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Prof. Dr. Ellen Kandeler

Linking Microbial Abundance and Function to Understand Nitrogen Cycling in Grassland Soils

Dissertation

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Kathleen Marie Regan
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Examination Committee

Chairperson:	Prof. Dr. M. Rotehutscord
Supervisor and reviewer:	Prof. Dr. E. Kandeler
Co-reviewer 1:	Prof. Dr. F. Rasche
Co-reviewer 2:	Prof. Dr. Y. Oelmann (Universität Tübingen)

I would like to dedicate this dissertation to my family, who have never questioned my pursuit of what have often appeared to be impossible goals.

This includes my mother and my husband's mother, who as women of a certain generation were both bold, each in her own way. They would be very proud.

And especially this is for my granddaughters Samara and Dove, so that they grow up to know they can be or do anything they choose. It is always worthwhile to pursue a dream.

Affidavit

Pursuant to Sec. 8(2) of the University of Hohenheim's doctoral degree regulations for Dr.sc.agr.

1. I hereby declare that I independently completed the doctoral thesis submitted on the topic "Linking Microbial Abundance and Function to Understand Nitrogen Cycling in Grassland Soils"

2. I only used the sources and aids documented and only made use of permissible assistance by third parties. In particular, I properly documented any contents which I used - either by directly quoting or paraphrasing - from other works.

3. I did not accept any assistance from a commercial doctoral agency or consulting firm.

4. I am aware of the meaning of this affidavit and the criminal penalties of an incorrect or incomplete affidavit.

I hereby confirm the correctness of the above declaration. I hereby affirm in lieu of oath that I have, to the best of my knowledge, declared nothing but the truth and have not omitted any information.

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1 Summary

Understanding the links between microbial communities and ecosystem processes is a major goal of ecosystem ecology. In terrestrial systems, above- and below-ground components are linked both spatially and temporally. Linkages are challenging to identify and quantify, however, due both to the extreme heterogeneity of soil and the fact that phylogenetically related microorganisms can be functionally different, while distantly or even unrelated microbes can perform similar functions. Because nitrogen availability is a limiting factor in many ecosystems, there is great interest in identifying such linkages as they influence nitrogen cycling microorganisms in soil. Most plants and animals cannot utilize atmospheric nitrogen directly, and depend on a complex community of microorganisms to transform atmospheric N_2 into biologically available forms. The series of nitrogen transformations: nitrogen fixation, ammonia oxidation, nitrification, and denitrification, are performed by diverse microorganisms possessing genes that code for particular enzymes which carry out the transformation steps. This thesis aimed to fill a knowledge gap by spatially and temporally characterizing relationships of 1) the soil microbial community, 2) the nitrogen cycling microbial community, and 3) a subset of members of the nitrogen cycling community, in relation to both abiotic soil conditions and plant growth. We aimed to identify linkages between abundance and function within the soil microbial community and in particular the nitrogen cycling component of that community. We chose an unfertilized grassland because, in contrast to more well-studied fertilized sites, unfertilized grasslands depend solely on soil-available nitrogen, most of which is derived from fixation of atmospheric nitrogen and subsequent nitrogen release through mineralization, and because nitrogen cycling in unfertilized perennial grasslands is considered to be both highly efficient and tightly coupled to plant growth. Links among above- and below-ground processes may therefore be identifiable in such an unfertilized grassland plot.

We addressed these goals in three studies. All used a biogeographic (spatially and temporally explicit) approach, sampling one 10 m x 10 m grassland plot six times over one growing season, 60 samples per date, total 360 samples. The first study identified above- and below-ground interactions among plants, soil microorganisms, and abiotic soil parameters as they changed over a season. The second examined interactions and potential niche differentiation between members of the nitrogen cycling microbial community, and the ways in which abiotic processes and changes in plant growth influenced their abundances and potential enzyme activities. The third study examined two microbial groups within the nitrogen cycling microbial community which perform interrelated steps in nitrogen transformation.

Results of the first study indicated that microbial community spatial structure was positively correlated with the local abiotic environment, i.e. physical and chemical soil properties, in spring and autumn, while the density and diversity of plants had an additional effect in the summer. Spatial relationships among plant and microbial communities were detected only in the early summer and autumn when aboveground biomass increase was most rapid and its influence on soil microbial communities greatest due to increased plant demand for nutrients. Individual properties varied in their degree of spatial structure over the season, but spatial structure was evident for most measured parameters in May and October, the dates associated with most rapid plant growth. Differential responses of Gram positive and Gram negative bacterial communities were detected both spatially and in response to seasonal shifts in soil nutrients. We concluded that spatial distribution patterns of soil microorganisms change over a season and that chemical soil properties are more important controlling factors than plant density or diversity.

In the second study, seasonal changes in abundance patterns of the nitrogen cycling community were detected, and were associated with changes in substrate availability related to plant growth stage. Only potential enzyme activities were strongly spatially structured at the studied scale and were strongest at sampling dates corresponding to periods of most active plant growth. Temporal variability in members of the N-cycling community versus the stability of their respective potential enzyme activities provided evidence of short-lived temporal niche partitioning and a degree of microbial functional redundancy.

The third study examined spatial and temporal interactions between ammonia-oxidizing archaea (AOA) and the nitrite oxidizing bacteria *Nitrobacter* and *Nitrospira.* Seasonally varying patterns in co-occurrence and spatial separation between the two nitrite-oxidizers provided evidence of niche differentiation, and these observations were linked to ammonium and nitrate availability. Further phylogenetic analysis indicated temporal shifts in *Nitrospira* community composition, indicating seasonal shifts in active members of this community.

In conclusion, this thesis demonstrated that microbial communities are subjected to many external structuring influences and that the relative importance of these influences is both context and microbial group dependent. Environmental properties were the main structuring agents, but there also appeared to be ephemeral but important shifts in those controls. The use of a biogeographical approach provided clear evidence of a complex, dynamic, but stable soil microbial community. Studies which changed the scale and/or frequency of sampling could yield further insights into the links between abundance and function in grassland soils. This

approach could also be fruitfully applied to more intensively managed grassland and cropland systems to characterize the complexity, stability, and resiliency of those nitrogen cycling microbial communities, information which could be useful for management decisions.

2 Zusammenfassung

Das Verstehen von Zusammenhängen zwischen mikrobiellen Gemeinschaften und Ökosystemprozessen ist eines der großen Ziele der Ökosystem-Ökologie. In terrestrischen Systemen sind ober- und unterirdische Komponenten räumlich und zeitlich miteinander verbunden. Deren Verbindungen zu identifizieren und zu quantifizieren ist jedoch eine Herausforderung, da der Boden einerseits sehr heterogen ist und andererseits phylogenetisch verwandte Mikroorganismen funktionell verschieden sein können, während entfernt oder nicht verwandte Mikroorganismen die gleichen Funktionen erfüllen können. Ein limitierender Faktor in vielen Ökosystemen ist die Stickstoffverfügbarkeit. Daher besteht großes Interesse daran Verbindungen, welche die am Stickstoffkreislauf beteiligten Mikroorganismen beeinflussen, zu identifizieren. Die meisten Pflanzen und Tiere können atmosphärischen Stickstoff nicht direkt nutzen und sind auf eine komplexe Gemeinschaft von Mikroorganismen angewiesen, die atmosphärisches N_2 in biologisch verfügbare Formen transformiert. Die Abfolge der Stickstofftransformationen von Stickstofffixierung über Ammoniakoxidation und Nitrifikation zu Denitrifikation wird von diversen Mikroorganismen durchgeführt. Diese Transformationsschritte werden durch genspezifische Enzymkodierung ermöglicht. Um die Zusammenhänge zwischen Abundanz und Funktion innerhalb der bodenmikrobiologischen Gemeinschaft und insbesondere den Mikroorganismen des Stickstoffkreislaufes zu identifizieren, sind Verbindungen 1) der gesamten bodenmikrobiologischen Gemeinschaft, 2) der am Stickstoffkreislauf beteiligten mikrobiellen Gemeinschaft und 3) einer Untergruppe der Mikroorganismengemeinschaft des Stickstoffkreislaufs räumlich und zeitlich charakterisiert und sowohl mit den abiotischen Bodeneigenschaften als auch mit dem Pflanzenwachstum in Beziehung gesetzt worden. Hierzu wählten wir eine ungedüngte Grünlandfläche aus. Diese Flächen sind, im Gegensatz zu den bereits gut untersuchten gedüngten Grünlandflächen, vollständig auf bodenverfügbaren Stickstoff angewiesen. Der Großteil des Stickstoffes stammt hierbei aus der Fixierung von atmosphärischem Stickstoff mit anschließender Stickstofffreisetzung durch Mineralisation. Zudem gelten Stickstoffkreisläufe in ungedüngtem Dauergrünland als hoch effizient und als eng mit dem Pflanzenwachstum verbunden. Verbindungen zwischen ober- und unterirdischen Prozessen könnten somit auf solch einer ungedüngten Grünlandfläche identifizierbar sein.

Wir untersuchten unsere Ziele im Rahmen von drei Studien. Alle nutzten einen biogeographischen (räumlich und zeitlich aufgelösten) Ansatz, wobei eine 10 m x 10 m Grünlandfläche sechsmal im Verlauf einer Vegetationsperiode beprobt wurde; 60 Proben an

jedem Beprobungszeitpunkt, insgesamt 360 Proben. Die erste Studie identifizierte ober- und unterirdische Interaktionen von Pflanzen, Bodenmikroorganismen und abiotischen Bodeneigenschaften, sowie deren Veränderungen im Jahresverlauf. Die Zweite untersuchte Interaktionen und potentielle Nischendifferenzierungen zwischen Mitgliedern der am Stickstoffkreislauf beteiligten mikrobiellen Gemeinschaft. Zudem wurde die Beeinflussung durch abiotische Prozesse und Veränderungen im Pflanzenwachstum auf die Abundanz und potentiellen Enzymaktivitäten dieser mikrobiellen Gemeinschaft analysiert. Die dritte Studie untersuchte zwei mikrobielle Gruppen, die innerhalb der am Stickstoffkreislauf beteiligten mikrobiellen Gemeinschaft wechselseitige Schritte in der Stickstofftransformation durchführen.

Die Ergebnisse der ersten Studie deuten darauf hin, dass die räumliche Verteilungsstruktur der mikrobiellen Gemeinschaft im Frühjahr und Herbst positiv mit der lokalen abiotischen Umwelt, das heißt den physikalischen und chemischen Bodeneigenschaften, korreliert, während die Pflanzendichte und -diversität einen zusätzlichen Effekt im Sommer zeigte. Räumliche Beziehungen zwischen Pflanzen und der bodenmikrobiologischen Gemeinschaft wurden nur im Frühsommer und Herbst detektiert, wenn die Zunahme an oberirdischer Pflanzenbiomasse am schnellsten und ihr Einfluss auf die bodenmikrobiologische Gemeinschaft durch den gestiegenen pflanzlichen Nährstoffbedarf am größten war. Einzelne Eigenschaften variierten in ihrem Ausmaß an räumlicher Struktur im Jahresverlauf. In Mai und Oktober, den Zeitpunkten mit dem schnellsten Pflanzenwachstum, wurden für die meisten gemessenen Parameter räumliche Strukturen detektiert. Gram positive und Gram negative Bakterien zeigten Differenzen sowohl in ihren räumlichen Verteilungsmustern als auch in ihren Reaktionen auf die jahreszeitliche Veränderung von Bodennährstoffen. Wir folgerten, dass sich räumliche Verteilungsmuster von Bodenmikroorganismen im Jahresverlauf ändern und dass chemische Bodeneigenschaften einen stärkeren Einfluss auf die Bodenmikroorganismen haben als Pflanzendichte oder -diversität. In der zweiten Studie wurden jahreszeitliche Schwankungen in den Abundanzmustern der mikrobiellen Gemeinschaft des Stickstoffkreislaufs detektiert, die mit Veränderungen der Substratverfügbarkeit, bedingt durch die Pflanzenwachstumsphasen, assoziiert waren. Nur die potentiellen Enzymaktivitäten waren auf der untersuchten Skala stark räumlich strukturiert. Am deutlichsten war ihre räumliche Strukturierung zu den Probennahmezeitpunkten mit dem stärksten Pflanzenwachstum ausgeprägt. Die zeitliche Variabilität der am Stickstoffkreislauf beteiligten Mikroorganismen verglichen mit der Stabilität ihrer entsprechenden potentiellen Enzymaktivitäten belegte das Vorhandensein kurzlebiger temporärer Nischendifferenzierung und eines gewissen Grades an funktioneller

Redundanz der Mikroorganismen. Die dritte Studie untersuchte die räumlichen und zeitlichen Interaktionen zwischen ammoniakoxidierenden Archaeen (AOA) und den nitritoxidierenden Bakterien *Nitrobakter* und *Nitrospira*. Sich im Jahresverlauf ändernde Muster zwischen gemeinsamem Auftreten und räumlicher Separation der zwei Nitritoxidierern deuteten auf eine Nischendifferenzierung hin. Diese Beobachtungen standen in Zusammenhang mit der Ammonium- und Nitratverfügbarkeit. Weitere phylogenetische Analysen deuteten eine zeitliche Verlagerung der *Nitrospira*-Gemeinschaftsstruktur an, was einen Hinweis auf jahreszeitliche Schwankungen der aktiven Mitglieder dieser Gemeinschaft gibt.

Schlussendlich demonstrierte diese Arbeit, dass mikrobielle Gemeinschaften von vielen externen, strukturierenden Einflüssen abhängen und dass die jeweilige Bedeutsamkeit dieser Einflüsse sowohl vom Kontext als auch von der mikrobiellen Gruppe abhängig ist. Umwelteigenschaften waren die wichtigsten strukturierenden Einflussgrößen, aber es hatte auch den Anschein, dass kurzzeitige, bedeutende Verschiebungen in diesen auftreten. Die Verwendung eines biogeographischen Ansatzes lieferte klare Beweise für eine komplexe, dynamische, aber stabile bodenmikrobiologische Gemeinschaft. Studien, die die Skala und/oder Frequenz der Beprobung verändern, könnten weitere Einblicke in die Frage der Zusammenhänge zwischen Abundanz und Funktion in Grünlandböden geben. Dieser Ansatz könnte auch erfolgreich in intensiver genutzten Grünlandflächen oder Ackerbausystem eingesetzt werden, um die Komplexität, Stabilität und Resilienz der dort am Stickstoffkreislauf beteiligten Bodenmikroorganismengemeinschaften zu charakterisieren, sowie um für Bewirtschaftungsentscheidungen nützliche Informationen zu gewinnen.

3 General Introduction

3.1 Linking microbial abundance and function

Understanding the links among microbial abundance, diversity and ecosystem function is a major goal of ecosystem ecology, but identifying linkages between microbial communities and ecosystem processes remains challenging (Carney & Matson 2005; Prosser *et al*., 2007; van der Heijden *et al*., 2008; Petersen *et al*., 2012; Wallenstein & Hall 2012; Graham *et al*., 2016). As a result, there continues to be much uncertainty about the relationship between microbial diversity and ecosystem function (Cavigelli & Robertson 2000; Hättenschwiler *et al*., 2005; Krause *et al*., 2014). In terrestrial systems, above- and below-ground components are linked both spatially and temporally (Reynolds *et al.*, 2003; Zak *et al.*, 2003; Wardle *et al.*, 2004; van der Heijden *et al*., 2008). The linkages, however, are challenging to identify and quantify due to the extreme heterogeneity of the soil environment (Coleman & Whitman 2005) and the fact that phylogenetically related microorganisms can be functionally different, while distantly or even unrelated microbes can perform similar functions (Torsvik *et al*., 2002; Cadotte *et al*., 2011; Flynn *et al*., 2011).

3.2 Relevance of grasslands to linkages

Grasslands are of particular importance with respect to links between microbial abundance and function. They are highly dynamic ecosystems covering almost 40% of global land area and representing 80% of agricultural land (Boval & Dixon 2012). They provide a wide range of ecosystem goods and services; feed and forage, soil stabilization, flood control, and potential sinks for carbon under a changing climate (Millenium Ecosystem Assessment 2005). Soil microbial communities form the basis of almost all the biogeochemical processes on which these ecosystem goods and services depend.

3.3 Scales and their relevance

3.3.1 *Spatial scales*

Many terrestrial studies have shown that soil microbial communities are structured at several spatial scales (Nunan *et al*., 2002, 2003; Franklin & Mills 2003; Ritz et al., 2004; Bru et al., 2011; Keil et al., 2011), indicating effects of environmental drivers such as land use and abiotic conditions. These studies indicate that just as drivers act upon and structure microbial communities at different scales, drivers at different scales also influence components of

microbial communities. Thus, scale is important both as a driver and as a response to drivers. Cultivation regimes, landscape gradients, edaphic factors, and topography have been shown to drive the spatial distribution of microbes at field scales (Ettema & Wardle 2002). Because nitrogen is one of the nutrients most often limiting plant biomass in terrestrial ecosystems, the functional microbial communities involved in the nitrogen cycle have been extensively studied and have served as model systems in microbial ecology (Kowalchuk & Stephen 2001; Philippot & Hallin 2005). Most spatial studies of the nitrogen cycling microbial community in particular have, to date, been done at field (Hallin *et al*., 2009; Enwall *et al*., 2010), and regional scales (Philippot *et al*., 2009; Petersen *et al*., 2012). Nitrifying microbial communities also have been spatially modeled at the micrometer scale (Grundmann *et al*., 2001). At this scale, small differences in substrate hotspots, soil aggregates, and fine roots can change the environment for microbes. Soil is an extremely heterogeneous environment due to differences in pore size and volume, particle size and distribution, and aggregate sizes. This physical complexity influences the likelihood of microorganisms encountering substrate and oxygen, as well as other microorganisms (Grundmann *et al*., 2001; Kuzyakov & Blagodatskaya 2015). These factors contribute to the creation of conditions for spatial ordering of microorganisms at small scales in soil.

3.3.2 *Temporal scales*

Microbial communities also show distinct and differing response patterns in time. 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 is highly variable, and changes among members of the microbial community vary depending on the choice of observed time scale. On the scale of hours to days, changes in patterns of microbial communities (Schmidt *et al*., 2007) and sometimes even community structure (Cruz-Martinez *et al*., 2012) have been detected, while over longer time periods, clear shifts in microbial community structure can change (Grayston *et al*., 2001; Bardgett *et al*., 2005; Dandie *et al*., 2008; Habekost *et al*., 2008; Lauber *et al*., 2013). During periods of vegetative plant growth, plant-derived exudates and availability of labile carbon act as drivers of microbial community structure and function (Houlden *et al*., 2008; Kuzyakov & Blagodatskaya 2015), while during plants' senescent phase, plant derived litter is the most important microbial driver through supply of carbon; both litter amount and quality strongly influence microbial performance in soil (Chapin *et al*., 2002; Wardle *et al*., 2004; Houlden *et al*., 2008; Kuzyakov & Xu 2013).

However, attempts to clarify the influence of plants on microbial communities have had mixed results. Balser and Firestone (2005) observed stable soil microbial communities in grasslands under season-long perturbations, but Habekost *et al*. (2008) observed temporal variation in the responses of different components of the microbial community to vegetation changes in a grassland over a season. Habekost *et al*. (2008) also showed that there were time lags between belowground responses to aboveground manipulations of plant functional groups. In other studied grassland plots, temporal shifts in soil microbial communities have not been clearly predictable (Lauber *et al*., 2013). Rather, interactions between the soil environment and diverse plant communities typical of grasslands were not easily teased apart due to their complexity. Bardgett *et al*. (2005) proposed that above- and below-ground communities operate at a hierarchy of temporal scales, from days to seasons to millennia, with different consequences for both ecosystem structure and function. In temperate grassland ecosystems, it has been shown that soil microbial communities are strongly influenced by vegetation type and site characteristics, with microbial community structure exhibiting high temporal variability as soil fertility changes over time (Grayston *et al*., 2001).

A rapid increase in the application of spatial methods over the last 20 years to both above- and below-ground communities has clarified interactions between plants and soil microorganisms at multiple spatial scales (Zak *et al*., 2003; Franklin & Mills 2007; Habekost, *et al*., 2008; Steffens *et al*., 2009; Kulmatiski & Beard 2011; Prosser 2012). These methods, coupled with traditional approaches, have expanded and improved our understanding of spatial relationships in the soil (Goovaerts 1998; Zak, *et al*., 2003), some specifically in grasslands (Ritz *et al*., 2004; Berner *et al*., 2011; Keil *et al*., 2011), making it possible to better characterize controls on soil microbiological processes (Bradford & Fierer 2012). But the nature and direction of these linkages, the role of soil microorganisms in mediating biogeochemical cycles, and plant-microbe interactions over time still have not been fully characterized (Schimel & Schaeffer 2012; Reynolds *et al*., 2003; Habekost *et al*., 2008; Kulmatiski & Beard 2011). Studies combining both spatial and temporal approaches, particularly at intermediate (cm to m^2) scales, are still rare. It is not yet known which spatial and temporal factors most strongly influence microbial communities at these scales, especially in systems such as grassland plots, where plant species heterogeneity is high and soil edaphic factors are stable.

3.4 Soil microbial community structure

One well established way to describe and quantify the soil microbial community is through the use of phospholipid fatty acid (PLFA) analysis. Phospholipids are present in the membranes of

all living cells and are the primary lipids making up cell membranes. Soil microbial community analysis using PLFA analysis is a robust method for identifying and quantifying different groups of bacteria and fungi (Frostegard & Baath 1996; Zelles 1999). Specific fatty acids, separated in a series of chemical extraction steps, are characteristic of, for example, Gram-positive and Gram-negative bacteria, as well as certain fungi (Ruess & Chamberlain 2010). More importantly, measuring changes in the signature fatty acids extracted through this method over a period of time can also provide insight into changes in the microbial community as a result of, for example, nutritional stress, changes in environmental conditions, or substrate availability (White *et al*., 1993; Zelles 1999; Flynn *et al*., 2011; Wixon & Balser 2013). Using PLFAs to characterize the living soil microbial community thus makes it possible to establish spatial and temporal linkages that may then be applied to understand processes which are mediated by subsets of that community (Ruess & Chamberlain 2010; Frostegard *et al*., 2011).

3.5 Nitrogen cycling community

The relationship between those soil microbes involved in nitrogen cycling and the larger soil microbial community is a question of more current interest (Schimel *et al*., 2005). Because some processes in soil, including but not limited to nitrogen cycling, are carried out by many organisms and often involve multiple steps, it is critical to characterize links between abundance and function at the level of the broader soil microbial community before examining specific processes such as nitrogen cycling (Schimel 1995).

3.5.1 *Nitrogen fixation and ammonia oxidation – nitrification*

Nitrogen availability is a limiting factor in many ecosystems. All organisms require nitrogen in order to live, as nitrogen is an essential component of amino acids needed for protein synthesis. Although Earth's atmosphere is 78% nitrogen gas (N_2), most plants and animals cannot utilize this nitrogen directly. Instead, they are dependent on a complex community of microorganisms to transform N_2 into biologically available forms they can then use. The series of nitrogen transformations: biological nitrogen fixation, ammonia oxidation, nitrification, and denitrification, are performed by a diverse community of microorganisms possessing genes that code for particular enzymes which carry out the different nitrogen transformation steps.

Simply stated, the overall reaction steps of biological nitrogen fixation, ammonia oxidation and nitrification include first, making atmospheric nitrogen biologically available through nitrogen fixation, a process carried out exclusively by prokaryotic bacteria. Some are free-living, others

are symbiotically associated with plants, but all contain the *nitrogenase* enzyme complex, which enables the conversion of N_2 gas to ammonia (NH_3). Nitrogenases are produced by the expression of a series of *nif* genes. The ammonia produced by fixation is oxidized by both ammonia-oxidizing archaea (AOA) and bacteria (AOB) (Treusch *et al*., 2005) to hydroxylamine and nitrite. This step is catalyzed by the expression of the functional gene *amo*A which encodes for the enzyme ammonia monooxygenase. Nitrite, a product of ammonia-oxidation, is in turn available as substrate for nitrite oxidizing bacteria, and under aerobic conditions is quickly further transformed to nitrate. There is generally considered to be a close relationship between the two processes, ammonia-oxidation and nitrite oxidation, as the first produces essential substrate for the second, which transforms nitrite, a potential toxin, into nitrate (Maixner *et al*., 2006). Nitrate can then be taken up by plants, further transformed by denitrification, or, because it is water soluble, can also be lost in runoff from soils.

3.5.2 *Denitrification*

The denitrification portion of the nitrogen cycle is a series of reduction steps comprising four stages: nitrate (NO_3^-) to nitrite (NO_2^-), to nitric oxide gas (NO), to nitrous oxide gas (N_2O), to dinitrogen gas (N_2). Each step is catalyzed by metallo-enzymes produced by specific genes which typically are expressed under low oxygen or anaerobic conditions (Zumft 1997). The first step, NO_3^- to NO_2^-, can be catalyzed by two different enzymes, Nar (membrane-bound NO_3^- reductase) and Nap (periplasmic NO_3^- reductase), encoded for by *nar* and *nap* genes, respectively, which differ in their location in the cell and in their biochemical properties (Zumft 1997). The second step transforms the reduced NO_2^- to its first gaseous form, NO, by two distinct types of *nir* genes that encode enzymes which are structurally different but functionally equivalent (Hallin & Lindgren 1999). They differ in that *nirK* encodes a copper-containing enzyme (Cu-*nir*), and *nirS* encodes enzymes with cytochromes c and d (cd_1-*nir*). The third step, from NO to N_2O, is catalyzed by *nor* genes, and the fourth step, from N_2O to N_2, by the *nos*Z gene, encoding N_2O-reductase (Zumft 1997). Not all microorganisms possess all the genes for complete denitrification, however, and many different microorganisms are capable of performing one or more of the denitrification steps. It has also recently been shown that organisms possessing the *nirK* gene more often do not have a corresponding *nosZ* gene than those possessing *nirS* (Philippot *et al*., 2011; Graf *et al*., 2014), and that some soil microorganisms have neither *nirK* nor *nirS*, but only *nosZ* (Sanford *et al*., 2012; Jones *et al*., 2014). The application of molecular techniques such as quantitative real-time polymerase chain reaction (qPCR) has made it possible to quantify specific functional genes and to use them as

markers for the presence and abundance of microorganisms involved in specific steps in the nitrogen cycle. Abundance of specific marker genes does not, however, indicate which members of the microbial community are active.

3.6 Potential enzyme activities

Potential nitrifying (PNA) and denitrifying enzyme activities (DEA) are measured to provide information on the maximum enzymatic capacity for nitrification or denitrification in a soil at optimal substrate concentrations (Stark & Firestone 1996; Koper *et al*., 2010). These potential activity measurements reflect the total capacity of the entire nitrifying or denitrifying microbial community in a soil by providing an estimate of the amount of functionally active enzymes present in that soil. Coupling potential activity measurements with abundance data indicates the functional potential of a microbial community (Patra *et al*., 2006; LeRoux *et al*., 2013). An accurate method of determining nitrifier enzyme biomass present in soil at the time of sampling is chlorate inhibition (Groffman 1987). Replicate incubations are carried out in the dark with and without addition of an AMO inhibitor, preventing the oxidation of nitrite to nitrate in the inhibited samples. Changes in inorganic N concentrations between the replicates are then used to infer nitrification rates (Belser & Mays 1980). DEA is similarly determined, with replicate incubations using acetylene to inhibit microbial reduction of N_2O to N_2. This is also followed by a rate calculation between inhibited and uninhibited samples (Smith & Tiedje 1979). These methods reflect the nitrifying or denitrifying enzymatic potential of the soil being sampled. Watson *et al*. (1994) demonstrated that DEA could be correlated with seasonal changes in soil conditions but their studies indicated that the strength of such correlations varied with sampling scale.

3.7 Phylogenetic analysis

Phylogenetic analysis is the study of evolutionary relationships. Identifying evolutionary connections at the genetic level has enabled researchers to identify divergences in those relationships as well as connections (Brinkman & Leipe 2001; Philippot *et al*., 2009; Jones *et al*., 2008). Thus, although many microorganisms are impossible to characterize at a species level taxonomically (Cohan 2002; Staley 2006; Cohan & Perry 2007), phylogenetic analysis makes it possible to detect evolutionary distinctions within a given microbial group of interest and to distinguish them metabolically or functionally, for example, with respect to nitrification (Bouskill *et al*., 2012) or environmental gradients (Flynn *et al*., 2011). This approach can be applied at different spatial and temporal scales to gain insight into who is active and when, as

well as to better understand preferential life strategies among prokaryotes in soils. The principle by which one type of phylogenetic analysis – Sanger sequencing (Sanger *et al*., 1977) - operates is by incorporating fluorescently labeled deoxyribonucleotide triphosphates (dNTPs) onto specially prepared DNA template strands which are then sequenced repeatedly. Illumina sequencing, which is currently used by many, has built on, improved, and elaborated the principles of the Sanger method, but uses modified dNTPs which contain terminators that block polymerization at a chosen point in order to add a single base at a time with a specific fluorescence to the DNA strand. Coupled with instrumentation advances, this makes it possible to generate high-throughput sequencing which can then be related to reference genomes and applied in a number of ways depending on the genomic question of interest (Bartram *et al*., 2011).

4 Outline of the Thesis

In terrestrial systems, above- and below-ground components are linked both spatially and temporally, but the dynamics of these linkages vary depending on the study scale (Reynolds *et al*., 2003; Zak *et al*., 2003; Wardle *et al*., 2004; van der Heijden *et al*., 2008). Factors shaping microbial communities at one scale may be neither important nor predictive at another. Linkages are also challenging to identify and quantify due both to the extreme heterogeneity of the soil environment (Coleman & Whitman 2005), and to the fact that phylogenetically related microorganisms can be functionally different, while distantly or even unrelated microbes can perform similar functions (Torsvik *et al*., 2002; Krause *et al*., 2014). In particular, studies of small-scale, seasonal variations in grassland microbial communities are lacking, especially those which comprehensively address changes in the abundances and function of microorganisms over time.

We chose a physically homogeneous grassland plot in an unfertilized temperate grassland soil to investigate controls on 1) the soil microbial community, 2) the nitrogen cycling members of that community, and 3) a subset of the nitrogen cycling microorganisms involved in a closely coupled set of nitrification steps. Unfertilized perennial grasslands with high plant diversity have been shown to have higher soil organic carbon, total nitrogen, and microbial carbon; greater food web complexity, more complex biological communities (Grayston *et al*.. 2001; Culman *et al*., 2010), and to use nitrogen more efficiently than those with less plant diversity or more intensive management such as croplands, especially in nutrient-limited soils (Zak *et al*., 2003; Kleinebecker *et al*., 2014). A physically homogeneous, unfertilized grassland plot therefore provided an opportunity to identify controls on abundance and function at the centimeter to meter scale in an environment where those processes are tightly linked. 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. It was 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 for a total of 360 samples. Sample pairs were separated by 50 cm to provide appropriate lag distances for later geostatistical analyses.

The first study investigated the relation of both spatial and temporal variation in soil microbial community structure to changes in plant growth stage and soil abiotic properties over one growing season. We hypothesized that by a temporally and spatially intensive examination of

an unimproved grassland plot 1) we could distinguish spatial changes in microbial distributions over time, and 2) that we could correlate these observed changes with stages of plant growth and soil abiotic properties. Results of the first study indicated that microbial community spatial structure was positively correlated with physical and chemical soil properties in spring and autumn, with additional influence of plant density and diversity in the summer. Spatial structure was evident for most measured properties at the dates associated with most rapid plant growth.

The second study built on what was learned in the first, using a similar biogeographical approach to characterize the microbial groups driving the inorganic nitrogen cycle in soil. The goal was to provide an explicit picture of both spatial and temporal dynamics of the microbial nitrogen cycling community at the plot scale. We addressed the following questions: 1) to what degree are different functional groups of microbes involved in N-cycling spatially correlated at the plot scale and how do these correlations change over a season? 2) Can the observed patterns be related to changes in abiotic characteristics or to changes in plant growth with associated changes in substrate availability? 3) What can the observed patterns tell us about grassland ecosystem N-cycling processes at the studied scale? The second study detected seasonal changes in abundance patterns of the nitrogen cycling microorganisms associated with changes in substrate availability due to plant growth stage, and strong spatial structure of potential enzyme activities at the dates of most rapid plant growth.

The goal of the third study was to determine the relationships among the microbial groups that oxidize ammonia (AOA and AOB) and the nitrite oxidizing bacteria *Nitrobacter* (NB) and *Nitrospira* (NS). Because these successive nitrification transformations steps are temporally and spatially interdependent, with the first step providing substrate for the second, they must be near enough one another in both space and time to complete these nitrification steps. Further, because there are two dominant groups that perform each of the steps, we hoped to identify spatial and/or temporal conditions on the plot that would favor one group or another, and if so, determine what factors influenced them. We hypothesized that the AOB-NB nitrifying network would dominate under high substrate concentrations in spring and summer, and the AOA-NS network under lower substrate conditions in autumn. Furthermore, we wanted to gain deeper and more detailed insight into the spatial dynamics of the metabolically active nitrite oxidizing bacteria NS and potential variability within the NS community, through its phylogenetic characterization – whether it is uniform in time and space and how it is linked to AOA on the plot.

5 Seasonal controls on grassland microbial biogeography: are they governed by plants, abiotic properties or both?*

Kathleen M. Regan[a*], Naoise Nunan[b], Runa S. Boeddinghaus[a], Vanessa Baumgartner[c], Doreen Berner[a], Steffen Boch[d], Yvonne Oelmann[e], Joerg Overmann[c], Daniel Prati[d], Michael Schloter[f], Barbara Schmitt[d], Elisabeth Sorkau[e], Markus Steffens[g], Ellen Kandeler[a], Sven Marhan[a]

[a] Institut für Bodenkunde und Standortslehre, Fachgebiet Bodenbiologie, Universität Hohenheim, Emil-Wolff-Str. 27, 70599 Stuttgart, Germany

[b] CNRS, BioEMCo, Campus AgroParisTech, 78850 Thiverval-Grignon, France

[c] DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen, Leibniz-Institut, Inhoffenstraße 7B, 38124 Braunschweig

[d] Institute of Plant Sciences and Botanical Garden, University of Bern, Altenbergrain 21, CH-3013 Bern, Switzerland

[e] Geoecology, Rümelinstraße 19-23, University of Tübingen, 72070 Tübingen

[f] Helmholtz Zentrum München Research Unit for Environmental Genomics, 85764 Neuherberg, Germany

[g] Lehrstuhl für Bodenkunde, Department für Ökologie und Ökosystemmanagement, Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt, Technische Universität München, 85350 Freising-Weihenstephan, Germany

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5.1 Abstract

Temporal dynamics create unique and often ephemeral conditions that can influence soil microbial biogeography at different spatial scales. This study investigated the relation between decimeter to meter spatial variability of soil microbial community structure, plant diversity, and soil properties at six dates from April through November. We also explored the robustness of these interactions over time. A historically unfertilized, unplowed grassland in southwest Germany was selected to characterize how seasonal variability in the composition of plant communities and substrate quality changed the biogeography of soil microorganisms at the plot scale (10 m × 10 m). Microbial community spatial structure was positively correlated with the local environment, i.e. physical and chemical soil properties, in spring and autumn, while the density and diversity of plants had an additional effect in the summer period. Spatial relationships among plant and microbial communities were detected only in the early summer and autumn periods when aboveground biomass increase was most rapid and its influence on soil microbial communities was greatest due to increased demand by plants for nutrients. Individual properties exhibited varying degrees of spatial structure over the season. Differential responses of Gram positive and Gram negative bacterial communities to seasonal shifts in soil nutrients were detected. We concluded that spatial distribution patterns of soil microorganisms change over a season and that chemical soil properties are more important controlling factors than plant density and diversity. Finer spatial resolution, such as the mm to cm scale, as well as taxonomic resolution of microbial groups, could help determine the importance of plant species density, composition, and growth stage in shaping microbial community composition and spatial patterns.

5.2 Introduction

All natural systems are temporally and spatially bounded and the defined spatial organization observed in many ecosystems suggests that spatial organization is of functional importance (Legendre *et al*., 2005). In terrestrial systems many studies have shown that soil microbial communities are structured at several spatial scales (Franklin & Mills, 2003; Ritz *et al*., 2004; Bru *et al*., 2011; Keil *et al*., 2011), indicating effects of environmental drivers such as land use and abiotic conditions. For example, Franklin and Mills (2003) found multi-scale variations in microbial community spatial structure (from 30 cm to >6 m) with high spatial heterogeneity due to soil properties, in a wheat field study using DNA fingerprinting. Ritz *et al*. (2004), in an unimproved grassland study, observed a high degree of spatial variation in community-level microbiological properties, but were not able to characterize overarching controlling factors.

Keil *et al*. (2011), in contrast, found that ammonia-oxidizing and denitrifying microorganisms were spatially structured in soils from 10 m x 10 m grassland plots. This was confirmed in a study by Berner *et al*. (2011), who found that spatial heterogeneity in grasslands at scales of 1-14 m was related to land use intensity; i.e., fertilization, mowing frequency, and grazing practices. Indeed, many studies indicate a close link between above and belowground components in terrestrial ecosystems (Reynolds *et al*., 2003; Zak *et al*., 2003; Wardle *et al*., 2004; van der Heijden *et al*., 2008). Plants may affect the soil microbial community directly via nutrient and water uptake, litter input, and root exudates, or indirectly, by changing composition or abundance of the decomposer community. Microbes may also have direct or indirect effects on plants; thus, understanding the patterns of interaction between plant and soil microbial communities is critical. However, the degree of coupling between plants and microbial communities has been hard to quantify in grasslands, probably due to the very high plant density (Ritz *et al*., 2004) and/or high plant species richness (Zak *et al*., 2003; Nunan *et al*., 2005). It is also possible that these interactions occur at scales that have not yet been identified.

The picture that emerges from the existing literature is that microbial communities are subjected to many external structuring influences and that the relative importance of these influences is both context and microbial group dependent (Martiny *et al*., 2006). Furthermore, many of the relationships are not particularly strong and it is therefore legitimate to ask whether they persist over time and through seasons. The vast majority of microbial spatial or biogeographic studies have been carried out at a single time point and those studies which have combined spatial and temporal approaches have yielded conflicting results. Zak *et al*. (2003), in a long term study, found that microbial composition and function were influenced by plant diversity, while Grayston *et al*. (2001) found plant productivity, temperature, and moisture to have the strongest effects on soil microbial community structure. However, Habekost *et al*. (2008) observed that distribution patterns of microbial communities in grassland soils changed with time, mainly in response to plant performance. Only a few studies have been carried out at the plot scale in grasslands or agricultural fields over multiple time points (Grayston *et al*., 2001; Habekost *et al*., 2008; Kulmatiski & Beard, 2011, Lauber *et al*., 2013). Coupled spatial characterization with temporal variability of soil microbial communities has been less often explored.

The goal of this study was to resolve some of this uncertainty by a detailed investigation of spatial patterns in microbial community structure to learn how the relationships between microbial communities and their local environment persist over time. Edaphic factors have been shown to exert the strongest influences on microbial community composition at regional and

continental scales (Fierer & Jackson, 2006; Lauber *et al*., 2008; Dequiedt *et al*., 2011; Griffiths *et al*., 2011; Sayer *et al*., 2013). A physically homogeneous grassland plot was used for this study, however. This provided an opportunity to assess what other factors could be identified at specific dates as drivers of spatial relationships of the microbial community to both the local soil environment and to changes in the plant community. One 10 m x 10 m plot in a grassland characterized by low land use was intensively sampled over a complete growing season, from early April, before plants had begun to actively grow, until November of that year when plant growth had ceased after a hard frost. Sampling times were selected to coincide with stages of plant growth in the permanent grassland; replicate samples were separated by 50 cm. Using a combination of conventional and spatial statistical approaches, we characterized above- and below-ground communities both temporally and spatially for each date. Our aim was to learn whether or not changes in microbial abundance, in microbial community structure, or in distributions of plants and microorganisms could be temporally and spatially distinguished.

We hypothesized that (i) by a temporally and spatially intensive examination of an unimproved grassland at the plot scale (10 m x 10 m) we could distinguish spatial changes in microbial biogeography, and (ii) this sampling approach would clarify the degree to which the microbial spatial structures we observed could be correlated with stages of plant growth and soil abiotic properties. We expected also to gain insight into the persistence of microbial spatial structure and the relationships of microbial communities with their environment.

5.3 Materials & Methods

5.3.1 *Site description*

The present study is part of a larger, interdisciplinary project of the German Biodiversity Exploratories (Fischer *et al*., 2010). Our study site is located near the village of Wittlingen, Baden-Württemberg, 48°25´0.01´´ N, 9°30´0.00´´ E, in the Swabian Alb, a limestone middle mountain range in southwest Germany. The study site is AEG31, within which a 10 m x 10 m grassland plot was established. Annual precipitation in 2011, the year in which this study was done, was 810 mm and average temperature was 8.1°C (Fig. S 5.1). The study site is managed at low intensity: no fertilizer is applied, it is mown once per year, and is briefly grazed by sheep for 1-2 weeks typically in late summer or early autumn. The soil type at the site is characterized as a Rendzic Leptosol (FAO classification), a calcareous, shallow AC-soil (typically 10 cm depth), with an average pH of 6.7, containing total 0.66 mg g^{-1} carbon (C) and 0.07 mg g^{-1} nitrogen (N). C/N ratios, pH, and soil texture were uniform over the sampling period.

5.3.2 *Sample design*

A 10 m x 10 m plot was established within this grassland and divided into 30 subplots (each 2m x 1.67m). Within each subplot six pairs of sample locations were randomly assigned, with one pair sampled at each of six dates over the growing season (Fig. S 5.2). Each sample pair per subplot for a given date was separated by 50 cm to provide appropriate lag distances for later geostatistical analyses (Fig. S 5.2). Sixty samples were collected at each date (two individual sample locations per subplot x 30 subplots). A total of 360 soil samples were collected over the season. Each sample location was assigned unique x and y coordinates with respect to the boundaries of the plot. Samples were collected in 2011: on April 5th at the beginning of the vegetation period, May 17th during the main growth phase, June 27th at around peak plant biomass, August 16th two weeks after the grassland was mown, October 5th, nine weeks after mowing and two weeks after it was lightly grazed, and November 21st after the first frost.

5.3.3 *Sampling – aboveground*

On each sampling date, before soil core samples were collected, 20 cm x 20 cm grids were centered over each of the sixty individual sampling points. Vegetation data and above ground biomass were collected from all grids. Above-ground biomass was harvested by cutting all plants at ground level. Biomass samples were sorted into litter (dead leaves and plant matter on the soil surface), grasses (Poaceae), legumes, forbs, bryophytes and *Rhinanthus minor*. The latter was separated because this species parasitizes other plants, and thus may affect the productivity of grasslands (Stein *et al*., 2009). Plants that remained rooted but had senesced were included in living plant biomass. The biomass samples were dried for 48 hours at 80°C and weighed to the nearest 10 mg. From these data total above ground biomass as a measure of grassland productivity was calculated. Furthermore, in May, June, and October all vascular plant species were recorded and their percentage of total ground cover was estimated, following the nomenclature of Wißkirchen and Häupler (1998).

5.3.4 *Sampling – belowground*

Belowground samples were collected with core augers (diameter 58 mm) to a depth of 10 cm. Two cores, one for bulk density and one for biogeochemical analyses, were collected adjacent to each other at each sampling point (Fig. S 5.2). The top one cm, consisting entirely of thatch, was removed from each soil core to avoid introducing surface plant residues into the soil.

Stones, roots, and soil macrofauna were removed in the field. Soil samples were stored at 4°C and sieved (< 5 mm) within 24 hours of collection, then subdivided for further analyses, with aliquots stored at 4°C or frozen at -20°C.

5.3.5 *Physical, chemical, biological soil properties*

Soil texture was determined by laser diffraction analysis (Beckman Coulter LS200 laser diffraction particle size analyzer, Beckman Coulter GmbH, Krefeld, Germany). To first determine the presence of carbonates in the samples they were tested using the Scheibler method (DIN ISO 10693:1997.05) for percent carbonate (CO_3^{-2}) determination in soil. Less than 0.1% CO_3^{-2} was detected; (for details see Appendix A: Methods Supplement 5.1). Soil pH was determined in 0.01 M $CaCl_2$ (soil to solution ratio w/v 1:2.5). Soil water content, reported as % soil dry weight, was determined gravimetrically after drying at 105°C overnight. Bulk density cores were weighed, lengths were measured, cores were dried for 3 days at 105°C, and re-weighed. Root biomass was determined in the bulk density cores; after flushing away the soil, roots were retrieved, dried at 60°C for 3 days and weighed.

Ammonium (NH_4^+) and nitrate (NO_3^-) were extracted with 1 M KCl from soil samples (soil to extractant ratio of 1:4 w/v). Soil suspensions were placed on a horizontal shaker for 30 min at 250 rpm, then centrifuged (30 min at 4400 xg). Concentrations of NH_4^+ and NO_3^- in extracts were measured colorimetrically with a Bran & Luebbe autoanalyzer (Bran & Luebbe, Norderstedt, Germany). To determine the bioavailable phosphorus (P) fractions in soil, the second step of the sequential P fractionation was used (Hedley *et al*., 1982). Five hundred mg of each soil sample were extracted with 0.5 M $NaHCO_3$ (adjusted to pH 8.5) and shaken for 30 min before decantation and filtration (13 P Munktell & Filtrak GmbH, Bärenstein, Germany). Inorganic P concentrations in the extracts were determined colorimetrically with a continuous flow analyzer (Murphy & Riley, 1962). Elemental C and N were analyzed with a MACRO CNS Elemental Analyzer (Elementar-Analysensysteme, GmbH, Hanau, Germany). Because < 0.1% carbonate was detected, total C was assumed to be organic C.

Microbial biomass carbon and nitrogen (C_{mic} and N_{mic}) were determined by chloroform fumigation extraction (Vance *et al*., 1987) with modifications (Keil *et al*., 2011). Extractable organic carbon and extractable organic nitrogen (EOC and EON) were calculated from the supernatants of the non-fumigated samples (Keil *et al*., 2011).

Two g of field moist soil were taken for lipid extraction and fractionation following the alkaline methylation method described in Frostegård *et al*. (1993 a). Samples were measured by gas chromatography (AutoSystem XL. PerkinElmer Inc., Massachusetts, USA) using a flame ionization detector, an HP-5 capillary column and helium as the carrier gas. Fatty acid nomenclature used was described by Frostegård *et al*. (1993 a, b). The following PLFA derived fatty acid methyl ethers (FAMEs) were used as indicators for Gram-positive bacteria (Gram+): a15:0, i15:0, i16:0 and i17:0; Gram-negative bacteria (Gram-): cy17:0 and cy19:0 (Ruess & Chamberlain, 2010). Total bacterial PLFAs were calculated as the sum of Gram+ and Gram- plus the FAME 16:1ω7 which is widespread in bacteria in general. Fungal biomass was represented by the PLFA 18:2ω6.

Bacterial cell numbers were determined using a protocol modified after Lunau *et al*. (2005) and counted by epifluorescence microscopy under blue excitation (Zeiss Axio Imager M2, filter set 38 HE eGFP; Göttingen, Germany) at a magnification of 1,000x. A minimum of 20 microscopic fields were counted for each sample (for details see Appendix A: Methods Supplement 5.1).

5.3.6 *Statistical analyses*

All statistical analyses were carried out in the R environment, (R Development Core Team 2012). Cell count data were log-transformed for all analyses to achieve homogeneity of variance. To test whether plant, microbial and abiotic variables exhibited seasonal changes, univariate, one-way ANOVAs with sampling date as a factor were calculated, followed by Tukey's HSD as post hoc test ($P < 0.05$).

To test whether variables exhibited spatial structure at a given date, semivariogram analyses were assessed using the gstat 2.4.0 Package (Pebesma 2004). Where non-random spatial patterns prevail, spatial structure can be measured by plotting empirical geostatistical functions (i.e. semivariogram functions). Semivariances tend to increase with distance of the sampling points until a plateau (sill) is reached, after which values fluctuate randomly about the sill. In stationary data, the sill equals the total sample variance. The distance at which the sill is reached is called the range and represents the maximum distance of spatial autocorrelation. Semivariograms usually exhibit a discontinuity at the origin, called the nugget effect, which is due either to non-measurable variation below the minimum sampling distance or to measurement error. Structural variance is that part of the total sample variance which is spatially auto-correlated. Empirical semivariograms were calculated for each variable to a maximum distance of 8 m, and a spherical, exponential or linear model was fitted based on RMSE and

visual control. Spherical and exponential models indicate that spatial structure occurs at the measured scale, whereas a linear model indicates spatial structure beyond that scale, i.e. a gradient through the plot. If only the nugget is apparent, no spatial structure can be detected at the sampled scale. The percent structural variance was calculated for spherical and exponential models by subtracting the nugget effect from the sill, and dividing the remaining, or partial, variance by the total sill. When a model could be fitted, a kriged map of the distribution of that property on the plot could be constructed. Maps were constructed using ArcGIS (ESRI 2010, Environmental Systems Research Institute, Redlands, CA, USA).

In order to determine how microbial communities were affected by spatial proximity and by environmental drivers, including root and litter mass, two approaches were used. In the first, principal components analysis (PCA) was used to reduce the dimensionality of the PLFA profiles, allowing the original samples to be scored on a small number of axes (principal components). Each of the principal components represents a distinct pattern of variation and can be considered to describe different aspects of the microbial community structure. Individual PLFAs were first normalized for every sampling date separately, then analysed for each date with PCA. The PLFA loadings for the first three axes for each date were then examined to determine which PLFAs were most strongly associated with which axes, and whether these varied by date. Sample scores along each axis were then extracted and used as ordinary variables in semivariogram analysis to determine the extent to which each axis of variation was spatially structured on the plot, and, by extension, the extent to which the PLFAs associated with that axis were spatially structured. The spatial patterns and the relationships of the principal components with the abiotic or biotic environment were then examined as described above for the univariate data.

The second approach consisted in examining how the whole community data varied as a function of spatial separation and how the community data was related to multivariate descriptors of the local environment. This was achieved using Mantel tests (Franklin & Mills, 2009) with the package ‘vegan’ (Oksanen *et al*., 2013). The Mantel test tests for the association between distance matrices. Distance or similarity matrices were calculated between all pair-wise combinations of samples for PLFA profiles, environmental conditions, plant biomass, plant species and geographic location. The distance matrices were constructed using the Euclidean distance. Euclidean distance was used because it is the metric that is usually used for PLFA and environmental data, as PLFA profiles generally have a linear response to environmental gradients. Four distance matrices were constructed for each sampling date: (i)

spatial distances among pairs of sampling points using the x-y coordinates; (ii) distances in abiotic measures of the environment (soil moisture, bulk density, texture, pH, soil organic C, soil total N, EOC, EON, NH_4^+, NO_3^-, and mineral P); (iii) distances in biomass of different plant functional groups (grasses, forbs and legumes); and (iv) distances in the PLFA profiles of soil microorganisms. The environmental variables were scaled to unit variance and zero mean to account for the different units of measurement. Mantel statistics were then calculated for all pairs of distance matrices using the default setting of 999 permutations in the R package 'vegan' (Oksanen *et al*., 2013).

5.4 Results

5.4.1 *Temporal patterns*

5.4.1.1 Plants and litter

Total plant, grass and forb biomass was lowest in April and peaked in June, before it was harvested by mowing in early August. Legume biomass was too low to be measured in April and May, but showed a marked increase after mowing and a peak in October (Fig. 5.1a). By November, after the first hard frost, biomass of all plant functional groups declined as a result of senescence (Fig 5.1a). In contrast, litter biomass decreased from April to June, and then steadily increased until November. Similarly, root biomass declined from April until August and increased to its highest level in November (Fig. 5.1b).

5.4.1.2 Abiotic soil properties

Bulk density changed slightly but significantly throughout the sampling period with highest bulk density in August (Table 5.1). Soil pH was relatively stable throughout the vegetation period, varying between 6.6 and 6.8 (Table 5.1).

Soil C and N content showed almost no differences over the sampling period (Table 5.1). Soil C/N ratios ranged from 10.0 to 10.3; with the lowest C/N ratios for the season recorded in June and the highest in August (Table 5.1). EOC differed significantly on most sampling dates with steadily decreasing values from April until October and a slight increase in November (Table 5.1, Fig. 5.1d). In contrast, EON was low in August, increased in October, and was lowest in November (Table 5.1, Fig. 5.1d).

Both mineral forms of nitrogen, NH_4^+ and NO_3^-, were highest in April and declined through May and June (Fig. 5.1c). Both increased after mowing in August, decreased in October, and

increased again in November. Bioavailable P was also highest in April, lowest in June and increased slightly from August through November (Table 5.1, Fig. 5.1c).

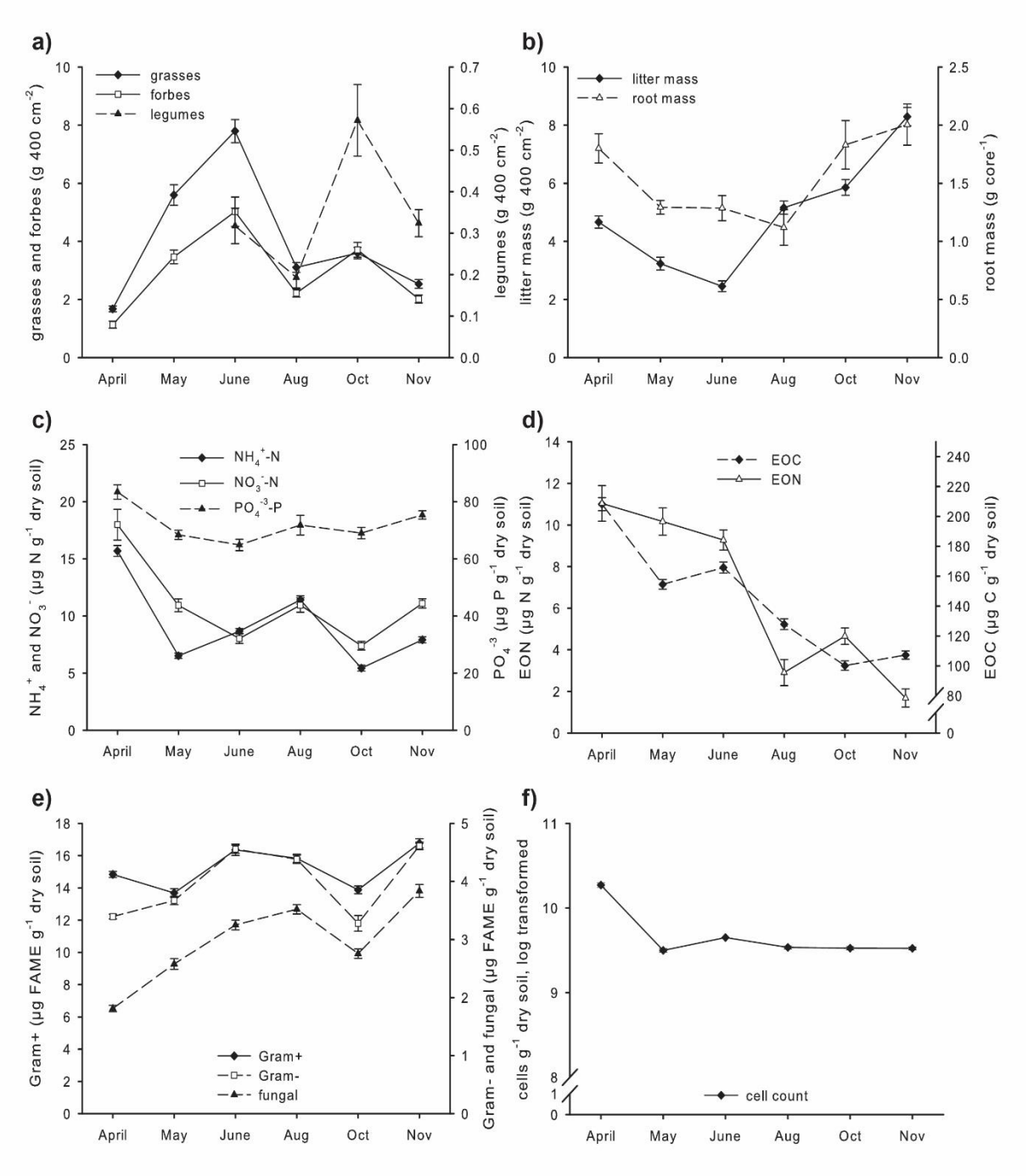

Fig. 5.1a-f Temporal changes in selected above- and below-ground properties for each sampling date; a) above ground plant biomass functional groups, b) litter mass and root mass, c) soil mineral nitrogen and phosphorus, d) extractable organic carbon and nitrogen, e) microbial fatty acids, and f) bacterial cell counts. Dotted lines indicate that the variable is scaled on the right Y axis. Error bars denote standard error. Cell count data were log-transformed for homogeneity of variance.
This is the corrected version from corrigendum Regan et al. (2015).

Table 5.1 Environmental, plant and microbial data measured at the six sampling dates in 2011. Means per sampling (n = 60) with standard deviation (SD). Letters indicate significant differences at P-values ≤ 0.05 obtained from Tukey's HSD test.
This is the corrected version from corrigendum Regan et al. (2015).

	5 Apr	SD	17 May	SD	27 Jun	SD	16 Aug	SD	5 Oct	SD	21 Nov	SD
Soil moist. (gravim. % H_2O)	57.56	(2.95) a	27.97	(1.71) e	36.81	(3.27) d	46.38	(2.36) b	26.79	(2.34) e	40.03	(2.35) c
Bulk density (g cm^{-3})	0.93	(0.12) b	0.83	(0.08) d	0.87	(0.07) cd	1.02	(0.07) a	0.91	(0.09) bc	0.86	(0.06) d
pH	6.65	(0.15) b	6.68	(0.15) b	6.78	(0.20) a	6.69	(0.24) ab	6.79	(0.19) a	6.78	(0.21) a
Soil organic C (mg g^{-1} dry soil)	65.4	(4.1) a	65.8	(5.0) a	65.2	(3.8) a	65.4	(4.3) a	65.1	(4.5) a	66.7	(3.2) a
Soil total N (mg g^{-1} dry soil)	6.5	(0.4) ab	6.5	(0.4) ab	6.5	(0.3) ab	6.4	(0.4) ab	6.4	(0.4) b	6.5	(0.3) a
C/N ratio	10.12	(0.20) bc	10.11	(0.25) c	9.97	(0.22) d	10.25	(0.26) a	10.24	(0.26) ab	10.20	(0.21) abc
EOC (µg g^{-1} dry soil)	208.25	(33.60) a	154.57	(25.37) b	165.78	(28.52) b	127.83	(28.13) c	100.19	(24.38) d	107.19	(21.64) d
EON (µg g^{-1} dry soil)	11.04	(6.63) a	10.17	(5.09) a	9.28	(3.75) a	2.91	(4.87) bc	4.66	(3.08) b	1.69	(3.39) c
NH_4^+-N (µg g^{-1} dry soil)	15.70	(3.66) a	6.52	(1.79) d	8.67	(1.83) c	11.41	(2.71) b	5.43	(2.03) d	7.91	(2.06) c
NO_3^--N (µg g^{-1} dry soil)	17.99	(10.43) a	10.93	(4.31) b	8.04	(3.38) c	10.96	(4.86) b	7.40	(2.90) c	11.11	(3.21) b
PO_4^{3-}-P (µg g^{-1} dry soil)	83.41	(19.50) a	68.39	(12.40) bc	64.88	(15.50) c	67.62	(15.20) bc	69.02	(15.30) bc	75.36	(11.40) ab
Plant biomass (g 20 x 20 cm)	2.81	(1.28) e	9.73	(2.99) b	13.54	(3.94) a	5.73	(1.58) d	8.27	(1.87) c	5.11	(1.24) d
Litter mass (g 20 x 20 cm)	116.71	(40.31) c	80.89	(42.96) d	61.46	(35.81) d	129.02	(44.43) bc	146.45	(52.47) b	207.33	(61.18) a
Root mass (g $core^{-1}$)	1.80	(0.98) ab	1.29	(0.46) bc	1.29	(0.84) bc	1.12	(1.19) c	1.83	(1.62) ab	2.01	(1.37) a
C_{mic} (µg g^{-1} dry soil)	1714.7	(156.4) b	1545.4	(234.6) c	1633.6	(189.9) bc	1702.0	(191.1) b	1570.4	(163.3) c	2036.5	(181.2) a
N_{mic} (µg g^{-1} dry soil)	269.2	(34.4) ab	215.9	(36.7) d	251.6	(40.0) bc	244.9	(35.6) c	213.4	(31.5) d	273.2	(34.6) a
Bacterial Cell Count (cells g^{-1} dry soil, data log transformed)	10.27	(0.20) a	9.50	(0.17) c	9.65	(0.19) b	9.53	(0.14) c	9.52	(0.16) c	9.52	(0.15) c
Bacterial PLFAs (µg g^{-1} dry soil)	24.43	(2.34) c	22.93	(3.37) cd	27.42	(4.13) ab	26.49	(3.12) b	22.22	(3.17) d	28.38	(3.41) a
Gram+ PLFAs (µg g^{-1} dry soil)	14.84	(1.46) bc	13.69	(2.10) d	16.36	(2.75) ab	15.83	(1.96) ab	13.88	(1.88) cd	16.76	(2.16) a
Gram- PLFAs (µg g^{-1} dry soil)	3.40	(0.32) bc	3.67	(0.55) b	4.56	(0.56) a	4.38	(0.55) a	3.28	(1.05) c	4.61	(0.50) a
Fungal PLFA (µg g^{-1} dry soil)	1.81	(0.42) d	2.58	(0.71) c	3.25	(0.65) b	3.52	(0.63) ab	2.76	(0.62) c	3.84	(0.85) a
Fungal/Bacterial ratio	0.07	(0.02) c	0.11	(0.03) b	0.12	(0.02) b	0.13	(0.02) a	0.12	(0.02) ab	0.14	(0.03) a

5.4.1.3 Soil-microbial community variables

Temporal patterns of both C_{mic} and N_{mic} were similar: mean values declined from April to May, increased slightly in June and August, declined again in October and were highest for both in November (Table 5.1). Bacterial PLFAs were highest in June, August, and November and lowest in October (Fig. 5.1e). Both Gram+ and Gram- bacteria exhibited a fluctuating pattern throughout the season, but Gram- bacteria did not decrease in May as did Gram+ (Fig. 5.1e). The fungal PLFA biomarker exhibited a different pattern from the bacterial markers. It was lowest in April, increased steadily to almost double its April value by August, declined in October and increased to its highest value of the season in November (Fig. 5.1e). This resulted in an increase in the fungal to bacterial ratio from August through November (Table 5.1). Total bacterial cell counts were significantly higher in April than at any other sampling date; from May through November there were no significant changes except in June, when cell counts increased slightly but significantly from all later sampling dates (log-transformed data) (Table 5.1, Fig. 5.1f).

5.4.2 *Univariate spatial patterns over time*

All measured variables showed spatial structuring on some of the sampling dates (Table S 5.1). However, the spatial structure changed over the sampling period, with little or no spatial structure in April, and more frequently detected spatial structure in both May and October. In addition, the ranges over which spatial structure was detected and the amount of variation explained varied considerably among properties, with spatial structures over distances of 2 to 8.6 m and explained variances between 11.4% and 94.1%. (Table S 5.1). Ranges for abiotic soil properties with fitted spherical models varied from 2.0 to 8.6 m. (Table S 5.1). Spatial structure in root biomass was evident only in November; with a range of 2.2 m. Plant functional groups exhibited no spatial structure until after the post-mowing sample collection. Forbs were first, in August, followed by grasses and legumes in October (Table S 5.1). C_{mic} and N_{mic} were spatially structured from June to October and May to October, respectively, but their ranges and percent structural variance differed (Table S 5.1). Total bacterial PLFAs exhibited spherical spatial structure in May, October, and November. Empirical variograms were also modeled for the individual PLFAs associated with either Gram+ or Gram- bacteria, as well as the single PLFA associated with fungi (Table S 5.1). Although PLFA 18:2ω6 can also be associated with fresh litter, its value did not vary with litter so we think it accurately represented fungi in this plot. The Gram+ and Gram- PLFAs consistently exhibited spatial structure in May and October. Other dates were more variable among both groups and this variability displayed no pattern

within or between groups (Table S 5.1). Although measured values did not vary significantly among these dates, kriged maps of the distributions of exemplary PLFAs that were spatially structured indicated that their distribution on the plot shifted from spring to autumn (Fig. 5.2 a-i).

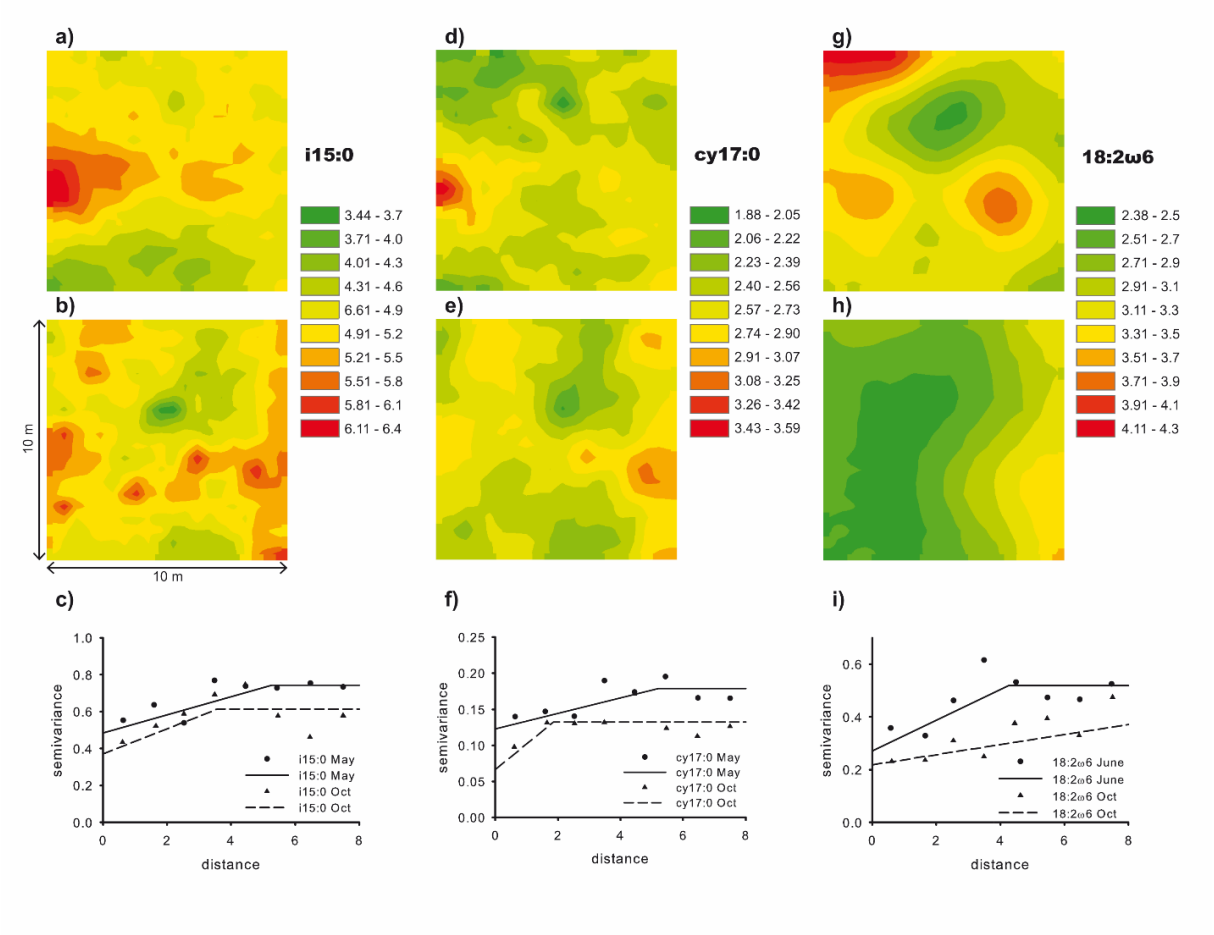

Fig. 5.2 a-i Kriged maps of exemplary Gram+, Gram- bacterial PLFAs and fungal PLFA. a) Gram+ i15:0 PLFA in May, b) Gram+ i15:0 PLFA in October, c) semivariograms used to create maps a & b; d); Gram- cy17:0 PLFA in May, e) Gram- cy17:0 PLFA in October, f) semivariograms used to create maps d & e; f) fungal PLFA 18:2ω6 in June, g) fungal PLFA 18:2ω6 in October, i) semivariograms used to create maps g & h. Dimensions of all maps are 10 m x 10 m.

The distances over which bacterial PLFAs exhibited spatial autocorrelation also became shorter as the season progressed (Fig. 5.2 c & f). Unlike the bacterial PLFAs, spherical spatial structure of the fungal PLFA was discernable only in June and August and its spatial autocorrelation increased; the model in October was linear and the variogram indicated that spatial autocorrelation extended past the limits of the plot (Table S 5.1, Fig. 5.2 h,i). The ranges in percent structural variance for the bacterial PLFAs were 23% in May and 42% in October, while for the fungal PLFA they were 47% in June. Percent structural variance for the fungal PLFA could not be calculated for October because the model was linear for that date (Table S 5.1).

Cell counts were spatially structured at our sampling scale at every date except April, at which date no model could be fitted. Their spatial structure began to emerge in May, and by June exhibited a spherical spatial structure which persisted through November (Table S 5.1).

5.4.3 *Changes in spatial patterns of microbial and plant community structure and environmental profiles over time*

5.4.3.1 Mantel statistic – relationships among communities

Mantel statistics were calculated to characterize spatial relationships among soil abiotic properties, plant functional groups and the microbial community (Table 5.2). Overall, abiotic soil properties exhibited strong spatial correlation throughout the year, except in November. In contrast, plant functional groups showed significant spatial structure only in April and November, whereas microbial community structure (PLFA profiles) exhibited weak spatial structure in April, August, and November (Table 5.2).

Table 5.2 Results of the Mantel tests including data spatial structure (spatial), abiotic properties (abiotic), plant functional groups (plant) and microbial community data (microbial) from the six sampling dates in 2011. Pearson correlations (r-values) with significance assessed by permutation test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns = not significant.

Sampling time	spatial/ abiotic	spatial/ plant	spatial/ microbial	abiotic/ plant	abiotic/ microbial	plant/ microbial
Apr	0.18^{***}	0.13^{**}	0.10^{*}	0.18^{*}	0.38^{***}	0.06^{ns}
May	0.16^{**}	-0.02^{ns}	0.05^{ns}	0.03^{ns}	0.38^{***}	0.05^{ns}
Jun	0.20^{***}	0.04^{ns}	-0.03^{ns}	0.04^{ns}	0.12^{*}	-0.08^{ns}
Aug	0.25^{***}	0.02^{ns}	0.09^{*}	-0.07^{ns}	0.27^{**}	-0.14^{ns}
Oct	0.12^{*}	-0.02^{ns}	0.01^{ns}	0.00^{ns}	0.35^{***}	-0.06^{ns}
Nov	0.06^{ns}	0.12^{**}	0.10^{*}	0.00^{ns}	0.27^{***}	-0.03^{ns}

A weak relationship between plant functional groups and abiotic soil properties was observed only in April (Table 5.2). In contrast, microbial community spatial structure was significantly correlated with abiotic soil properties throughout the year, especially in spring and autumn but, though still significant, the correlation was weakest in June and August. At no sampling date was microbial community spatial structure significantly correlated to plant functional groups. Plant species composition, which was available for three of the six sampling dates, was not significantly related to microbial community spatial structure (results not shown).

5.4.3.2 PCA – distinctions within the microbial community

To take a closer look at the different groups of the microbial community, principal components analysis (PCA) was used to analyze individual PLFAs associated with the microbial community for each sampling date (Table 5.3). The first three principal components (PC) together accounted for 57-67% of total PLFA variance over the six sampling dates for all PLFAs. The PC scores of the first three PCs were also spatially modeled (Table S 5.1).

Table 5.3 Correlations of scores of principal component analyses for microbial communities (using the microbial PLFA data) with abiotic properties, root and litter mass and plant functional groups at each of the six sampling dates in 2011. Only significant ($P < 0.01$) correlations of properties with each of the three PC-axes are shown. Properties in italics indicate negative correlations.

Sampling date	PLFAs			Abiotic parameters		
	PC1	PC2	PC3	PC1	PC2	PC3
April	a15.0		i17.0		PO_4^{3-}-P	
May	i15.0		cy17.0	*NO_3^--N*		pH
	18.2ω6					NO_3^--N
June	a15.0	i16.0			*pH*	*pH*
	i15.0	i17.0			*C*	
	18.2ω6	cy19.0			*Total N*	
			18.2ω6		*PO_4^{3-}-P*	
					EOC	
		cy17.0	i16.0		*EON*	
Aug	i15.0				*Soil moisture*	Forb mass
	18.2ω6				*N_{min}*	
	a15.0	i16.0	cy17.0		*PO_4^{3-}-P*	
Oct	i15.0	i17.0		*Root mass*	Total N	NH_4^+-N
	18.2ω6	cy19.0			N_{min}	EOC
	a15.0		i16.0		EON	EON
Nov	i15.0		cy17.0	*PO_4^{3-}-P*	*NO_3^--N*	NH_4^+-N
			cy19.0		*PO_4^{3-}-P*	EOC
						EON

In April and May, there was little or no spatial structure to the variance in principal components. In June and August, there was more evidence of spatial structure in the second PC, but that structure was no longer evident in autumn (Table S 5.1). Scores of the first three PCs were then correlated with soil environmental and abiotic properties to determine which were significantly correlated with each PC at each date (Table 5.3). In April and May there were few significant relationships (Table 5.3). Visual inspection of the PC loadings over the entire season indicated that PC1 was mainly associated with PLFAs indicative of Gram+ bacteria and fungi, while PC2

and PC3 were mainly associated with those of Gram- bacteria and this discrimination held throughout the season (Table 5.3). In June, of the seven PLFAs associated with particular subsets of the microbial community, two were more strongly related to measured soil properties. Gram- bacterial PLFAs were always associated with PCs that were strongly correlated to soil abiotic properties (Table 5.3). Furthermore, the correlations in PC2 were mainly negative, while those in PC3 were more often positive (Table 5.3). PLFA i16:0 was associated with PC2, and fungal PLFA 18:2ω6 with PC3 (Table 5.3). However, PC2 and PC3 together accounted for only 31% of the observed variance at this date. There were no apparent trends in the relationships between the microbial community and plants, although forbs were positively correlated with PC3 in August, and root biomass with PC1 in October.

5.5 Discussion

The overall structure of the microbial community was strongly related to the local abiotic environment throughout the sampling period, as indicated by the Mantel statistics (Table 5.2). Our results confirm the findings of others who have looked at soil microbial biogeography in croplands and grasslands at broader scales (Ettema & Wardle, 2002; Fierer & Jackson, 2006; Martiny *et al*., 2006; Lauber *et al*., 2008; Fierer *et al*., 2009; Ranjard *et al*., 2010; Dequiedt *et al*., 2011; Griffiths *et al*., 2011; Martiny *et al*., 2011). However, although the relationship between soil abiotic properties and the microbial community persisted throughout the year, the strength of that relationship varied, suggesting that it was not constant over time, and that other factors also influenced microbial community composition. The fact that subsets of the microbial community, as differentiated by PCA that discriminated PLFAs associated with Gram+ and Gram- bacteria and with saprotrophic fungi, were related to different environmental variables at specific times, tends to confirm this (Table 5.3). Gram+ bacteria exhibited little relationship to measured soil properties, and the sole correlation we could identify for Gram+ bacteria was only negatively related to root mass and only at one date. This suggests that Gram- bacterial PLFAs may have been more influenced by belowground processes than were Gram+ PLFAs (Table 5.3). This discrimination between Gram+ and Gram- bacterial responses to belowground processes, furthermore, persisted over time (Table 5.3).

Kriged maps of the changes in distributions of exemplary Gram+ and Gram- bacterial PLFAs showed that Gram+ bacteria varied more across the site than did Gram- bacteria at the two dates shown (Fig. 5.2 a-d). One must use caution in interpreting changes in PLFAs; shifts can indicate changes in populations of microbes, in species composition, or in physiological adaptations of existing populations as a response to environmental stress (Wixon & Balser, 2013). However,

the decline in Gram+ and the increase in Gram- bacterial numbers in May suggest differential responses to increased competition with plants for soil nutrients (Fig. 5.1e). In addition, Gram- bacteria may have been able to take advantage of root exudates more rapidly than Gram+ bacteria at this date. Increases in the PLFAs associated with Gram- bacteria under conditions of increasing environmental stress have been measured by Moore-Kucera and Dick (2008). Conversely, a slight increase in June in soil moisture and in EOC would have reduced the environmental stress on bacteria, resulting in our observed increases in all PLFAs at this date (Fig. 5.1d- f), as well as in the bacterial cell counts, which measure only the active portion of the bacterial community (Table 5.1, Fig. 5.1f). These observations support the findings of Lennon *et al*. (2012), who, using a taxonomic approach, linked functional traits of microbial groups to their responses to a moisture gradient. In their study, different members of the microbial community, characterized by the coarse taxonomic classifications of Gram+ or Gram-, demonstrated varying degrees of tolerance and resilience to small changes in environmental stresses over the season.

Spatial structure at this scale and at specific times suggests that extremely local processes were influencing the properties we modeled (Table S 5.1). Exemplary Gram+ and Gram- PLFA maps in May and October indicated that, although the range of values did not differ much from one time point to the other, distributions of the bacteria shifted somewhat on the plot (Fig. 5.2a, b & d, e). The shift of Gram+ PLFA i15:0 from a cosmopolitan to a patchy distribution from May to October (Fig. 5.2a, b) was possibly due to competition with plants for soil nutrients. When nutrients are rate limiting, as may have been the case for Gram+ bacteria by late in the season, their growth could have been confined to "hotspots" in which nutrients were accessible (Nunan *et al*., 2003). In contrast, the overall pattern of distribution was more uniform for Gram- PLFA cy17:0 (Fig. 5.3 d, e). Both Gram+ and Gram- PLFAs were low in the same regions in October, perhaps reflecting a process we were not able to capture at our sampling scale. The distribution of the fungal PLFA in October was almost uniformly low on the plot as compared to the more variable bacterial PLFAs, suggesting bacteria may have been able to take advantage either of different resources or of the same resources to a greater degree than fungi were at this time (Fig. 5.2 b, e, h). The correspondence between our observed low fungal and higher bacterial distributions on the plot in October could have been due to competitive strategies for resources between bacteria and fungi described by de Boer *et al*. (2005). The ranges of spatial structuring in plant, abiotic, and microbial properties which we were able to characterize on the plot late in the season suggests that the local belowground environment had changed in tandem with seasonal aboveground processes, resulting in a much more structured microbial community at

the scale of this study. This can be seen in the development of more spherical spatial models of most parameters toward the end of the vegetation period (October). The fact that much of the spatial structure at our sampling scale was no longer detectable by November also supports this claim (Table S 5.1).

Our study in a low land use intensity grassland could not detect any effect of the biomass of plant functional groups on the structure of the microbial community (Table 5.2), even though plants have been shown to exert a strong effect on soil microbial communities when different plant communities such as deciduous or coniferous forests are being compared (Wardle *et al*., 2004). This could have been because plant functional groups exhibited no spatial structure themselves and were not correlated with abiotic soil conditions over most of the growing season (Table 5.2, Table S 5.1). Our results are consistent with Fierer and Jackson (2006) and Sayer *et al*. (2013) who were not able to identify direct links between microbial and plant community composition or stage of plant growth. But they are in contrast to Reynolds *et al*. (2003) and Kulmatiski and Beard (2011), perhaps because many studies on plant-soil feedbacks concentrate on particular dominant species. Our studied grassland was a species-rich community with between 12 and 20 plant species per 20 cm x 20 cm, without a single dominant plant species. In grasslands roots are also very dense and enmeshed; microbial communities may therefore be affected by many plants at once, reinforcing the lack of dominance of individual species.

Despite the absence of spatial variability, plant biomass varied strongly over the season. Our plot was mowed in early August, two weeks before August sample collection. Biomass removal by cutting or mowing is known to increase root exudation (Kuzyakov *et al*., 2002) and several studies have shown positive effects of plant defoliation on microbial biomass and/or activity (Mawdsley & Bardgett, 1997; Macdonald *et al*., 2006). Therefore, we had expected to see an effect of mowing on bacterial PLFAs due to increased exudation of simple carbon compounds (Paterson & Sim, 2000). However, we saw a negative response; both groups of bacterial PLFAs declined in August. Exudates may have been depleted by the time of our sample collection; their turnover rate in soil can occur in hours to days (Bais *et al*., 2006; Drake *et al*., 2013). Therefore, two weeks after mowing may have been too late to see a positive response in the bacteria. EOC was also low in August, suggesting that available carbon might have been limiting at this date. EON was low as well, and there is evidence that nitrogen availability can be a rate-limiting step in microbial uptake of root exudates (Zhou *et al*., 2012; Drake *et al*., 2013). The fungal PLFA associated with saprotrophic fungi often increases after mowing in

response to increased C input to the soil from exudation, and fungi can also take advantage of the recalcitrant carbon in litter (Bardgett *et al*., 1996; Denef *et al*., 2009). The fungal PLFA in our study did increase in August and so did litter (Fig. 5.1 b,e). Increased litter could have contributed to the fungal PLFA increase we observed in August. Therefore changes we observed in both bacterial and fungal PLFAs at this date could not be clearly related to mowing. Although evidence of direct linkages between above- and below-ground processes could not be established in our study, indirect links were indicated by the relationship of changing substrate availability to changes in microbial PLFA abundances and distributions (Fig. 5.1c-e). The differential responses of Gram+ and Gram- bacteria suggest a need for a deeper look (for example, using pyrosequencing) into the members of these communities, to learn whether our observations hold at a finer scale of taxonomic resolution.

5.6 Conclusions

Over the season, the physical soil structure of this unplowed, unfertilized grassland was homogeneous. Dense root penetration throughout the soil meant that we could not identify individual plant effects at this site. This is in agreement with other studies of unfertilized grasslands, in which direct links between above- and belowground properties have proven elusive (Ritz *et al*., 2004). Nevertheless, it is clear that a complex combination of interactions was operative at the scale of our study. We identified variability in microbial community composition through a close analysis of PLFA data, and showed that the controls on that variability differed over the season. Environmental properties were the main structuring agents of the microbial community, as they are at larger scales. However, although this relationship persisted over time, individual components of environmental properties varied with season, and those differences may be hypothetically related, albeit indirectly, to changes in plant growth. Changes in soil nutrient status, for example, were directly related to plant growth, and could have served to integrate a number of related processes, similar to the integrating effect of pH at the landscape scale. This in turn masked more ephemeral – but important - shifts in controls on microbial spatial distribution and community composition. We demonstrated not only evidence of the complexity of microbial communities in grassland soils but also the importance of a temporal component to the characterization of soil microbial biogeography.

5.7 Acknowledgments

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environmental offices of Baden-Wuerttemberg, (according to § 72 BbgNatSchG). We thank the manager of the Swabian Alb Exploratory, Swen Renner, and Jörg Hailer for their work in maintaining the plot and project infrastructure; Simone Pfeiffer and Christiane Fischer for giving support through the central office, Michael Owonibi for managing the central data base, and Markus Fischer, Eduard Linsenmair, Dominik Hessenmöller, Jens Nieschulze, Ingo Schöning, François Buscot, Ernst-Detlef Schulze, Wolfgang W. Weisser, and the late Elisabeth Kalko for their role in setting up the Biodiversity Exploratories project. N. Nunan acknowledges funding received from the PHC (Partenariats Hubert Curien) programme of the French ministry of foreign affairs (project n°: 28184WH).We further thank Sabine Rudolph, Daniel Dann and Annegret Wahl for their technical support in the laboratory.

5.8 Supplementary material

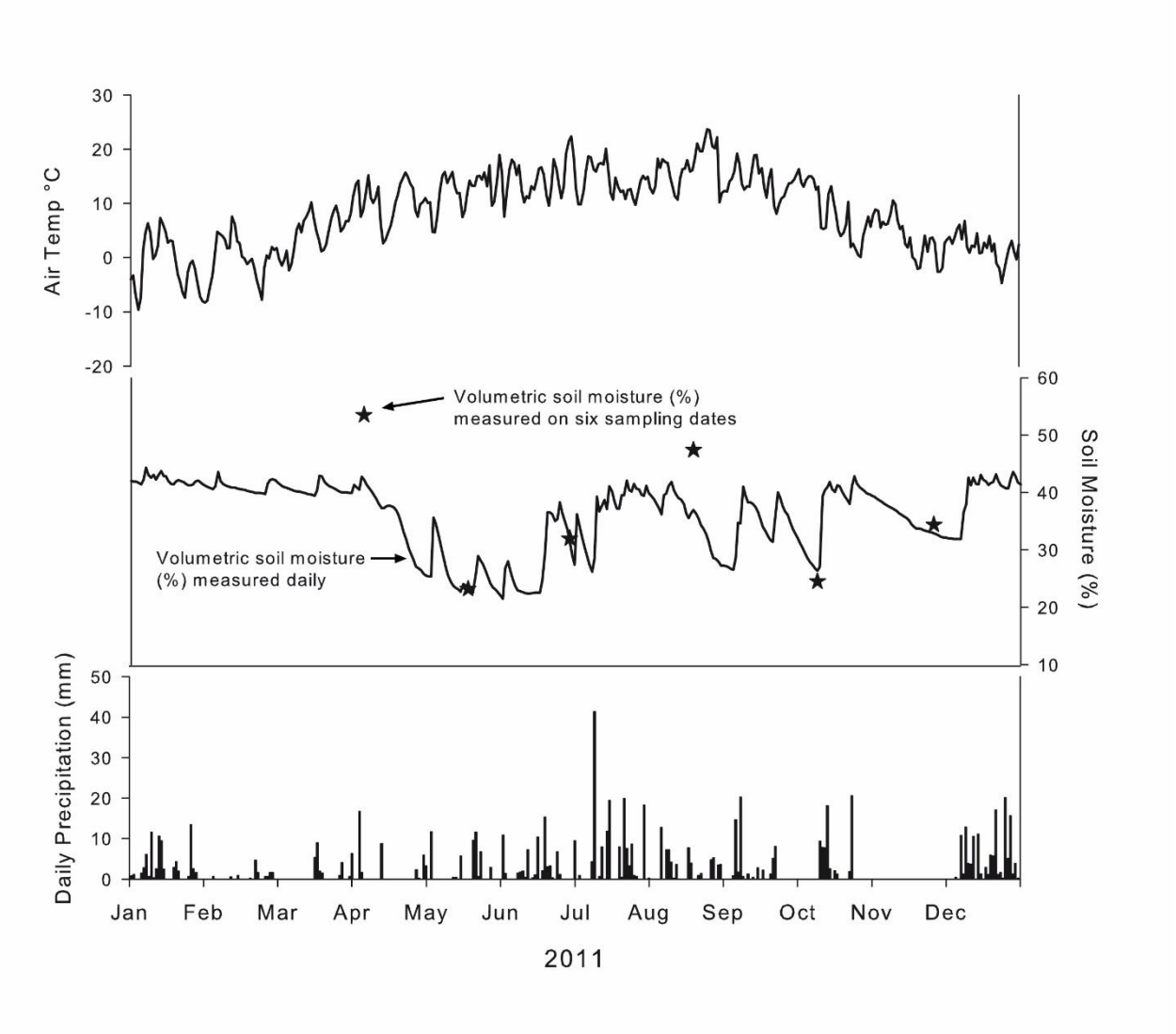

Fig. S 5.1 Daily precipitation, volumetric soil moisture and daily air temperatures provided by the German Weather Service from station located at Münsingen, the station nearest the plot. Soil moisture measurements for the six sampling dates are indicated by asterisks. Discrepancies between the weather station and our data are likely due to the slightly different locations of the weather station and the sampled plot.

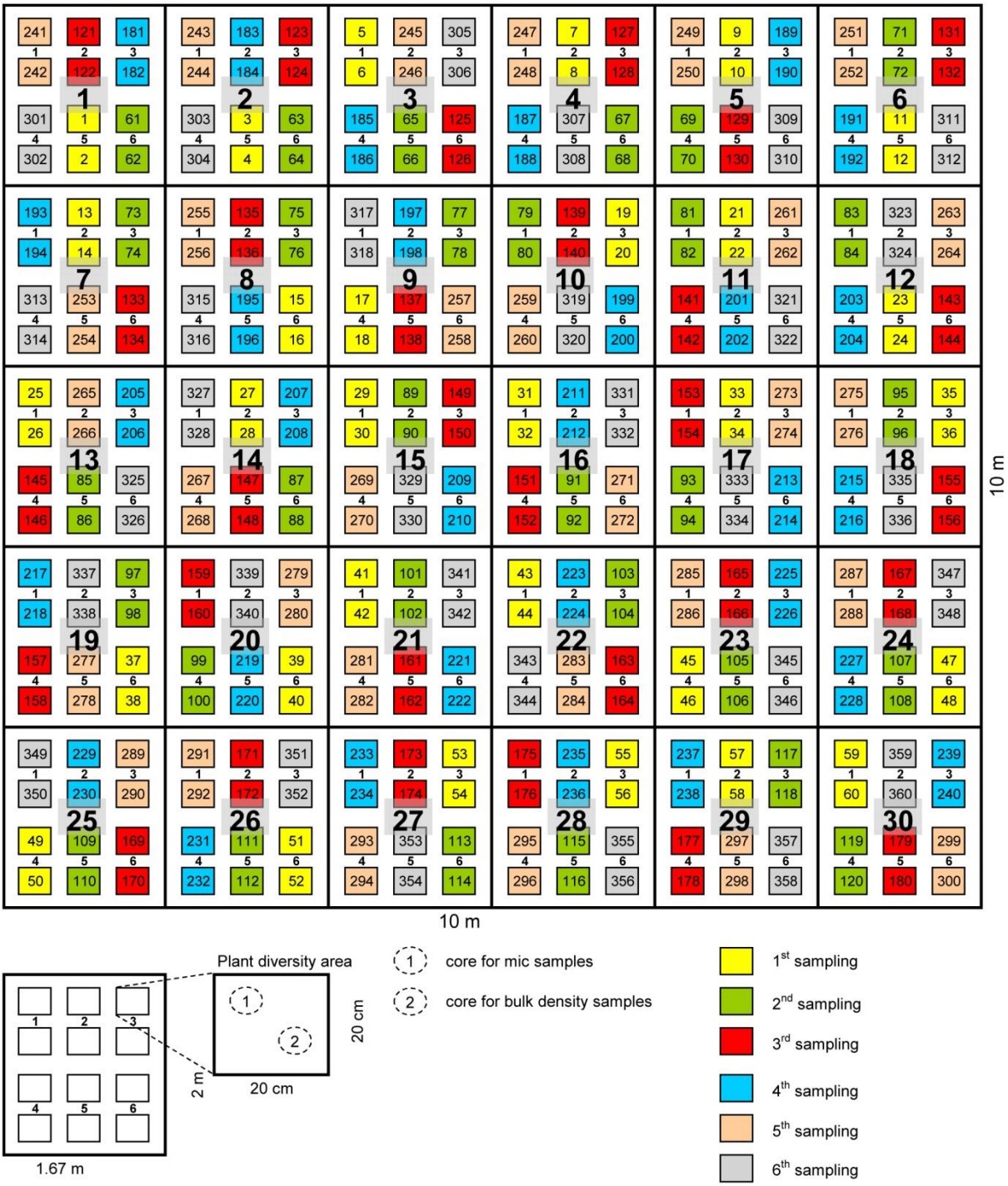

Fig. S 5.2 Sample design of the study site.

Methods Supplement

Details of soil sample preparation for texture analysis

Integrated samples from half the area of each subplot yielded 60 total samples for soil texture analysis.Samples were then air-dried and sieved (< 2 mm). All organic material present in the samples was destroyed by adding reagent grade 30% hydrogen peroxide (H_2O_2) in a 1:1 v/v ratio with deionized water (total 100 mL) to 5 g soil. After addition of H_2O_2, samples were held at room temperature overnight, then heated slowly in a water bath to 90°C (about 10°C h^{-1}) at which temperature they were held up to 24 hours, until all foaming had stopped. Samples were then cooled to room temperature, centrifuged at 1500 xg for 20 minutes, and the supernatant decanted. Two hundred mL deionized water was added to each sample and conductivity of the solution checked to confirm that it was below 40 µS.

Details of cell count method

Samples for the enumeration of bacterial cells were fixed immediately after sampling in the field (flash frozen in liquid N_2). From each soil sample, 0.1 g was transferred to a sterile screw cap Eppendorf tube and 1 mL of 10 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer (pH 5.5) containing 1% (v/v) glutaraldehyde was added. Samples were stored at 4°C in the dark. For counting, subsamples were diluted between 30 and 150 times, depending on initial cell concentrations, using MES buffer containing 30% (v/v) methanol. Dilutions were dispersed by two rounds of sonication for 10 min at 35°C (model RK 100H, 35 kHz, 4 x 80 W per period; Bandelin Electronic, Berlin, Germany). One hundred µL of the cell suspension were transferred to a 15 mL Falcon tube, then 9.9 mL 3-(N-morpholino) propanesulfonic acid (MOPS) buffer (2 mM, pH 7.0) were added and the samples stained with 2 µL SYBR green I (10.000x concentrate in DMSO; Molecular Probes, Eugene, OR) and held in the dark for 15 minutes on a rotary shaker. Stained samples were filtered on black 0.2-µm-pore-size Isopore™ membrane filters (25 mm diameter, Millipore, Billerica, MA, USA). After drying, filters were embedded in DABCO solution (25 mg of 1,4-diazabicyclo [2.2.2] octane in 1 mL of PBS buffer [130 mM NaCl, and 30 mM Na-phosphate, pH 7.4] plus 9 mL of glycerol)

Table S 5.1 Fitted semi-variogram model details for selected abiotic and biotic variables measured in the study.
NA = no model could be fit, % SV = percentage of structural variance, model abbreviations: Lin = linear, Sph = spherical, Nug = nugget, Exp = exponential.

Abiotic		Date						Abiotic		Date					
Parameter	Details	Apr	May	Jun	Aug	Oct	Nov	Parameter	Details	Apr	May	Jun	Aug	Oct	Nov
Soil moisture	Model	Lin	NA	NA	Lin	Sph	Sph	PO_4^{3-}-P	Model	Exp	Sph	Lin	NA	Sph	Nug
	Nugget	3.02	—	—	3.69	2.64	3.70		Nugget	306.879	55.179	25.42	—	117.076	124.234
	Sill	—	—	—	—	5.70	5.60		Sill	402.84	162.613	—	—	229.826	—
	p-Sill	—	—	—	—	3.06	1.90		p-Sill	95.96	107.43	—	—	112.75	—
	Range	—	—	—	—	5.14	2.98		Range	8.64	3.853	—	—	5.253	—
	% SV	—	—	—	—	53.7	33.9		% SV	23.8	66.1	—	—	49.1	—
pH	Model	NA	Sph	Lin	Lin	Lin	Sph	% C	Model	NA	Sph	Lin	NA	Sph	NA
	Nugget	—	0.011	0.026	0.02	0.031	0.033		Nugget	—	0.118	0.103	—	0.049	—
	Sill	—	0.023	—	—	—	0.044		Sill	—	0.273	—	—	0.189	—
	p-Sill	—	0.01	—	—	—	0.01		p-Sill	—	0.16	—	—	0.14	—
	Range	—	7.54	—	—	—	2.713		Range	—	6.59	—	—	2.02	—
	% SV	—	52.2	—	—	—	25.0		% SV	—	56.8	—	—	74.1	—
NH_4^+-N	Model	Nug	Lin	NA	NA	Sph	Lin	% N	Model	Lin	Sph	Lin	Lin	Nug	Nug
	Nugget	14.69	2.75	—	—	1.939	2.836		Nugget	0.001	0.001	0.001	<0.001	0.001	<0.001
	Sill	—	—	—	—	4.428	—		Sill	—	0.002	—	—	—	—
	p-Sill	—	—	—	—	2.49	—		p-Sill	—	0.00	—	—	—	—
	Range	—	—	—	—	2.038	—		Range	—	5.4	—	—	—	—
	% SV	—	—	—	—	56.2	—		% SV	—	50.0	—	—	—	—
NO_3^--N	Model	Nug	Sph	Nug	Lin	Sph	Lin	EOC	Model	Nug	Nug	Nug	Nug	Sph	NA
	Nugget	120.69	15.855	12.35	24.02	7.118	12.324		Nugget	1200	657.88	800	800	401.42	—
	Sill	—	18.962	—	—	8.033	—		Sill	—	—	—	—	547.44	—
	p-Sill	—	3.11	—	—	0.91	—		p-Sill	—	—	—	—	146.02	—
	Range	—	4.537	—	—	3.275	—		Range	—	—	—	—	3.324	—
	% SV	—	16.4	—	—	11.4	—		% SV	—	—	—	—	26.7	—

Biotic Parameter	Details	Date Apr	May	Jun	Aug	Oct	Nov
Roots	Model	Nug	Nug	NA	NA	Nug	Sph
	Nugget	0.847	0.246	—	—	4.51	0.609
	Sill	—	—	—	—	—	1.95
	p-Sill	—	—	—	—	—	1.34
	Range	—	—	—	—	—	2.235
	% SV	—	—	—	—	—	68.8
C_{mic}	Model	NA	NA	Sph	Sph	Sph	NA
	Nugget	—	—	19279	31447	12730	—
	Sill	—	—	37247	36758	26332	—
	p-Sill	—	—	17968	5311	13602	—
	Range	—	—	3.664	2.996	2.939	—
	% SV	—	—	48.2	14.4	51.7	—
N_{mic}	Model	NA	Sph	Sph	Sph	Sph	Lin
	Nugget	—	938.25	976.44	989.86	592.67	812.15
	Sill	—	1421.07	1710.38	1292.14	1032	—
	p-Sill	—	482.82	733.94	302.28	439.33	—
	Range	—	4.961	3.999	7.05	3.734	—
	% SV	—	34.0	42.9	23.4	42.6	—
Cell count	Model	NA	Exp	Sph	Sph	Sph	Sph
log transformed	Nugget	—	0.023	0.017	0.010	0.013	0.016
	Sill	—	0.03	0.021	0.02	0.026	0.269
	p-Sill	—	.007	0.004	0.010	0.013	0.253
	Range	—	6.89	5.344	2.352	3.820	5.910
	% SV	—	23.33	19.05	50.00	50.00	94.05
Litter	Model	Nug	NA	Exp	Sph	Sph	Sph
	Nugget	3.58	—	1.21	1.54	1.44	0.16
	Sill	—	—	2.23	3.15	4.82	0.43
	p-Sill	—	—	1.02	1.61	3.38	0.27
	Range	—	—	7.32	2.41	2.98	5.91
	% SV	—	—	45.74	51.11	70.12	62.36

Biotic Parameter	Details	Date Apr	May	Jun	Aug	Oct	Nov
Grasses	Model	Lin	Nug	Lin	Nug	Sph	Sph
	Nugget	0.58	14.05	9.55	10.10	1.82	0.16
	Sill	—	—	—	—	2.37	0.43
	p-Sill	—	—	—	—	0.55	0.27
	Range	—	—	—	—	5.27	5.91
	% SV	—	—	—	—	23.17	62.36
Forbs	Model	Lin	Nug	Lin	Sph	Sph	Sph
	Nugget	12.28	4.15	11.10	0.90	1.95	0.16
	Sill	—	—	—	1.39	4.08	0.43
	p-Sill	—	—	—	0.49	2.13	0.27
	Range	—	—	—	4.65	3.07	5.91
	% SV	—	—	—	35.35	52.21	62.79
Legumes	Model	NA	NA	NA	Lin	Sph	NA
	Nugget	—	—	—	1.30	0.16	—
	Sill	—	—	—	—	0.43	—
	p-Sill	—	—	—	—	0.27	—
	Range	—	—	—	—	5.91	—
	% SV	—	—	—	—	62.79	—

Lipid Parameter	Details	Date Apr	May	Jun	Aug	Oct	Nov
Total bacterial PLFAs	Model	Nug	Sph	Nug	NA	Sph	Sph
	Nugget	5.343	9.056	15.984	—	6.067	9.064
	Sill	—	11.79	—	—	10.453	11.934
	p-Sill	—	2.73	—	—	4.39	2.87
	Range	—	5.398	—	—	3.351	5.911
	% SV	—	23.2	—	—	42.0	24.0

Lipid		Date					
Parameter	Details	Apr	May	Jun	Aug	Oct	Nov
Gram(+)	Model	Nug	Sph	Nug	Lin	Sph	Nug
i15:0	Nugget	0.4115	0.485	0.477	0.517	0.37	0.831
	Sill	—	4.451	—	—	0.61	—
	p-Sill	—	3.97	—	—	0.24	—
	Range	—	5.26	—	—	3.6	—
	% SV	—	89.1	—	—	39.3	—
Gram(+)	Model	Lin	Nug	Nug	Lin	Sph	Nug
a15:0	Nugget	0.208	0.48	1.885	0.331	0.253	0.538
	Sill	—	—	—	—	0.477	—
	p-Sill	—	—	—	—	0.22	—
	Range	—	—	—	—	3.647	—
	% SV	—	—	—	—	47.0	—
Gram(+)	Model	Nug	Sph	Nug	Exp	Sph	Lin
i16:0	Nugget	0.087	0.097	0.486	0.101	0.062	0.119
	Sill	—	0.176	—	0.153	0.121	—
	p-Sill	—	0.08	—	0.05	0.06	—
	Range	—	4.45	—	8.31	4.442	—
	% SV	—	44.9	—	34.0	48.8	—
Gram(+)	Model	Lin	Sph	Sph	Exp	Sph	Exp
i17:0	Nugget	0.018	0.055	0.154	0.027	0.028	0.044
	Sill	—	0.072	0.168	0.047	0.044	0.057
	p-Sill	—	0.02	0.01	0.02	0.02	0.01
	Range	—	4.607	3.937	7.53	4.232	8.376
	% SV	—	23.6	8.3	42.6	36.4	22.8
Gram(-)	Model	Lin	Sph	Sph	Sph	Sph	Sph
cy17:0	Nugget	0.047	0.123	0.271	0.077	0.066	0.13
	Sill	—	0.175	0.38	0.157	0.132	0.192
	p-Sill	—	0.05	0.11	0.08	0.07	0.06
	Range	—	5.215	3.901	7.386	1.866	5.648
	% SV	—	29.7	28.7	51.0	50.0	32.3

Lipid		Date					
Parameter	Details	Apr	May	Jun	Aug	Oct	Nov
Gram(-)	Model	Nug	Sph	Nug	Sph	Sph	Lin
cy19:0	Nugget	0.011	0.027	0.066	0.006	0.012	0.016
	Sill	—	0.005	—	0.014	0.019	—
	p-Sill	—	-0.02	—	0.01	0.01	—
	Range	—	1.526	—	6.757	5.083	—
	% SV	—	0.0	—	57.1	36.8	—
Fungal	Model	Nug	NA	Sph	Sph	Lin	NA
18:2ω6	Nugget	0.201	—	0.271	0.285	0.218	—
	Sill	—	—	0.517	0.405	—	—
	p-Sill	—	—	0.25	0.12	—	—
	Range	—	—	4.256	2.878	—	—
	% SV	—	—	47.6	29.6	—	—
PC1	Model	NA	NA	Nug	NA	Sph	NA
	Nugget	—	—	0.592	—	0.363	—
	Sill	—	—	—	—	0.59	—
	p-Sill	—	—	—	—	0.227	—
	Range	—	—	—	—	3.93	—
	% SV	—	—	—	—	38.5	—
PC2	Model	NA	Sph	Sph	Sph	NA	NA
	Nugget	—	0.246	0.348	0.342	—	—
	Sill	—	0.61	0.58	0.57	—	—
	p-Sill	—	0.364	0.235	0.23	—	—
	Range	—	1.48	4.97	4.21	—	—
	% SV	—	59.7	40.3	40.2	—	—
PC3	Model	NA	Sph	NA	NA	Sph	NA
	Nugget	—	0.49	—	—	0.382	—
	Sill	—	0.58	—	—	0.55	—
	p-Sill	—	0.093	—	—	0.17	—
	Range	—	5.47	—	—	6.88	—
	% SV	—	16.0			30.8	—

6 Spatial and temporal dynamics of nitrogen fixing, nitrifying and denitrifying microbes in an unfertilized grassland soil*

Kathleen Regan[a]*, Barbara Stempfhuber[b], Michael Schloter[b], Frank Rasche[c], Daniel Prati[d], Laurent Philippot[e], Runa S. Boeddinghaus[a], Ellen Kandeler[a], Sven Marhan[a]

[a] Institute of Soil Science and Land Evaluation, Soil Biology Section, University of Hohenheim, Emil-Wolff-Str. 27, 70599 Stuttgart, Germany

[b] Helmholtz Zentrum München Research Unit for Environmental Genomics, Ingolstädter Landstraße 1, 85764 Neuherberg, Germany

[c] Institute of Plant Production and Agroecology in the Tropics and Subtropics, University of Hohenheim, D-70593 Stuttgart, Germany

[d] Institute of Plant Sciences and Botanical Garden, University of Bern, Altenbergrain 21, CH-3013 Bern, Switzerland

[e] INRA, UMR 1347 Agroécologie, F-21065 Dijon cedex, France

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6.1 Abstract

The microbial groups of nitrogen fixers, ammonia oxidizers, and denitrifiers largely drive the inorganic nitrogen cycle in temperate terrestrial ecosystems. Their spatial and temporal dynamics, however, vary depending on the studied scale. The present study aimed to fill a knowledge gap by providing an explicit picture of spatial and temporal dynamics of a subset of these soil microorganisms at the plot scale. We selected an unfertilized perennial grassland, where nitrogen cycling is considered to be efficient and tightly coupled to plant growth. At six times over one growing season 60 soil samples were taken from a 10 m x 10 m area and abundances of marker genes for total archaea and bacteria (16S rRNA), nitrogen fixing bacteria (*nifH*), ammonia oxidizing archaea (*amoA* AOA) and bacteria (*amoA* AOB), and denitrifying bacteria (*nirS*, *nirK* and *nosZ*) were determined by qPCR. Potential nitrification activity (PNA) and denitrifying enzyme activity (DEA) were determined. Seasonal changes in abundance patterns of marker genes were detected, and were associated with changes in substrate availability associated with plant growth stages. Potential nitrification and denitrification enzyme activities were strongly spatially structured at the studied scale, corresponding to periods of rapid plant growth, June and October, and their spatial distributions were similar, providing visual evidence of highly localized spatial and temporal conditions at this scale. Temporal variability in the N-cycling communities versus the stability of their respective potential activities provided evidence of both short-lived temporal niche partitioning and a degree of microbial functional redundancy. Our results indicate that in an unfertilized grassland, at the meter scale, abundances of microbial N-cycling organisms can exhibit transient changes, while nitrogen cycling processes remain stable.

6.2 Introduction

Soils are challenging environments to study because of their extreme structural and microbial heterogeneity, and yet soil microorganisms are important drivers of soil quality and ecosystem function, depending both on local microbial adaptation and on interactions with plants and other soil biota (Bardgett 2005). Recent estimates indicate that in addition to a large number of fungi, protists, and other micro-eukaryotes, one gram of soil may harbor more than one 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 critical topic in microbial soil ecology. Different influences on the soil microbiome and its functions have been identified depending on the scale under investigation (Franklin and Mills, 2009). Spatial studies of influences on the composition of the microbial community have to

date been done mainly at the scale of fields (Hallin *et al*., 2009; Enwall *et al*., 2010) and regions (Philippot *et al*., 2009; Drenovsky *et al*., 2010). At field scales, typically in the range of hectares, cultivation regimes such as fertilizer application, tillage practices, landscape gradients, land use history, edaphic factors such as soil texture and pH, and vegetation composition affect community composition and spatial distribution of microbes by, for example, influencing their access to nutrients and moisture (Ettema and Wardle, 2002; Ritz *et al*., 2004; Martiny *et al*., 2006). At regional and landscape scales, typically in the range of km, factors such as soil type, climate, and precipitation regimes influence the composition of microbial communities and their functional traits through differences in soil physicochemical properties (Lauber *et al*., 2008; Bru *et al*., 2011; Dequiedt *et al*., 2011). Both field and regional scales are characterized by heterogeneity of vegetation, soil, microclimate, land-use history, and in the case of regional scales, of underlying geology. As scale increases, the interactions of factors such as soil type, climate, land management, or pollution, rather than of individual compounds, contribute to the composition of the soil microbiome (Grayston *et al*., 2001; Bardgett *et al*., 2005; Fierer and Jackson, 2006). In contrast, the plot scale, ranging typically from centimeter to meter, is characterized by homogeneity of these factors. For example, Grundmann *et al*. (2001), using a modeling approach, demonstrated significant differences in microbial communities which catalyze processes of nitrification in different soil compartments at the sub-millimeter scale. At this scale, individual substrates or physicochemical properties have been identified as drivers of microbial community development. In a multi-scale study, Franklin and Mills (2003) demonstrated that small variations in soil properties at scales from 30 cm to greater than 6 m contributed to shaping subsets of microbial communities in soil. Thus, studies at these small spatial scales make it possible to detect influences that may be obscured under more heterogeneous conditions, but which must be identified in order to understand interactions among microorganisms.

Microbial communities also show distinct and differing response patterns in time. As a consequence, the concept of highly localized and concentrated areas of microbial activity, known as hotspots (Parkin 1987; Nunan *et al*., 2003) has been expanded to include hot moments (Groffman *et al*., 2009; Kuzyakov and Blagodatskaya, 2015). The duration of hot moments is highly variable, and changes among members of the microbial community vary depending on the choice of observed time scale. On the scale of hours to days, activity patterns of microbial communities (Schmidt *et al*., 2007) and sometimes even community structure (Cruz-Martinez *et al*., 2012) have been detected, while over longer time periods, clear shifts in microbial community structure can occur (Grayston *et al*., 2001; Bardgett *et al*., 2005; Dandie *et al*., 2008; Habekost *et al*., 2008; Lauber *et al*., 2013).

Plants can be considered as both architects of spatial heterogeneity and as drivers of temporal heterogeneity in soils. For example, growing roots change the physico-chemical environment of soils, thereby introducing small scale heterogeneity. In grassland soils, as a result of intensive plant growth, plants can change the physico- chemical conditions of the entire upper 10 cm of a soil (Mueller *et al*., 2013). During periods of vegetative growth, plant-derived exudates and availability of labile carbon act as drivers of microbial community structure and function (Houlden *et al.*, 2008; Kuzyakov and Blagodatskaya, 2015), while during plants' senescent phase, plant litter and decaying root material become the most important supply of carbon for microbial communities. Therefore, both the amount and quality of carbon from exudates and litter vary substantially over the season, and this variation strongly influences microbial performance in soil (Chapin et al., 2002; Wardle *et al.*, 2004; Houlden *et al.*, 2008; Kuzyakov and Xu, 2013).

Previous studies in which both temporal and spatial dynamics have been investigated have often focused on either phylogenetic aspects of microbial communities (De Boer and Kowalchuk, 2001; Gubry-Rangin *et al.*, 2011; Pasternak *et al.*, 2013; Graf *et al.*, 2014) or on a single functional group of microorganisms involved in soil N cycling, such as denitrifiers (Dandie *et al.*, 2008; Groffman 2012). In particular, studies of small-scale, seasonal variations in grassland microbial communities are lacking, especially those which comprehensively address changes in the abundances of microorganisms involved in different soil N-cycling processes together with their potential activities. Our goal was to fill a knowledge gap in the relationships between abundance and function in the soil nitrogen cycling microbial community at this scale. We selected an unfertilized perennial grassland with high plant diversity, where nitrogen cycling is considered to be highly efficient and tightly coupled to plant growth (Culman *et al.*, 2010). In an initial study on this plot, we provided a biogeographical overview of microbial communities by documenting their small-scale spatial and temporal variability in relation to abiotic soil characteristics and plant biomass using PLFA analysis (Regan *et al*., 2014). This was followed by a characterization of spatial interactions between archaeal ammonia-oxidizers and nitrite-oxidizing bacteria, a specific group of organisms involved in two tightly coupled steps in N-cycling (Stempfhuber *et al*., 2016).

In this study we provide an explicit picture of spatial and temporal changes in abundances of nitrogen fixing bacteria, ammonia oxidizers (archaeal and bacterial) and bacterial denitrifiers as well as the potential enzyme activities of the latter two in order to address the following questions: 1) To what degree are different functional groups of microbes involved in the N-cycle spatially correlated with each other at the plot scale and how do these correlations change over a season? 2) Can the observed patterns be related to changes in abiotic characteristics such as soil moisture and N

availability or to changes in plant growth associated with land management and the resulting changes in substrate availability? 3) What can the observed patterns tell us about grassland ecosystem N-cycling processes at the studied scale?

Spatial and temporal changes in microbial populations involved in N-fixation (*nifH*), ammonia-oxidation (AOA and AOB), and denitrification (*nirK, nirS*, and *nosZ*), and total bacterial and archaeal abundances as well as potential nitrification and denitrification activities were investigated at the plot scale of 10 m x 10 m at six dates over one growing season. Sampling times were selected to coincide with plant growth stages and management activities, from before active plant growth had started in the spring until after plant senescence following frost in autumn. Data were analyzed for geostatistical relationships with previously published soil biogeochemical data on nutrient distributions and changes in biomass of plant functional groups (Regan *et al.*, 2014). While it is known that archaeal *nifH* (Francis *et al*., 2005; Dos Santos *et al*., 2012) archaeal *nirK* (Bartossek *et al*., 2010; Long *et al*., 2015), and archaeal *nosZ* (Rusch 2013) have also been identified, they have to date most often been studied in manipulated or extreme environments, and less often in temperate, unfertilized grasslands. Their exact mechanisms and routes of archaeal denitrification are also still being investigated (Wallenstein *et al*., 2006; Lund *et al*., 2012; Pajares and Bohannan 2016). They were therefore not included in this study.

6.3 Materials and Methods

6.3.1 *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 Schwäbische Alb, 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 Rendzic 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.

6.3.2 *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.

6.3.3 *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 in another aliquot of the same soil sample as described in Regan *et al.* (2014).

6.3.4 *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 (Stempfhuber *et al.*, 2016); 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 (10 pmol, 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. For details on thermal profiles and on the standards used, we refer to Table S 6.1.

6.3.5 *Potential enzyme activities*

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 (napthylenediamine 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 and 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.

6.3.6 *Statistical analyses*

All statistical analyses were carried out in the R environment (R Development Core Team 2012). To test whether marker gene abundances and potential 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 $P< 0.05$. Correlations were calculated for each date and for the entire season for all measured variables. Correlations of measured marker genes with mean air temperature for every sampling date were also calculated. Air temperature was used instead of soil temperature because it has been shown to have a high statistical correlation to net nitrogen mineralization (Lee *et al.*, 2013).

To test whether spatial autocorrelation could be determined in marker 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. Data were log-transformed when necessary to achieve normality of distribution. 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).

To test whether the measured marker genes and potential enzyme activities representing the nitrogen cycling microbial community were correlated to other aspects of the community and to multivariate descriptors of the environment as a function of spatial separation, Mantel tests were employed. A Mantel test calculates pair-wise correlations between similarity or distance matrices among samples (Franklin and Mills 2007). 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 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 for all pairs of distance matrices were tested for significance using the default settings of 999 permutations in the R package 'vegan' (Oksanen *et al.*, 2013).

6.4 Results

6.4.1 *Temporal dynamics*

Abundances of both archaeal and bacterial 16S rRNA genes were significantly different ($P < 0.05$) between sampling dates (Table 6.1). Archaeal 16S rRNA gene abundance increased from April to May, decreased from May to October, and increased again at the last sampling time point in November. Changes in abundance of the bacterial 16S rRNA gene contrasted with that of the archaeal 16S rRNA gene, decreasing at the first three sampling dates, and increasing in August, after which its abundance remained high for the duration of the season. The high values of bacterial 16S rRNA and denitrifying genes measured on this plot are consistent with other grassland studies in the same and nearby regions (Regan *et al*., 2011; Keil 2015). Over the entire season, abundance of the archaeal 16S rRNA gene was two orders of magnitude lower than that of its bacterial counterpart.

Table 6.1 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	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

Abundance of the *nifH* gene, used as a marker for nitrogen fixing bacteria, was highest in May and lowest in August and October; at the other sampling times values did not differ significantly from one another (Fig. 6.1a). Abundance data of ammonia-oxidizing archaeal (AOA) and bacterial (AOB) genes have previously been described by Stempfhuber *et al*. (2016). In brief, the archaeal *amoA* gene was three to four orders of magnitude more abundant than that of its bacterial counterpart AOB, and did not vary significantly at the first three sampling dates (April-June) (Fig. 6.1a). AOA abundance was lowest in August and highest in November, while the abundance of AOB varied inversely from that of AOA over most of the sampling period.

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 was significantly highest in November (Fig. 6.2a). Abundance of *nirS* harboring denitrifiers, in contrast, was highest in April and lowest in May and October, different from those harboring *nirK* (Fig. 6.2a). Abundance of *nosZ* harboring denitrifiers was also significantly highest in April (Fig. 6.2a). 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. 6.2a). All gene abundances are reported on the basis of g^{-1} DW.

Potential 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 and not significantly different from one another (Fig. 6.1b). In contrast to PNA, DEA was more variable across dates, but did not exhibit a clear seasonal pattern. Furthermore, because it was also spatially more variable than PNA, as indicated by the larger standard errors, only the lowest activity in August differed significantly from the highest activities in June and October (Fig. 6.2b).

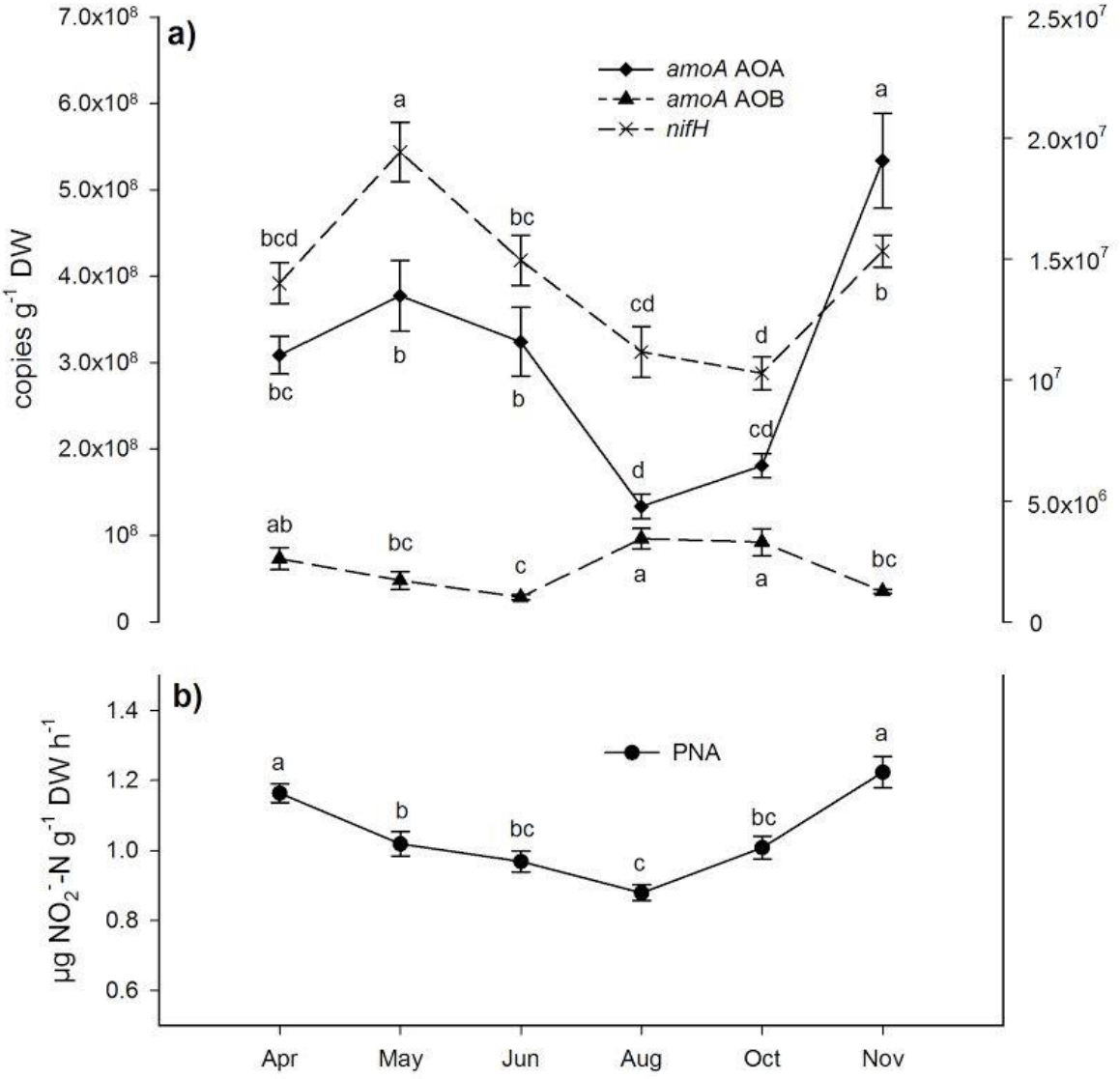

Fig. 6.1 a & b **a)** Nitrogen fixing marker gene nifH and ammonia-oxidizing marker genes amoA of archaea (AOA) and bacteria (AOB), expressed as copies g^{-1} dry soil (DW). Solid line indicates that values are plotted on the left hand Y axis (AOA only). Broken lines indicate values are plotted on the right hand Y axis (both AOB and nifH); and **b)** Potential nitrification enzyme activity (PNA). Units are µg NO_2^--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 ($P< 0.05$). Data of AOA and AOB were taken from Stempfhuber *et al*. (2016).

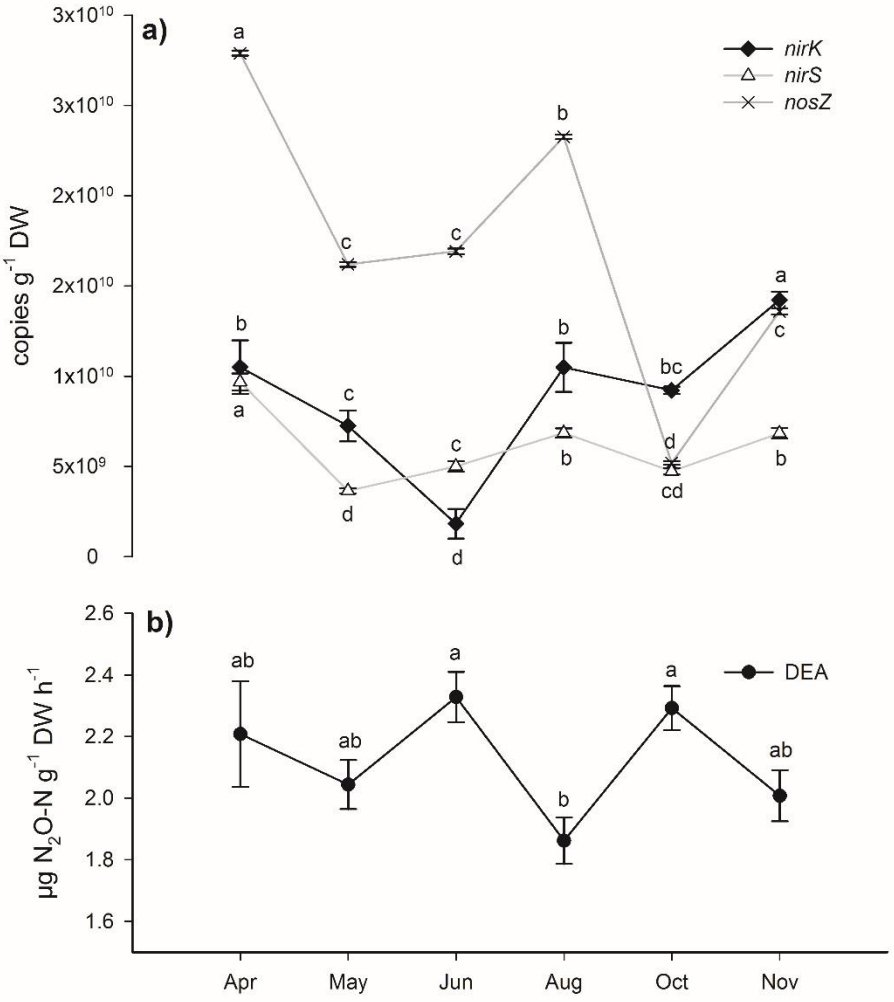

Fig. 6.2 a & b **a)** Denitrifying marker genes *nirK*, *nirS* and *nosZ* expressed as copies g^{-1} dry soil (DW); and **b)** Potential denitrifying enzyme activity (DEA). Units are µg N_2O-N g^{-1} dry soil (DW) h^{-1}. Different letters close to the points indicate significant differences between sampling dates for each measured gene. Differences were determined by one-way ANOVA followed by Tukey's HSD test ($P< 0.05$).

6.4.2 *Correlations between biotic and abiotic soil properties over time*

Soil abiotic properties at this studied plot have previously been described in detail (Regan, *et al.*, 2014, 2015). 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. S 6.1). Only *nirS* and *nosZ* exhibited correlations with soil moisture (Fig. 6.3 a, b). Neither 16S rRNA genes (archaeal and bacterial) nor archaeal or bacterial *amoA* genes, *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. 6.3 c-e). No significant correlations between marker gene abundances and mean air temperature for any sampling date could be determined (data not shown).

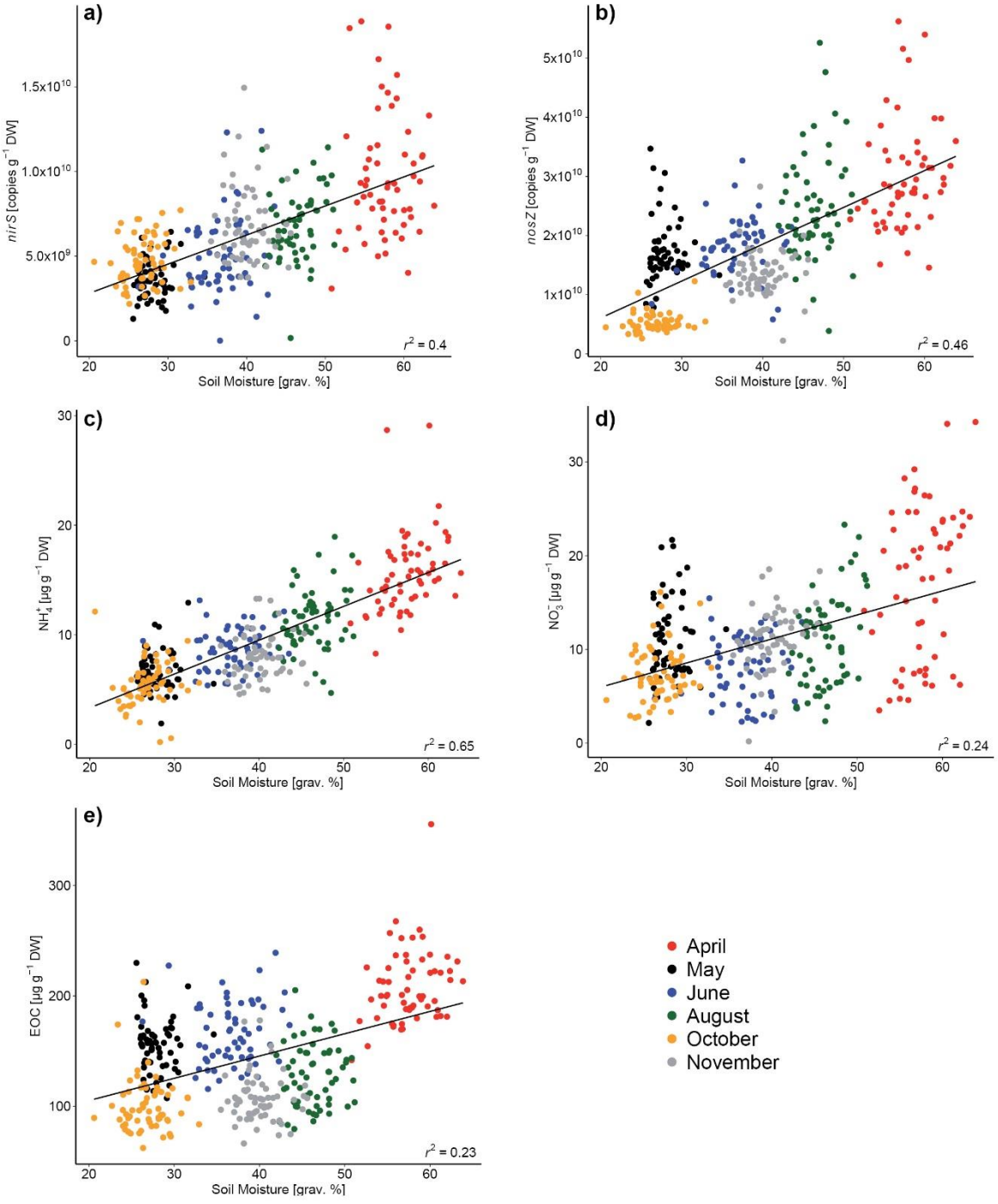

Fig. 6.3 a – e Scatterplots of Pearson correlations between denitrifying marker genes **a)** *nirS*, **b)** *nosZ*; **c)** NH_4^+; **d)** NO_3^-; **e)** EOC with soil moisture over the entire season. Sample dates are indicated by symbol color.

Correlations of the different marker genes and potential enzyme activities with their putative substrates were also examined. Correlations between AOA and NH_4^+ were negative and between AOA and NO_3^- were positive in October (see also Stempfhuber *et al.*, 2016, Fig. 7.5), but both correlations were weaker when calculated over the entire sampling period (data not shown). Among

the denitrifier marker genes, only *nirS* and *nosZ* exhibited weak positive relationships to NO_3^-, and *nosZ* was also positively correlated with EOC (Fig. 6.4 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. 6.4 d,e).

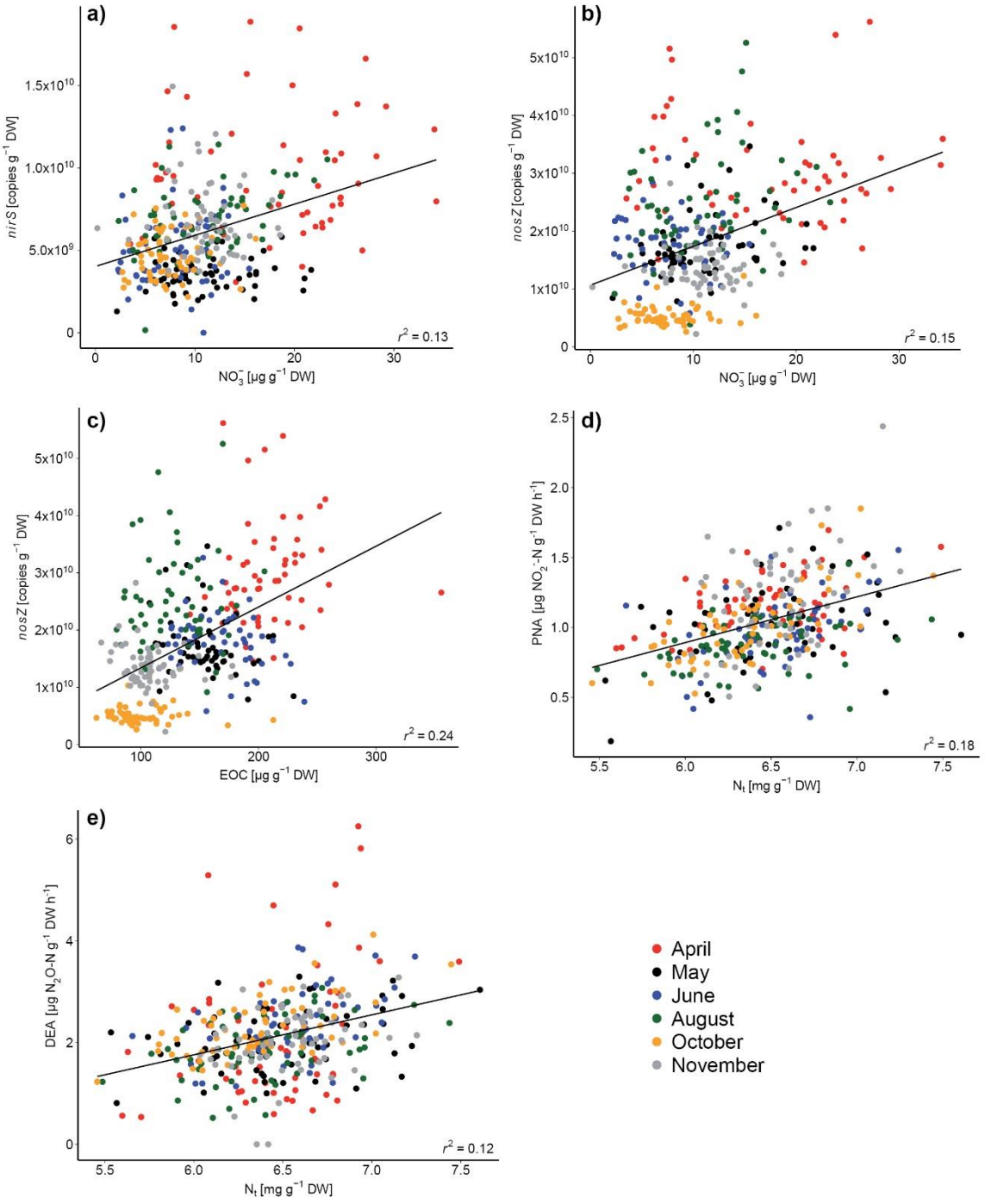

Fig. 6.4 a – e Scatterplots of Pearson correlations between denitrifying marker genes **a)** *nirS* and **b)** *nosZ* with NO_3^-, **c)** of *nosZ* with EOC, and correlations of potential activity measurements **d)** PNA and **e)** DEA with total soil nitrogen. Sample dates are indicated by symbol color.

No significant associations could be determined between PNA and abundance patterns of AOA or AOB, calculated for each date or for the entire season. There were also no correlations between *nirK* and DEA, *nirS* and DEA, or the sum *of nirK* and *nirS* with DEA (data not shown).

6.4.3 *Spatial structure*

Geostatistical analyses of abundances of all marker genes, whereby empirical variograms were determined from measured data, also indicated weak spatial structure at our sampling scale for most of the measured marker genes. High nugget values suggested that some measured genes were spatially structured at a smaller scale than that of our measurements (data not shown). Log-transformed values of AOA and AOB, however, exhibited weakly spherical structure at some dates although they were not spatially structured at the same dates (see Stempfhuber *et al*., 2016, Fig. 7.2).

Potential enzyme activity measurements could be modeled at this scale at several sampling dates. 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. 6.5 a-d). Patterns of PNA and DEA were similar to one another in June and again in October, but differed between the dates.

6.4.4 *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, as reported in Regan *et al*. (2014). The marker genes measured in this study were not spatially correlated at our sampling scale at any date. Potential enzyme activities were correlated with space only in November (Table 6.2).

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 potential enzyme activities were spatially correlated only in October. No spatial correlations between soil abiotic properties and plant functional groups or soil abiotic properties and the analyzed marker genes could be determined. Soil abiotic properties and potential enzyme activities, however, were spatially correlated at all dates (Table 6.2).

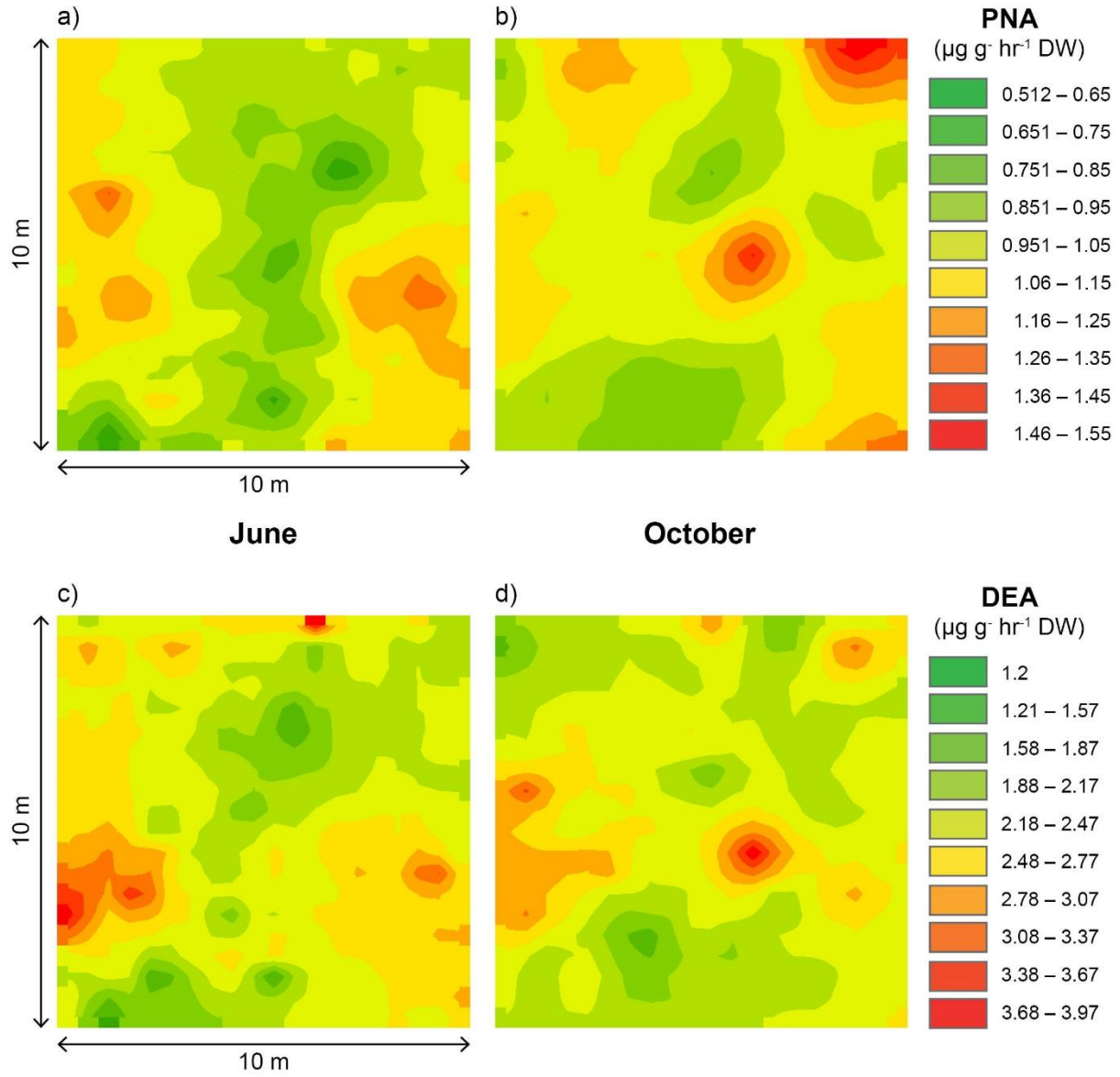

Fig. 6.5 a – d Kriged maps of potential nitrification enzyme activity (PNA) in ***a)*** June and ***b)*** October, and of denitrifying enzyme activity (DEA) also in ***c)*** June and ***d)*** October.

Table 6.2 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. Only values shown in bold are significant. **P* < 0.05, ***P* < 0.01, ****P* < 0.001

Date	Space/ Abiotic	Space/ Plants	Space/ Genes	Space/ Enzymes	Abiotic/ Plants	Abiotic/ Genes	Abiotic/ 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

6.5 Discussion

We investigated the spatio-temporal response patterns of nitrogen fixing bacteria (*nifH*), archaeal (AOA) and bacterial (AOB) ammonia oxidizers (*amoA*), and bacterial denitrifiers (*nirK*, *nirS* and *nosZ*), in soil of a temperate grassland managed at low land use intensity. Examined in conjunction with the potential enzyme activities of nitrifiers and denitrifiers, these functional groups and their potential activities provided an opportunity to fill a knowledge gap in understanding differences in nitrogen cycling microbes' habitat preferences, potential enzyme activities, and responses to changes in plant growth stages at the m^2 scale.

6.5.1 *Spatio-temporal dynamics*

6.5.1.1 Nitrogen fixation

Highest abundance of N_2-fixing bacteria harboring the *nifH* gene was observed in May, decreasing by almost 50% between May and October and increasing slightly in November. This is in agreement with Pereira e Silva *et al*. (2011), who demonstrated that up to 60% of the community composition of diazotrophs can shift seasonally. High *nifH* abundance in May could be explained by measured low NH_4^+ values at that date, creating favorable conditions for *nifH*-gene harboring bacteria in May, since biological N-fixation is known to be inhibited when NH_4^+ levels in soil are high (Pereira e Silva *et al.*, 2013). Their study also demonstrated that both abundance and composition of N-fixing communities varies seasonally. However, in contrast to their findings, on our plot in October *nifH* abundance was lowest when NH_4^+ concentration in soil was lowest. N-fixation is an energetically expensive process, requiring adequate available carbon. The availability of C was presumably low on the plot in October, as EOC values were lowest (Regan *et al.*, 2014, 2015), and this may have limited *nifH* abundance at that date. Overall, none of the other measured abiotic properties showed similar temporal patterns or correlations with *nifH* abundances, nor was spatial structure on the plot detectable for *nifH*.

6.5.1.2 Ammonia oxidizing archaea and bacteria

AOA exhibited a temporal pattern similar to that of *nifH*, resulting in positive associations between *nifH* and AOA in April, August, and October. This close interaction was also observed by Tsiknia *et al*. (2015) who described a linear response of AOA abundance to *nifH* abundance in natural, non-agricultural ecosystems at the watershed scale. They suggest that this is evidence of the important role of diazotrophs in providing N to natural (unfertilized) ecosystems. However, this relationship was not detected in another study of grassland sites in the same region of Germany as ours (Meyer

et al., 2013).

In accordance with previous studies (Leininger *et al.*, 2006; Prosser and Nicol, 2012; Daebeler *et al*., 2015), AOA microorganisms on our plot were more abundant than their bacterial counterpart. The concentration of NH_4^+ in soil has been identified as an important factor driving the relative distributions of AOA and AOB. Pure cultures have shown that AOA have a high affinity for NH_4^+ and are able to grow under oligotrophic conditions. In the investigated grassland, abundance of AOA varied almost inversely with measured NH_4^+ concentrations at all sampling dates, while that of AOB varied positively with NH_4^+ (Fig.6.1.a, Fig. S 6.2), suggesting NH_4^+-driven niche separation between AOA and AOB, a finding supported by other studies (Prosser and Nicol, 2012; Marusenko 2013). This was also seen in their divergent spatial patterns, identified by Stempfhuber *et al.* (2016). It is well accepted that plants can strongly influence microbial communities and their functional traits in the rhizosphere by the excretion of exudates, providing carbon to soil microorganisms (Philippot *et al.*, 2013; Berg *et al*., 2014). However, both amount and quality of root exudates vary at different stages of plant growth (Harrison *et al.*, 2007; Ziegler *et al.*, 2013; Huang *et al.*, 2014). Also, as NO_3^- or NH_4^+ uptake by plants can result in nitrogen depletion in soil, competition with plants for NO_3^- or NH_4^+ can also influence nitrogen cycling microbial communities (Boudsocq *et al.*, 2012; Kuzyakov and Xu, 2013; Cantarel *et al.*, 2015; Moreau *et al*., 2015). Plants also have varying nitrogen preferences and uptake strategies (Harrison *et al*., 2007), which can impact the abundances of some ammonia-oxidizers (Hatzenpichler 2012). This is most pronounced at the plant-soil interface during periods of most rapid plant growth, typically in May, when competition for the same nitrogen sources are most intense (Kuzyakov and Xu 2013). Our data suggest that AOA were able to out-compete AOB when NH_4^+ availability was low in May, in accord with previous studies (Di *et al*., 2010), but that soil the environment had changed by October, making it possible for AOB to compete more effectively for the limited NH_4^+ at this date. For example, in a detailed characterization of spatial interactions of ammonia-oxidizers and nitrite-oxidizing bacteria in the same grassland plot, Stempfhuber *et al*. (2016) found that AOA abundance varied in concert with the bacterial nitrite oxidizer *Nitrospira*, which was also low in October.

Abundance of AOB was high in April, August and October. In April NH_4^+ was highest, likely due to N mineralization over the winter. There was no competition with plants for available NH_4^+ at this date since plant growth had not yet begun. NH_4^+ also rose following mowing in August, with correspondingly high AOB. In October NH_4^+ was low, which should have been unfavorable to AOB, but it has also been shown that AOB abundance increases in the presence of legumes (Malchair *et al.*, 2010; Le Roux *et al*., 2013) and legumes had reappeared on the plot by October.

The effect of legumes is thought to be related to changing ammonium levels in soil, even if only in microsites, leading to small and locally higher NH_4^+ levels (Temperton *et al.*, 2007), and which can be enough to induce a shift in AOB community composition (Avrahami *et al.*, 2003).

The spatial structures of AOA and AOB were weakly detectable at some dates (For details see Stempfhuber *et al*., 2016, Fig. 2), but were not the dates with highest measured marker gene values, suggesting that factors influencing spatial structure are not necessarily the same as those that determine abundance of microbial groups. This is consistent with the findings of Regan *et al*. (2014) which identified the most robust spatial structures of both NH_4^+ and NO_3^- on this plot at dates of high plant growth but low inorganic nitrogen concentrations. Others have also reported stronger spatial connections between microbial communities with life history and dispersal strategies (Bissett *et al.*, 2010), or soil physico-chemical properties, land use, and management (Franklin and Mills, 2009; Enwall *et al*., 2010; Bru *et al.*, 2011; Dequiedt *et al*., 2011) than with abundances.

6.5.1.3 Linking nitrifiers with denitrifiers

In unfertilized soils, denitrifiers depend on nitrifiers, as the latter produce nitrate required by denitrifiers through microbial oxidation of NH_4^+ (Prosser 1989). Close spatial and temporal interaction with nitrifiers can therefore be advantageous for denitrifiers to avoid competition with plants for available, but limited, nitrate. Some AOA harbor *nirK* genes (Inatomi and Hochstein, 1996; Ichiki *et al*., 2001; Lund *et al*., 2012), and some nitrifying bacteria can also denitrify (Bothe *et al.*, 2007). This underscores the close coupling between microbes involved in the two processes (Boudsocq *et al.*, 2012; Kuzyakov and Xu, 2013). Similar to ammonia-oxidizers, the abundances of denitrifiers, in particular those harboring *nirS* and *nosZ* genes, also appeared to be influenced, albeit weakly, by substrate availability of nitrate and EOC respectively (Fig.6.4a-c). All measured denitrifying marker genes exhibited similar directions in abundance patterns over the entire sampling period except in June, when *nirS* and *nosZ* increased while *nirK* decreased (Fig.6.2a). Studies have indicated that when the community structures of *nirK* or *nirS* type denitrifiers were examined along environmental gradients, they changed along those gradients (Santoro *et al*., 2006; Smith and Ogram, 2008; Mosier and Francis, 2010). Cain *et al*. (1999) demonstrated that soil nitrogen is highly spatially variable at the cm to m scale, with short-duration areas of high resource availability. Due to the limited mobility of soil microbes, competition as a major structuring force is restricted to the small scales where competitors co-occur (Ettema and Wardle, 2002). It is therefore possible that between May and June, during a phase when plant growth was strongest and competition for substrate between plants and microorganisms as well as between denitrifiers was greatest, a microsite-scale temporal environmental gradient existed that contributed to observed

differences in *nirK* and *nirS* abundances in June. Other studies have indicated that spatial variation in environmental parameters may result in niche partitioning, and that this may be a factor in determining community composition of different *nir*-type denitrifiers (Philippot *et al*., 2009; Enwall *et al*., 2010, Keil *et al*., 2011).

An increase in soil moisture triggers both microbial activity and reduced transport of oxygen (Cook and Orchard 2008), resulting in decreased oxygen availability, promoting the growth of microbes capable of using alternative electron acceptors such as NO_3^-. AOA varied inversely with soil moisture, while changes in AOB abundance did not follow a pattern related to soil moisture. AOB appeared to vary more in concert with the denitrifiers (Fig. 6.1a, Fig. S6.1). All quantified marker genes for denitrification changed corresponding to soil moisture and soil NO_3^- content with the exception of one date, for *nirK* only (Fig. 6.2a, Fig. S6.1). This suggests that *nirS* and *nos*Z harboring denitrifiers were more sensitive to soil moisture than *nirK* and that *nirK* was more responsive to some environmental factor we could not identify. The similarity in responses of *nirS* and *nosZ* harboring denitrifiers to soil moisture also supports recent findings that *nosZ* is more closely associated with microbes harboring *nirS* than *nirK* (Graf *et al*., 2014; Jones *et al*., 2014). No spatial structure could be detected for any of the denitrifiers at the studied scale at any date.

6.5.2 *Linking temporal dynamics of functional microbial communities and potential enzyme activities*

PNA changed in concert with AOB abundance early in the season, but changed in direction similar to AOA (and *nifH*) later in the year, further supporting the concept of seasonal niche partitioning and at least some degree of functional redundancy between the two microbial groups of ammonia oxidizers. In general, correlations between gene abundances and potential activities have proven difficult to determine, with conflicting results depending on the environment studied (Prosser and Nicol, 2012; Le Roux *et al*., 2013), and determinations of those relationships have been weakest in grasslands (Meyer *et a*l., 2013; Rocca *et al*., 2015). However, Stempfhuber *et al*. (2014) identified correlations of PNA with both AOA and AOB in mineral grassland soils of the same region as this study plot.

We could not identify any temporal relationship between PNA and plant growth stages. DEA, however, was highest at the dates corresponding to the appearance of legumes on the plot, June and October. Measured values were not significantly different from those at most other sampling dates, but similar associations of DEA with legumes have been shown as well by Le Roux *et al*. (2013) in an experimental grassland. After accounting for increased plant species richness and other factors,

legume presence remained a significant influence on DEA, and their observations held at different levels of legume abundance.

In contrast to the marker genes, both PNA and DEA were spatially structured at the scale of our plot at some dates. The dates with the strongest spatial structure in potential enzyme activities corresponded to periods of rapid plant growth and high plant biomass, but not with correspondingly highest gene abundances or substrate concentrations (Rocca *et al.*, 2015). Potential enzyme activities represent the integrated activity of living microbes over time, e.g., on the order of days; direct relationships between substrate availability, which can be highly variable over short time periods, and potential activities have proven more difficult to determine (Le Roux *et al*., 2013).

Kriged maps of the spatial distributions of both PNA and DEA showed areas of similar minima and maxima (Fig. 6.5 a-d) and congruent changes in both from one date to the other. The finding of Stempfhuber *et al*. (2014), correlating PNA to NO_3^- rather than to NH_4^+, may explain the similarity in spatial distributions of PNA and DEA in June and again in October and indicate that DEA relies on the availability of NO_3^- produced by ammonia oxidizers and nitrifiers in close proximity to denitrifiers.

Spatial structure at the May and October sampling 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 is consistent with Kuzyakov and Blagodatskaya (2015), who specifically defined hotspots and hot moments as highly ephemeral.

6.6 Conclusions

Unfertilized perennial grasslands with high plant diversity have been shown to have higher soil organic carbon, total nitrogen, and microbial carbon; greater food web complexity; and more complex biological communities than more intensively managed croplands (Grayston *et al.*, 2001; Culman *et al.*, 2010). Grasslands such as these have also been shown to use nitrogen more efficiently than those with less plant diversity, especially in nutrient-limited soils (Zak *et al.*, 2003; Kleinebecker *et al.*, 2014). Understanding nitrogen cycling processes in detail and at multiple scales is therefore especially critical in grassland ecosystems managed at low intensity. Conclusions about controls on nitrogen cycling have also differed with changes in scale and with different subsets of the microbial community. Factors shaping microbial communities at one scale may be neither important nor predictive at another; it is necessary, therefore, to investigate the distribution of

microorganisms and of their activities at various spatial scales (Turner 1989; Nunan *et al*., 2002; Franklin and Mills 2009). Our study provides evidence for habitat preferences of nitrogen cycling microbes and of their responses to different stages of plant growth. We found clear seasonal changes in patterns of abundance of the measured marker genes and could associate these with changes in substrate availability related to plant growth stages. The most striking result was that small and ephemeral changes in soil environmental conditions can result in changes in these microbial communities, but the changes may not reflect potential process rates, suggesting short-term niche partitioning and functional redundancy. This was indicated by the relatively stable potential enzyme activity measurements over the sampling period as compared to microbial abundances. In addition, we provided evidence of a dynamic relationship between microorganisms and plants, an important mechanism controlling ecosystem N-cycling.

6.7 Acknowledgements

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6.8 Supplementary Materials

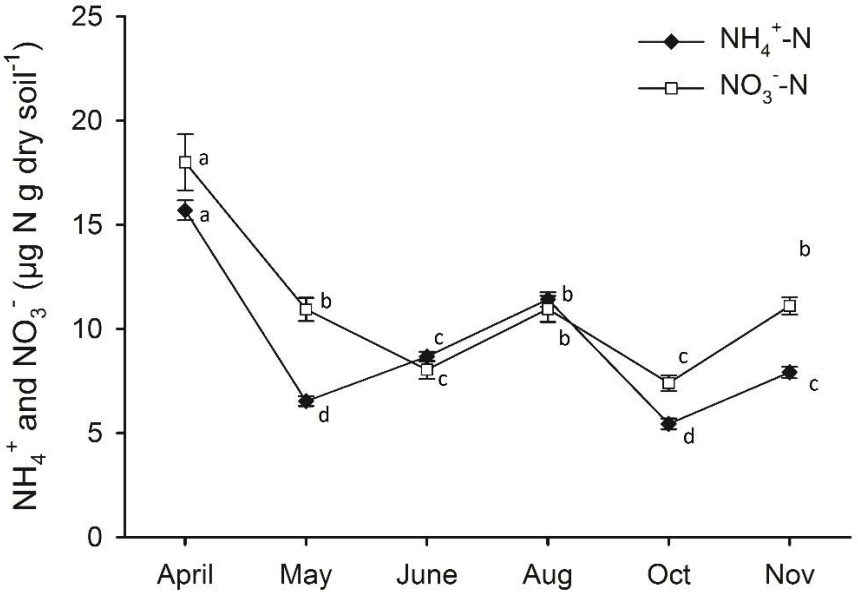

Fig. S 6.1 Mean values of NH_4^+ and NO_3^- for all sample dates. Values are expressed as µg g^{-1} dry soil. Error bars indicate standard error. Lower case letters close to the points indicate significant differences between sampling dates for each measured gene. Differences were determined by one-way ANOVA followed by Tukey's HSD test ($P< 0.05$). Data were taken from Regan et al. (2014).

Table S 6.1 Thermal details of measured gene abundances**.**

Target/Primers	Sequence(5'-3')	Thermal Conditions	Reference
16S rRNA[1]	*Pseudomonas aeruginosa*		Lopez-Gutierrez *et al*., 2004
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			Nicol *et al*., 2005 f
rSAf(i)[2]	*Methanobacterium sp.*	94°C / 20 s, 55°C / 60 s, 72°C / 30 s, 5 cycles	Bano *et al*., 2004 r
958r[3]		94°C / 20 s, 50°C / 60 s, 72°C / 30 s, 35 cycles	
***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	Leininger *et al.*, 2006
CrenamoA616r48x[5]	GCC ATC CAB CKR TAN GTC CA		Schauss *et al*., 2009
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	Rotthauwe *et al*., 1997
amoA-2r	CCC CTC KGS AAA GCC TTC TTC		
nifH[7]	*Sinorhizobium meliloti*	95°C / 45 s, 55°C / 45 s, 72°C / 45 s, 40 cycles	Rösch *et al*., 2002
nifHf			
nifHr			
nirK[8]	*Sinorhizobium meliloti*		Henry *et al*., 2004
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		Throbäck *et al*., 2004
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		Henry *et al*., 2006
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,	
nosZ2r	CAK RTG CAK SGC RTG GCA GAA	72°C / 30 s, 40 cycles	

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

Barbara Stempfhuber[1*+], Tim Richter-Heitmann[2+], Kathleen M. Regan[3+], Angelika Kölbl[4], Pia Kaul[5], Sven Marhan[3], Johannes Sikorski[5], Jörg Overmann[5], Michael W. Friedrich[2], Ellen Kandeler[3], Michael Schloter[1]

[1] Environmental Genomics, Helmholtz Zentrum München, German Research Centre for Environmental Health, Neuherberg, Germany

[2] Faculty of Biology/Chemistry, University of Bremen, Bremen, Germany

[3] Institute of Soil Science and Land Evaluation, University of Hohenheim, Stuttgart-Hohenheim, Germany

[4] Lehrstuhl für Bodenkunde, Technische Universität München, Freising, Germany

[5]Leibniz-Institute DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany

+ These authors contributed equally to this work

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7.1 Abstract

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 m². 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 onwards, indicating its important role in nitrite oxidation.

7.2 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 & Schwartz 2008; Zhang *et al*., 2010) or bacterial ammonia-oxidizer (Di *et al*., 2009; Jia & 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 & 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 & 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 & 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 & 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 (WWTP) (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 & Garnier 2005; Kim & 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 & Garnier 2005; Ke *et al*., 2013; Ollivier *et al*., 2013; Placella & 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 & 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 & 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 & 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 also act 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 nitrite oxidizing bacteria as affected by time and space, and to link these to the presence of AOA and AOB.

7.3 Experimental Procedures

7.3.1 *Study site description and sampling design*

The experiment was performed in the frame of the 'German Biodiversity Exploratories' (www.biodiversity-exploratories.de; (Fischer *et al*., 2010)), a large interdisciplinary study aimed at improving our understanding 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 810mm. 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 x 10 m plot was divided into 30 subplots (each 2 m x 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 x 6 dates). Dates were chosen to correspond to stages of plant growth on the plot. Per date, 2 samples were collected from the upper 10 cm soil horizon from each of the 30 subplots within the 10 x 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 (5mm) 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).

7.3.2 *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 minutes. 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.

7.3.3 *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 Table S 7.1. Serial dilutions of the plasmids containing fragments of the marker genes (Table S 7.1) 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.

7.3.4 *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, USA). Besides the specific binding sites 341f (Muyzer *et al*., 1993) and 515R (Lane 1991), the primers contained the Illumina adapter sequence as well as the 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, 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 the sequencing at the Helmholtz Center for Infectious Diseases, Braunschweig, Germany. Two samples (one in April, one in June, respectively) 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 & Godzik 2006; Fu *et a*l., 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). Exclusively OTUs affiliated with those genera were then extracted from the 16S rRNA dataset. For reference sequences, the RDP-Classifier (with 16S rRNA training set 10), BLAST (Altschul *et al*., 1990) (versus the Nucleotide collection (nr/nt)), 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 nitrite-oxidizing bacterium, 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.

7.3.5 *Statistics*

Statistical analyses were performed using R (R Core Team, 2014; http://www.R-project.org). 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 correction according to the Akaike Information Criterion (AIC) was 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 & 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 is homogeneously distributed or the spatial distribution is independent of the scale chosen (see Table S 7.3) 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.

7.4 Results

7.4.1 *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) (Table S 7.2, Figure 7.1). 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 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 of AOA ($p<0.001$) and NB ($p<0.05$) between October and November, and for NB in early spring ($p>0.01$).

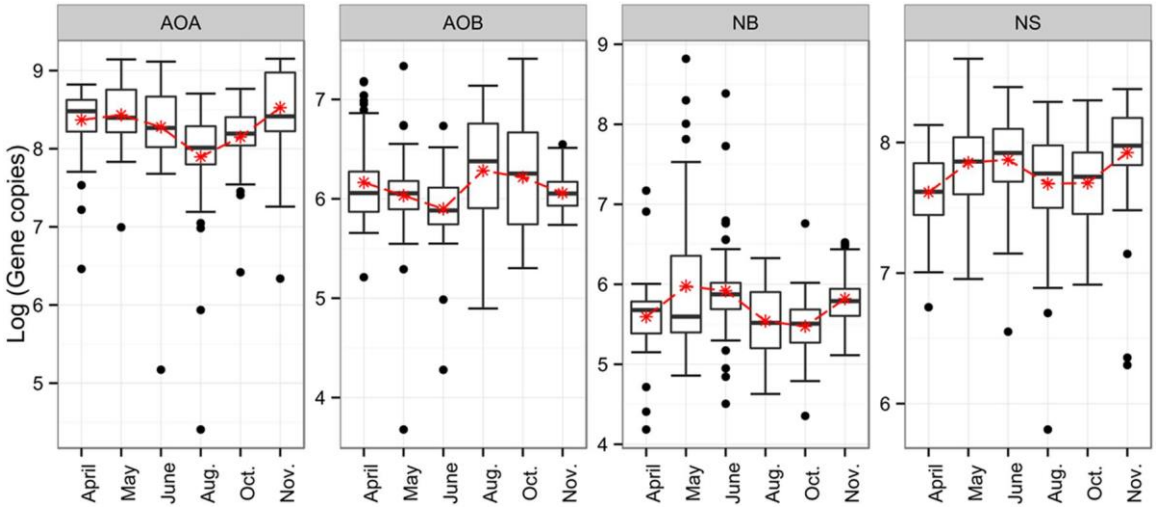

Fig. 7.1 Boxplots for seasonal dynamics of ammonia- and nitrite-oxidizers. Depicted are gene copy numbers. AOA = ammonia-oxidizing archaea, AOB = ammonia-oxidizing bacteria, NB = Nitrobacter-like, NS = Nitrospira-like

7.4.2 *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 m², geostatistical semivariogram analyses were conducted. Table S 7.3 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 (Table S 7.3). 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 7.2). 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 7.2 A6), to large patches with hotspots in April (Figure 7.2 A1), and finally more homogeneous structures in August (Figure 7.2 A4) 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 (Figure 7.2 B1-6). 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 (Figure 7.2 C4-5) 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 (Figure 7.2 D2-3).

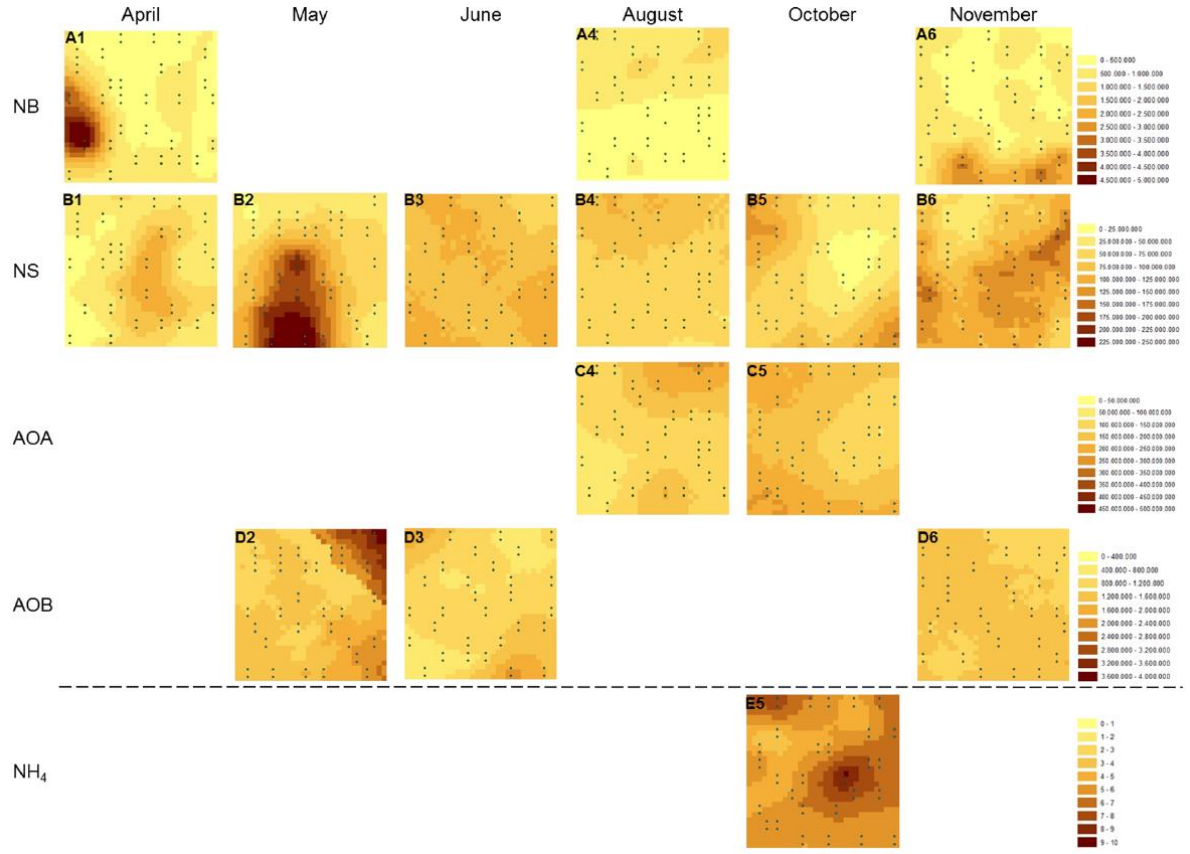

Fig. 7.2 Spatial distribution of selected variables. Kriged maps were constructed for gene abundances of (A) *nxrA* gene (NB), (B) 16S rRNA genes (NS), (C) *amoA* gene (AOA), (D) *amoA* gene (AOB) and for soil ammonium content (E) at different sampling dates (1 - 6). Gene abundances are given in gene 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.

Spatial variability was more homogeneous in November. Figure 7.2 E5 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.

7.4.3 *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 ($p < 0.01$), 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 (Figure S 7.1). 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 OTU 01, which was not significant ($p = 0.06$). Interestingly, the activities of OTUs 01 and 02 both declined during the late season sampling dates, whereas OTU 03 remained stable, thus increasing its abundance compared to the other *Nitrospira* OTUs (Figure S7.1).

NS 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 (Figure S 7.2). According to 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 (Figure S 7.2). At the beginning and towards 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 mild correlation between nitrate and the *Nitrobacter*_OTU was also found in October.

A phylogenetic tree was constructed based on the Neighbor Joining algorithm (Figure 7.3) 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) (Table S 7.4). 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 *Candidatus 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 Fig.S 5.2 in Regan *et al*., 2014) that exhibited the most pronounced dynamics in 16S rRNA gene abundances for NS-like NOB (Figure 7.4C). We compared shifts in the relative activity of OTUs by plotting their relative abundances against each other, setting the total abundance to 1 (Figure 7.4A). 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 7.4B), it was especially pronounced in this location, suggesting spatial heterogeneity of species distribution.

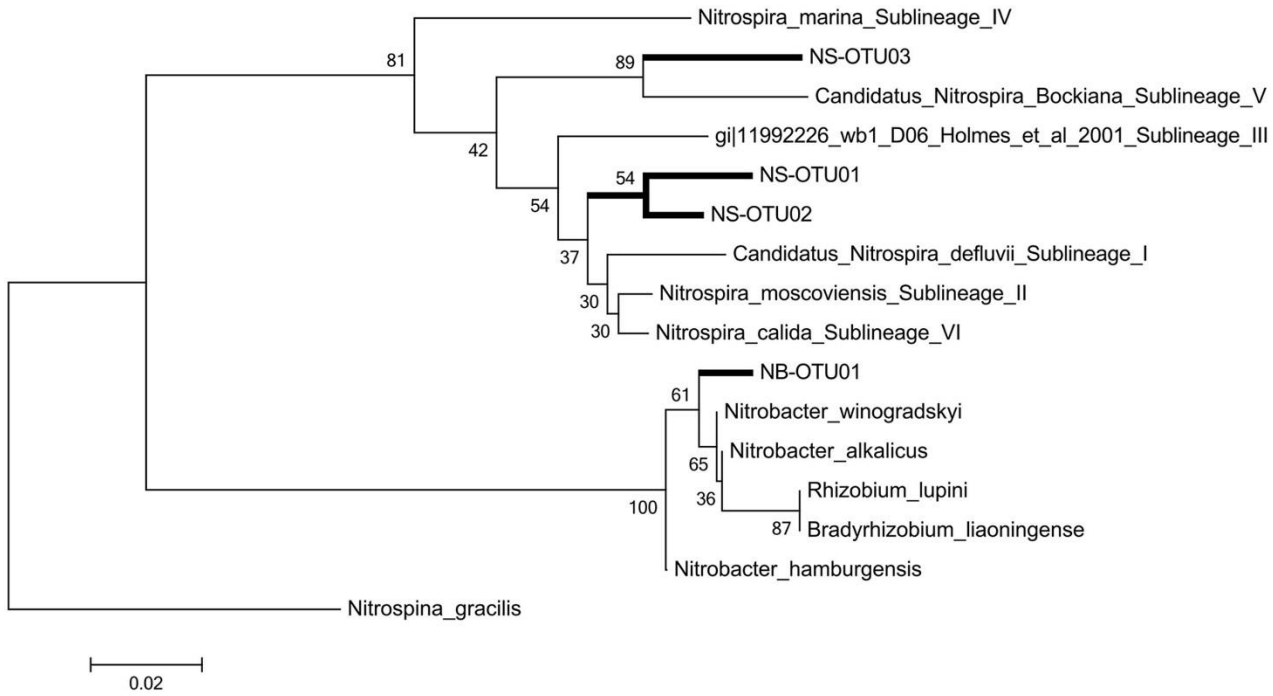

Fig. 7.3 Phylogenetic tree. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0,39985022 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) and are shown 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.

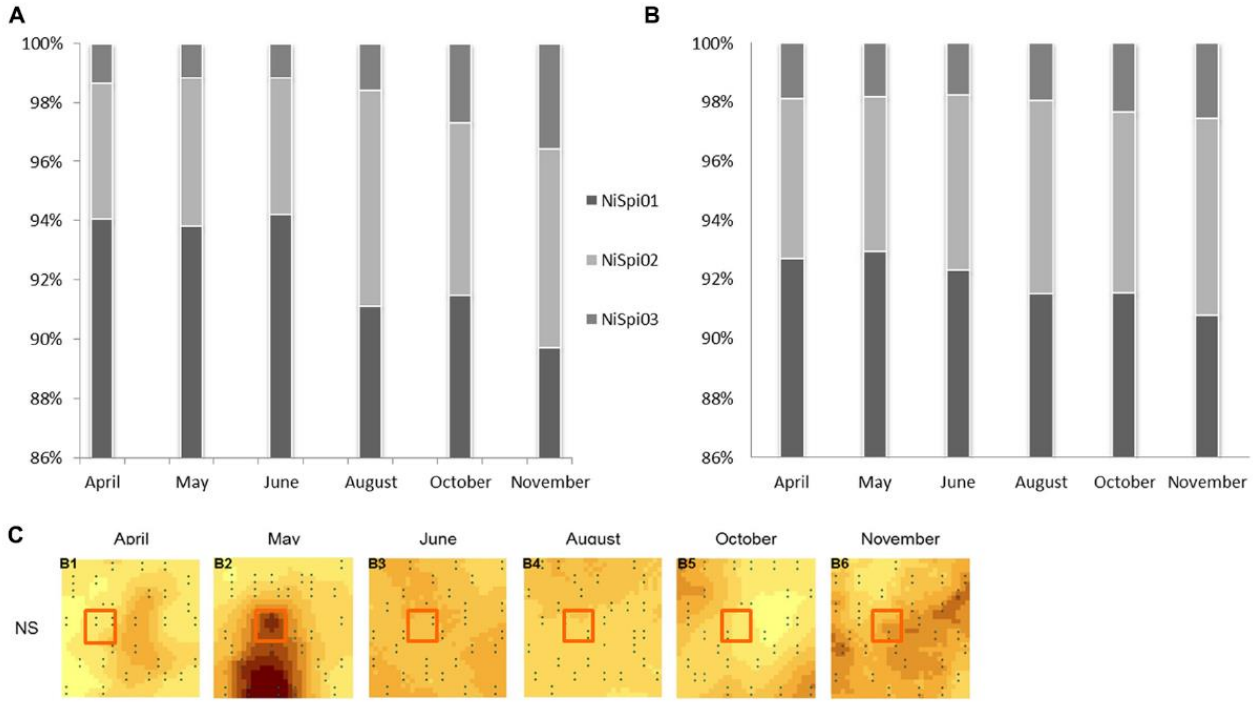

Fig. 7.4 Relative abundances of NS-assigned OTUs. Columns display the relative abundances of *Nitrospira*-like NOB OTUs 01-03 over the season. The total abundance of NS-assigned OTUs was set to 100%. NS = *Nitrospira*-like nitrite-oxidizing bacteria. Barcharts depict either relative abundances within one selected subplot (A) or represent the complete dataset (B). The location of the selected subplot is indicated by the red square (C).

7.5 Discussion

7.5.1 *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 & Firestone 2013; Daebeler *et al*., 2014). Marginally higher abundances of NS-assigned 16S rRNA sequences on the RNA level (Figure S 7.1), compared with lower *Nitrospira* rRNA 16S gene abundances on the DNA level during autumn (Table S 7.2) 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 & 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 (Table S 7.2), corresponding to previous studies (Leininger et al., 2006; Adair & 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 & Nicol 2008; Jia & Conrad 2009). The high standard deviations in gene 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.

7.5.2 *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*., 2009; Krause *et al*., 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 (Figure 7.2 A1, B1), whereas homogeneous and congruent abundance patterns for both NS and NB were found in August, indicating co-occurrence at the same spatial scale (Figure 7.2 A4, 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 (Figure 7.2 A6, 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 7.2) 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 & 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 & 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).

7.5.3 *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 7.3, see Table S 7.4 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 *Candidatus* N. bockiana with 94% similarity (Figure 7.3), and similar substrate preferences that hold true for *Candidatus* N. bockiana as cultured representative may also apply to other members of sublineage V (Lebedeva *et al*., 2008), such as the incapability 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 & 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 7.4). 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 & Debouzie 2000; Maixner *et al*., 2006).

7.5.4 *Spatial interactions of nitrifying organisms*

Studies on nitrifiers at spatial ranges from µm (Maixner *et al*., 2006) to the landscape scale (Grundmann & 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 & 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 (Table S 7.3) (Steffens *et al.*, 2009). The ranges of spatial dependence of the abundance data in this study (Table S 7.3) were, however, similar to spatial autocorrelations ranging from 1.4 – 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 & 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 & Conrad, 2009; Di *et al*., 2010; Wertz *et al*., 2012; Ke *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 7.2, Table S 7.5), 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 catalyse 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 *et al*., 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 7.5), when congruent spatial patterns of AOA and NS were most pronounced (Figure 7.2, Figure 6, Table S 7.5) and all investigated molecular markers were highly correlated, with each other, which was observed only in October (Figure S 7.3). Several significant, positive pairwise correlations were detected in October. Correlations between nitrate and NS OTU03, AOA and NS, and NS and NS OTUs 01 and 02, respectively, were all found to be significant at $p_{adjusted}<0.05$, 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 (Table S 7.5).

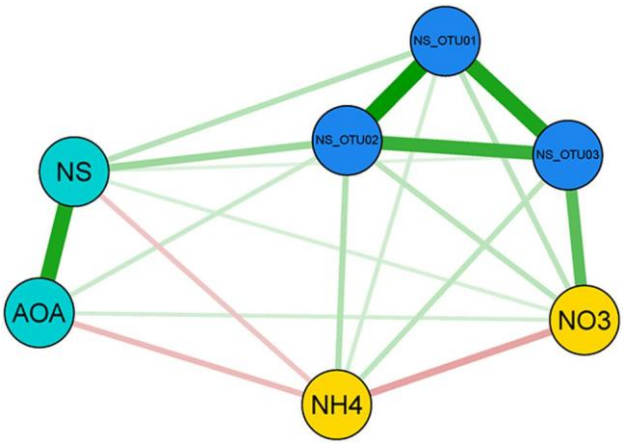

Fig. 7.5 Network analysis of interactions between NS-assigned OTUs, gene abundances and nitrification-associated nitrogen-pools in October. Depicted are pearson correlations between three parameter groups for sampling date October: gene abundances (light blue circles), *Nitrospira* OTUs 01-03 (dark blue 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).

Nitrate concentration was positively connected most clearly with OTU03 in October (r = 0.42) (Figure S 7.2), which hints at the active participation of sublineage V (Figure 7.3) in the production of nitrate and for subsequent nitrite oxidation from August on (Figure 7.4). 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 (Figure S 7.2). The positive correlation of AOA and nitrate (Figure 7.5) 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 (Figure 7.2 C5, E5), indicating consumption of ammonia as substrate by AOA (Schleper & Nicol 2010; Ke *et al*., 2013). The negative correlation of nitrate and ammonium (r = 0.233) (Figure 7.5, Figure S 7.2) 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 catalyse complete nitrification (commamox) that have not been assessed by our study of spatial interaction patterns (Daims *et al*., 2015; van Kessel *et al*., 2015).

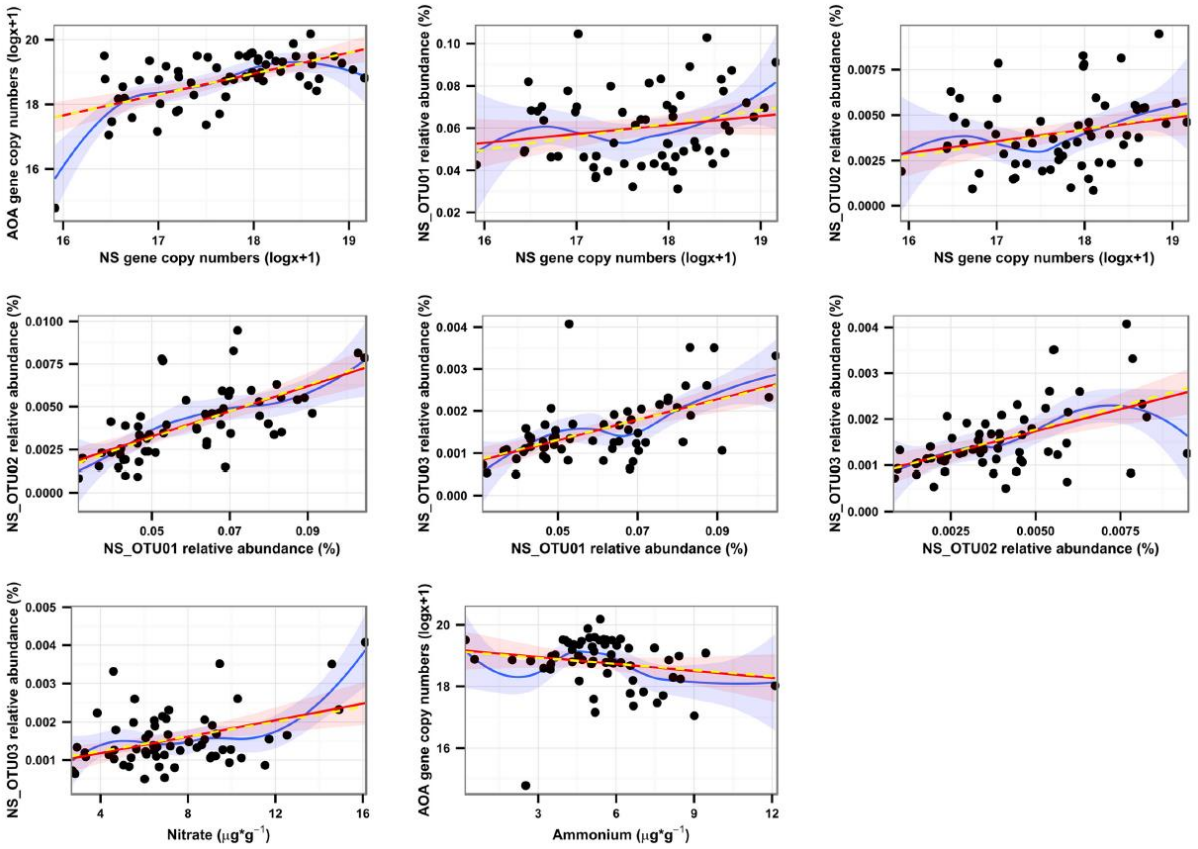

Fig. 7.6 Univariate linear models between pairs of variables in October. Pairs of variables were selected from the network analysis (figure 4) to show additional support for our conclusions after accounting for spatial autocorrelation. Red lines indicate the uncorrected, gaussian regression models, whereas 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.

Different growth strategies, such as potential mixotrophy or heterotrophy may obscure the interactions between AOA and NS. Consequently, the utilization of alternative substrates (Prosser & Nicol 2008; Tourna *et al*., 2011; Prosser & Nicol 2012) 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 could increase the competitiveness (Rogers & Casciotti 2010; Lehtovirta-Morley *et al*., 2014) 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 & 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 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; Patra *et al*., 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 *et al*., 2001; Patra *et al*., 2006; Le Roux *et al*., 2008; Kuzyakov & Xu 2013). The heterogeneous ammonium distribution may also be linked to plant diversity, as strong spatial distribution patterns of legumes were observed mainly in October at the site (Regan *et al*., 2014).

This study presents evidence for both temporal and spatial correlation of ammonia-oxidizing archaea 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 one-year study. Thus, it might be interesting 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 might exist between functional guilds of nitrification and play an important role for the stabilization of nitrifier networks mainly in fertilized soils.

7.6 Acknowledgments

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7.7 Supplementary Experimental Procedures

7.7.1 *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).

7.7.2 *Geostatistical analysis*

Geostatistical semivariogram analyses were carried out using the g-stat package (Pebesma, 2004) for the R environment (R 3.0.2, R Development CoreTeam, 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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7.8 Supplementary Figures and Tables

7.8.1 *Supplementary Figures*

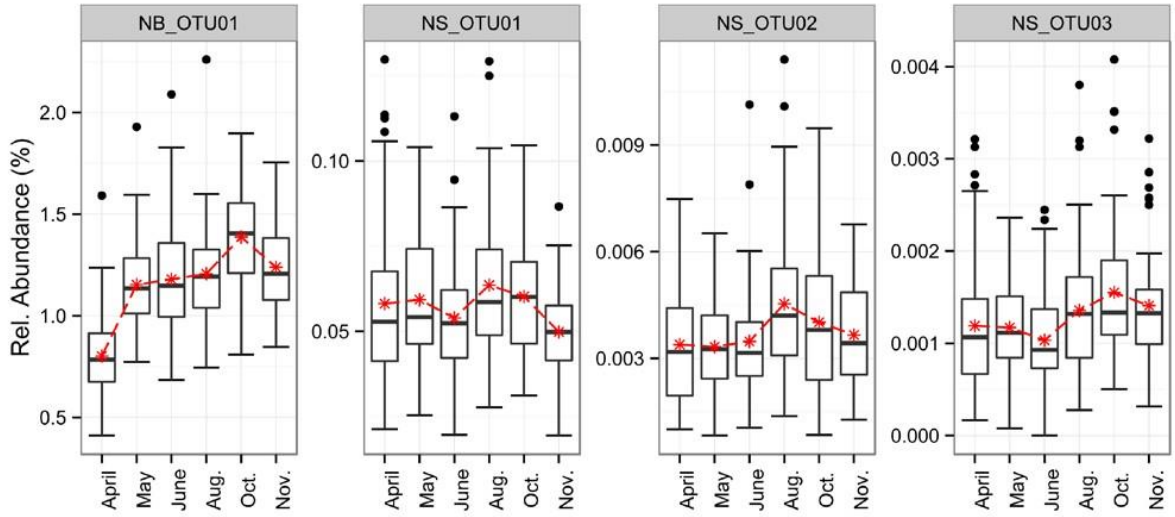

Fig. S 7.1 Boxplots for seasonal dynamics of nitrite-oxidizing bacteria associated OTUs, showing relative read abundances.

Fig. S 7.2 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.

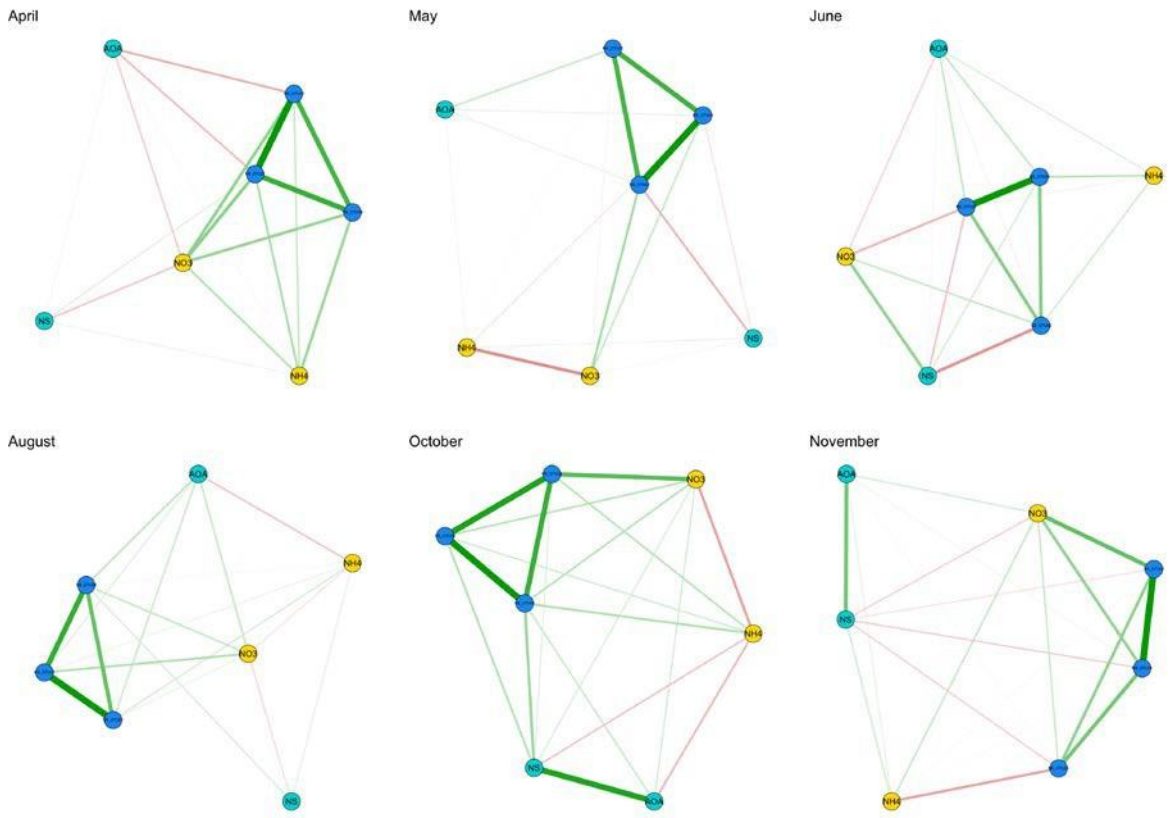

Fig. S 7.3 Network analysis of interactions between NS-assigned OTUs, gene abundances and nitrification-associated nitrogen- pools. Depicted are Pearson correlations between three parameter groups for all sampling dates: gene abundances (light blue circles), *Nitrospira* OTUs 01-03 (dark blue circles) 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).

7.8.2 *Supplementary Tables*

Table S 7.1 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 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
nxrA (NB)	*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 (NS)	*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

Table S 7.2 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	**Date**					
(copies g^{-1} soil dw)	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)	$2.58\ 10^6 \pm 3.54\ 10^6$	$1.68\ 10^6 \pm 2.84\ 10^6$	$1.02\ 10^6 \pm 8.19\ 10^5$	$3.46\ 10^6 \pm 3.25\ 10^6$ ***/***	$3.30\ 10^6 \pm 4.33\ 10^6$	$1.27\ 10^6 \pm 6.41\ 10^5$

Table S 7.3 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

Table S 7.4 *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*	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*	Lebedeva et al., 2008	OTU03
Sublineage VI	*Nitrospira calida*	Lebedeva et al., 2011	OTU01 / OTU02

Table S 7.5 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	

8 Final Conclusions

This thesis characterized spatial and temporal relationships of the soil microbial community, the nitrogen cycling microbial community, and a subset of the nitrogen cycling community with soil abiotic properties and plant growth stages in an unfertilized temperate grassland. Unfertilized perennial grasslands depend solely on soil-available nitrogen and in these environments nitrogen cycling is considered to be both highly efficient and tightly coupled to plant growth. Unfertilized perennial grasslands with high plant diversity, such as ours, have also been shown to have higher soil organic carbon, total nitrogen, and microbial carbon; greater food web complexity; and more complex biological communities than more intensively managed grasslands or croplands. This made the choice of study plot especially well-suited for characterizing the relationships we sought to identify, and made it possible to detect spatial and temporal patterns at a scale that has heretofore been under-examined.

The first study used a combination of abiotic, plant functional group, and PLFA measurements together with spatial statistics to interpret spatial and temporal changes in the microbial community over a season. We found that its overall structure was strongly related to the abiotic environment throughout the sampling period (Table 5.2). The strength of that relationship varied, however, indicating that it was not constant over time and that other factors also influenced microbial community composition. PLFA analysis combined with principal components analysis made it possible to discern changes in abundances and spatial distributions among Gram-positive and Gram-negative bacteria as well as saprotrophic fungi. Modeled variograms and kriged maps of the changes in distributions of exemplary lipids of both bacterial groups also showed distinct differences in their distributions on the plot, especially at stages of most rapid plant growth (Fig. 5.2a-d). Although environmental properties were identified as the main structuring agents of the microbial community, components of those environmental properties varied over the season, suggesting that plant growth stage had an indirect influence, providing evidence of the complexity and dynamic nature of the microbial community in a grassland soil.

The second study took the same analytical approach, this time applying it to abundances of key members of the soil nitrogen cycling community. Marker genes for total archaea and bacteria (16S rRNA), nitrogen fixing bacteria (*nifH*), ammonia oxidizing archaea (*amoA* AOA) and bacteria (*amoA* AOB), and denitrifying bacteria (*nirS*, *nirK* and *nosZ*) were quantified by qPCR. Potential nitrification activity (PNA) and denitrifying enzyme activity (DEA) were also

determined. We found clear seasonal changes in the patterns of abundance of the measured genes and could associate these with changes in substrate availability related to plant growth stages. Most strikingly, we saw that small and ephemeral changes in soil environmental conditions resulted in changes in these microbial communities, while at the same time, process rates of their respective potential enzyme activities remained relatively stable (Figs. 6.1 a&b, 6.2 a&b). This suggests both short term niche-partitioning and functional redundancy within the nitrogen cycling microbial community. The seasonal changes in abundances we observed also provided additional evidence of a dynamic relationship between microorganisms and plants, an important mechanism controlling ecosystem nitrogen cycling.

To determine the relationships among the microbial groups that oxidize ammonia (AOA and AOB) and the nitrite oxidizing bacteria *Nitrospira* and *Nitrobacter* (NOB), and to characterize the interdependence of AOA and NOB in this grassland plot, the third study determined spatial and temporal interactions between AOA, AOB and NOB. These steps are related in both space and time, as the ammonia-oxidizers provide the necessary substrate for nitrite-oxidizers. Using a combination of spatial statistics and phylogenetic analysis, our data indicated seasonally varying patterns of niche differentiation between the two bacterial groups, *Nitrospira* (NS) and *Nitrobacter* (NB) in April (Figs.7.2 A1& B1), but more homogeneous patterns by August (Figs. 7.2 A4 & B4), which may have been due to different strategies for adapting to changes in substrate concentrations resulting from competition with plants. We then asked a further question: was the microbial structure at sampling sites with high NS gene abundances fundamentally different from those with low NS gene abundances? Using a phylogenetic approach, the operational taxonomic unit (OTU) composition of NS was analyzed (Fig. 7.4). Community composition did not change over the first half of the season, but by the second half, the relative proportion of a particular OTU (OTU 03) had increased significantly. This suggested an intraspecific competition within the NS and the possible importance of OTU 03 in nitrite oxidation at a specific period of time. Observed positive correlations between AOA and *Nitrospira* further suggested that in this unfertilized grassland plot, the nitrification process may be predominantly performed by these groups, but is restricted to a limited timeframe (Fig. 7.5).

In conclusion, the results of this suite of studies provided a detailed spatial and temporal characterization of soil microbial communities, including members of the nitrogen cycling community, at the cm to m scale in an unfertilized temperate grassland soil. It is clear that temporal dynamics create unique and often ephemeral conditions that influence soil

biogeography at the studied scale, and that by combining spatial and temporal approaches it was possible to identify influences that thus far have proven elusive. Soil microbial communities are clearly structured by a combination of factors and the relative importance of those factors can change over very short time periods. Clear seasonal changes with concomitant spatial structure at the sampling times most strongly associated with rapid plant growth on the plot provided indirect evidence of linkages between above- and belowground processes. This was seen in all three of the studies.

Because factors shaping microbial communities at one scale may be neither important nor predictive at another, it is important to apply this approach to different scales as well. Finer spatial resolution, such as the mm to cm scale, could provide insight into spatial patterns that may exist at a scale smaller than that of our sampling scheme. A smaller sampling scale would also make it possible to characterize microbial hotspots and hot moments in more detail. The phylogenetic approach could also be profitably applied at smaller scales to learn whether the factors we identified were also operative at those scales. However, by investigating an unfertilized perennial grassland plot, we were able to identify influences that are obscured at larger scales and in more highly managed grassland systems. At this scale, the soil microbial community as a whole, while dynamic, also appeared to be robust and resilient to seasonal changes.

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Curriculum Vitae

Personal Information

Name:	Kathleen Marie Regan
Date of Birth:	September 13, 1949
Place of Birth:	Tiffin, Ohio USA
Address:	289 Barlows Landing Road P O Box 1072 Pocasset, MA 02559 USA

Education

2016	Ph.D. Agricultural Sciences (Doktor der Agrarwissenschaften) 2016. Cum laude. Title: "Linking Microbial Abundance and Function to Understand Nitrogen Cycling in Grassland Soils" December 16, 2016. University of Hohenheim, Institute of Soil Science and Land Evaluation, University of Hohenheim, Stuttgart, D-70593, Germany
2009	M.Sc. Soil Biology, 2009. With distinction. University of Hohenheim, Institute of Soil Science and Land Evaluation, University of Hohenheim, Stuttgart, D-70593, Germany
2002	B.A. Philosophy, 2002. Cum laude. Wellesley College, Wellesley, MA., USA
1979	Assoc.Sc. Applied Research, 1979. Summa cum laude. Ohio State University Agricultural Technical Institute, Wooster, OH., USA

Professional Positions/Employment History

2014-present:	Editor. Provide technical and substantive content and language edits to scientific manuscripts, proposals, presentations, technical manuals, educational materials
2010-2014:	University of Hohenheim, doctoral candidate in microbial ecology of soil
2007-2009:	University of Hohenheim, M.Sc. student
2004-2006:	Tufts University, Medford, MA. Laboratory manager, Department of Civil & Environmental Engineering
2002-2004:	Public Conversations Project, Watertown, MA. Program Coordinator
2000-2002:	Wellesley College, Wellesley, MA. Full-time student
1985-2000:	Ecosystems Center of Marine Biological Laboratory. 1) Research Assistant II; 2) Laboratory Manager; 3) Arctic Long Term Ecological Research (LTER) Project Assistant Coordinator

Publications

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Oral Presentations

Deutsche Bodenkundliche Gesellschaft (German Soil Science Society) Rostock, Germany Sept. 2013: Soil Microbial Communities in grasslands – spatial and temporal patterns at the local scale

Ecological Society of America Minneapolis, MN Aug. 2013: Soil microbial communities in grasslands – above-below ground links and seasonal variation

Deutsche Bodenkundliche Gesellschaft (German Soil Science Society) Berlin, Germany Sept. 2011: Soil microbial communities in grasslands – early results of combined spatial/temporal approach

Deutsche Bodenkundliche Gesellschaft (German Soil Science Society) Frauenchimsee, Germany Sept 2010: Can differences in microbial abundances help explain enhanced N_2O emissions in a permanent grassland under elevated atmospheric CO_2?

Posters

Biodiversity Exploratories, Feb. 2013 Mainz, Germany
International Society for Microbial Ecology (ISME), Copenhagen, Denmark Aug. 2012
European Soil Science Societies, 4th International Congress, Bari, Italy July 2012
Biodiversity Exploratories, Feb. 2012 Mainz, Germany
Biodiversity Exploratories, Feb. 2011 Bad Blankenburg, Germany

Professional Memberships

American Association for the Advancement of Science (AAAS)
Ecological Society of America (ESA)
International Society for Microbial Ecology (ISME)
Soil Science Society of America (SSSA)

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