{-1}, decreasing over the year. Extractable organic nitrogen ranged from 1.69 µg g^{-1} to 11.04 µg g^{-1} with lowest values in August and November. Highest values for NH_4^+ and NO_3^- have been detected in April (15.70 µg g^{-1} and 17.99 µg g^{-1}, respectively), lowest values in October (5.43 µg g^{-1} and 7.40 µg g^{-1}, respectively). Soil moisture was dynamic over the year, exhibiting highest water content in April (57.56%) and lowest soil moisture contents in May and October (27.97% and 26.79%). For further information see Regan et al. (2014) and the corrigendum Regan et al. (2015).

At each sampling date, before soil cores were collected, 20 cm x 20 cm grids were centered over each of the 60 sampling points. Aboveground biomass was removed from each grid by cutting all visible plants at ground level. Samples were then sorted into the following categories: litter (dead leaves and other dead plant matter on soil surface), grasses (*Poaceae*), legumes, forbs, bryophytes, and *Rhinanthus minor*. Vegetation coverage for all sampling dates for the three most abundant plant categories (grasses, forbs, legumes) and litter mass was calculated as g per 400 cm^2 grid. Grasses dominated at the first three sampling dates, but were rather low in abundance from August to November. Forbs followed the pattern of grasses, but with lower abundance than grasses in May and June. Legumes were the only plant group to increase after mowing, appeared from June on and were highest in October. Litter mass exhibited a different pattern; it declined from April to June, increased after mowing and continued to increase at each of the last three sampling dates. Details can be found in Regan et al. (2014).

Supplementary Material

Geostatistical analysis

Geostatistical semivariogram analyses were carried out using the g-stat package (Pebesma, 2004) for the R environment (R 3.0.2, RDevelopmentCoreTeam, 2008). Nugget (intercept at the origin), sill (the plateau at which the distance based variance values levels off, representing the maximum semivariance) and range (maximal distance of spatial autocorrelation) have been determined by semivariogram analyses and used for calculation of measures of spatial dependence and variance. P-sill is defined as the part of total variance that is spatially explained. The degree of spatial dependence is calculated by dividing nugget by the sum of nugget and sill (Fortin and Dale, 2005; Steffens et al., 2011). Values below 25% resulting from low nugget values related to maximal semivariance, indicate the presence of spatial dependency, whereas high values above 75% would point to the absence of spatial dependency (Cambardella et al., 1994; Steffens et al., 2009; Steffens et al., 2011). Our results (values below 49%) indicated a spatial dependence for all measured variables at sampling dates when a model could be fitted (Steffens et al., 2009). An appropriate model was fitted to each experimental semivariogram using the gstat fitting routine. Spherical models were tested first, as they would best explain spatial dependencies (Berner et al., 2011). Furthermore, exponential models were tested if no spherical model could be fitted. The fitting of a geostatistical model indicated that the selected spatial scale of the study might be appropriate to describe the spatial distribution of the observed parameters (Steffens et al., 2009). Step widths between 0.6 and 0.8 m were applied.

The display of spatial distribution of AOA and AOB variables reflects the rather weak spatial dependence after fitting of different settings to construct kriged maps. Nevertheless, the maps display a rough distribution of variables, for visual comparison of e.g. co-localization and hence are essential for gaining deeper insights in the interaction of functional groups under observation.

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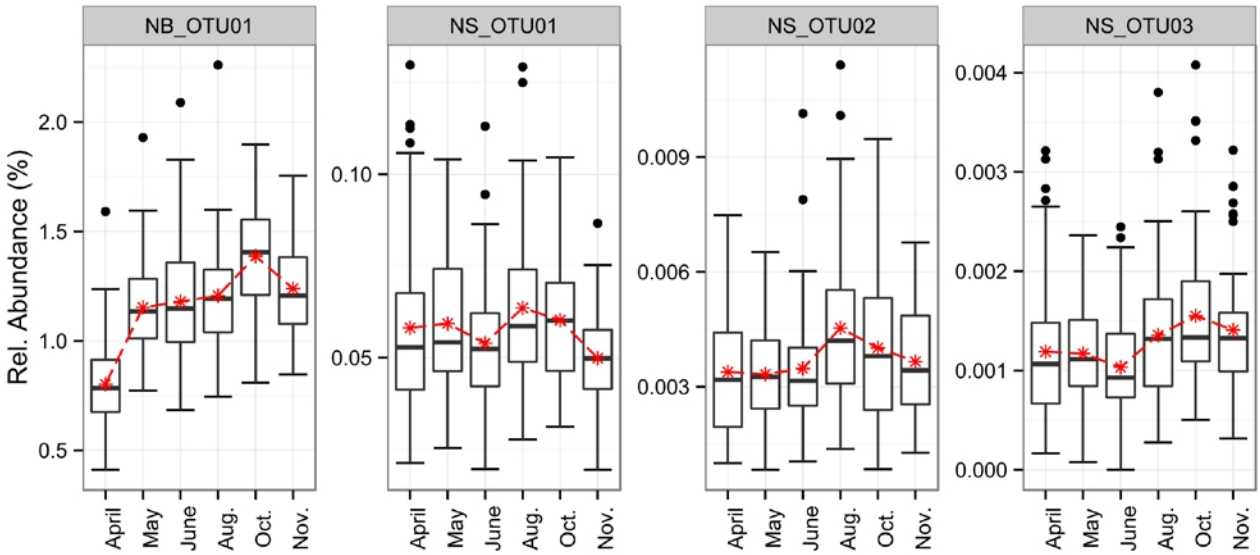
2 Supplementary Figures and Tables

2.1 Supplementary Figures



Supplementary Figure S1. Boxplots for seasonal dynamics of nitrite-oxidizing bacteria associated OTUs, showing relative read abundances.

Supplementary Figure S2. The diagram shows pairwise comparisons of NS-like-nitrite oxidizing bacteria-associated OTU-abundances as well as nitrate and ammonium concentrations in the soil. Each row/column represents one of the 5 parameters, with the diagonal showing density plots. The lower triangle of the plot matrix consists of scatterplots, with the corresponding Pearson correlation coefficients appearing in the upper triangle. Data is always colored according to the sample dates. The scale for the OTU is representing percentage abundances; for ammonium and nitrate, concentrations (given in µg N per g soil dry weight) are used.

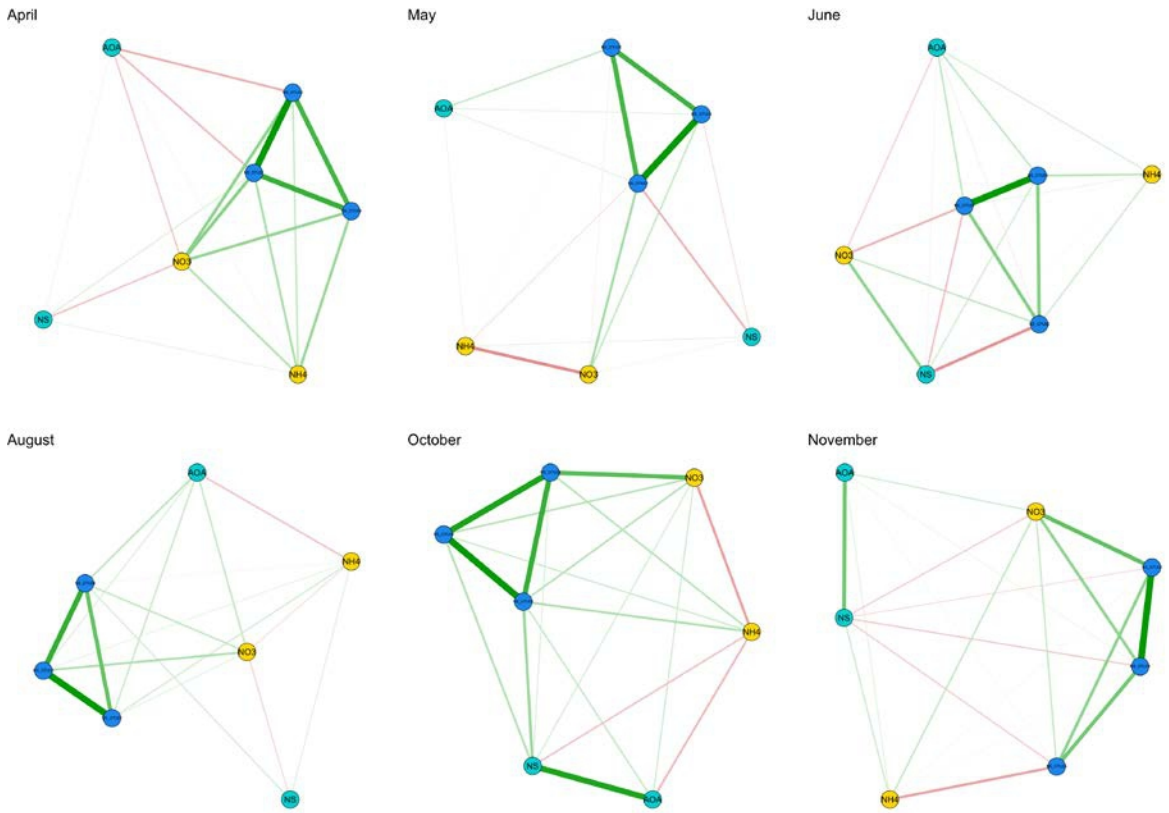


Figure S3: **Network analysis of interactions between NS-assigned OTUs, gene abundances and nitrification-associated nitrogen-pools**.

Depicted are Pearson correlations between three parameter groups for all sampling dates: gene abundances (light blue circles), *Nitrospira* OTUs 01-03 (dark blue circles) and nitrate and ammonium concentrations (yellow circles), respectively. Edges between the nodes are weighted according to the correlation strength. Positive coefficients are colored in green, negatives are displayed in red. AOA = Ammonia-oxidizing archaea, NS = *Nitrospira*-like nitrite-oxidizing bacteria (NOB).

2.2 Supplementary Tables

Supplementary Table S1. Thermal profiles, primer and standards used for real-time PCR quantification of the following genes: *amoA* (AOA), *amoA* (AOB), *nxrA* (NB) and 16S rRNA genes (NS). AOA = ammonia-oxidizing archaea, AOB = ammonia-oxidizing bacteria, NB = *Nitrobacter*-like, NS = *Nitrospira*-like.

Target gene	Standard source	Primer	Primer reference	Thermal profile	No. of cycles
amoA (AOA)	Fosmid clone 54d9	amo19F	Leininger et al., 2006	94°C/45 s, 55°C/45 s, 72°C/45 s	40
		CrenamoA16r48x	Schauss et al., 2009		
amoA (AOB)	*Nitrosomonas sp.*	amoA1F	Rotthauwe et al., 1997	94°C/60 s, 58°C/60 s, 72°C/60 s	40
		amoA2R	Rotthauwe et al., 1997		
nxrA (NB)	*Nitrobacter hamburgensis*	F1norA	Poly et al., 2008	94°C/30 s, 55°C/30 s, 72°C/30 s	40
	X14 (DSMZ 10229)	R2norA	Wertz et al., 2008		
16S rRNA gene (NS)	*Nitrospira* 16S rRNA gene	Nspra 675f	Graham et al., 2007	94°C/30 s, 64°C/30 s, 72°C/60 s	40
	Accession No. FJ529918	Nspra 746r	Graham et al., 2007		

Supplementary Table S2. Gene abundances for *amoA* (AOA), *amoA* (AOB), *nxrA* (NB) and 16S rRNA genes (NS) in copy numbers per g of soil (dry weight) at different sampling dates. Values represent mean values for the complete plot at the respective date including corresponding standard deviations. AOA = ammonia-oxidizing archaea, AOB = ammonia-oxidizing bacteria, NB = *Nitrobacter*-like, NS = *Nitrospira*-like. Asterisks indicate transitions between months which are statistically significant based on two models (i) linear Gaussian mixed models with time as random effect and corrected for spatial autocorrelation, but not for heteroscedasticity; and (ii) generalized linear models under the negative binomial distribution with correction for heteroscedasticity, but not for temporal and spatial random effects. Significance levels: * < 0.05, ** < 0.01, *** < 0.001, corrected for multiple testing.

Gene abundances (copies g^{-1} soil dw)	**Date**					
	April	May	June	August	October	November
nxrA (NOB-NB)	$8.07\ 10^5 \pm 2.10\ 10^6$	$1.97\ 10^7 \pm 8.90\ 10^7$ **/*	$6.04\ 10^6 \pm 3.19\ 10^7$	$5.25\ 10^5 \pm 4.80\ 10^5$ **/***	$4.53\ 10^5 \pm 7.38\ 10^5$	$9.12\ 10^5 \pm 8.40\ 10^5$ */***
16S rRNA gene (NOB-NS)	$5.19\ 10^7 \pm 3.38\ 10^7$	$1.01\ 10^8 \pm 9.71\ 10^7$ -/**	$9.46\ 10^7 \pm 5.88\ 10^7$	$6.79\ 10^7 \pm 4.99\ 10^7$	$6.37\ 10^7 \pm 4.57\ 10^7$	$1.05\ 10^8 \pm 5.99\ 10^7$ -/**
amoA (AOA)	$3.08\ 10^8 \pm 1.69\ 10^8$	$3.78\ 10^8 \pm 3.18\ 10^8$	$3.24\ 10^8 \pm 3.08\ 10^8$	$1.15\ 10^8 \pm 1.11\ 10^8$ ***/**	$1.81\ 10^8 \pm 1.08\ 10^8$	$5.34\ 10^8 \pm 4.26\ 10^8$ ***/***
amoA (AOB)	$2.58\ 10^6 \pm 3.54\ 10^6$	$1.68\ 10^6 \pm 2.84\ 10^6$	$1.02\ 10^6 \pm 8.19\ 10^5$	$3.46\ 10^6 \pm 3.25\ 10^6$ ***/***	$3.30\ 10^6 \pm 4.33\ 10^6$	$1.27\ 10^6 \pm 6.41\ 10^5$

Supplementary Table S3. Variogram parameters of gene abundances for *amoA* (AOA), *amoA* (AOB), *nxrA* (NB) and 16S rRNA genes (NS) at different sampling dates. Nugget, sill and range values are derived from fitted spherical models; step widths between 0.6 and 0.8 m were applied. Data sets to which no model could be fit ted, are indicated with "-". AOA = ammonia-oxidizing archaea, AOB = ammonia-oxidizing bacteria, NB = *Nitrobacter*-like, NS = *Nitrospira*-like.

Gene	Variogram details	Date					
		April	May	June	August	October	November
nxrA (NOB-NB)	Nugget	1.60	-	-	100.36	-	0.12
	p-Sill	2.96	-	-	261.59	-	0.95
	Sill	4.56	-	-	361.95	-	1.07
	Range [m]	4.5	-	-	7.7	-	12.3
	Nugget / (Nugget + Sill)[a]	26.0	-	-	21.7	-	10.1
	p-Sill / Sill[b]	64.9	-	-	72.3	-	88.8
16S rRNA gene (NOB-NS)	Nugget	171.19	3700.50	2966.92	217.77	351.60	208.79
	p-Sill	1233.20	7522.09	433.67	88.89	4930.48	103.14
	Sill	1404.39	11222.59	3400.59	306.66	5282.08	311.93
	Range [m]	4.6	8.1	7.7	2.9	21.2	1.2
	Nugget / (Nugget + Sill)[a]	10.9	24.8	46.6	41.5	6.2	40.1
	p-Sill / Sill[b]	87.8	67.0	12.8	29.0	93.3	33.1
amoA (AOA)	Nugget	-	-	-	8997.87	8439.89	-
	p-Sill	-	-	-	5171.11	3292.38	-
	Sill	-	-	-	14168.98	11732.27	-
	Range [m]	-	-	-	12.9	4.9	-
	Nugget / (Nugget + Sill)[a]	-	-	-	38.8	41.8	-
	p-Sill / Sill[b]	-	-	-	36.5	28.1	-
amoA (AOB)	Nugget	-	280.39	0.39	-	-	0.40
	p-Sill	-	36.93	0.22	-	-	0.01
	Sill	-	317.32	0.61	-	-	0.41
	Range [m]	-	9.1	7.2	-	-	2.3
	Nugget / (Nugget + Sill)[a]	-	46.9	39.0	-	-	49.4
	p-Sill / Sill[b]	-	11.6	36.1	-	-	2.4

[a] Nugget / (Nugget + Sill) [%] = indicator for spatial distribution

[b] p-Sill / Sill [%] = degree of spatial dependence

Supplementary Table S4. *Nitrospira*-like NOB sublineages. All known *Nitrospira*-like sublineages are listed with cultured / enriched representatives and the respective publication citation. OTUs detected in this study that could be affiliated to different sublineages according to their phylogeny are listed in the last row.

Sublineage	Representative species	Publication	affiliated OTU (this study)
Sublineage I	Ca. *Nitrospira defluvii*	Spieck et al., 2006	OTU01 / OTU02
Sublineage II	*Nitrospira moscoviensis*	Ehrich et al., 1995	OTU01 / OTU02
Sublineage III	16S rRNA clones	Holmes et al., 2001	
Sublineage IV	*Nitrospira marina*	Watson et al., 1986	
Sublineage V	Ca. *Nitrospira bockiana*	Lebedeva et al., 2008	OTU03
Sublineage VI	*Nitrospira calida*	Lebedeva et al., 2011	OTU01 / OTU02

Supplementary Table S5. Correlation matrix for gene abundance data. Pearson correlation coefficients are given for each sampling date and across the complete season to display putative linear correlations of nitrifier abundances. AOA = ammonia-oxidizing archaea, AOB = ammonia-oxidizing bacteria, NB = *Nitrobacter*-like, NS = *Nitrospira*-like.

Date		AOA	AOB	NB	NS
April	AOA		0.255	0.576	0.055
	AOB	0.255		0.506	0.104
	NB	0.576	0.506		0.116
	NS	0.055	0.104	0.116	
May	AOA		-0.432	-0.008	-0.009
	AOB	-0.432		0.233	-0.147
	NB	-0.008	0.233		-0.089
	NS	-0.009	-0.147	-0.089	
June	AOA		0.433	0.159	-0.010
	AOB	0.433		0.265	-0.037
	NB	0.159	0.265		-0.158
	NS	-0.010	-0.037	-0.158	
August	AOA		0.523	0.473	-0.021
	AOB	0.523		0.176	-0.111
	NB	0.473	0.176		0.278
	NS	-0.021	-0.111	0.278	
October	AOA		0.239	0.561	0.574
	AOB	0.239		0.203	0.160
	NB	0.561	0.203		0.579
	NS	0.574	0.160	0.579	
November	AOA		-0.309	0.136	0.398
	AOB	-0.309		0.301	-0.112
	NB	0.136	0.301		0.123
	NS	0.398	-0.112	0.123	
all	AOA		0.092	0.290	0.196
	AOB	0.092		0.134	-0.084
	NB	0.290	0.134		0.153
	NS	0.196	-0.084	0.153	

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PLOS ONE

RESEARCH ARTICLE

Influence of Commonly Used Primer Systems on Automated Ribosomal Intergenic Spacer Analysis of Bacterial Communities in Environmental Samples

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Abstract

Due to the high diversity of bacteria in many ecosystems, their slow generation times, specific but mostly unknown nutrient requirements and syntrophic interactions, isolation based approaches in microbial ecology mostly fail to describe microbial community structure. Thus, cultivation independent techniques, which rely on directly extracted nucleic acids from the environment, are a well-used alternative. For example, bacterial automated ribosomal intergenic spacer analysis (B-ARISA) is one of the widely used methods for fingerprinting bacterial communities after PCR-based amplification of selected regions of the operon coding for rRNA genes using community DNA. However, B-ARISA alone does not provide any taxonomic information and the results may be severely biased in relation to the primer set selection. Furthermore, amplified DNA stemming from mitochondrial or chloroplast templates might strongly bias the obtained fingerprints. In this study, we determined the applicability of three different B-ARISA primer sets to the study of bacterial communities. The results from *in silico* analysis harnessing publicly available sequence databases showed that all three primer sets tested are specific to bacteria but only two primers sets assure high bacterial taxa coverage (1406f/23Sr and ITSF/ITSReub). Considering the study of bacteria in a plant interface, the primer set ITSF/ITSReub was found to amplify (*in silico*) sequences of some important crop species such as *Sorghum bicolor* and *Zea mays*. Bacterial genera and plant species potentially amplified by different primer sets are given. These data were confirmed when DNA extracted from soil and plant samples were analyzed. The presented information could be useful when interpreting existing B-ARISA results and planning B-ARISA experiments, especially when plant DNA can be expected.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

Bacterial automated ribosomal intergenic spacer analysis (B-ARISA) is a widely used, culture-independent, molecular technique for analyzing bacterial diversity and community structure in various types of habitats, including both terrestrial and aquatic ecosystems [1–5]. B-ARISA is a PCR-based method that estimates the number of bacterial operational taxonomic units (OTUs) based on the length heterogeneity of the 16S-23S ribosomal intergenic spacer region (IGS) [1], [2]. This method is highly sensitive, reliable and reproducible [3, 4]. Considering the length of the bacterial IGS region (100–1500 bps), B-ARISA can potentially discriminate at least 700 bacterial OTUs (using a 2 bp window for binning), so this method may be suitable to use for a large number of samples collected over a range of locations and at different times [4]. However, B-ARISA alone does not provide any taxonomic information and the results may be severely biased in relation to the primer set selection [2].

Thus, the aim of this study was to compare the coverage and specificity of three primer sets *in silico* and *in vitro*, mainly to investigate their applicability for studies of bacterial communities at the plant–soil interface: 1406f/23Sr [1], ITSF/ITSReub [2] and S-D-Bact-1522-b-S-20/L-D-Bact-132-a-A-18 [6]. We used the updated databases from December 2012 to March 2014 and improved B-ARISA PCR conditions [7, 8]. In addition, we evaluated the primer sets in a more meaningful way by examining both forward and reverse primers together (with 1 to 3 mismatches) instead of evaluating each primer separately. Furthermore, we evaluated, for the first time the specificity of these three B-ARISA primer sets to bacteria. To this end, we also tested whether the primer sets would amplify (*in silico*) chloroplast, mitochondrial, fungal, plant and invertebrate sequences.

Materials and Methods

Ethics Statement

Field work permits were issued by the responsible environmental offices of the state of Baden-Württemberg, Germany (according to § 72 BbgNatSchG).

In silico testing

To determine the most valuable primer set for the B-ARISA technique, ecoPCR software (http://www.grenoble.prabi.fr/trac/ecoPCR) [9, 10] was used for theoretical sequence amplification by virtual PCR using data from four sets of databases (S1 Databases, S1 Table, S2 Table, S3 Table, S4 Table). The two primer sets (1406f/23Sr and ITSF/ITSReub) that produced the best results from ecoPCR were evaluated further for their coverage and specificity to bacteria using the FastM and ModelInspector tool, implemented in the Genomatix software suite (http://www.genomatix.de/solutions/genomatix-software-suite.html). Some archaeal sequences were also contained in some databases.

In vitro testing

Ten soil samples were obtained from a long-term soil fertilization experiment that has been running for 110 years in Bad Lauchstädt, Germany [11], where different levels of fertilizer application have been compared. Furthermore, wood samples were taken from 10 different logs of two tree species in the Schwäbische Alb Biodiversity Exploratory (five samples from European beech, *Fagus sylvatica* and five samples from Norway spruce, *Picea abies*) [12]. DNA extracts from all samples were processed with B-ARISA as described by Cardinale et al. [2] for primer set ITSF/ITSReub, and as described by Borneman and Triplett [1] modified according

to Yannarell et al. [7] and Frossard et al. [8] for primer set 1406f/23Sr (for more details about the material, methods and statistical analysis, see S1 Methods).

Results

Coverage and specificity of primer sets revealed by the ecoPCR software

Proportions of bacterial taxa virtually amplified by different primer sets for different levels of bacterial taxonomic classification (from phylum to species) in the prokaryote Whole Genome Sequences database (wgs-embl-pro) retrieved from EMBL are presented in Table 1. Primer set 1406f/23Sr achieved a much higher proportion of bacterial taxa virtually amplified than the other two primer sets at all levels of taxonomic classification when either zero or one mismatch was allowed. For Genome Sequence Scan, High Throughput Genome Sequencing and Standard sequence classes of prokaryotes retrieved from the EMBL (embl-pro) and constrained bacterial 16S-23S spacer (ncbi-bact-spacer) databases, the 1406f/23Sr and ITSF/ITSReub primer sets amplified similar percentages of the bacterial species (zero and one mismatch), with proportions much higher than achieved using the S-D-Bact-1522-b-S-20/L-D-Bact-132-a-A-18 primer set (S1 Table and S2 Table). Fig. 1 shows the total number of bacterial taxa virtually amplified by each primer set allowing zero to three mismatches on both forward and reverse primers from the wgs-embl-pro database. The total numbers of bacterial species or sequences with a positive virtual amplification (separated by phylum) for each primer set from the embl-pro, ncbi-bact-spacer and wgs-embl-pro databases are shown in S1 Table, S2 Table, S3 Table. Overall, the 1406f/23Sr and ITSF/ITSReub primer sets perform better than the S-D-Bact-1522-b-S-20/L-D-Bact-132-a-A-18 primer set by amplifying more bacterial species and sequences in all databases especially when zero and one mismatches was allowed.

We tested whether the three primer sets are able to amplify *in silico* the non-target sequences of chloroplast (ncbi-chloro), mitochondria (ncbi-mito), fungi (embl-fun), plant (embl-pln) and invertebrates (embl-inv) (S4 Table). The S-D-Bact-1522-b-S-20/L-D-Bact-132-a-A-18 primer set was most specific for bacterial sequences, only amplifying the IGS region from 4 out of 4945 and 5 out of 115186 species represented in the embl-pln database, respectively for Chlorophyta and Streptophyta. This primer set was unlikely to amplify any chloroplast, mitochondrial, fungal or invertebrate sequences when zero to three mismatches were allowed, except for chloroplast sequences where only one species of Chlorophyta was virtually amplified when 3 mismatches were allowed. The 1406f/23Sr and ITSF/ITSReub primer sets were also specific to bacterial sequences with zero or one mismatch. Primer set 1406f/23Sr virtually amplified 5 out of 27 Chlorophyta (ncbi-chloro), 1 out of 2 Chromerida (ncbi-chloro),

Table 1. Proportion of bacterial taxa with positive virtual amplification in the wgs-embl-pro database revealed by ecoPCR.

Taxonomic rank	No. of taxa	Primer set 1406F/23Sr		Primer set ITSF/ITSReub		Primer set S-D-Bact-1522-b-S-20/L-D-Bact-132-a-A-18	
		0 mismatch	1 mismatch	0 mismatch	1 mismatch	0 mismatch	1 mismatch
phylum	19	84.2	89.5	36.8	68.4	0	52.6
class	32	78.1	87.5	43.8	59.4	0	46.9
order	72	83.3	91.7	44.4	63.9	0	31.9
family	163	79.8	87.1	49.7	64.4	0	34.4
genus	483	65.4	72.7	33.3	48.9	0	16.8
species	1389	48.2	56.7	28.9	40.3	0	12.0

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	No. of species	Primer set 1406F/23Sr				Primer set ITSF/ITSReub				Primer set S-D-Bact-1522-b-S-20/L-D-Bact-132-a-A-18			
		0 mismatch	1 mismatch	2 mismatches	3 mismatches	0 mismatch	1 mismatch	2 mismatches	3 mismatches	0 mismatch	1 mismatch	2 mismatches	3 mismatches
Bacterial phyla:													
Actinobacteria	209	99	133	139	189	103	128	134	134	0	94	121	131
Aquificae	2	2	2	2	2	0	1	1	1	0	1	2	2
Bacteroidetes	126	45	80	84	95	7	15	16	16	0	1	34	64
Chlamydiae	5	4	4	4	4	0	0	0	0	0	0	4	4
Chlorobi	1	1	1	1	1	1	1	1	1	0	0	0	1
Chloroflexi	2	1	1	1	2	0	1	1	1	0	0	0	1
Cyanobacteria	26	23	23	24	24	0	1	21	21	0	0	8	21
Deinococcus-Thermus	2	0	0	0	1	0	0	0	1	0	0	0	0
Firmicutes	379	147	155	172	204	84	117	129	133	0	42	82	145
Fusobacteria	9	0	0	2	5	0	0	0	0	0	0	0	1
Lentisphaerae	1	1	1	1	1	0	0	0	0	0	0	1	1
Planctomycetes	6	0	0	0	6	0	0	0	0	0	0	0	0
Proteobacteria	582	336	357	359	450	204	290	294	294	0	21	194	289
Spirochaetes	28	1	2	2	5	0	0	0	1	0	2	2	2
Synergistetes	7	3	6	6	6	0	1	1	1	0	1	1	3
Tenericutes	18	2	14	16	17	0	0	2	2	0	0	1	8
Thermotogae	3	0	2	2	2	2	2	2	2	0	2	2	2
Verrucomicrobia	6	2	4	4	5	1	1	1	1	0	1	4	4
Archaeal phyla:													
Crenarchaeota	3	0	0	0	0	0	0	3	3	0	0	0	0
Euryarchaeota	19	0	0	0	8	0	0	3	8	0	0	0	0
Thaumarchaeota	1	0	0	0	0	0	0	0	1	0	0	0	0

Fig 1. Number of potentially amplified species for different prokaryotic phyla in the wgs-embl-pro database revealed by ecoPCR by increasing mismatches allowed on both forward and reverse primers. The overlaid heatmap (white = 0, darkest = maximum number of sequences) illustrates rising anticipated amplification success with increasing mismatches and was applied per row (i.e. heatmap is proportional to the sequence number available per phylum). The in-cell bar illustrates the relative contribution of the phyla in the used database.

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2 out of 5 Euglenida (ncbi-chloro), 16 out of 4945 Chlorophyta (embl-pln) and 2 out of 115186 Streptophyta (embl-pln) species. Primer set ITSF/ITSReub amplified only 8 out of 115186 Streptophyta (embl-pln) and 1 out of 1339 Echinodermata (embl-inv) species. The plant sequences amplified *in silico* by the primer sets 1406f/23Sr and ITSF/ITSReub with zero and one mismatch are presented in S1 Sequences and most of them match with bacterial sequences when blasted against GenBank. When three mismatches were allowed, the 1406f/23Sr and ITSF/ITSReub primer sets amplified *in silico* more chloroplast, fungal and plant species; in addition, ITSF/ITSReub also amplified more invertebrate species. Nevertheless, the proportions of non-bacterial species amplifiable by these two primer sets were very low even when three mismatches were allowed.

Coverage and specificity of primer sets revealed by the Genomatix software suite

The total number of virtually amplified sequences using each primer set and the number of different genera to which these sequences belong was analyzed. Genera have been grouped into the corresponding bacterial phyla. Representatives of the phyla Chloroflexi, Deinococcus-Thermus, Gemmatimonades and Planctomycetes were only covered by primer set 1406f/23Sr (S5 Table). Primer set ITSF/ITSReub, however, seems not to amplify sequences belonging to any representative of these phyla. The number of sequences and of genera within each phylum varied between the primer sets tested. A table containing a detailed list of the genera included in the analysis can be found in S6 Table and S7 Table. Twenty one amplifiable sequences were found with primer set1406f/23Sr and 12 sequences with ITSF/ITSReub in the Genomatix database for plant-assigned sequences (S5 Table). Primer set 1406f/23Sr resulted in sequence hits

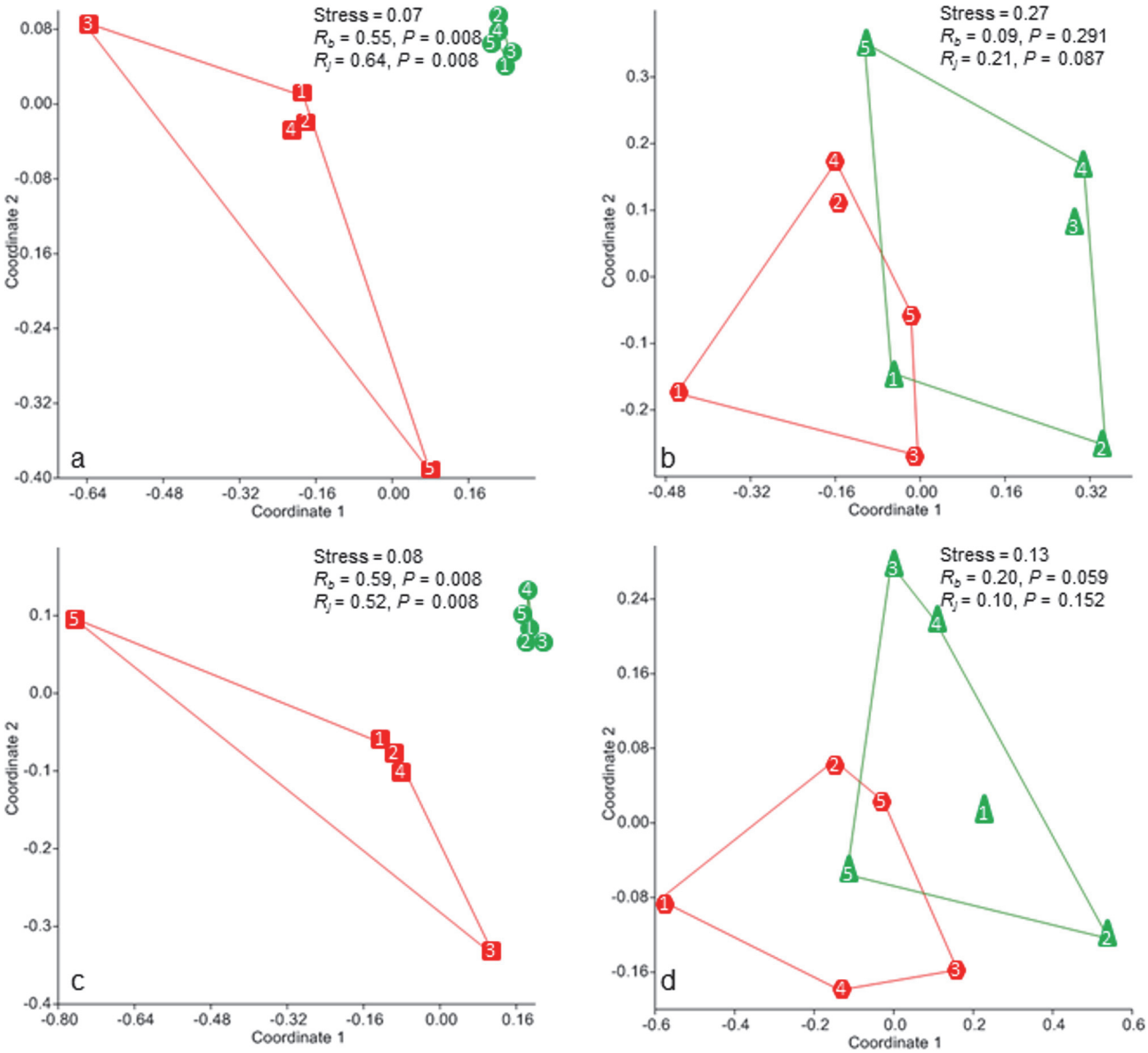

Fig 2. NMDS ordination plots of bacterial community structure in soil (a, c) and wood (b, d) samples using different primer sets: 1406f/23Sr (a, b) and ITSF/ITSReub (c, d). Stress values from the NMDS ordinations and R_{ANOSIM} based on Bray-Curtis (R_b) and Jaccard (R_j) distance measures are shown on the right. Square = fertilized soil, circle = unfertilized soil, hexagon = *Picea abies*, triangle = *Fagus sylvatica*. Each number (1 to 5) represents one individual replicate.

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for green algae (Chlorophyta) to a large extent, whilst red algae (Rhodophyta) and sequences of genus *Zea* were only found for the set ITSF/ITSReub. *Paulinella*, belonging to Rhizaria, could be amplified by both primer sets. In both cases, most sequences were identified as plastid / chloroplast sequences. Besides a few sequences without known genus and not reported in the result tables, the ITS1F/ITSReub primer pair could amplify *Methanocella* of the domain Archaea.

In vitro testing

The figures for bacterial richness and community structure obtained using the 1406f/23Sr and ITSF/ITSReub primer sets were similar (S8 Table; Fig. 2). The correlation between the two

primer sets with respect to OTU richness and Shannon diversity was significant for plant samples (OTU richness: $r = 0.67$, $P = 0.03$; Shannon diversity $r = 0.89$, $P = 0.0007$) but not for soil samples (OTU richness: $r = -0.10$, $P = 0.43$; Shannon diversity $r = 0.24$, $P = 0.46$). When we examined the results for each primer set in order to determine the effects of fertilization (unfertilized *vs*. fertilized soil) and tree species (Norway spruce *vs*. European beech) on bacterial richness and community structure, similar results were obtained regardless of the primer set used.

Discussion and Conclusions

Under changing conditions attributed to the rapid database expansions and new software tools for analyzing the specificity of primer systems, in our study we could show that the 1406f/23Sr and ITSF/ITSReub can be considered as the most promising primer sets for B-ARISA. However those results have to be interpreted in the light of the paucity of the publicly available sequence databases. The wgs-embl-pro was used as the most accurate database to estimate the amplification potential of the IGS region as all tested species were supposed to have the same chance to be virtually amplified by the different primer sets. This is equally true for the non-target ncbi-chloro and ncbi-mito databases as they contain full genomes of chloroplast and mitochondria, respectively. For the other databases however, most of the sequences do not cover the IGS region (embl-pro, -pln, -fun, -inv) or do not contain the region targeted by the primers (ncbi-bact-spacer) resulting in low virtual amplification rates, even for the targeted prokaryotic phyla (S1 Table and S2 Table).

For the primer set ITSF/ITSReub, we could confirm its high coverage and specificity for bacteria when 0 to 1 mismatch was allowed, as obtained by Cardinale et al. [2]. However, for the primer set 1406F/23Sr, current bioinformatics as well as advances in analytical methodology reveal contrasting results compared to a similar study carried out on a datasets almost a decade older [2]. An improvement in the soil DNA extraction method and/or different PCR conditions we used for our B-ARISA for the 1406f/23Sr primer set could also have increased the quality of the B-ARISA fingerprints obtained. Cardinale et al. [2] reported that with the 1406f/23Sr primer set no B-ARISA peak from soil samples (including natural and polluted soil) could be obtained. However, in our study we found that the 1406f/23Sr primer set was quite able to amplify bacterial DNA templates in natural and fertilized soil and the numbers of B-ARISA peaks (OTUs) obtained by 1406f/23Sr and ITSF/ITSReub were not significantly different. When we examined the results for each primer set in order to determine the effects of fertilization (unfertilized vs fertilized soil) and tree species (Norway spruce *vs*. European beech) on bacterial richness and community structure, similar results were obtained regardless of the primer set used. We could also show that, if primers used have comparable properties based on *in silico* analysis, the data obtained for diversity and richness of bacterial communities based on ARISA were highly similar, independent of the studied habitat, which has also been postulated by others [4]. However, we suggest that the bias of each primer set should be taken into consideration when selecting a suitable primer set for each particular experiment. We list bacterial genera and plant species potentially amplified by primer sets 1406F-23Sr and ITSF/ITSFReub; this information could be useful when interpreting existing B-ARISA results and planning B-ARISA experiments involving samples containing plant material.

In conclusion, we consider that B-ARISA is still a powerful tool for analyzing bacterial communities, especially for simple communities originating from a restricted area or a controlled system with known bacterial community composition and biases. Using B-ARISA to investigate complex bacterial communities may still be valuable as it can provide a quick snapshot of bacterial richness and community composition before applying more sensitive approaches

such as amplicon sequencing. The usefulness of B-ARISA patterns can also be seen in the study of Gobet et al. [13] where they were ecologically coherent with the data obtained from 454 pyrosequencing.

Supporting Information

S1 Table. Number of species with positive virtual amplification for different prokaryotic phyla in the embl-pro database (41 phyla, 1200281 sequences) revealed by ecoPCR.
(XLSX)

S2 Table. Number of species with positive virtual amplification for different bacterial phyla in ncbi-bac-spacer database (19 phyla, 37134 sequences) revealed by ecoPCR.
(XLSX)

S3 Table. Number of amplified sequences for different prokaryotic phyla in wgs-embl-pro database (21 phyla, 178462 sequences) revealed by ecoPCR.
(XLSX)

S4 Table. Non-target species amplifiable by the different primer sets. Table shows the potential ability of primer sets to amplify mitochondrial, chloroplast, fungal, plant and invertebrate sequences.
(XLSX)

S5 Table. Number of amplifiable sequences from different bacterial, archaeal, plant and invertebrate phyla and genera revealed by Genomatix software suite.
(XLSX)

S6 Table. List of bacterial genera included for Genomatix analysis.
(XLS)

S7 Table. Bacterial genera potentially amplified by primer sets 1406f/23Sr (a) and ITSF/ITSFReub (b).
(XLSX)

S8 Table. B-ARISA analyses in soil and wood samples for 1406f/23Sr and ITSF/ITSReub primer sets.
(XLS)

S1 Databases.
(DOC)

S1 Methods.
(DOC)

S1 Sequences.
(DOC)

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Author Contributions

Conceived and designed the experiments: WP FB MS DK. Performed the experiments: WP BS GL. Analyzed the data: WP BS GL. Contributed reagents/materials/analysis tools: WP BS GL DF TR. Wrote the paper: WP BS GL MS DK.

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S1 Databases

ecoPCR analyses were performed on four sets retrieved from databases to test coverage and specificity of different B-ARISA primer sets. Though the plant databases actually include other groups of organisms such as algae and protozoa, we simply used the term “plant” to refer to the original name of each database.

First, all Genome Sequence Scan, High Throughput Genome Sequencing and Standard sequence classes of fungi, invertebrates, plants and prokaryotes were retrieved from the EMBL release 118 of December 2013 (embl-fun, embl-inv, embl-pln, embl-pro).

Second, Whole Genome Sequences of prokaryotes were retrieved from the EMBL release 114 of December 2012, and only one entry per species name was conserved for further analysis (wgs-embl-pro).

Third, a more constrained database was retrieved from the NCBI GenBank nucleotide database using the key search “((16S-23S spacer) AND "bacteria"[porgn:__txid2]) AND 200:1000000[Sequence Length]” on March 2013 (Release 194, ncbi-bact-spacer) in order to restrict the database to the potential target sequences (i.e. bacterial intergenic spacer) of the analysed primer pairs.

Finally, the available full chloroplast and mitochondrial genomes were retrieved from the NCBI Organelle Genome Ressources on March 2014 (ncbi-chloro and ncbi-mito).

S1 Methods

Material and methods

Soil and plant samples

Ten soil samples were obtained from the Static Fertilization Experiment in Bad Lauchstädt (Northeastern Germany), a long-term soil fertilization experiment running for over 110 years, (Merbach and Schulz 2012). Five soil samples belong to control (unfertilized soil) and the rest belongs to samples that were treated with manure and chemical fertilized soil (20 t/ha). In addition, ten wood samples were taken from ten different logs of two different tree species (5 samples from European beech, *Fagus sylvatica* and another 5 samples from Norway spruce, *Picea abies*), all from the Schwäbische Alb Biodiversity Exploratory (Fischer et al., 2010). The logs from these two tree species were comparable in length, diameter and decay class. The samples taken from the same log were combined, homogenized, ground into a fine powder with the aid of liquid nitrogen using a swing mill (Retsch, Haan, Germany) and stored at -80 ºC.

DNA extraction and B-ARISA

DNA was extracted from 250 mg homogenized soil or 100 mg homogenized wood samples, using the ZR Soil Microbe DNA MiniPrep kit (Zymo Research, Irvine, CA, USA), according to the manufacturer's instructions. The presence and quantity of genomic DNA was checked using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Dreieich, Germany) and the extracts were then stored at -20ºC. B-ARISA was performed in duplicate reactions using 1µl DNA template solution (20 ng template as determined by NanoDrop) under the conditions described by Cardinale et al. (2004) (primer set ITSF/ITSReub) and Borneman and Triplett (1997) modified according to Frossardet al. (2012) (primer set 1406f/23Sr). For the primer set 1406f/23Sr, briefly, the PCR mixture (20 µl) contained 1 µl DNA template (~20 ng DNA template as determined by NanoDrop); 10 µM of primer 1406f (5'-TGYACACACCGCCCGT-3') labeled with FAM at 5'-end and an unlabeled 23Sr primer (5'-GGGTTBCCCCATTCRG-3'); 4 µl FIREPol 5x Master Mix (Solis BioDyne, Tartu, Estonia); and water to 20 µl. PCR was carried out with an initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 35 s, 55 °C for 45 s and 72 °C for 2 min, with a final extension at 72 °C for 5 min. The PCR products were purified using a PCRExtract Mini Kit (5PRIME, Hamburg, Germany). A standardized quantity of DNA (40 ng of DNA, as determined by NanoDrop) was mixed with 14 µl of deionized Hi-Di formamide (Applied Biosystems, Foster City, CA, USA) and 0.1 µl of internal size standard Map Marker 1500 ROX (50-1500 bp) (BioVentures, Inc, Murfreesboro, TN, USA). The mixture was denatured for 5 min at 95^{o}C and chilled on ice for at least 10 min before being further processed on a capillary sequencer (ABI PRISM 3730xl Genetic Analyzer, Applied Biosystems). The two independent PCR replicates were highly correlated ($\rho = 0.84$, $P < 0.0001$; data not shown). All peaks of the fragments between 200 and

1500 bp that appeared in two technical PCR replicates were used for further analyses (Frossardet al., 2012). Operational taxonomic unit (OTU) binning was carried out using an interactive custom binning script (Ramette, 2009) in R version 2.14.1 (The R Foundation for Statistical Computing, 2011-2012) (binning size = 2 bp, cut off = 0.09%). Double DNA normalization steps before the initial PCR and the separation of DNA fragments via capillary electrophoresis make this standard F-ARISA robust for inferring changes in community structure (Ramette, 2009).

Primer pairs targeting the bacterial 16S-23S intergenic spacer

	Forward	Reverse	Reference
1406f/23Sr	TGYACACACCGCCCGT	GGGTTBCCCCATTCRG	Borneman and Triplett, 1997
ITSF/ITSReub	GTCGTAACAAGGTAGCCGTA	GCCAAGGCATCCACC	Cardinale et al., 2004
S-D-Bact-1522-b-S-20/L-D-Bact-132-a-A-18	TGCGGCTGGATCCCCTCCTT	CCGGGTTTCCCCATTCGG	Ranjard et al., 2000

Statistical analysis

B-ARISA fingerprint data were analyzed using the PAST program (Hammer et al., 2001). OTU richness of different treatments in soil and wood samples were analyzed using the paired t-test incorporating the Jarque-Bera JB test for normality and the *F* test for the equality of group variances, while the bacterial community structures were assessed using one-way ANOSIM based on Bray-Curtis and Jaccard distance measures. Non-metric multidimensional scaling (NMDS) based on Bray-Curtis distance measure was performed to visualize the bacterial community structure of different treatments.

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S1 Sequences

Fragment of the plant sequences *in silico* amplified in the embl-pln databases at 0 and 1 mismatch allowed. Sequences are annotated with their original sequence identifier, their species name, their phylum (if available or “None”) and the primer pair which virtually amplified it.

>FJ789639 *Brassica rapa* Streptophyta 1406f/23Sr
cacaccatgggagttggattcactcgaaggcgttgagctaaccgtaaggaggcaggcgac
cacagtgggtttagcgactggggtgaagtcgtaacaaggtagccgtaggggaacctgcgg
ctggatcacctcctttctaaggatcgtgacgaaagcgtcagtgctagacactgaaagagc
ttcgtcatttccaaagaacatagccgccgtcctcatgtcccttcatcactagagattagc
gcagttcgctgcgctgatagctgagcaggctcaagcgcctctggctgctaacgcagcctg
atttggcagctgggccggtagctcaggtggttagagcgcacgcctgataagcgtgaggtc
ggaggttcaactcctccccggcccaccagcatttggtgaggggctttagctcagctggga
gagcggttgctttgcaagcatcaggtcatcggttcgatcccgataagctccaccatttgc
tgcgcaaatggtcttttgatttgggtccgctgcataagcagcgacggccgttcggccttg
cgaaccttcggttcgttggagcgccaatcaagcgatctgccagatactctagttgatgaa
gatagcggtttaccagccaaggctggtgatatggctcgcacaagcgagcctctttgacat
tgtgaatgggtttttaatcgatgccgtggcgacatggttcggttttggtgtttccgca

agggagcatccggaaagcgggcgatgttgtacacacaagattatctggctgagtttaata
accacaccgatacagctttcgacaaatgctacccagtattgtcgttggtggtgtggactc
tcaagcgtgaggtaagggcatctggtgaatgccttggcatgtacaggcgatgaaggacgt
ggcacgctgcgataagcgtgggggagccgtgagcaggctttgatcccgcgattt

>GU799579 *Carica papaya* Streptophyta 1406f/23Sr
cacaccatgggagtgggttgcaaaagaagtaggtagcttaaccttcgggagggcgcttac
cactttgtgattcatgactggggtgaagtcgtaacaaggtaaccgtaggggaacctgcgg
ttggatcacctccttaccttaaagaagcgttctttgcagtgctcacacagattgtctgat
agaaagtgaaaagcaaggcgtttacgcgttgggagtgaggctgaagagaataaggccgtt
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ttttcgtgtccccttcgtctagaggcccaggacaccgccctttcacggcggtaacagggg
ttcgaatcccctaggggacgccacttgctggtttgtgagtgaaagtcacctgccttaata
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ctgaaaattgaaacactgaacaacgaaagttgttcgtgagtctctcaaattttcgcaaca
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tggatgccctggcagtcagaggcgatgaaggacgtgctaatctgcgataagcgtcggtaa
ggtgatatgaaccgttataaccggcgattt

>AF393605 *Pedinomonas minor* Chlorophyta 1406f/23Sr
cacaccacgggagctggctatgcccaaagtcgttaccccaaccgtttggagggggatgcc
taaggcagagctagtgactggggtgaagtcgtaacaaggtagccgtactggaaggtgcgg
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tgagcataaaaacaaatccatggtcaaaggacatacggattacggtggatacctaggcac
ttagagtcgatgaagggcgtggaaaccaacgaaatgcttcggggagctggaaacaagcta
cgatccggagattc

>AM084273 *Paulinella chromatophora* None 1406f/23Sr
cacaccatggaagttggccatgcccgaagtcgttacttcaacccttgtggaggaggacgc
cgaaggtggggctgatgactggggtgaagtcgtaacaaggtagccgtaccggaaggtgcg
gctggatcacctcctaacagggagacaaaatgtgttactaatgtttggctacttatattg
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cctgggccattagctcaggtggttagagcgcacccctgataagggtgaggtccctggttc
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ctgcgataagtctcggggagctggaaacacgctttgatccgggaattt

>CP000815 *Paulinella chromatophora* None 1406f/23Sr
cacaccatggaagttggccatgcccgaagtcgttacttcaacccttgtggaggaggacgc
cgaaggtggggctgatgactggggtgaagtcgtaacaaggtagccgtaccggaaggtgcg
gctggatcacctcctaacagggagacaaaatgtgttactaatgtttggctacttatattg
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cctgggccattagctcaggtggttagagcgcacccctgataagggtgaggtccctggttc
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ctgcgataagtctcggggagctggaaacacgctttgatccgggaattt

>CP000815 *Paulinella chromatophora* None 1406f/23Sr
cacaccatggaagttggccatgcccgaagtcgttacttcaacccttgtggaggaggacgc
cgaaggtggggctgatgactggggtgaagtcgtaacaaggtagccgtaccggaaggtgcg
gctggatcacctcctaacagggagacaaaatgtgttactaatgtttggctacttatattg
ccattctggtatcctgtcaccttaggtcgatcggtatcttatttcgtaagtctggatttt
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cctgggccattagctcaggtggttagagcgcacccctgataagggtgaggtccctggttc
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acaactacataggcaaaattagaagaataagtttcttatgggggcttacctaaataatag
tagtccatttgttagactattgctataaggataagcttctagccagaataaaatttgatg
ttttgatataaatgcctgcttttaatttgattaaaacaggccaaaatcatgatattagtg
aatctattttgatgatctgccctcgtgattttgaagtttatcaaaaaattaatctggtta
agctacaaagagctcacggtggataccttggcacacagaggcgatgaaggacgtggttac
ctgcgataagtctcggggagctggaaacacgctttgatccgggaattt

>DQ291132 *Oltmannsiellopsis viridis* Chlorophyta 1406f/23Sr
cacaccatggaagttggttccgcccgaagtcgtggatctaacctttggaagaaagcgcc
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agtgctttttgtttaacagctacctttgaggtagcctttggctattatgttcctcccccc

gggaaggttcccagggggaggaacataatggttttaatatttgcggtggcgtgaatcgat
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gtctttgcacggcagatgtcagcggttcgaatccgcttatatccacctgaaatatatttc
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caattagcttagctagttgggtggggcatccaaagatcaactagctaatttaaagtttgc
tagtggttttgtggtcaaatgacaataggcttatggtggatacctaggcacccaaagttg
atgaagggcgcggaaaccggcgaaacgcttcggggagttggaaacacgctttgatccgaa
gatac

>DQ291132 *Oltmannsiellopsis viridis* Chlorophyta 1406f/23Sr
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tacggtgtaactagtaactatggtgaagtcgtaacaaggtatccgtactggaaggtgtgg
attggaatacctcctttaaaggacaaaataatgcgtagcgaagtccttttgactttgct
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>FJ968740 *Pedinomonas minor* Chlorophyta 1406f/23Sr
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>FN563093 *Crustomastix stigmatica* Chlorophyta 1406f/23Sr
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>ED495993 *Sorghum bicolor* Streptophyta ITSF/ITSFReub
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tggtgtttccgcaagggagcatccggaaagcgggcgatgttgtacacacaagattatctg
gctgagtttaataaccacaccgatacagctttcgacaaatgctacccagtattgtcgttg
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>GU799579 *Carica papaya* Streptophyta ITSF/ITSFReub
ggggaacctgcggttggatcacctccttaccttaaagaagcgttctttgcagtgctcaca
cagattgtctgatagaaagtgaaaagcaaggcgtttacgcgttgggagtgaggctgaaga
gaataaggccgttcgctttctattaatgaaagctcaccctacacgaaaatatcacgcaac
gcgtgataagcaattttcgtgtccccttcgtctagaggcccaggacaccgccctttcacg
gcggtaacaggggttcgaatcccctaggggacgccacttgctggtttgtgagtgaaagtc
acctgccttaatatctcaaaactcatcttcgggtgatgtttgagatatttgctctttaaa
aatctggatcaagctgaaaattgaaacactgaacaacgaaagttgttcgtgagtctctca
aattttcgcaacacgatgatgaatcgaaagaaacatcttcgggttgtgaggttaagcgac
taagcgtacac

>AM084273 *Paulinella chromatophora* None ITSF/ITSFReub
ccggaaggtgcggctggatcacctcctaacagggagacaaaatgtgttactaatgtttgg
ctacttatattgccattctggtatcctgtcaccttaggtcgatcggtatcttatttcgta
agtctggattttgtaattcagctatcatttcagttcctaaactttgtctaagtcatgccc
cggcaagggtctcctgggccattagctcaggtggttagagcgcacccctgataagggtga
ggtccctggttcaagtccaggatggcccattcgtgtttgggggtttagctcagttggtag
agcgcctgctttgcaagcaggatgtcagcggttcaaatccgctaacctccattgaccaaa
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ttagaattcagcatttttcttacttagactggtcaaactaaaagtaaaaccagtctaagt
aagaaaaatgctgaactccttgtaatgagactaaatcttagttttcattagagatagttc
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cctaaataatagtagtccatttgttagactattgctataaggataagcttctagccagaa
taaaatttgatgttttgatataaatgcctgcttttaatttgattaaaacaggccaaaatc
atgatattagtgaatctattttgatgatctgccctcgtgattttgaagtttatcaaaaaa
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>CP000815 *Paulinella chromatophora* None ITSF/ITSFReub
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ctacttatattgccattctggtatcctgtcaccttaggtcgatcggtatcttatttcgta
agtctggattttgtaattcagctatcatttcagttcctaaactttgtctaagtcatgccc
cggcaagggtctcctgggccattagctcaggtggttagagcgcacccctgataagggtga
ggtccctggttcaagtccaggatggcccattcgtgtttgggggtttagctcagttggtag
agcgcctgctttgcaagcaggatgtcagcggttcaaatccgctaacctccattgaccaaa
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atgatattagtgaatctattttgatgatctgccctcgtgattttgaagtttatcaaaaaa
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>CP000815 *Paulinella chromatophora* None ITSF/ITSFReub
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agtctggattttgtaattcagctatcatttcagttcctaaactttgtctaagtcatgccc
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cctaaataatagtagtccatttgttagactattgctataaggataagcttctagccagaa
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>DQ369902 *Zea mays* Streptophyta ITSF/ITSFReub
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gacccaccaattttgtgtgggaaacgcctgtagaaatacggggccatagctcagctggga
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c

>DQ369903 *Zea mays* Streptophyta ITSF/ITSFReub
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gtacac

>DQ369905 *Zea perennis* Streptophyta ITSF/ITSFReub
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tgaagacatcaacaagacatcaaacttttataaccatgtcattagacgtgtgttcttag
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>DQ369907 *Zea diploperennis* Streptophyta ITSF/ITSFReub
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cagattgtctgataaaaagtaatgagcaagacggctgcgaagtcgtgacactacccgtgt
ccccttcgtctagcggttaggactccgccctttcacggcggcaacaggggttcgaatccc
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tgacttaaccgtcatgtttgagatattgctctttaacaatccggaacaagctgaaaattg
aaacgacgtgttggttcatttctccgtaataggaaatgaataacaacatgttcgagtctc
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agcgtacac

>DQ369909 *Zea mays* Streptophyta ITSF/ITSFReub
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ctctttaaaaatctggatcaagctgaaaattgaaacgacacatctttaatggtgtgttcg
agtctctcaaattttcgcaatcagaagtgaaacatcttcgggttgtgaggttaagcgact
aagcgtacac

>DQ369911 *Zea mays* Streptophyta ITSF/ITSFReub
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cagattgtctgatagaaaacgagcagtaaaaccttataggcttgtagctcaggtggttag
agcgcacccctgataagggtgaggtcggtggttcaagtccactcaggcctaccaaatttt
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>DQ369913 *Zea mays* Streptophyta ITSF/ITSFReub
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acacgaattgcttgattcattgaagaagacgatagaagcagctttaagctccaagctgat
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aagatagactgaacgttactttcactggtaacggatcaggctaaggtaaaatttgtaagt
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>HF562234 *Chondrus crispus* None ITSF/ITSFReub
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ggggtatagctcagctggtagagcgctgcctttgcaaggcagatgtcagcggttcgagtc
cgcttatctccacaaagttaagttgtacttctcaaataaaatttttattatttgataataa
aaaaaagtaaagtaagtaagagcttac

>JF810595 *Nitella hyalina* Streptophyta ITSF/ITSFReub
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agaagtagggctgcgccctgctatatccaaaataatgcattg

>KC894740 *Grateloupia taiwanensis* None ITSF/ITSFReub
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tacatcccagatcgaaaatataagagctcttaatttaagtgttctaagaaatttgagtaa
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aagtaaatttaaggcttac

>X54299 *Antithamnion sp.* None ITSF/ITSFReub
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tcttatcctaggtcgtaattttcgggctattagctcagttggttagagcgcacccctgat
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tggtagagcgctgcctttgcaaggcagatgtcagcggttcgagtccgcttatctccacgc
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attttatttatagttttatgtataataaaatatcaagtcattaagggcttac

>Z29521 *Chondrus crispus* None ITSF/ITSFReub
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taaccttagatcgtatattaaataatttttaagggctattagctcagatggttagagcgc
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ggggtatagctcagctggtagagcgctgcctttgcaaggcagatgtcagcggttcgagtc
cgcttatctccacaaagttaagttgtacttctcaaataaaattttattatttgataataa
aaaaaagtaaagtaagtaagagcttac

Figure Legends Tables:

Table S1. Number of species with positive virtual amplification for different prokaryotic phyla in the embl-pro database (41 phyla, 1200281 sequences) revealed by ecoPCR.

The overlaid heatmap (white = 0, darkest = maximum number of sequences) illustrates rising anticipated amplification success with increasing mismatches and was applied per row (i.e. heatmap is proportional to the sequence number available per phylum). The in-cell bar illustrates the relative contribution of the phyla in the used database.

Table S2. Number of species with positive virtual amplification for different bacterial phyla in ncbi-bac-spacer database (19 phyla, 37134 sequences) revealed by ecoPCR.

The overlaid heatmap (white = 0, darkest = maximum number of sequences) illustrates rising anticipated amplification success with increasing mismatches and was applied per row (i.e. heatmap is proportional to the sequence number available per phylum). The in-cell bar illustrates the relative contribution of the phyla in the used database.

Table S3. Number of amplified sequences for different prokaryotic phyla in wgs-embl-pro database (21 phyla, 178462 sequences) revealed by ecoPCR.

The overlaid heatmap (white = 0, darkest = maximum number of sequences) illustrates rising anticipated amplification success with increasing mismatches and was applied per row (i.e. heatmap is proportional to the sequence number available per phylum). The in-cell bar illustrates the relative contribution of the phyla in the used database.

Table S4. Non-target species amplifiable by the different primer sets. Table shows the potential ability of primer sets to amplify mitochondrial, chloroplast, fungal, plant and invertebrate sequences. The in-cell bar illustrates the relative contribution of the taxonomic groups in the used database.

Table S5. Number of amplifiable sequences from different bacterial, archaeal, plant and invertebrate phyla and genera revealed by Genomatix software suite.

Table S6. List of bacterial genera included for Genomatix analysis.

Table S7. Bacterial genera potentially amplified by primer sets 1406f/23Sr (a) and ITSF/ITSFReub (b).

Table S8. B-ARISA analyses in soil and wood samples for 1406f/23Sr and ITSF/ITSReub primer sets.

	No. of species	1406f/23Sr				ITSF/ITSReub				S-D-Bact-1522-b-S-20/L-D-Bact-132-a-A-18			
		0 mismatch	1 mismatches	2 mismatches	3 mismatches	0 mismatch	1 mismatches	2 mismatches	3 mismatches	0 mismatch	1 mismatches	2 mismatches	3 mismatches
Bacterial phyla:													
Acidobacteria	452	7	8	8	8	6	7	7	7	0	0	7	7
Actinobacteria	42746	165	202	207	216	219	259	264	265	7	169	210	223
Aquificae	244	11	13	13	13	7	11	11	11	0	6	13	13
Armatimonadetes	4	1	1	1	1	0	1	1	1	0	1	1	1
Bacteroidetes	10499	46	80	85	86	18	26	27	28	0	0	21	57
Caldiserica	1	1	1	1	1	0	0	1	1	0	0	1	1
Calescamantes	1	0	0	0	0	0	0	0	0	0	0	0	0
Chlamydiae	121	14	16	17	17	6	6	6	6	0	0	13	16
Chlorobi	176	9	9	10	10	10	10	10	10	0	0	1	9
Chloroflexi	158	10	11	11	13	0	1	3	3	0	1	10	11
Chrysiogenetes	4	1	1	1	1	1	1	1	1	0	0	0	1
Cloacimonetes	1	0	1	1	1	0	0	1	1	0	0	1	1
Cyanobacteria	5852	268	270	273	273	0	57	282	309	0	4	18	69
Deferribacteres	31	1	4	4	4	0	1	1	1	0	0	3	4
Deinococcus-Thermus	540	6	6	6	7	0	1	6	8	0	1	6	6
Dictyoglomi	8	2	2	2	2	0	2	2	2	0	0	2	2
Elusimicrobia	3	0	0	1	1	0	0	1	1	0	0	0	0
Fibrobacteres	4	1	1	1	1	0	0	0	0	0	0	1	1
Firmicutes	39314	266	286	299	302	214	345	375	378	1	77	190	303
Fusobacteria	178	0	1	3	6	0	0	1	1	0	0	0	2
Gemmatimonadetes	29	1	1	1	1	0	0	0	0	0	1	1	1
Gracilibacteria	7	0	0	0	0	0	0	0	0	0	0	0	0
Ignavibacteriae	2	1	2	2	2	1	1	2	2	0	0	1	2
Lentisphaerae	11	0	0	0	0	0	0	0	0	0	0	0	0
Marinimicrobia	15	0	0	0	0	0	0	0	0	0	0	0	0
Nitrospinae	23	0	0	0	0	0	0	0	0	0	0	0	0
Nitrospirae	61	4	5	5	5	1	3	4	4	0	1	3	5
None*	24184	35	51	55	57	32	35	45	46	0	23	27	31
Omnitrophica	1	0	0	0	0	0	0	0	0	0	0	0	0
Planctomycetes	411	0	6	7	13	0	1	6	7	0	0	3	6
Proteobacteria	109336	624	691	703	711	476	730	763	766	1	85	496	634
Spirochaetes	1349	1	17	17	19	2	3	11	11	0	7	17	18
Synergistetes	84	2	6	6	7	0	3	3	3	0	1	2	4
Tenericutes	1989	50	122	132	136	0	0	80	86	0	0	67	176
Thermodesulfobacteria	21	2	2	2	2	0	0	0	0	0	0	0	2
Thermotogae	123	4	20	20	20	12	12	13	13	0	9	17	20
Verrucomicrobia	400	2	3	5	7	1	1	2	2	0	2	4	5
Archaeal phyla:													
Crenarchaeota	315	0	0	2	29	0	1	37	37	0	0	1	1
Euryarchaeota	2706	0	0	0	60	0	2	84	107	0	0	0	0
Korarchaeota	2	0	0	0	0	0	0	1	1	0	0	0	0
Nanoarchaeota	1	0	0	0	0	0	0	0	0	0	0	0	0
Thaumarchaeota	11	0	0	0	0	0	0	0	3	0	0	0	0
Total (% of total species)	241918	0,63	0,76	0,79	0,84	0,42	0,63	0,85	0,88	0,00	0,16	0,47	0,67

Table S1

	No. of species	1406f/23Sr				ITSF/ITSReub				-b-S-20/L-D-Bact-132-a-A-18			
		0 mismatch	1 mismatches	2 mismatches	3 mismatches	0 mismatch	1 mismatches	2 mismatches	3 mismatches	0 mismatch	1 mismatches	2 mismatches	3 mismatches
Acidobacteria	5	4	4	4	4	3	4	4	4	1	4	4	4
Actinobacteria	587	49	53	53	53	116	131	134	135	2	54	62	66
Aquificae	35	0	0	0	0	0	0	0	0	0	0	0	0
Armatimonadetes	1	1	1	1	1	1	1	1	1		1	1	1
Bacteroidetes	85	9	13	14	14	2	3	3	3	0	0	1	8
Chlamydiae	16	12	13	13	13	3	3	3	3	0	0	12	14
Chloroflexi	5	2	2	2	2	1	1	2	2	0	1	2	2
Cyanobacteria	810	114	114	114	115	0	36	156	163	0	0	7	21
Fibrobacteres	1	1	1	1	1	0	1	1	1	0	0	0	0
Firmicutes	591	58	66	71	71	125	199	221	221	1	29	65	91
Fusobacteria	16	0	0	0	3	0	0	1	1	0	0	0	1
Gemmatimonadetes	3	2	2	2	2	2	2	2	2	1	3	3	3
Nitrospirae	8	2	2	2	2	1	3	3	3	0	2	2	2
None*	135	38	44	45	46	31	42	50	51	1	18	34	43
Planctomycetes	78	2	6	8	8	1	3	7	8	0	0	4	6
Proteobacteria	2060	179	198	201	204	245	351	357	358	4	51	186	228
Spirochaetes	54	1	2	2	2	1	1	2	2	0	2	3	3
Synergistetes	1	1	1	1	1	0	0	0	0	0	0	0	0
Tenericutes	795	38	91	98	102	0	0	75	79	0	0	65	165
Verrucomicrobia	3	1	2	2	2	0	1	1	1	0	1	1	2
Total (% of total species)	5289	9,72	11,63	11,99	12,21	10,06	14,79	**19,34**	**19,63**	0,19	3,14	8,55	12,48

* species without phylum annotation

Table S2

	No. of sequences	1406f/23Sr				ITSF/ITSReub				S-D-Bact-1522-b-S-20/L-D-Bact-132-a-A-18			
		0 mismatch	1 mismatches	2 mismatches	3 mismatches	0 mismatch	1 mismatches	2 mismatches	3 mismatches	0 mismatch	1 mismatches	2 mismatches	3 mismatches
Bacterial phyla:													
Acidobacteria	71	2	2	2	2	0	3	3	3	0	0	3	3
Actinobacteria	38430	134	191	201	458	154	195	204	204	0	141	182	196
Aquificae	779	7	7	7	8	0	2	2	2	0	1	7	7
Bacteroidetes	7448	79	166	175	224	18	29	30	30	0	1	67	122
Chlamydiae	104	4	4	4	4	0	0	0	0	0	0	4	4
Chlorobi	47	1	1	1	1	1	1	1	1	0	0	0	1
Chloroflexi	157	4	4	4	5	0	4	4	4	0	0	0	4
Cyanobacteria	2960	35	35	37	47	0	1	34	34	0	0	13	33
Deinococcus-Thermus	39	0	0	0	1	0	0	0	1	0	0	0	0
Firmicutes	39769	329	351	406	471	192	309	332	336	0	96	201	327
Fusobacteria	652	0	0	3	6	0	0	0	0	0	0	0	2
Lentisphaerae	81	4	4	4	4	0	0	0	0	0	0	4	4
Planctomycetes	1924	0	0	0	16	0	0	0	0	0	0	0	0
Proteobacteria	80470	634	667	673	1039	283	463	467	468	0	30	336	530
Spirochaetes	2162	1	2	2	5	0	0	0	1	0	2	2	2
Synergistetes	421	3	9	9	11	0	1	1	1	0	1	1	4
Tenericutes	269	2	15	17	18	0	0	3	3	0	0	1	9
Thermotogae	69	0	2	2	2	2	2	2	2	0	2	2	2
Verrucomicrobia	147	2	4	4	8	1	1	1	1	0	1	4	4
Archaeal phyla:													
Crenarchaeota	631	0	0	0	0	0	0	3	3	0	0	0	0
Euryarchaeota	831	0	0	0	10	0	0	3	8	0	0	0	0
Thaumarchaeota	1	0	0	0	0	0	0	0	1	0	0	0	0
Total (% of total sequences)	178462	0,70	0,82	0,87	1,31	0,36	0,57	0,61	0,62	0,00	0,15	0,46	0,70

Table S3

	No. of species	1406f/23Sr				ITSF/ITSReub				S-D-Bact-1522-b-S-20/L-D-Bact-132-a-A-18			
		0 mismatch	1 mismatches	2 mismatches	3 mismatches	0 mismatch	1 mismatches	2 mismatches	3 mismatches	0 mismatch	1 mismatches	2 mismatches	3 mismatches
ncbi-mito (mitochondrial database from Metazoa), 3943 sequences													
Acanthocephala	6	0	0	0	0	0	0	0	0	0	0	0	0
Annelida	15	0	0	0	0	0	0	0	0	0	0	0	0
Arthropoda	695	0	0	0	0	0	0	0	0	0	0	0	0
Brachiopoda	4	0	0	0	0	0	0	0	0	0	0	0	0
Bryozoa	7	0	0	0	0	0	0	0	0	0	0	0	0
Chaetognatha	5	0	0	0	0	0	0	0	0	0	0	0	0
Chordata	2611	0	0	3	13	0	0	0	0	0	0	0	0
Cnidaria	83	0	0	0	0	0	0	0	0	0	0	0	0
Ctenophora	2	0	0	0	0	0	0	0	0	0	0	0	0
Echinodermata	32	0	0	0	0	0	0	0	0	0	0	0	0
Entoprocta	2	0	0	0	0	0	0	0	0	0	0	0	0
Hemichordata	4	0	0	0	0	0	0	0	0	0	0	0	0
Mollusca	169	0	0	0	0	0	0	0	0	0	0	0	0
Nematoda	80	0	0	0	0	0	0	0	0	0	0	0	0
Nemertea	9	0	0	0	0	0	0	0	0	0	0	0	0
Onychophora	4	0	0	0	0	0	0	0	0	0	0	0	0
Placozoa	5	0	0	0	0	0	0	0	0	0	0	0	0
Platyhelminthes	57	0	0	0	0	0	0	0	0	0	0	0	0
Porifera	49	0	0	0	0	0	0	0	0	0	0	0	0
Priapulida	2	0	0	0	0	0	0	0	0	0	0	0	0
Rotifera	3	0	0	0	0	0	0	0	0	0	0	0	0
Tardigrada	2	0	0	0	0	0	0	0	0	0	0	0	0
Xenacoelomorpha	1	0	0	0	0	0	0	0	0	0	0	0	0
Xenoturbellida	1	0	0	0	0	0	0	0	0	0	0	0	0
Total (% of total species)	3848	0,00	0,00	0,08	0,34	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
ncbi-chloro (chloroplast database), 498 sequences													
Apicomplexa	7	0	0	0	0	0	0	0	0	0	0	0	0
Bacillariophyta	6	0	0	1	5	0	0	0	0	0	0	0	0
Chlorophyta	27	2	5	19	19	0	0	6	10	0	0	0	1
Chromerida	2	1	1	1	1	0	0	1	1	0	0	0	0
Euglenida	5	0	2	3	4	0	0	0	0	0	0	0	0
Eustigmatophyceae	6	0	0	6	6	0	0	0	0	0	0	0	0
None*	25	1	1	15	18	0	3	9	11	0	0	0	0
Phaeophyceae	3	0	0	0	3	0	0	0	0	0	0	0	0
Streptophyta	406	0	0	11	12	0	0	14	15	0	0	0	0
Xanthophyceae	1	0	0	1	1	0	0	0	0	0	0	0	0
Total (% of total species)	488	0,82	1,84	11,68	14,14	0,00	0,61	6,15	7,58	0,00	0,00	0,00	0,20
embl-fun (fungal database), 1287591 sequences													
Ascomycota	50855	0	0	1	18	0	0	1	7	0	0	0	0
Basidiomycota	23108	0	0	0	2	0	0	0	11	0	0	0	0
Blastocladiomycota	43	0	0	0	0	0	0	0	0	0	0	0	0
Chytridiomycota	409	0	0	0	0	0	0	0	0	0	0	0	0
Cryptomycota	3	0	0	0	0	0	0	0	0	0	0	0	0
Entomophthoromycota	102	0	0	0	0	0	0	0	0	0	0	0	0
Glomeromycota	1100	0	0	0	0	0	0	0	0	0	0	0	0
Microsporidia	818	0	0	1	3	0	0	0	0	0	0	0	0
Monoblepharidomycota	30	0	0	0	0	0	0	0	0	0	0	0	0
Neocallimastigomycota	305	0	0	0	0	0	0	0	0	0	0	0	0
None*	9442	0	0	0	0	0	0	0	9	0	0	0	0
Total (% of total species)	86215	0,00	0,00	0,00	0,03	0,00	0,00	0,00	0,03	0,00	0,00	0,00	0,00

Table S4

embl-pln (plant database), 18132367 sequences													
Aurearenophyceae	2	0	0	0	0	0	0	0	0	0	0	0	0
Bacillariophyta	1334	0	0	1	5	0	0	2	24	0	0	0	0
Bolidophyceae	7	0	0	0	0	0	0	0	0	0	0	0	0
Chlorophyta	4945	7	16	52	62	0	0	21	37	0	0	0	4
Eustigmatophyceae	135	0	0	6	6	0	0	0	0	0	0	0	0
None*	10511	1	1	27	41	0	4	90	116	0	0	0	0
Phaeophyceae	1076	0	0	0	4	0	0	0	12	0	0	0	0
Pinguiophyceae	7	0	0	0	0	0	0	0	0	0	0	0	0
Streptophyta	115186	1	2	13	55	3	8	53	172	0	0	4	5
Xanthophyceae	148	0	0	1	1	0	0	1	1	0	0	0	0
Total (% of total species)	133351	0,01	0,01	0,07	0,13	0,00	0,01	0,13	0,27	0,00	0,00	0,00	0,01
embl-inv (invertebrate database), 3913563 sequences													
Acanthocephala	130	0	0	0	0	0	0	0	0	0	0	0	0
Annelida	3675	0	0	0	0	0	0	3	69	0	0	0	0
Apicomplexa	4536	0	0	0	2	0	0	14	16	0	0	0	0
Arthropoda	132792	0	0	0	12	0	0	289	690	0	0	0	0
Brachiopoda	213	0	0	0	0	0	0	0	1	0	0	0	0
Bryozoa	351	0	0	0	1	0	0	0	13	0	0	0	0
Chaetognatha	52	0	0	0	0	0	0	1	1	0	0	0	0
Chordata	350	0	0	0	0	0	0	0	8	0	0	0	0
Chromerida	2	1	1	1	1	0	0	1	1	0	0	0	0
Cnidaria	4670	0	0	0	0	0	0	354	390	0	0	0	0
Ctenophora	53	0	0	0	0	0	0	25	25	0	0	0	0
Cycliophora	7	0	0	0	0	0	0	0	0	0	0	0	0
Echinodermata	1339	0	0	1	1	0	1	1	4	0	0	0	0
Entoprocta	24	0	0	0	0	0	0	0	0	0	0	0	0
Euglenida	265	0	2	3	6	0	0	0	0	0	0	0	0
Gastrotricha	175	0	0	0	0	0	0	0	0	0	0	0	0
Gnathostomulida	26	0	0	0	0	0	0	0	0	0	0	0	0
Haplosporidia	49	0	0	0	0	0	0	0	0	0	0	0	0
Hemichordata	48	0	0	0	0	0	0	0	0	0	0	0	0
Kinorhyncha	49	0	0	0	0	0	0	0	0	0	0	0	0
Loricifera	2	0	0	0	0	0	0	0	0	0	0	0	0
Mollusca	13006	0	0	0	0	0	0	7	209	0	0	0	0
Myzostomida	46	0	0	0	0	0	0	0	0	0	0	0	0
Nematoda	5337	0	0	0	5	0	0	33	258	0	0	0	0
Nematomorpha	42	0	0	0	0	0	0	0	0	0	0	0	0
Nemertea	333	0	0	0	0	0	0	0	0	0	0	0	0
None*	6874	0	0	2	12	0	1	15	38	0	0	0	0
Onychophora	128	0	0	0	0	0	0	0	0	0	0	0	0
Orthonectida	1	0	0	0	0	0	0	0	0	0	0	0	0
Picozoa	5	0	0	0	0	0	0	0	0	0	0	0	0
Placozoa	95	0	0	0	0	0	0	0	0	0	0	0	0
Platyhelminthes	4313	0	0	0	1	0	0	4	275	0	0	0	0
Porifera	1943	0	0	0	0	0	0	31	153	0	0	0	0
Priapulida	8	0	0	0	0	0	0	0	0	0	0	0	0
Rhombozoa	10	0	0	0	0	0	0	0	0	0	0	0	0
Rotifera	350	0	0	0	0	0	0	11	12	0	0	0	0
Tardigrada	419	0	0	0	0	0	0	0	0	0	0	0	0
Xenacoelomorpha	185	0	0	0	0	0	0	0	0	0	0	0	0
Xenoturbellida	2	0	0	0	0	0	0	0	0	0	0	0	0
Total (% of total species)	181905	0,00	0,00	0,00	0,02	0,00	0,00	0,43	1,19	0,00	0,00	0,00	0,00

* species without higher taxon annotatio

Table S4

	1406f/23Sr		ITSF/ITSReub	
	No. of sequences	No. of genera	No. of sequences	No. of genera
Bacteria:				
Acidobacteria	3	2	2	1
Actinobacteria	150	20	161	22
Aquificae	1	1	1	1
Bacteroidetes	41	7	30	3
Chlamydiae	7	4	7	4
Chlorobi	7	1	6	1
Chloroflexi	4	2	-	-
Cyanobacteria	62	10	22	3
Deferribacteres	2	1	2	1
Deinococcus-Thermus	1	1	-	-
Firmicutes	590	28	648	29
Gemmatimonades	1	1	-	-
Planctomycetes	1	1	-	-
Proteobacteria*:				
α-Proteobacteria	203	36	207	32
β-Proteobacteria	139	20	145	21
γ-Proteobacteria	692	47	692	41
δ-Proteobacteria	41	9	34	7
ε-Proteobacteria	13	4	20	3
Spirochaetes	1	1	2	1
Tenericutes	5	1	3	1
Thermotogae	6	2	2	1
Plants/Algae:				
Chlorophyta	18	12	-	-
Cercozoa (Rhizaria)	3	1	3	1
Streptophyta	-	-	7	3
Rhodophyta	-	-	2	2
Others:				
Annelida (Metazoa)	-	-	-	-
Euryarchaeota (Archaea)	-	-	2	1

* Due to the large numbers of genera within the Proteobacteria, this phylum was subdivided into its respective classes.

Table S5

	1406f/ 23Sr	Genus	ITSF/ ITSReub
Bacterial phyla:			
Acidobacteria	1	*Candidatus Koribacter*	2
	2	*Candidatus Solibacter*	-
Actinobacteria	1	*Acidothermus*	1
	11	*Arthrobacter*	11
	21	*Bifidobacterium*	17
	30	*Corynebacterium*	31
	-	*Curtobacterium*	8
	2	*Frankia*	2
	4	*Kineococcus*	-
	9	*Kitasatospora*	9
	3	*Kocuria*	2
	2	*Microbacterium*	2
	-	*Microlunatus*	1
	1	*Micromonospora*	1
	25	*Mycobacterium*	27
	2	*Nocardia*	2
	2	*Nocardioides*	2
	-	*Rathayibacter*	1
	13	*Rhodococcus*	14
	3	*Rothia*	3
	1	*Rubrobacter*	1
	3	*Salinispora*	3
	12	*Streptomyces*	18
	4	*Thermobifida*	4
	1	*Tropheryma*	1
Aquificae	1	*Hydrogenobacter*	1
Bacteroidetes	13	*Bacteroides*	15
	3	*Capnocytophaga*	-
	3	*Cytophaga*	-
	6	*Flavobacterium*	-
	7	*Parabacteroides*	7
	8	*Porphyromonas*	8
	1	*Salinibacter*	-
Chlamydiae	2	*Candidatus Fritschea*	2
	3	*Chlamydia*	2
	1	*Chlamydophila*	2
	1	*Waddlia*	1
Chlorobi	7	*Chlorobium*	6
Chloroflexi	2	*Anaerolinea*	-
	2	*Roseiflexus*	-
Cyanobacteria	4	*Anabaena*	-
	1	*Aphanizomenon*	-
	1	*Gloeobacter*	-
	4	*Microcystis*	-
	4	*Nostoc*	-
	11	*Prochlorococcus*	4
	32	*Synechococcus*	16
	2	*Synechocystis*	2
	1	*Thermosynechococcus*	-
	2	*Trichodesmium*	-
Deferribacteres	2	*Deferribacter*	2
Deinococcus-Thermu	1	*Deinococcus*	-
Firmicutes	9	*Alkaliphilus*	10
	99	*Bacillus*	95
	15	*Brevibacillus*	15
	3	*Caldicellulosiruptor*	3
	4	*Carboxydothermus*	4
	28	*Clostridium*	68
	6	*Desulfitobacterium*	6
	8	*Desulfotomaculum*	8
	11	*Erysipelothrix*	-
	8	*Finegoldia*	8

	18	*Geobacillus*	19
	-	*Halobacillus*	42
	82	*Lactobacillus*	73
	7	*Lactococcus*	7
	4	*Leuconostoc*	4
	4	*Macrococcus*	4
	4	*Melissococcus*	4
	1	*Moorella*	1
	7	*Oceanobacillus*	9
	2	*Oenococcus*	2
	17	*Paenibacillus*	-
	3	*Pasteuria*	-
	7	*Pediococcus*	2
	2	*Pelotomaculum*	2
	12	*Solibacillus*	12
	-	*Sporolactobacillus*	6
	83	*Staphylococcus*	67
	119	*Streptococcus*	120
	3	*Syntrophomonas*	3
	-	*Tuberibacillus*	3
	-	*Virgibacillus*	27
	24	*Weissella*	24
Gemmatimonades	1	*Gemmatimonas*	-
Planctomycetes	1	*Candidatus Brocadia*	-
Proteobacteria:			
α-Proteobacteria	39	*Acetobacter*	43
	4	*Acidiphilium*	4
	51	*Agrobacterium*	33
	3	*Azorhizobium*	3
	9	*Azospirillum*	9
	6	*Bartonella*	7
	3	*Bradyrhizobium*	11
	3	*Brucella*	3
	1	*Candidatus Pelagibacter*	-
	2	*Chelativorans*	2
	1	*Erythrobacter*	1
	-	*Gluconacetobacter*	1
	3	*Gluconobacter*	6
	3	*Granulibacter*	3
	1	*Hyphomonas*	-
	1	*Jannaschia*	1
	3	*Magnetococcus*	3
	2	*Magnetospirillum*	2
	2	*Maricaulis*	2
	2	*Mesorhizobium*	2
	1	*Methylobacterium*	1
	2	*Nitrobacter*	2
	3	*Novosphingobium*	3
	4	*Ochrobactrum*	-
	4	*Paracoccus*	4
	1	*Phenylobacterium*	-
	3	*Rhizobium*	20
	11	*Rhodobacter*	11
	9	*Rhodopseudomonas*	7
	8	*Rhodospirillum*	8
	1	*Roseobacter*	1
	3	*Ruegeria*	3
	5	*Silicibacter*	5
	3	*Sinorhizobium*	-
	3	*Sphingobium*	3
	2	*Sphingomonas*	2
	1	*Sphingopyxis*	1
β-Proteobacteria	8	*Acidovorax*	8
	9	*Bordetella*	9
	77	*Burkholderia*	77
	6	*Candidatus Accumulibacter*	6

	1	*Collimonas*	1
	4	*Cupriavidus*	4
	4	*Dechloromonas*	4
	2	*Janthinobacterium*	2
	1	*Methylibium*	1
	2	*Methylobacillus*	2
	4	*Neisseria*	4
	1	*Nitrosomonas*	1
	1	*Nitrosospira*	1
	3	*Polaromonas*	3
	1	*Polynucleobacter*	1
	7	*Ralstonia*	7
	1	*Ramlibacter*	1
	2	*Rhodoferax*	2
	-	*Taylorella*	6
	2	*Thiobacillus*	2
	3	*Verminephrobacter*	3
γ-Proteobacteria	5	*Acinetobacter*	5
	14	*Actinobacillus*	12
	19	*Aeromonas*	-
	4	*Alcanivorax*	4
	2	*Alkalilimnicola*	2
	16	*Arsenophonus*	13
	2	*Candidatus Blochmannia*	2
	1	*Candidatus Hamiltonella*	-
	1	*Candidatus Portiera*	23
	5	*Candidatus Regiella*	-
	1	*Candidatus Serratia*	-
	5	*Chromohalobacter*	5
	2	*Cobetia*	2
	9	*Colwellia*	-
	1	*Coxiella*	-
	4	*Dichelobacter*	3
	-	*Edwardsiella*	25
	7	*Enterobacter*	24
	7	*Erwinia*	7
	83	*Escherichia*	84
	9	*Francisella*	9
	23	*Haemophilus*	23
	1	*Haererehalobacter*	2
	5	*Hahella*	5
	96	*Halomonas*	111
	2	*Halorhodospira*	2
	8	*Klebsiella*	8
	4	*Legionella*	4
	2	*Marinobacter*	3
	8	*Marinomonas*	-
	3	*Nitrosococcus*	3
	7	*Pantoea*	7
	7	*Photorhabdus*	7
	-	*Piscirickettsia*	1
	1	*Proteus*	1
	5	*Pseudoalteromonas*	-
	45	*Pseudomonas*	41
	13	*Psychrobacter*	13
	10	*Psychromonas*	10
	1	*Saccharophagus*	1
	14	*Salmonella*	14
	-	*Serratia*	1
	117	*Shewanella*	108
	28	*Shigella*	28
	7	*Sodalis*	7
	3	*Thiomicrospira*	3
	41	*Vibrio*	12
	2	*Wigglesworthia*	-
	7	*Xanthomonas*	22

	35	*Yersinia*	35
δ-Proteobacteria	2	*Anaeromyxobacter*	2
	2	*Bdellovibrio*	-
	12	*Desulfovibrio*	12
	4	*Geobacter*	4
	4	*Myxococcus*	-
	6	*Pelobacter*	6
	8	*Polyangium*	7
	2	*Syntrophobacter*	2
	1	*Syntrophus*	1
ε-Proteobacteria	5	*Arcobacter*	-
	11	*Campylobacter*	13
	3	*Nitratiruptor*	-
	4	*Sulfurimonas*	4
	3	*Sulfurovum*	-
	3	*Wolinella*	3
Spirochaetes	1	*Spirochaeta*	2
Tenericutes	-	*Acholeplasma*	3
	5	*Mycoplasma*	-
Thermotogae	4	*Thermosipho*	-
	2	*Thermotoga*	2

Table S6

Bacterial phyla:

Acidobacteria	Actinobacteria	Aquificae	Bacteroidetes	Chlamydiae	Chlorobi	Chloroflexi	Cyanobacteria
Candidatus Koribacter ab	*Acidothermus* ab	*Hydrogenobacter* ab	*Bacteroides* ab	*Candidatus Fritschea* ab	*Chlorobium* ab	*Anaerolinea* a	*Anabaena* a
Candidatus Solibacter a	*Arthrobacter* ab		*Capnocytophaga* a	*Chlamydia* ab		*Roseiflexus* a	*Aphanizomenon* a
	Bifidobacterium ab		*Cytophaga* a	*Chlamydophila* ab			*Gloeobacter* a
	Corynebacterium ab		*Flavobacterium* a	*Waddlia* ab			*Microcystis* a
	Curtobacterium b		*Parabacteroides* ab				*Nostoc* a
	Frankia ab		*Porphyromonas* ab				*Prochlorococcus* ab
	Kineococcus a		*Salinibacter* a				*Synechococcus* ab
	Kitasatospora ab						*Synechocystis* ab
	Kocuria ab						*Thermosynechococcus* a
	Microbacterium ab						*Trichodesmium* a
	Microlunatus b						
	Micromonospora ab						
	Mycobacterium ab						
	Nocardia ab						
	Nocardioides ab						
	Rathayibacter b						
	Rhodococcus ab						
	Rothia ab						
	Rubrobacter ab						
	Salinispora ab						
	Streptomyces ab						
	Thermobifida ab						
	Tropheryma ab						

						Pro
Deferribacteres	Deinococcus-Thermus	Firmicutes	Gemmatimonades	Planctomycetes	α-Proteobacteria	β-Proteobacteria
Deferribacter ab	*Deinococcus* a	*Alkaliphilus Bacillus* ab	*Gemmatimonas* a	*Candidatus Brocadia* a	*Acetobacter* ab	*Acidovorax* ab
		Brevibacillus ab			*Acidiphilium* ab	*Bordetella* ab
		Caldicellulosiruptor ab			*Agrobacterium* ab	*Burkholderia* ab
		Carboxydothermus ab			*Chelativorans* ab	*Candidatus Accumulibacter* ab
		Clostridium ab			*Azorhizobium* ab	*Collimonas* ab
		Desulfitobacterium ab			*Azospirillum* ab	*Cupriavidus* ab
		Desulfotomaculum ab			*Bartonella* ab	*Dechloromonas* ab
		Erysipelothrix ab			*Bradyrhizobium* ab	*Janthinobacterium* ab
		Finegoldia a			*Brucella* ab	*Methylibium* ab
		Geobacillus ab			*Candidatus Pelagibacter* a	*Methylobacillus* ab
		Halobacillus ab			*Erythrobacter* ab	*Neisseria* ab
		Lactobacillus b			*Gluconacetobacter* b	*Nitrosomonas* ab
		Lactococcus ab			*Gluconobacter* ab	*Nitrosospira* ab
		Leuconostoc ab			*Granulibacter* ab	*Polaromonas* ab
		Macrococcus ab			*Hyphomonas* a	*Polynucleobacter* ab
		Melissococcus ab			*Jannaschia* ab	*Ralstonia* ab
		Moorella ab			*Magnetococcus* ab	*Ramlibacter* ab
		Oceanobacillus ab			*Magnetospirillum* ab	*Rhodoferax* ab
		Oenococcus ab			*Maricaulis* ab	*Taylorella* b
		Paenibacillus ab			*Mesorhizobium* ab	*Thiobacillus* ab
		Pasteuria a a			*Methylobacterium* ab	*Verminephrobacter* ab
		Pediococcus ab			*Nitrobacter* ab	
		Pelotomaculum ab			*Novosphingobium* ab	
		Solibacillus ab			*Ochrobactrum* a	
		Sporolactobacillus b			*Paracoccus* ab	
		Staphylococcus ab			*Phenylobacterium* a	
		Streptococcus ab			*Rhizobium* ab	
		Syntrophomonas ab			*Rhodobacter* ab	
		Tuberibacillus b b			*Rhodopseudomonas* ab	
		Virgibacillus ab			*Rhodospirillum* ab	
		Weissella			*Roseobacter* ab	
					Ruegeria ab	
					Silicibacter ab	
					Sinorhizobium a	
					Sphingobium ab	
					Sphingomonas ab	
					Sphingopyxis ab	

teobacteria

γ-Proteobacteria		δ-Proteobacteria		ε-Proteobacteria		Spirochaetes		Tenericutes		Thermotogae	
Acinetobacter	ab	*Anaeromyxobacter*	ab	*Arcobacter*	a	*Spirochaeta*	ab	*Acholeplasma*	b	*Thermosipho*	a
Actinobacillus	ab	*Bdellovibrio*	a	*Campylobacter*	ab			*Mycoplasma*	a	*Thermotoga*	ab
Aeromonas	a	*Desulfovibrio*	ab	*Nitratiruptor*	a						
Alcanivorax	ab	*Geobacter*	ab	*Sulfurimonas*	ab						
Alkalilimnicola	ab	*Myxococcus*	a	*Sulfurovum*	a						
Arsenophonus	ab	*Pelobacter*	ab	*Wolinella*	ab						
Candidatus Blochmannia	ab	*Polyangium*	ab								
Candidatus Hamiltonella	a	*Syntrophobacter*	ab								
Candidatus Portiera	ab	*Syntrophus*	ab								
Candidatus Regiella	a										
Candidatus Serratia	a										
Chromohalobacter	ab										
Cobetia	ab										
Colwellia	a										
Coxiella	a										
Dichelobacter	ab										
Edwardsiella	b										
Enterobacter	ab										
Erwinia	ab										
Escherichia	ab										
Francisella	ab										
Haemophilus	ab										
Haererehalobacter	ab										
Hahella	ab										
Halomonas	ab										
Halorhodospira	ab										
Klebsiella	ab										
Legionella	ab										
Marinobacter	ab										
Marinomonas	a										
Nitrosococcus	ab										
Pantoea	ab										
Photorhabdus	ab										
Piscirickettsia	b										
Proteus	ab										
Pseudoalteromonas	a										
Pseudomonas	ab										
Psychrobacter	ab										
Psychromonas	ab										
Saccharophagus	ab										
Salmonella	ab										
Serratia	b										
Shewanella	ab										
Shigella	ab										
Sodalis	ab										
Thiomicrospira	ab										
Vibrio	ab										
Wigglesworthia	a										
Xanthomonas	ab										
Yersinia	ab										

Table S7

Environmental sample	1406f/23Sr			ITSF/ITSReub		
	Number of OTUs ± SD	Range of OTU size (bp)	Shannon diversity (mean± SD)	Number of OTUs ± SD	Range of OTU size (bp)	Shannon diversity (mean± SD)
Natural soil (unfertilized soil)	41.00±9.06 a	385 - 1093	3.47±0.23 a	70.20±17.09 a	229 - 1297	3.90±0.23 a
Fertilized soil	39.80±11.28 a	383 - 1053	3.40±0.28 a	59.80±22.64 a	221 - 1301	3.70±0.51 a
Norway Spruce wood	48.20±15.34 a	478 - 1236	3.55±0.36 a	65.40±29.62 a	270 - 1300	3.65±0.64 a
European Beech wood	64.40±19.44 a	446 - 1346	3.58±0.47 a	69.80±29.84 a	234 - 1298	3.42±0.76 a

***Different letters represent statistically significant differences ($P < 0.05$) after the paired t-test . Jarque-Bera JB test was used to test for normality in all datasets

Table S8

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G List of publications and manuscripts

List of Manuscripts / Publications (included or related to this study):

- Allan, E., Manning, P., Alt, F., Binkenstein, J., Blaser, S., Blüthgen, N., Böhm, S., Grassein, F., Hölzel, N., Klaus, V.H., Kleinebecker, T., Morris, E.K., Oelmann, Y., Prati, D., Renner, S.C., Rillig, M.C., Schaefer, M., Schloter, M., Schmitt, B., Schöning, I., Schrumpf, M., Solly, E., Sorkau, E., Steckel, J., Steffen-Dewenter, I., Stempfhuber, B., Tschapka, M., Weiner, C.N., Weisser, W.W., Werner, M., Westphal, C., Wilcke, W., Fischer, M., 2015b. Land use intensification alters ecosystem multifunctionality via loss of biodiversity and changes to functional composition. Ecology Letters 18, 834-843. (published)

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- Beckervordersandforth, R., Tripathi, P., Ninkovic, J., Bayam, E., Lepier, A., Stempfhuber, B., Kirchhoff, F., Hirrlinger, J., Haslinger, A., Lie, D.C., Beckers, J., Yoder, B., Irmler, M., Götz, M., 2010. In Vivo Fate Mapping and Expression Analysis Reveals Molecular Hallmarks of Prospectively Isolated Adult Neural Stem Cells. Cell Stem Cell 7, 744-758. (published)