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INFLUENCE OF LAND USE ON ABUNDANCE, FUNCTION AND
SPATIAL DISTRIBUTION OF N-CYCLING MICROORGANISMS IN
GRASSLAND SOILS

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Heidelberg, am 04.11.2015

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1 SUMMARY

Microorganisms are important players of matter cycling in soils. They perform crucial functions for many nutrients and resources such as carbon (C), nitrogen (N), and phosphorous (P) at the abiotic-biotic interface. Grasslands are diverse ecosystems, which cover approximately 30% of the Earth's land surface. In Germany, grasslands comprise about 30% of the agricultural land, whereby these grasslands are mainly used as meadows and pastures.

Given the diverse range of effects of nitrogen-cycling microorganisms on ecosystem functioning, this thesis focuses on the influence of land use on N-cycling microorganisms in grassland soils. The objective of this thesis was to investigate how different intensities of land use contribute to variations in the abundance, function and spatial distribution of microorganisms in grassland ecosystems involved in the nitrogen cycle (N cycle), but also on soil biogeochemical properties (e.g. pH, mineral N, microbial C and N), as well as on enzyme activities involved in the carbon-, nitrogen-, and phosphorous cycle. The activity and performance of nitrifying and denitrifying microorganisms in soils is known to be controlled by a number of different factors, including biogeochemical properties of the soil, soil type, climate, and soil structure among others. Moreover, anthropogenic activities such as land-use management are likely to have controlling effects on N-cycling microorganisms in grassland soils.

Little is known so far about the interactions between soil biogeochemical properties and land use in grassland soils on the performance and spatial distribution of nitrifying and denitrifying microorganisms. Furthermore, there is still a lack of knowledge of how denitrifying microorganisms in grasslands respond to effects of climate change, thereby contributing to the emission of substantial amounts of the greenhouse gas nitrous oxide (N₂O).

The objective of this thesis was tackled in three studies. All study sites that were investigated as part of this thesis were preselected and assigned according to study region and land use within the framework of the "*Exploratories for Functional Biodiversity Research – The Biodiversity Exploratories*" of the DFG (*Deutsche Forschungsgemeinschaft*) priority program 1374. The basis of understanding the data obtained from these studies resulted from a combination of classical soil biological methods to determine and describe the ecological environment, and modern techniques. These comprise quantitative PCR (*polymerase chain reaction*), enzyme assays using fluorogenic substrates, and gas chromatography measurements to assess potential emissions of nitrous oxide.

In the first two studies, for the first time, geostatistics on replicated grasslands was developed and applied in order to assess spatial relationships of land-use intensity, soil

biogeochemical properties, and the abundance and activity of soil microorganisms in grassland ecosystems.

The first study addressed the question whether land-use intensity influences soil biogeochemical properties, as well as the abundance and spatial distributions of ammonia-oxidizing and denitrifying microorganisms in grasslands of the *Schwäbische Alb*. To address this question, for the first time a geostatistical approach on replicated grassland sites (10 m × 10 m), belonging to either unfertilized pastures (n = 3) or fertilized mown meadows (n = 3), representing low and high land-use intensity, was applied. Results of this study revealed that grassland management, in terms of land-use intensity, changed spatial patterns of both soil biogeochemical properties and N-cycling microorganisms at the plot scale (10 m × 10 m). For soil biogeochemical properties, spatial heterogeneity decreased with higher land-use intensity, but increased for ammonia oxidizers and *nirS*-type denitrifiers. This suggests that other factors, both biotic and abiotic than those measured, are driving the spatial distribution of these microorganisms at the plot scale. Another outcome of the geostatistical analysis indicated spatial coexistence for ammonia oxidizers (*amoA* ammonia-oxidizing archaea and *amoA* ammonia-oxidizing bacteria) and nitrate reducers (*napA* and *narG*), but niche partitioning between *nirK*- and *nirS*-type denitrifiers.

The second study aimed at whether land-use intensity contributes to spatial variation in microbial abundance and function in grassland ecosystems of the *Schwäbische Alb* assigned to either low (unfertilized pastures, n = 3), intermediate (fertilized mown pastures, n = 3), or high (fertilized mown meadows, n = 3) land-use intensity. Geostatistics was applied to address plot-scale (10 m × 10 m) spatial heterogeneity and autocorrelation of soil biogeochemical properties, microbial biomass and enzymes involved in C, N, and P cycle. Geostatistical analyses revealed spatial autocorrelations (*p-Range*) of chemical soil properties located within the maximum sampling distance of the investigated plots (14 m), whereas a greater variation of *p-Ranges* of soil microbiological properties provided evidence of spatial heterogeneity at multiple scales. An expected decrease in small-scale spatial heterogeneity due to fertilizer amendment and mowing practices in high land-use intensity could not be confirmed for microbiological soil properties. Finding smaller spatial autocorrelations for most of the investigated properties indicated increased habitat heterogeneity at smaller scales under high land-use intensity.

In the third study, the effects of warming and drought on the abundance of denitrifier marker genes, the potential denitrification activity and subsequently the N₂O emission potential from three grassland ecosystems distributed across Germany (*Schwäbische Alb*, Hainich, and Schorfheide) was investigated. The degree of land use was defined individually for each grassland site through a land-use index that integrated mowing, grazing and fertilization at the sites over the last three years before sampling of the soil.

It was tested whether the microbial community response on warming and drought depended on soil organic carbon content, water holding capacity or soil acidity to uncover effects of these more static site properties in interaction with grassland land use, the study region and climate change treatment. It was further tested to which extent the N₂O emission potential was influenced by more dynamic properties, e.g. the actual water content, the availability of organic carbon and nitrate or the size of the denitrifier community itself.

Warming effects in grasslands enhanced the potential denitrification performance of denitrifying microorganisms. While differences among the study regions were mainly related to soil chemical and physical properties, the land-use index was a stronger driver for potential denitrification, and grasslands with higher land use also had greater potentials for N₂O emissions. The total bacterial community (16S rRNA gene abundance) did not respond to experimental warming or drought treatments, displaying resilience to minor and short-term effects of climate change. In contrast, the denitrifier community tended to be influenced by the experimental treatments: *nirS* and *nirK*-type denitrifiers were more influenced by drought in combination with land-use index and pH, while the *nosZ* abundance was influenced by the drought treatment. The results indicate that warming and drought affected both, the denitrifying communities and the potential denitrification in grassland soils, but these effects are overruled by study region and site-specific land-use index.

This thesis finally gives novel insights into the performance of N-cycling microorganisms in grassland ecosystems. The spatial distribution of soil biogeochemical properties is strongly dependent on land-use intensity, as in return is the spatial distribution of nitrifying and denitrifying microorganisms and the ecosystem services they perform. Yet, future work will be necessary to fully understand the interrelating factors that make up the ecosystem function and ecosystem service that is provided by N-cycling soil microorganisms at multiple scales.

2 ZUSAMMENFASSUNG

Mikroorganismen nehmen entscheidende Funktionen an der Schnittstelle zwischen der abiotischen und der biotischen Umwelt ein und sind Schlüsselkomponenten der in Böden stattfindenden Stoffkreisläufe. Dabei sind sie am Umsatz vieler wichtiger Ressourcen und Nährstoffe wie beispielsweise dem Kohlenstoff- (C), Stickstoff- (N) und Phosphor- (P) Kreislauf beteiligt. Grünlandflächen sind diverse Ökosysteme, die etwa 30% der weltweiten Landoberfläche bedecken. Innerhalb von Deutschland nehmen Grünländer etwa 30% der landwirtschaftlich genutzten Fläche ein, wobei die Nutzung dieser Flächen hauptsächlich aus Wiesen und Weiden besteht.

Diese Arbeit befasst sich aufgrund ihrer vielfältigen Ökosystem-relevanten Funktionen mit Stickstoff-umsetzenden Mikroorganismen in Grünlandböden. Dabei liegt der Fokus insbesondere darauf, wie sich die Landnutzungsintensität von Grünländern auf die Abundanz, Funktion und räumliche Verteilung dieser Mikroorganismen auswirkt. Darüber hinaus wurde auch der Einfluss der Bewirtschaftungsform von Grünländern auf biogeochemische Bodeneigenschaften (z.B. pH, mineralischer N, mikrobieller C und N) und typische Enzymaktivitäten des C-, N- und P-Kreislaufs untersucht.

Die Aktivität und Leistungsfähigkeit nitrifizierender und denitrifizierender Bodenmikroorganismen hängt von einer Vielzahl verschiedener Faktoren ab und umfasst beispielsweise biogeochemische Bodeneigenschaften, klimatische Bedingungen, sowie Bodenart und Bodenstruktur. Zusätzlich zu diesen Faktoren sind es anthropogene Faktoren wie das Landnutzungsmanagement, welche die Stickstoff umsetzenden Bodenmikroorganismen regulierend beeinflussen. Wie sich das Zusammenwirken von biogeochemischen Bodeneigenschaften und Landnutzungsintensität auf die *Performance* und räumliche Verteilung nitrifizierender und denitrifizierender Bodenmikroorganismen in Grünlandböden auswirkt, ist bis zum heutigen Tag nicht ausreichend geklärt. Darüber hinaus sind Auswirkungen des Klimawandels auf die mikrobielle Denitrifikation in Grünlandböden, die eine wichtige Quelle des Treibhausgases Di-Stickstoff-Monoxid (N_2O) darstellen, nur ungenügend untersucht.

Die Fragestellung dieser Arbeit wurde in drei Studien bearbeitet. Die Einteilung aller untersuchten Flächen (Untersuchungsgebiete, Beurteilung und Einteilung der Landnutzungsintensitäten) geschah im Voraus durch das Gebietsmanagement im Rahmen der „Biodiversitäts-Exploratorien“ des DFG (Deutsche Forschungsgemeinschaft) Schwerpunktprogrammes 1374. Die Datengrundlage dieser Arbeit bildete eine Kombination klassischer bodenbiologischer Untersuchungsmethoden zur Charakterisierung des „Lebensraums Boden“ und modernen Techniken, um die Abundanz und Funktion der Bodenmikroorganismen zu erfassen. Diese modernen Methoden umfassen die quantitative

PCR (*polymerase chain reaction*), den Einsatz fluorogener Substrate in Enzymanalysen und die Messung von N₂O mittels Gaschromatographie. Im Rahmen der ersten beiden Studien wurde erstmals ein geostatistischer Ansatz entwickelt und angewendet, bei dem auf replizierten Untersuchungsflächen die räumlichen Zusammenhänge von Landnutzungsintensität, biogeochemischer Bodeneigenschaften, sowie der Abundanz und Aktivität von Bodenmikroorganismen in Grünland-Ökosystemen untersucht wurde.

Die erste Studie sollte klären, wie sich die Landnutzungsintensität auf biogeochemische Bodeneigenschaften, sowie die Abundanz und räumliche Verteilung von Nitrifizierern und Denitrifizierern in Grünländern der Schwäbischen Alb auswirkt. Erstmals wurde ein geostatistischer Ansatz gewählt, bei dem Grünlandflächen (10 m × 10 m) untersucht wurden, die replizierten Nutzungsintensitäten zugeordnet waren: ungedüngte Weiden (n = 3) sowie gedüngte Mähwiesen (n = 3). Die Ergebnisse dieser Studie zeigen, dass die räumliche Verteilung der biogeochemischen Bodeneigenschaften und die der Stickstoff umsetzenden Mikroorganismen auf der untersuchten Plot-Ebene (10 m × 10 m) durch die Landnutzungsintensität beeinflusst wurden. Die räumliche Heterogenität nahm für die untersuchten Bodenparameter mit zunehmender Landnutzungsintensität ab. Im Gegensatz dazu konnte eine Zunahme der räumlichen Heterogenität bei Ammoniak oxidierenden Mikroorganismen und Denitrifizierern des *nirS*-Typs bei steigender Landnutzungsintensität gezeigt werden. Diese Ergebnisse lassen vermuten, dass innerhalb der Plot-Skala (10 m × 10 m) andere Faktoren (abiotisch und biotisch) als jene, die in dieser Studie erfasst wurden, die räumliche Verteilung dieser Mikroorganismen bedingen. Weiterhin wurde im Rahmen dieser Studie festgestellt, dass Nitrifizierer (*amoA* Ammoniak oxidierende Archaea und *amoA* Ammoniak oxidierende Bakterien) und Nitrat-Reduzierer (*napA* und *narG* Denitrifizierer) koexistieren, während *nirK*- und *nirS*-Denitrifizierer unterschiedliche ökologische Nischen besetzen.

Die zweite Studie befasste sich mit der Frage, ob Landnutzungsintensität zur räumlichen Variabilität mikrobieller Abundanz und Funktion in Grünlandböden der Schwäbischen Alb beiträgt. Der Landnutzung wurde in dieser Studie drei Stufen zugeordnet: niedrig (ungedüngte Weiden, n = 3), mittel (gedüngte Mähweiden, n = 3) und hoch (gedüngte Mähwiesen, n = 3). Wie in der ersten Studie wurde ein geostatistischer Ansatz gewählt, um auf Plot-Ebene (10 m × 10 m) die räumliche Heterogenität und Abhängigkeit von biogeochemische Bodeneigenschaften, mikrobieller Biomasse, sowie von Enzymen des C-, N-, und P-Kreislaufes zu untersuchen. Die geostatistische Analyse der chemischen Bodeneigenschaften ergab räumliche Autokorrelationen (*p-Range*), die innerhalb der maximalen Beprobungsdistanz der Untersuchungsflächen lagen (14 m). Eine größere Variation der *p-Range* Werte lieferte den Beweis für räumliche Heterogenität auf mehreren Ebenen für mikrobiologische Bodenparameter. Nicht bestätigt wurde hingegen die erwartete

Abnahme kleinskaliger räumlicher Heterogenität von mikrobiellen Bodeneigenschaften durch den Einsatz von Dünger und Mahd bei hoher Landnutzungsintensität. Für die meisten untersuchten Kenngrößen wurde eine geringere räumliche Autokorrelation bei hoher Landnutzungsintensität gefunden. Dies lässt den Schluss zu, dass bei hoher Landnutzungsintensität eine erhöhte, kleinskalige Habitat-Heterogenität vorliegt.

In der dritten Studie wurde untersucht, wie sich experimentelle Bodenerwärmung im Frühjahr und Trockenphasen im Sommer auf die Abundanz von Markergenen denitrifizierender Bakterien sowie das N₂O-Emissionspotenzial (potentielle Denitrifikation) in drei Grünlandökosystemen in Deutschland auswirken. Das Studiendesign wurde in replizierten Grünlandflächen mit unterschiedlicher Landnutzung umgesetzt ("Biodiversitäts Exploratorien": *Schwäbische Alb*, *Hainich* und *Schorfheide*). Die Landnutzung wurde für jedes untersuchte Grünland durch einen individuellen Landnutzungsindex beschrieben, der Mahd, Beweidung und Düngung während der letzten drei Jahre vor der Probennahme berücksichtigte. Weiterhin wurde untersucht, ob die von Erwärmung und Dürre induzierten Effekte von eher statischen Standorteigenschaften, wie zum Beispiel dem Gehalt des organischen Kohlenstoffs, der Wasserhaltekapazität des Bodens oder des pH-Wertes in Zusammenhang mit der Landnutzung abhängen. Außerdem wurde in dieser Studie untersucht, in wie weit die potentielle Denitrifikation von mehrheitlich dynamischeren Standorteigenschaften, wie beispielsweise dem aktuellen Wassergehalt, der Verfügbarkeit von organischem Kohlenstoff und Nitrat, oder der Abundanz der Denitrifizierer selbst beeinflusst ist.

Bodenerwärmung der Grünlandflächen steigerte die Aktivität der denitrifizierenden Mikroorganismen und führte zu erhöhter potentieller Denitrifikation. Die Unterschiede zwischen den „Exploratorien“ waren hauptsächlich auf bodenchemischen und bodenphysikalischen Eigenschaften begründet. Folglich war der Landnutzungsindex ein starker bestimmender Faktor für die potentielle Denitrifikation und Flächen mit einem höheren Flächennutzungsindex hatten dementsprechend ein erhöhtes Potential für N₂O-Emissionen. Die mikrobielle Gemeinschaft (16S-rRNA Markergene) pufferte Effekte der experimentellen Erwärmung oder Trockenheit. Im Gegensatz dazu zeigten die denitrifizierenden Bodenmikroorganismen eine Tendenz, auf die experimentellen Behandlungen zu reagieren: Denitrifikanten vom *nirS*- und *nirK*-Typ wurden vermehrt durch die Trockenheit in Kombination mit Landnutzungsindex und pH-Wert beeinflusst, während die *nosZ*-Denitrifizierer Abundanzen eine Reaktion auf die Sommertrockenheit zeigten. Die Ergebnisse dieser Studie zeigen, dass denitrifizierende Mikroorganismen sowohl auf Erwärmung und Dürre reagierten und dass Grünlandböden mit höherem Landnutzungsindex auch ein erhöhtes Potential zu N₂O-Emissionen zeigten als Flächen mit niedrigerem

Nutzungsindex. Der Grad der Ausprägung dieser Effekte war dabei hauptsächlich und der Untersuchungsregion und dem Landnutzungsindex der Grünlandflächen abhängig.

Diese Arbeit erweitert die Kenntnisse über die Funktion und Rolle von Stickstoff umsetzenden Mikroorganismen in Grünland-Ökosystemen. Die räumliche Verteilung von biogeochemischen Bodenparametern wird von der Landnutzungsintensität beeinflusst, was sich wiederum auf die räumliche Verteilung der Nitrifizierer und Denitrifizierer sowie deren Ökosystemleistungen auswirkt. Um die vielfältigen und vielschichtigen Ökosystemfunktionen, die von Stickstoff umsetzenden Bodenmikroorganismen bewältigt werden auf allen Ebenen zu verstehen, sind jedoch weitere Untersuchungen notwendig, um beispielsweise die zeitliche Variabilität dieser Funktionen besser verstehen zu können

3 INTRODUCTION

3.1 SPATIAL HETEROGENEITY OF SOILS AND LAND-USE MANAGEMENT

Soil microorganisms are essential for ecosystem functioning and key drivers for the nutrient cycling in soils (Millenium Ecosystem Assessment, 2005; van der Heijden *et al.*, 2008). For example, they are main actors of the carbon and nitrogen cycle (e.g. Tiedje *et al.*, 1989; Hogberg *et al.*, 2001). Besides, they are involved in processes such as soil generation and fertility, detoxification and waste decomposition, as well as water purification (Kremen, 2005). Soils represent a particularly heterogeneous habitat (e.g. Brussaard, 1997; Ettema & Wardle, 2002; Nannipieri *et al.*, 2003; van der Heijden *et al.*, 2008), and multiple factors are assumed to control spatial patterns of microorganisms and their functions.

The challenge of understanding the function of soil microorganisms is to evaluate the factors that are likely regulating their activity. These factors comprise e.g. land-use management, biological interactions, soil physicochemical properties, soil structure, and other abiotic factors such as climate, which can be in addition spatially and temporally heterogeneous (Ettema & Wardle, 2002). Spatial heterogeneity in soils has been determined at various scales, ranging from the millimeter and particle size range, to the centimeter and meter scale of plot-size areas, to scales as large as the landscape and regional level. Controlling factors of spatial heterogeneity may vary between these scales, and can also be interlacing. While the spatial dependence of biogeochemical soil properties is well known at different hierarchical levels, the spatial dependence of microbial communities performing nutrient cycling (processing and recovery) in soils has not yet been sufficiently clarified.

Processing and recovery of nutrients is one essential function of microbial activity in soils. This functional activity requires a diverse number of extracellular enzymes, making them a suitable estimator of microbial activity and diversity (Caldwell, 2005; Sinsabaugh *et al.*, 2008). Due to the substrate specificity of soil enzymes, measuring potential activities of enzymes involved in nutrient cycling in soils can give insight into conversion processes of organic and inorganic compounds. The disposability of these compounds is dependent on land-use management, which in return determines the spatial distribution of soil microorganisms. Extracellular soil enzyme activity can be linked to wide range of characters, such as soil biogeochemical properties (Amador *et al.*, 1997) or community structure (Waldrop *et al.*, 2000), and the scale of spatial resolution herein ranges from landscape scale (Decker *et al.*, 1999; Gallo *et al.*, 2004; Waldrop *et al.*, 2004) to the size of soil particle fractions (Kandeler *et al.*, 1999). Using fluorogenic substrates facilitates the identification of functional microbial diversity in soils (Marx *et al.*, 2001). For example, Tscherko *et al.* (2004) used this promising technique to assess the soil microbial activity in primary succession of alpine ecosystems. Many studies investigated the spatial dependence of chemical-physical

soil properties at scales ranging from < 1m to several hundreds of meters, and even to the landscape scale (Ettema & Wardle, 2002; Schöning *et al.*, 2006; Wang *et al.*, 2009), but the spatial dependence of functional microbial communities is less clear (Saetre & Bååth, 2000). Spatial analysis of microbial habitat characteristics and soil microbial communities is well established in distinct microhabitats (e.g. rhizo- and detritosphere), and data about the factors responsible for determination of the patterns of soil microbial properties and bacterial community structure in grasslands are diverse. For example, Ritz and colleagues (2004) reported that land-use intensity, namely fertilizer application, affected soil properties and therewith plant species composition and the spatial distribution of microbial communities in grazed grasslands. By contrast, Kennedy *et al.* (2004) identified chemical soil properties to be more important for bacterial community structure than plant rhizosphere effects in natural, unimproved, and fertilized grassland ecosystems. Wallenius and colleagues (2011) investigated effects of land use on soil enzyme activities and bacterial communities involved in carbon, phosphorous, and the sulphur cycle, including investigations at the plot scale and horizontal spatial structure. Bissett *et al.* (2011) investigated the effect of land use on soil microbial communities and their function, focusing on bacteria, archaea, and fungi in agricultural and grasslands. Like others (Lauber *et al.*, 2008; Drenovsky *et al.*, 2010), these studies provided evidence that changes in land use influence the biogeography of soil microorganisms.

The spatial distribution of nitrifying and denitrifying microorganisms has been investigated at scales ranging from millimeters (Grundmann & Debouzie, 2000) to the landscape level (Bru *et al.*, 2011). Patra *et al.* (2005) showed that sheep grazing affected N-fixing, nitrifying, and denitrifying microorganisms in grassland soils, inducing changes in size, composition, and structure of these communities. In cattle pastures, Philippot and colleagues (2009) demonstrated that the intensity of cattle grazing, together with soil properties, strongly influenced spatial patterns of both relative abundance and activities of denitrifying bacteria.

Despite this, information is still limited to date about how land-use intensity of grasslands influences the spatial distribution of microbial communities involved in N-cycling. Intensification of land use was shown to cause changes in plant diversity, nutrient status, and microbial community structure in native grasslands (Steenwerth *et al.*, 2006). The importance of soil properties as key criterion for the performance of soil microorganisms was demonstrated by Enwall *et al.* (2010), who investigated the spatial patterns of community structure, size, and activity of denitrifying communities in integrated and organic crop production systems. They observed habitat selection between *nirK*- and *nirS*-type denitrifiers, with copper being a strong driver of the abundance of *nirK*-type denitrifiers, while nitrate and clay content determined the *nirS* denitrifier community structure. As a result, niche differentiation to avoid competitive exclusion between denitrifiers having the two types of

nitrite reductases was suggested. Despite these approaches, information about whether different land-use intensities of a single ecosystem type (*i.e.* grasslands) influence the spatial structure of microbial properties (e.g. abundance and activity) is still elusive. This provides possibilities to a closer investigation of this important ecosystem type. For a better approximation of microbial parameters (abundances) and soil biogeochemical properties in terms of geostatistical analyses, all data acquired in the first two studies presented in this thesis were expressed on an area basis (m^{-2} and 10 cm soil depth), which was proposed by e.g. Bolton *et al.* (1993) and Doran & Parkin, 1996. These studies are unique concerning geostatistical analyses of microbial abundances, function, and soil biogeochemical properties on replicated grassland sites, taking into account the microbial habitat by expressing the data at an area basis.

3.2 MICROBIAL NITROGEN CYCLE IN SOILS

Soils are the major interface for nitrogen transformations in the N-cycle, having strong interactions with the biosphere and the atmosphere. An overview of the N-cycle and processes influenced by microbial activity is presented in Figure 1. Biological N-fixation through microbes is the prominent way of molecular N_2 to enter the biogeochemical N-cycle (Orme-Johnson, 1985; Halbleib & Ludden, 2000), providing some estimated 90-150 Tg N year^{-1} to global ecosystems (Galloway, 1998). The anaerobic reduction of molecular N_2 to ammonium (NH_4^+) is catalyzed by a nitrogenase protein complex, and highly conserved *nifH* genes are common target molecules to assess N-fixing communities in soils (Zehr & Turner, 2001; Zehr *et al.*, 2003; Raymond *et al.*, 2004). *Vice versa*, organic bound N is mineralized to NH_4^+ in the process of ammonification (Hart *et al.*, 1994; Schimel & Bennett, 2004). As important parts of the N-cycle, microbial nitrification and denitrification can be responsible for N-losses through nitrate (NO_3^-) leaching or greenhouse gas emissions in the form of nitrous oxide (N_2O) (Philippot *et al.*, 2007). During nitrification, NH_4^+ is first aerobically oxidized to nitrite (NO_2^-) by the enzyme ammonia-mono-oxidase (Amo), which can be produced by both, ammonia-oxidizing archaea (AOA) or *amoA* ammonia-oxidizing bacteria (AOB) genes having the responsible *amoA* functional gene, respectively (Rotthauwe *et al.*, 1997; Treusch *et al.*, 2005).

Denitrification is a facultative respiratory pathway, which is widely spread among prokaryotes. Under oxygen limiting conditions, microorganisms can successively reduce nitrate (NO_3^-) to nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O), and finally to molecular N_2 , thus effectively closing the nitrogen cycle.

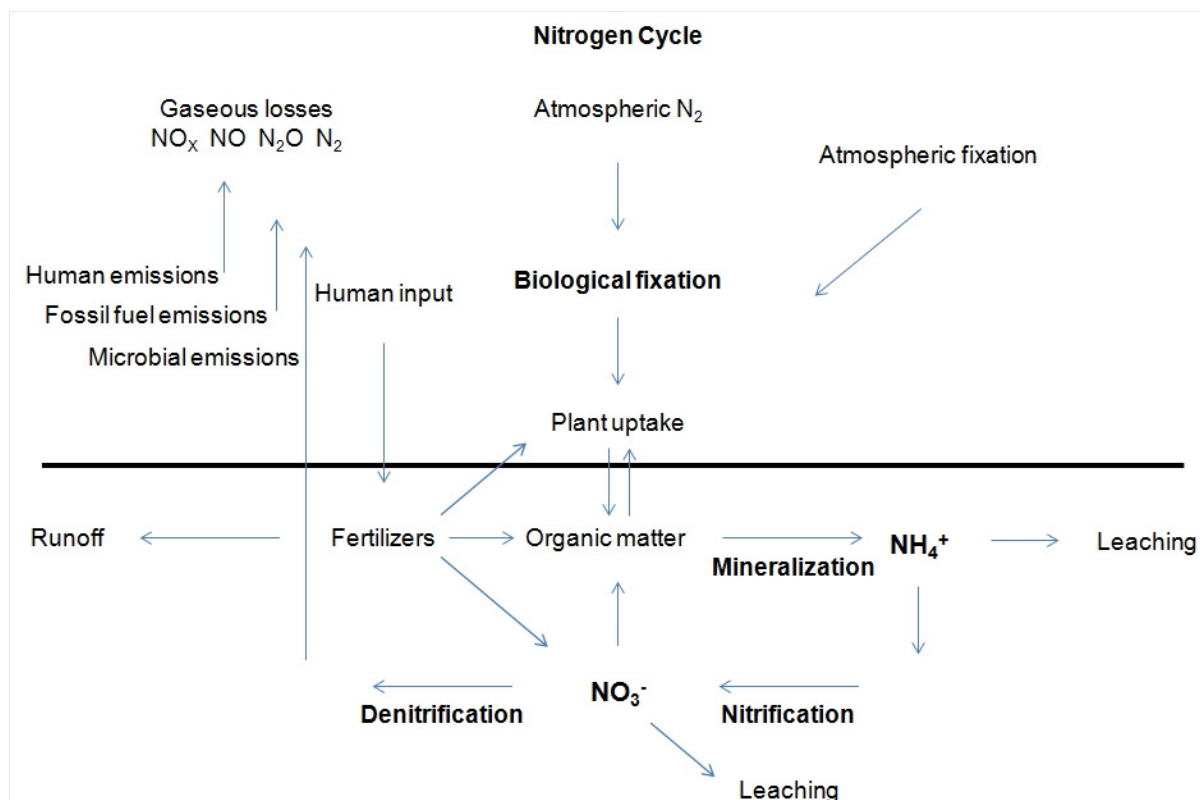


FIGURE 1: Schematic overview of the N cycle with focus on components influenced by microorganisms in bold. (modified after Cavigelli *et al.*, 1998; Jetten, 2008).

The reduction of NO_3^- to NO_2^- is catalyzed by either one of the nitrate reductases, the periplasmatic encoded by *napA*, or the membrane bound encoded by *narG* genes. Microorganisms can either harbor the *nap* or the *nar* genes, or both nitrate reductase genes. The reduction of NO_2^- to gaseous NO is the defining step of denitrification, thus distinguishing denitrifiers from other nitrate respiring microorganisms (Jones *et al.*, 2008). This reaction is catalyzed by either a multi copper-dependent *nirK* or a *nirS* nitrite reductase containing a cytochrome *cd₁* active site. To date, no microorganism has been identified having both of the nitrite reductases at a time, although functional redundancy of the two enzymes has been demonstrated by Glockner *et al.* (1993). Nitric oxide is further reduced to N_2O by the nitric oxide reductases *cNor* or *qNor* (Zumft, 1997). The reduction of nitrous oxide, a potent greenhouse gas, to N_2 gas effectively closes the nitrogen cycle. This reaction is exclusively catalyzed by the nitrous oxide reductase, *Nos*. N_2O is finally reduced to N_2 by the nitrous oxide reductase encoded by *nosZ*.

Functional marker genes of denitrification are usually induced and expressed under anaerobic conditions, and even low O_2 concentrations inhibit denitrification efficiency, potentially increasing the production of NO and N_2O as products of denitrification. In addition, some denitrifying microorganisms are lacking the *nosZ* gene, expressing a truncated

denitrification enzyme apparatus, thus producing N_2O as final product (Zumft, 1997; Philippot, 2002). The end product of complete denitrification, N_2 , then forms the major part of the atmosphere. Relating functional gene abundances to the enzymes they encode for, can give important information on the actual performance of these microorganisms.

In a review by Torsvik & Øvreås (2002), the importance, difficulties and biases of linking microbial gene diversity in soils to ecosystem functions becomes clear. Therefore, it is further necessary to correlate gene abundances and subsequent enzyme data to other biogeochemical properties, soil structure, plant diversity, climate data, and spatial heterogeneity and many in order to complete our knowledge about the functional diversity of N-cycling soil microorganisms. Furthermore, N_2O as intermediate or end product of denitrification has strong implications for atmospheric trace gas emissions contributing to climate change.

3.3 THE PERFORMANCE OF N-CYCLING SOIL MICROORGANISMS IN THE LIGHT OF CLIMATE CHANGE

One important ecosystem function with global relevance is the production and consumption of greenhouse gases from soils by soil microorganisms. Until now however, there is still a lack in understanding the factors controlling microbial ecosystem functions in soils (Mooney *et al.*, 2009; Gärdenäs *et al.*, 2011). Microbial community composition and activity is sensitive to altered environmental conditions through climate change (Bardgett *et al.*, 2008; Sheik *et al.*, 2011), and positive feedback mechanisms of increased temperatures on microbial activity has been reported by e.g. Heimann and Reichenstein (2008) and other groups. Microbial processes within the N-cycle are thereby likely respond to climate change (Vitousek *et al.*, 1997).

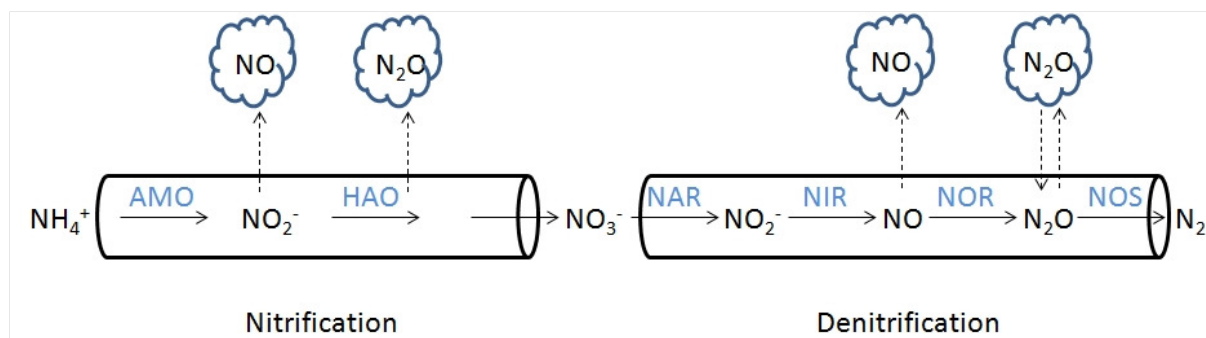


FIGURE 2: Schematic of nitrogen transformations in nitrification and denitrification and potential gaseous N-losses. (AMO = Ammonium monooxygenase, HAO = Hydroxylamine oxidoreductase, NAR = Nitrate reductase, NIR = Nitrite reductase, NOR = Nitric oxide reductase, NOS = Nitrous oxide reductase) (modified after Davidson *et al.*, 1991).

Microbial nitrification and denitrification in soils are the two key processes responsible for the production of nitrous oxide (N_2O) (Figure 2) (e.g. Firestone *et al.*, 1980; Conrad, 1996; Bremner, 1997; Barnard *et al.*, 2005). Apart from carbon dioxide (CO_2) and methane (CH_4), N_2O is one of the most noteworthy greenhouse gases contributing to climate change, as well as to the depletion of the ozone layer, having a global warming potential about 300 times higher compared to that of CO_2 (Forster *et al.*, 2007; Ravishankara *et al.*, 2009). In this context, microbial denitrification is of primary interest, as it is considered to be the main contributor to N_2O emissions from soils (Baggs & Bateman, 2005). In complete microbial denitrification, molecular N_2 is finally released into the atmosphere, effectively closing the N-cycle (Knowles, 1982).

Functional genes of denitrification are usually induced and expressed under anaerobic conditions, and even low O_2 concentrations inhibit denitrification efficiency, potentially increasing the production of NO and N_2O as products of denitrification (Zumft, 1997; Bollmann & Conrad, 1998). In addition, some denitrifying microorganisms are lacking the *nosZ* gene, expressing a truncated denitrification enzyme apparatus, thus producing N_2O as final product (Philippot, 2002). Also, Philippot *et al.* (2011) revealed a causal relationship of denitrifiers lacking the *nosZ* gene and potential N_2O emissions, demonstrating the importance of microorganisms involved in N-cycling and their contribution to N_2O production.

Other determinants driving whether or not N_2O is emitted or further reduced to N_2 are known to depend on soil physical and chemical properties in soil, such as temperature, soil water content, oxygenation status, mineral N content, carbon supply, pH, and plant diversity (e.g. Smith *et al.*, 2003; Niklaus *et al.*, 2006; Jones *et al.*, 2007; Cuhel *et al.*, 2010). Microbial denitrification was furthermore shown to be dependent on land management (Flechard *et al.*, 2007). Barnard and colleagues (2005) identified grasslands to represent a substantial source for terrestrial N_2O emissions. Regarding grassland ecosystems, there are indications that different types of land-use management (e.g. fertilizer application, mowing practices, grazing)

contribute in variations of N₂O fluxes (Mosier *et al.*, 1991; Velthof *et al.*, 1996; Oenema *et al.*, 1997).

For example, Hartmann *et al.* (2013) investigated effects of summer drought and N deposition by cattle in two grasslands differing in climate and management. They found that N-cycling and related potential N₂O emissions in these grasslands were influenced by complex interacting factors, including the drought, nitrogen supply, pH and the uptake of nutrients by plants. They concluded, that the spatial heterogeneity of the investigated pastures was an important regulator that needs to be considered when investigating effects of drought and nitrogen inputs on N-cycling and related N₂O emissions in grazed grasslands.

The importance of biological controls such as the plant diversity on potential denitrifying enzyme activities was also shown in study by Le Roux *et al.* (2013). They demonstrated that denitrifying enzyme activities in temperate grassland were more influenced by the plant species community composition (e.g. species richness, legume/non-legume) than by the denitrifier abundance. In contrast, the nitrifying enzyme activity was more depending on the abundance of ammonia oxidizing nitrifying soil microorganisms.

The results of a study manipulating the soil microbial community by Philippot *et al.* (2013) indicate that altering both, the size of the microbial community and the availability of resources may alter the potential denitrification in soils. The loss of soil microbial diversity in soils affects nitrogen cycling, and functional redundancy of soil microorganisms may be overstated in certain cases. Consequently, potential denitrification in soils depends on various biotic and abiotic factors, which are not always mitigated by the diverse functional abundance of soil microorganisms. Recently, Jurburg & Salles (2015) also discussed that the buffering capacity of the soil microbial community may be over-estimated, and that changes in biodiversity and climate contribute to alterations of the soil microbial community and the ecosystem services they provide.

Beside land-use type, the influence of climate change, with expected changes of temperature and precipitation and consequently the soil water content is likely to control N turnover processes in grassland soil (Vitousek *et al.*, 1997; Melillo *et al.*, 2002; Singh *et al.*, 2010). Both soil temperature and soil water content were shown to affect soil microbial activity and soil aeration status, thus directly controlling conditions for the emission of gaseous compounds from soils e.g. (Skiba & Smith, 2000; Dobbie & Smith, 2001; Horváth *et al.*, 2010). Schaufler *et al.* (2010) studied greenhouse gas emissions from four different soils at different SWC and rising temperatures in the laboratory and found that N₂O emissions were better correlated with high soil water content (ranging from 60% to 80% water filled pore space) than to temperature. Barnard and colleagues (2005) reviewed relationships of global change, nitrification, and denitrification, pointing that only limited knowledge about interactions of climate change factors (elevated CO₂, N addition, warming) and denitrification

is available. In a two year experiment, Cantarel *et al.* (2011) investigated major drivers of climate change (warming, summer drought, and elevated CO₂) in extensively managed grassland. They concluded that N₂O responses to climate change mainly depended on temperature effects.

The importance of understanding the factors controlling nitrous oxide emissions from soils was also highlighted in a review by Butterbach-Bahl *et al.* (2013): They outlined the importance of a better understanding of the composition and diversity of soil microbial communities across a variety of soils in different climates and under different land use, as well as plant - microbe interactions in the rhizosphere, which may provide a key to better understand the variability of N₂O fluxes at the soil - atmosphere interface. They concluded, that although there is an increasing amount of literature reporting N₂O gas exchange data from both, field measurements and laboratory incubation experiments, there is still a lack of understanding N₂O budgets at larger regional or even at a global scale. In the light of spatially and temporally variables that influence N₂O emissions from soils, the factor of modeling approaches needs to become a major focus for further research in the field, even more that these processes are in addition increasingly altered by climate change.

Altogether, the abundance, function spatial distribution of N-cycling microorganisms in grassland soils depends on multiple interacting abiotic and biotic factors, which are variable at multiple spatial and temporal scales. The results of this thesis add further details to a better understanding of the processes involved, but further research will still be necessary to gain a full insight in all of these complex processes.

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5 OBJECTIVE OF THE THESIS

This thesis applies three studies to assess the role of grassland land-use intensity on (i) the abundance, activity, and spatial distribution of microorganisms involved in the C- and N-cycle, and (ii) the abundance and performance of denitrifying microorganisms in answer to implications of climate change scenarios.

The first study addressed the influence of land-use intensity on the spatial distribution of soil biogeochemical characteristics, as well as the spatial distribution of nitrifying and denitrifying microorganisms. So far, most studies applied single-site investigations per treatment. We therefore developed a geostatistical approach investigating replicated grassland sites, belonging to either low or high land-use intensity in the *Schwäbische Alb*. Current research states that management practices such as fertilizer application, mowing practices, and grazing influences soil biogeochemical properties, subsequently influencing soil microbial performance. We therefore hypothesized that high land-use intensity results in reduced spatial heterogeneity of soil biogeochemical properties and subsequently reduced spatial heterogeneity of soil microorganisms in comparison with grasslands assigned to low land-use intensity. We assessed functional genes involved in N-cycling and soil properties as a function of land-use intensity. For data analyses, we developed a linear mixed effect model with geostatistical covariance structure. Ordinary kriging was used to map spatial distributions of ammonia-oxidizing and denitrifying communities of the study sites.

The second study was used to provide a functional link between the spatial distribution of soil biogeochemical properties and extracellular enzyme activities as a measure of microbial activity potentials in soils. Selected grassland sites were located in the *Schwäbische Alb*, belonging to low, intermediate, and high land-use intensity. Several soil physical and chemical properties known to regulate microbial activity were assessed. Soil microbial properties were reflected by microbial biomass and enzymes involved in C-, N-, and P-cycling. We hypothesized that grasslands belonging to high land-use intensity reveal management intensification by decreased spatial heterogeneity of microbial processes and decreased soil habitat diversity in comparison to grassland sites of low land-use intensity. Again, a geostatistical approach on replicated sites was used to analyze the spatial parameters determined in this study. Soils form a highly structured and heterogeneous environment for microbial functioning. We therefore expressed our geostatistical model data obtained from the two studies mentioned above on two bases for interpretation: Classically, data was expressed per gram dry soil, mainly for a better comparison of the data with other studies. Additionally, we included bulk density into the model calculation, which allowed a more realistic estimation of both soil biogeochemical properties and gene abundances at an area basis (m^{-2} and 10 cm soil depth).

Climate change has strong implications on microbial performance in terrestrial ecosystems. Another set of hypotheses therefore addressed to potential nitrous oxide (N₂O) emissions from denitrification in grassland soils under different land use-types. The experimental setup targeted the following hypotheses: (i) Increasing soil temperatures in spring stimulate microbial activity, resulting in increased potential greenhouse gas emissions, while (ii) reducing precipitation in summer results in lowered soil water content and subsequent better aeration of the soils, thus reducing the potential of N₂O production in these treatments. Furthermore (iii), sites belonging to high land-use types were attributed having a greater potential of producing nitrous oxide gas under beneficial conditions through more abundant and more easily available C- and N sources than sites at medium and low land-use types.

6 INFLUENCE OF LAND-USE INTENSITY ON SPATIAL DISTRIBUTION OF N-CYCLING MICROORGANISMS IN GRASSLAND SOILS

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Abstract

A geostatistical approach using replicated grassland sites (10 m × 10 m) was applied to investigate the influence of grassland management, *i.e.* unfertilized pastures and fertilized mown meadows representing low and high land-use intensity (LUI), on soil biogeochemical properties and spatial distributions of ammonia-oxidizing and denitrifying microorganisms in soil. Spatial autocorrelations of the different N-cycling communities ranged between 1.4 and 7.6 m for ammonia oxidizers and from 0.3 m for *nosZ*-type denitrifiers to scales > 14m for *nirK*-type denitrifiers. The spatial heterogeneity of ammonia oxidizers and *nirS*-type denitrifiers increased in high LUI, but decreased for biogeochemical properties, suggesting that biotic and/or abiotic factors other than those measured are driving the spatial distribution of these microorganisms at the plot scale. Furthermore, ammonia oxidizers (*amoA* ammonia-oxidizing archaea and *amoA* ammonia-oxidizing bacteria) and nitrate reducers (*napA* and *narG*) showed spatial coexistence, whereas niche partitioning was found between *nirK*- and *nirS*-type denitrifiers. Together, our results indicate that spatial analysis is a useful tool to characterize the distribution of different functional microbial guilds with respect to soil biogeochemical properties and land-use management. In addition, spatial analyses allowed us to identify distinct distribution ranges indicating the coexistence or niche partitioning of N-cycling communities in grassland soil.

Keywords

Grassland, land-use intensity, ammonia oxidizers, denitrifiers, geostatistics

Running title

Spatial distribution of N-cycling soil microorganisms

7 LAND-USE INTENSITY MODIFIES SPATIAL DISTRIBUTION AND FUNCTION OF SOIL MICROORGANISMS IN GRASSLANDS

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Abstract

The aim of the present study was to investigate whether land-use intensity (LUI) contributes to spatial variation in microbial abundance and function in grassland ecosystems. At one time point, three sites at low (unfertilized pastures), at intermediate (fertilized mown pastures) and at high (fertilized mown meadows) LUIs were selected in southern Germany. Within each of these nine grassland sites, 54 soil samples (0 – 10 cm) were taken in a 10 × 10 m area in spring. Plot-scale spatial dependence and autocorrelation of soil biogeochemical properties, microbial biomass and enzymes involved in C-, N- and P-cycling were analyzed. Applying geostatistics (exponential or spherical model) revealed that most chemical and microbiological properties showed at least a moderate spatial autocorrelation. Chemical soil properties (e.g. C_{org} , N_t , pH) were characterized by practical ranges (pRange) of between 1 and 14 m, whereas soil microbiological properties showed a greater variation of pRanges, providing evidence of spatial heterogeneity at multiple scales. The expected decrease in small-scale spatial heterogeneity in high LUI could not be confirmed for microbiological soil properties, because sampling in early spring might have reduced the influence of growing plants and fertilization. However, microbial biomass carbon was significantly greater in high LUIs, indicating that the benefit to soil microbial populations from the long-term increase in substrate and nutrient availability in fertilized grasslands is independent from factors affecting spatial structures in the short-term.

Keywords Soil enzyme activities; Microbial biomass; Spatial distribution; Land use; Grassland; Geostatistics

Running title

Spatial patterns of microorganisms in grasslands

Introduction

A central goal in both microbial ecology and biogeography is to quantify factors explaining variation in microbial abundance and diversity in different ecosystems (Ramette and Tiedje, 2007). In comparison to aquatic systems, soils are particularly heterogeneous, and multiple environmental factors are assumed to control spatial patterns of microorganisms and their functions. Soil heterogeneity results from the interaction of a hierarchical series of interrelated variables that fluctuate at many different spatial and temporal scales (Ettema and Wardle, 2002). Although spatial dependence of chemical and physical soil properties is well known at scales ranging from decimeters to several hundred meters (e.g. Schöning et al., 2006; Don et al., 2007; Wang et al., 2009), the spatial dependence of microbial communities is less clear (Saetre and Bååth, 2000).

For example, multi-scale comparisons have found evidence for nested scales of spatial structure: microbial biomass and activity in agricultural and shrub-steppe ecosystems were spatially dependent at scales < 1 m, nested within variations at the landscape scale (Ettema and Wardle, 2002). Other studies comparing microbial communities in forest and agricultural sites provide evidence that land-use changes influence the biogeography of soil microorganisms (Lauber et al., 2008; Drenovsky et al., 2010; Bru et al., 2011, Wallenius et al., 2011). Lauber et al. (2008) concluded, from a study of arable, grassland, and forest sites, that specific changes in soil properties, and not necessarily land-use type itself, determined the microbial community structure across a given landscape.

Information about whether different land-use intensities of a single ecosystem type influence the spatial structure of microbial properties (e.g. abundance and activity) is still elusive. It has been shown that land-use intensification of native grasslands change plant diversity, status of nutrients, and consequently microbial community structure (Steenwerth et al., 2006). In raised peat bogs for example, land-use intensification changed microbial respiration and microbial biomass C (Brake et al., 1999). The composition of the plant community was determined as a factor that influences the spatial patterns of soil microbial properties in temperate upland grassland at scales from one to a few meters (Ritz et al., 2004). In contrast, Kennedy et al. (2004) found in natural, unimproved, and fertilized grassland ecosystems that chemical soil properties (soil lime and nitrogen status) were more important controls on bacterial community structure than were plant rhizosphere effects.

Previous studies of land-use intensity focused mainly on physicochemical characteristics (Murphy et al., 2006) and general characteristics of soil microbiota (community composition, biomass, and respiration activities) (Brake et al., 1999; Lauber et al., 2008). However, the functioning of microorganisms in terrestrial ecosystems relies mainly on the activity of extracellular enzymes (Caldwell, 2005), which makes them a good estimator of microbial

decomposition activity and functional diversity (Sinsabaugh et al., 2002). The application of fluorogenic substrates (Marx et al., 2001) offers a high throughput method to identify the functional diversity of microorganisms in soils under different land-use intensities and some studies have already applied this technique to grassland soils (Mayr et al., 1999; Tscherko et al., 2004). In addition, classical standardized methods are available to follow the degradation of organic compounds by soil enzymes (Schinner et al., 1996).

The aim of the present study was to clarify whether grassland land-use intensity influences the spatial distribution of soil microorganisms and their functions. We selected grassland sites of low (unfertilized pastures), intermediate (fertilized mown pastures), and high (fertilized mown meadows) land-use intensities (LUIs) to assess the influence of land-use intensity on spatial patterns of soil microbial biomass, enzymes involved in C-, N- and P-cycling, and several soil physical and chemical properties (e.g., bulk density, pH, soil organic carbon and mineral N content), which are known to regulate microbial activity.

We assume that the heterogeneity of the soil and thus the diversity of microbial habitats is altered under long-term differences in grasslands' fertilizer inputs and mowing practices. We hypothesized that in grasslands with high LUI, a higher and more homogenous nutrient input (i.e. high LUI = mechanical fertilization versus low LUI = patchy fertilization by animal dung) and hence reduced plant diversity will decrease the spatial heterogeneity of microbial processes by decreasing soil habitat diversity in comparison to low LUI grasslands. For the first time, we used a geostatistical approach with replicated sites ($n = 3$) comprising three LUIs, which allowed us a sound geostatistical analysis of the spatial parameters determined.

Materials and Methods

Sampling sites

The sites investigated in the present study form part of the interdisciplinary project of the German Biodiversity Exploratories (see: www.biodiversity-exploratories.de). An overview of the German Biodiversity Exploratories is given by Fischer et al. (2010a, b). The sites in this study represent continuously managed grassland ecosystems and are located in the biosphere area Schwäbische Alb, a limestone middle mountain range in southwest Germany. The area is characterized by an average annual precipitation of 800-930 mm a^{-1} and an average mean annual temperature of 6-8 °C. The nine investigated grassland sites consist of three unfertilized pastures, classified as low land-use intensity (low LUI, labeled AEG 7 - 9), three fertilized mown pastures (intermediate LUI, labeled AEG 4 - 6) and three fertilized mown meadows (high LUI, labeled AEG 1-3). An overview of the sites (LUI, notation, location and soil type) is shown in Table 1.

Table 1

Plot description: land-use intensity (LUI), land-use type and management, Plot IDs, altitude and coordinates of the nine investigated grassland sites.

LUI	Land-use type and management	Plot ID	Altitude (m a.s.l.)	Latitude	Longitude
Low	Pasture, Unfertilized, Sheep	AEG 7	795 m	48°23'29"	9°22'37"
		AEG 8	760 m	48°25'22"	9°29'32"
		AEG 9	745 m	48°23'41"	9°30'10"
Intermediate	Mown pasture, Fertilized, Cattle, horse	AEG 4	660 m	48°22'51"	9°25'08"
		AEG 5	715 m	48°23'45"	9°26'21"
		AEG 6	710 m	48°24'05"	9°26'30"
High	Mown meadow, Fertilized, Mown 2 or 3 times	AEG 1	690 m	48°23'53"	9°20'31"
		AEG 2	750 m	48°22'37"	9°28'22"
		AEG 3	810 m	48°24'32"	9°31'57"

Soil sampling

Soil sampling was conducted in a 10 × 10 m² area within each of the nine grassland sites according to the method of Ritz et al. (2004) and Schöning et al. (2006). Within this area, nine points were marked to form a central grid with 2.5 m between each point (Fig. 1). Starting from every grid point, a transect with five subsequent steps was constructed, resulting in 54 samples per grassland site (Keil et al., 2011). The transect consisted of incrementally decreasing distances (1.5 m, 1 m, 0.5 m, 0.25 m and 0.125 m) moving at a random angle at each step.

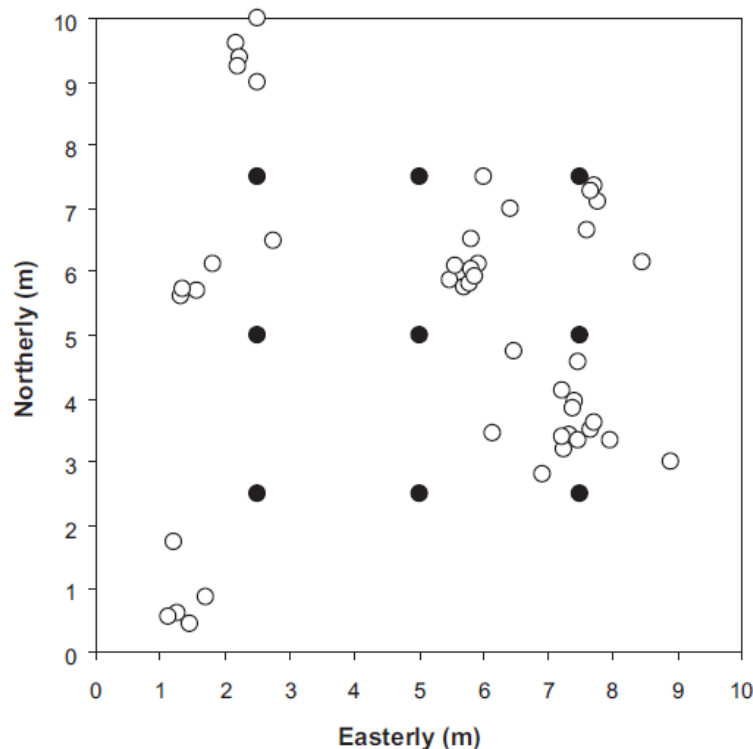


Fig. 1. Sampling design showing location of sampling points for site AEG 2, filled circles represent the nine grid points. Starting from every grid point a transect with five subsequent steps moving at a random angle at incrementally increasing distances was laid.

Using a core auger, two intact soil samples of 58 mm diameter and 100 mm depth were taken at each sampling point. Aboveground plant material and the top 1 cm of the soil, which consisted mostly of plant roots and litter, were excluded. Soil cores were transported under cooled conditions and frozen at -24 °C immediately after returning to the laboratory. One of the two soil cores per sampling point was subsequently dried (105°C for three days) and used for bulk density

determination; the other was used for physical, chemical and microbiological analyses. For this, the soil was sieved (< 5 mm) to exclude roots, stones and soil macrofauna. Field sampling of all nine grasslands was carried out within one week in the middle of April 2008. Since previous studies had shown that the magnitude of differences in soil biological

properties was seasonally consistent, measures of microbiological properties were made at one sampling date only (Kandeler and Eder, 1993; Bardgett and McAlister, 1999).

Physical and chemical soil properties

Soil pH was determined with a glass electrode in the supernatant of a soil suspension using 1:2.5 mixtures of soil and 0.01 M CaCl_2 . Soil water content (SWC) was determined gravimetrically for each sample after drying at 105 °C for one day. Soil organic C (C_{org}) and total N (N_t) contents were measured with an elemental analyzer (Leco C/N 2000, Leco Corporation, St. Joseph, USA). Nitrate (NO_3^-) and ammonium (NH_4^+) were extracted with 1 M KCl from the fresh soil by using a soil to extractant ratio (w/v) of 1 to 4. Soil suspensions were shaken on a horizontal shaker (30 min at 250 rpm) then centrifuged (30 min at 4400 g). Concentrations of NO_3^- and NH_4^+ in the supernatant were measured on an autoanalyzer (Bran & Luebbe, Norderstedt, Germany).

Microbiological soil properties

Physical, chemical and basic microbiological data (C_{mic} , N_{mic} , extractable organic carbon (EOC) and extractable nitrogen (EN)) for low and high LUI were published by Keil et al. (2011). The additional measurements necessary to complete the data set for intermediate LUI samples were done as follows:

Xylanase activity was measured by incubating 0.3 g of fresh soil with a 5 ml solution of substrate (1.7 % w/v xylan from oat spelts suspended in 2 molar acetate buffer, pH 5.5) and 5 ml of 2 M acetate buffer (pH 5.5) for 24 h at 50 °C (Schinner et al., 1996).

Urease activity was determined by incubating 1 g of fresh soil with 1.5 ml of 0.08 M substrate (urea) solution at 37 °C for 2 h (Schinner et al., 1996). Released ammonium was extracted with 12 ml of a 1 M potassium chloride/0.01 M hydrochloric solution and determined by a modified Berthelot reaction.

The activities of the following enzymes were measured according to Marx et al. (2001): β -D-glucosidase (EC 3.2.1.21), xylosidase (EC 3.2.1.37), β -N-acetylglucosaminidase (EC 3.2.1.52) and phosphatase (EC 3.1.3.1) using fluorescent 4-methylumbelliferone substrates (4-MUF; Sigma-Aldrich, St. Louis, USA). Substrates were dissolved in 300 μl dimethyl sulphoxide and brought to 10 ml total volume with autoclaved water to obtain a 10 mM stock solution. Working solutions (1 mM) were prepared with autoclaved MES buffer (0.1 M MES-buffer 2-[N-morpholino]ethanesulphonic acid, pH 6.1). Standards were dissolved in methanol and water (v:v = 1:1) to a concentration of 10 mM and then diluted to a final concentration of 10 μM . After preparation of the solutions, 1 g fresh soil from each sample was dispersed in 50 ml of autoclaved deionised water by an ultrasonic disaggregator (50 J s⁻¹ for 120 s). Fifty μl aliquots of soil suspension, 50 μl of autoclaved MES-buffer, and 100 μl substrate solution

were then added to each microwell (black PP 96 well microplate, Greiner Bio-one GmbH, Frickenhausen, Germany). Standards (0, 10, 20, 50, 80, 120 μl) were mixed with 50 μl soil suspension and buffer (150, 140, 130, 100, 70, 30 μl) to give final concentrations of 0, 100, 200, 500, 800 and 1200 pmol/well. The microplates were preincubated at 30 °C in the dark for 30 min. Fluorescence was measured after 0, 30, 60, 120 and 180 min by a microplate reader (excitation at 360 nm, emission at 460 nm, Microplate Fluorescence Reader FLX 800, Bio-Tek Instruments Inc., Winooski, VT, USA). Enzyme activities were expressed as the increasing release rates of MUF ($\text{nmol g}^{-1} \text{ h}^{-1}$). For details see Poll et al. (2006).

Statistical analyses

Results from this study were expressed on an areal basis of soil for the top 10 cm soil layer (m^{-2}) to account for a realistic estimation of soil biogeochemical properties in terms of geostatistical analyses and microbial abundances (Bolton et al., 1990, 1993; Doran and Parkin, 1996). For better comparison with other studies data were also given on oven-dry soil (g^{-1} soil dw).

ANOVAs were performed for each parameter to test whether the average values differed between the three LUIs. Differences between LUIs were determined by Tukey post-hoc tests. Multivariate analyses [Multi-Dimensional Scaling (MDS) in combination with discriminant function analysis (DFA); (Egert et al., 2004)] including the standardized average per site of all parameters were performed to test whether the sites clustered according to the three LUIs. ANOVA and MDS-DFA were carried out with STATISTICA 6.0 (Stat Soft, Tulsa, USA) and a statistical probability of $P < 0.05$ was considered significant.

To answer the question of whether or not different LUIs resulted in differences in the homogeneity/heterogeneity of each of the measured variables, we fitted two linear mixed models with geostatistical covariance structure; i.e., one reduced model with the same set of covariance parameters for the different land-use intensities, and a full model where each level of land-use intensity had a separate set of covariance parameters.

For one specific variable the linear mixed model can be written as:

$$(1) y_{ijk} = \delta_i + p_{ij} + t_{ijk} + \varepsilon_{ijk},$$

where y_{ijk} represents the k -th measurement of a soil sample coming from the j -th grassland plot which was farmed at the i -th intensity. The term δ_i references the i -th fixed effect for treatment (LUI), p_{ij} represents the ij -th random plot effect which is distributed as $p_{ij} \sim N(0, \sigma_p^2)$, t_{ijk} is the spatial trend effect, and the residual errors terms ε_{ijk} are

distributed as $\varepsilon_{ijk} \sim N(0, \sigma_R^2)$. In spatial modeling the error variance σ_R^2 is usually denoted as nugget effect. The spatial trend effect t_{ijk} is used to model the covariance of each pair of observations (m, n) with locations (x_m, y_m) and (x_n, y_n) , depending on their Euclidean distance

$$(2) \ h = \sqrt{(x_m - x_n)^2 + (y_m - y_n)^2}$$

The covariance function can be written as

$$(3) \ C(h) = \sigma_V^2 \cdot \rho(h)$$

where σ_V^2 denotes the variance parameter which is usually called the sill or scale parameter, and $\rho(h)$ corresponds to the correlation function which determines the spatial dependency among observations as a function of distance h . We considered three such correlation functions; Gaussian, exponential, and spherical correlation (Schabenberger and Pierce, 2002). Among these, we chose the best fitting full model and confined the analysis to this model.

The full linear mixed model that we considered fits a separate covariance, respective, correlation function to each level of LUI; i.e. a separate nugget effect, a separate sill, and a separate range. Thus, there are six additional parameters fitted compared to the reduced model.

We used a restricted maximum likelihood (REML) approach to fit both the reduced and the full mixed model. The fixed effect part of both models is equal. Therefore, we were able to use a likelihood ratio test (LRT) to infer whether the covariance structure of the full model fit significantly better than the reduced model (Schabenberger and Pierce, 2002). The resulting P-values were adjusted for multiple testing by the Bonferroni correction method controlling the family-wise error rate (FWER). If the LRT was significant, we could infer that there were differences in the way spatially located observations were correlated among the three levels of LUI. We used the statistical software SAS (version 9.2; SAS institute, Cary, NC, USA), specifically the MIXED procedure, to fit both models for each variable.

The sampling locations of the 54 soil samples per site were randomly chosen and gave a rather coarse picture of the existing conditions regarding specific variables. In order to smooth the data, we used the covariance parameter estimates obtained from fitting the linear mixed models, and used them as parameters for an ordinary Kriging (OK) procedure (PROC

KRIGE2D of the SAS system), which yielded estimates of $y_{ijk} - e_{ijk}$. This procedure is equivalent to a best linear unbiased prediction (BLUP) of the same quantity based on mixed model (1) (Robinson, 1991). We used KRIGE2D instead of the MIXED procedure because of computational speed.

The spatial covariance model, i.e. the type of correlation function and covariance parameter estimate, determines the way optimal weights are calculated for OK (Isaaks and Srivastava, 1989). Spatial dependence and autocorrelation were described by a distinct set of spatial parameters (Table 3) which were calculated for each LUI when the model was able to distinguish between the three LUIs. The ratio of partial sill to total sill (pSill/Sill), expressed as a percentage, was used to classify spatial dependence. A ratio of < 25 % indicated weak spatial dependence, between 25 and 75 % indicated moderate spatial dependence, and > 75 % indicated strong spatial dependence (Cambardella et al., 1994). The practical range (pRange) is expressed in meters and was used as an indicator for the scale of spatial autocorrelation.

To characterize the spatial pattern of soil microorganisms as well as their habitat, we calculated all data based on both soil dry weight (g^{-1} soil dw) (Table S1) and on surface area of the soil (m^{-2}) (Fig. 2, Table S2).

Spearman correlations were calculated both for all LUIs together and separately for low, intermediate and high LUIs to determine pair-wise relationships between the parameters. Resulting P-values were adjusted for multiple testing by the Bonferroni-correction method as described for the LRT. Both correlation analysis and plotting of kriged maps were performed using the software package R (version 2.9.1, R Development Core Team, Vienna, Austria).

Results

Physical, chemical and microbiological soil properties

Across the nine sites, pH ranged from 5.22 to 7.09, with significantly ($P = 0.037$) lower values for grasslands under intermediate LUI (Fig. 2; Table S1). Calculated on an area basis, the NO_3^- content decreased ($P = 0.028$) and N_t tended to decrease ($P = 0.087$) with decreasing LUI (Fig. 2).

Microbial biomass (C_{mic}) was significantly higher ($P = 0.031$) for high than for low LUIs with values for intermediate LUIs falling between low and high LUIs (Fig. 2). Enzymes involved in carbon-cycling (β -glucosidase, β -N-acetylglucosaminidase and xylosidase) showed increasing activities with increasing LUI, with significant differences for β -glucosidase ($P = 0.037$) and xylosidase ($P = 0.016$) between the low and high LUIs. In contrast, the activity of xylanase did not respond to LUI. Phosphatase activity was the only parameter in which

intermediate LUI showed a significant effect with higher values than low or high LUI ($P = 0.002$; Fig. 2). Urease activity tended to increase with increasing LUI (Fig. 2).

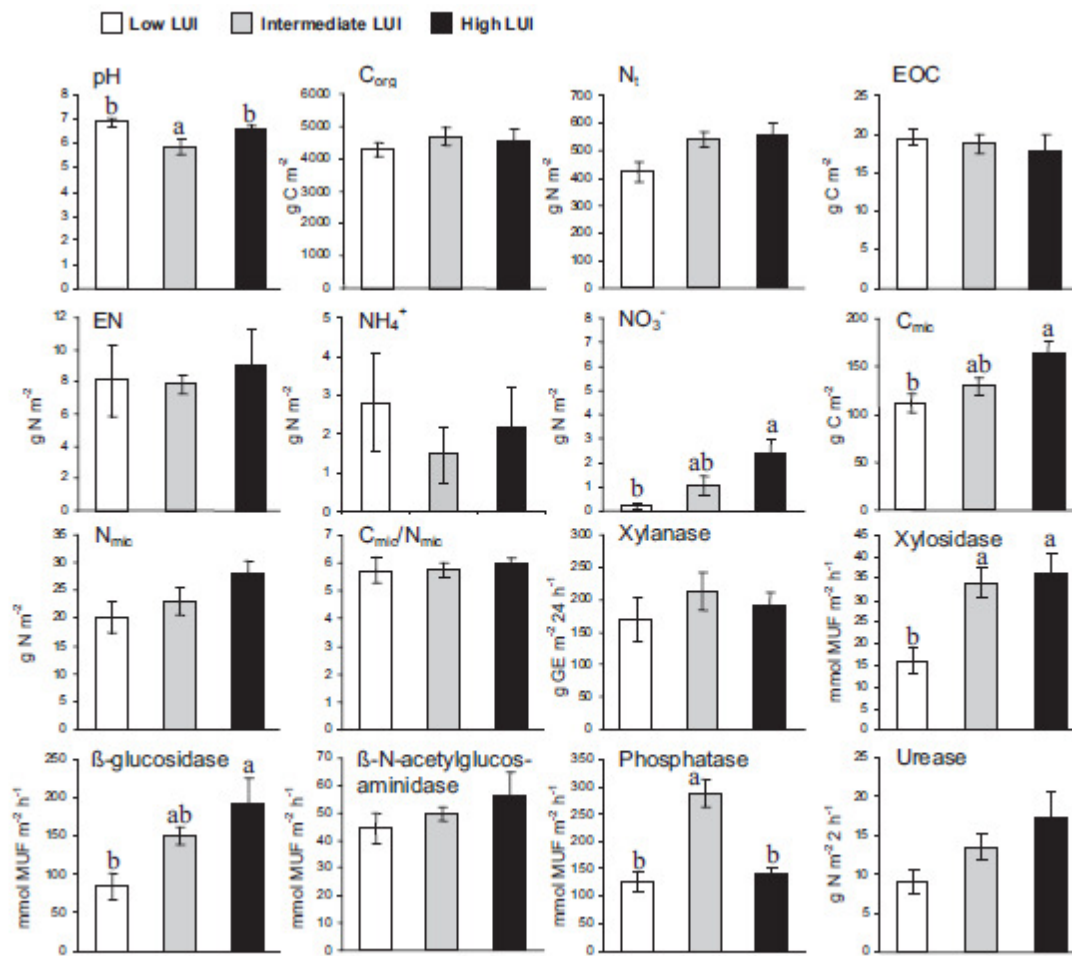


Fig. 2. Average values of all measured parameters related to area (m^{-2}) for the three land-use intensities (LUI), low, intermediate and high LUI. Mean \pm S.E. ($n=3$). Bars without or sharing the same letter are not significantly different (LSD-test; $P < 0.05$). Physical, chemical and basic microbiological data (C_{mic} , N_{mic} , EOC and EN) for low and high LUI were taken from Keil et al. (2011).

Multivariate analyses (MDS-DFA), including all parameters (except soil water content) distinguished significant differences between low and the two other LUIs but not between intermediate and high LUIs for both data sets, on both a dry soil basis (g^{-1}) and on an area basis (m^{-2}) (Fig. 3, Table 2).

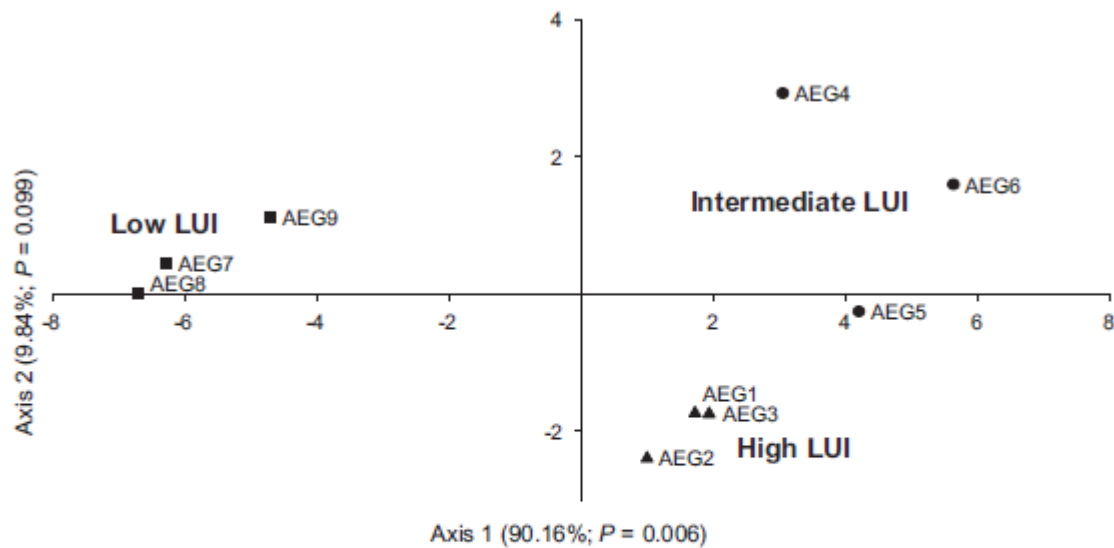


Fig. 3. MDS-DFA analysis including all measured parameters related per area (m^{-2}) for low (squares), intermediate (circles) and high (triangles) land-use intensity (LUI) grassland sites. Single plots are indicated by AEG 1–9 (see also Table 1). Significance of the whole model was $F_{(8,6)} = 7318$; $P < 0.013$. Axis 1 ($P = 0.006$) represents 90.16%, Axis 2 ($P = 0.099$) 9.84% of the total variance.

Separation of the sites and LUI according to Axis 1, which explained 90.2% of the variance, was mainly related to differences in pH, xylosidase and phosphatase activities (Table 2). Also, significant relationships of NO_3^- , C_{mic} and N_{mic} to Axis 2 were indicated, but separation according to Axis 2 was not significant ($P = 0.099$).

Table 2

Results of multivariate MDS-DFA analysis with standardized area-related (m^{-2}) data: (a) F - and P -values; indicating the separation between the low, intermediate and high land-use intensity (LUI) sites; (b) Pearson correlation of parameters with Axes 1 and 2; parameters responsible for the separation of the three LUIs according to the respective axes are indicated by significant correlations and written in bold.

(a) <i>F</i> -/ <i>P</i> -values	Low LUI	Intermediate LUI	High LUI
Low LUI		0.017	0.036
Intermediate LUI	19.57		0.164
High LUI	11.50	3.53	

(b) Parameter	Axis 1	Axis 2
pH	-0.682*	-0.612
Organic carbon (C _{org})	0.345	-0.249
Total nitrogen (N _t)	0.605	-0.361
Ammonium (NH ₄ ⁺)	-0.574	-0.264
Nitrate (NO ₃ ⁻)	0.508	-0.727*
Extractable organic carbon (EOC)	-0.270	0.278
Extractable nitrogen (EN)	-0.001	-0.307
Microbial biomass carbon (C _{mic})	0.424	-0.784*
Microbial biomass nitrogen (N _{mic})	0.364	-0.776*
β-Glucosidase	0.614	-0.564
β-N-Acetylglucosaminidase	0.346	-0.426
Xylosidase	0.807**	-0.402
Phosphatase	0.706*	0.658
Urease	0.476	-0.645
Xylanase	0.298	0.234

* $P < 0.05$.

** $P < 0.01$.

Geostatistics

Because separation according to LUI was slightly more pronounced when parameters were calculated per m⁻², results of geostatistical analyses are shown only for area-related data (Table 3).

For most of the parameters, except soil water content, xylanase and β -N-acetylglucosaminidase activity, the full model fitted significantly better than the reduced model, indicating significant differences in spatial dependence and in a distinct set of spatial parameters. Nugget, pSill, and pRange were calculated for each LUI. Spatial variability was generally best explained by exponential or spherical models (Table 3).

Table 3

Results of geostatistical analyses of area (m⁻²) related data for low, intermediate (intermed.) and high land-use intensity (LUI) using exponential (exp) or spherical (sph) spatial models. No separation between LUIs was possible with reduced (red) models.

Parameter	Spatial model	P-value (Bonferroni)	Model used	LUI	Nugget (C ₀)	pSill (Model variance)	Sill	pSill/Sill ratio (%)	pRange (m) P < 0.05
pH	sph	0.226	full	Low	0.021	0.092	0.113	81	7.18
				Intermed.	0.024	0.058	0.082	71	5.27
				High	0.017	0.036	0.053	68	11.84
SWC	sph	1	red	NA	0.103	0.167	0.270	62	7.09
				Low	0	0.297	0.297	100	0.36
				Intermed.	0.203	0.065	0.273	24	6.58
Organic carbon (C _{org})	sph	<0.001	full	High	0.442	0.074	0.516	14	2.12
				Low	0.200	0.220	0.420	52	0.86
				Intermed.	0.242	0.107	0.349	31	5.90
Total nitrogen (N _t)	sph	0.001	full	High	0.545	0.084	0.629	13	2.14
				Low	0.131	0.112	0.243	46	6.55
				Intermed.	0.045	0.031	0.076	41	5.39
Extractable organic carbon (EOC)	exp	<0.001	full	High	0.107	0.094	0.201	47	20.42
				Low	1.838	3.771	5.609	67	7.82
				Intermed.	0.729	0.450	1.179	38	14.45
Extractable nitrogen (EN)	sph	<0.001	full	High	1.915	2.286	4.201	54	1.93
				Low	1.368	3.469	4.837	72	10.43
				Intermed.	0.289	0.688	0.977	70	5.48
Ammonium (NH ₄ ⁺)	sph	<0.001	full	High	1.965	0.897	2.862	31	3.89
				Low	0.892	2.527	3.419	74	5.17
				Intermed.	7.666	28.036	35.702	79	6.17
Nitrate (NO ₃ ⁻)	sph	<0.001	full	High	47.881	42.086	89.967	47	5.03
				Low	0.021	0.027	0.048	56	82.55
				Intermed.	0.023	0.040	0.063	64	21.29
Microbial biomass carbon (C _{mic})	exp	<0.001	full	High	0.062	0.064	0.126	51	19.74
				Low	0.140	0.153	0.293	52	36.86
				Intermed.	0.105	0.105	0.210	50	5.43
Microbial biomass nitrogen (N _{mic})	sph	<0.001	full	High	0.225	0.173	0.398	43	3.31
				Low	0.326	0.042	0.368	11	4.79
				Intermed.	0.176	0.818	0.994	82	3.59
C _{mic} /N _{mic}	sph	<0.001	full	High	0.346	0.616	0.962	64	3.26
				NA	0.215	0.104	0.319	33	2.28
				Low	0.135	0.007	0.142	5	0.95
Xylanase	exp	1	red	Intermed.	0.334	0.241	0.575	42	13.37
				High	0.580	0.375	0.955	39	2.28
				Low	3.980	8.055	12.035	67	232.95
β -Glucosidase	exp	<0.001	full	Intermed.	8.546	4.429	12.975	34	24.63
				High	19.727	35.845	55.572	65	69.51
				Low	0.879	0.493	1.372	36	3.08
β -N-Acetylglucosaminidase	exp	0.870	red	NA	0.051	0.031	0.082	38	2.41
				Low	0.214	0.041	0.255	16	3.18
				Intermed.	0.046	0.029	0.075	39	2.28
Phosphatase	sph	<0.001	full	High	3.290	5.565	8.855	63	44.90
				Low	5.733	9.745	15.478	63	73.82
				Intermed.	8.409	4.493	12.902	35	2.83

NA, not applicable.

Chemical properties

Most chemical properties showed a spatial autocorrelation (pRange) smaller than the maximum lag distance of 14 m within the 10 × 10 m² area (Table 3). Soil pH showed similar structural variance in the three LUIs, with the smallest pRange in the intermediate LUI. Spatial dependence of C_{org} was strong in the low LUI plots, which was indicated by a high

explained structural variance (high pSill to sill ratio at short pRanges < 1 m; Table 3). In intermediate and high LUIs structural variance of C_{org} was only weakly to moderately explained by the spatial model, with moderate ranges between two and seven meters. Kriging was used to map the spatial pattern of C_{org} in all sites, visualizing the differences in its spatial distribution between the LUIs (Fig. 4). In high LUI sites EOC showed a large spatial autocorrelation (pRange > 14 m), but spatial autocorrelation of EN was small (pRange = 1.93 m). Structural variance of NH_4^+ was explained in low and intermediate LUI sites highly and moderately, respectively, but in contrast to C_{org} and N_t , the pRange was largest in the low LUI.

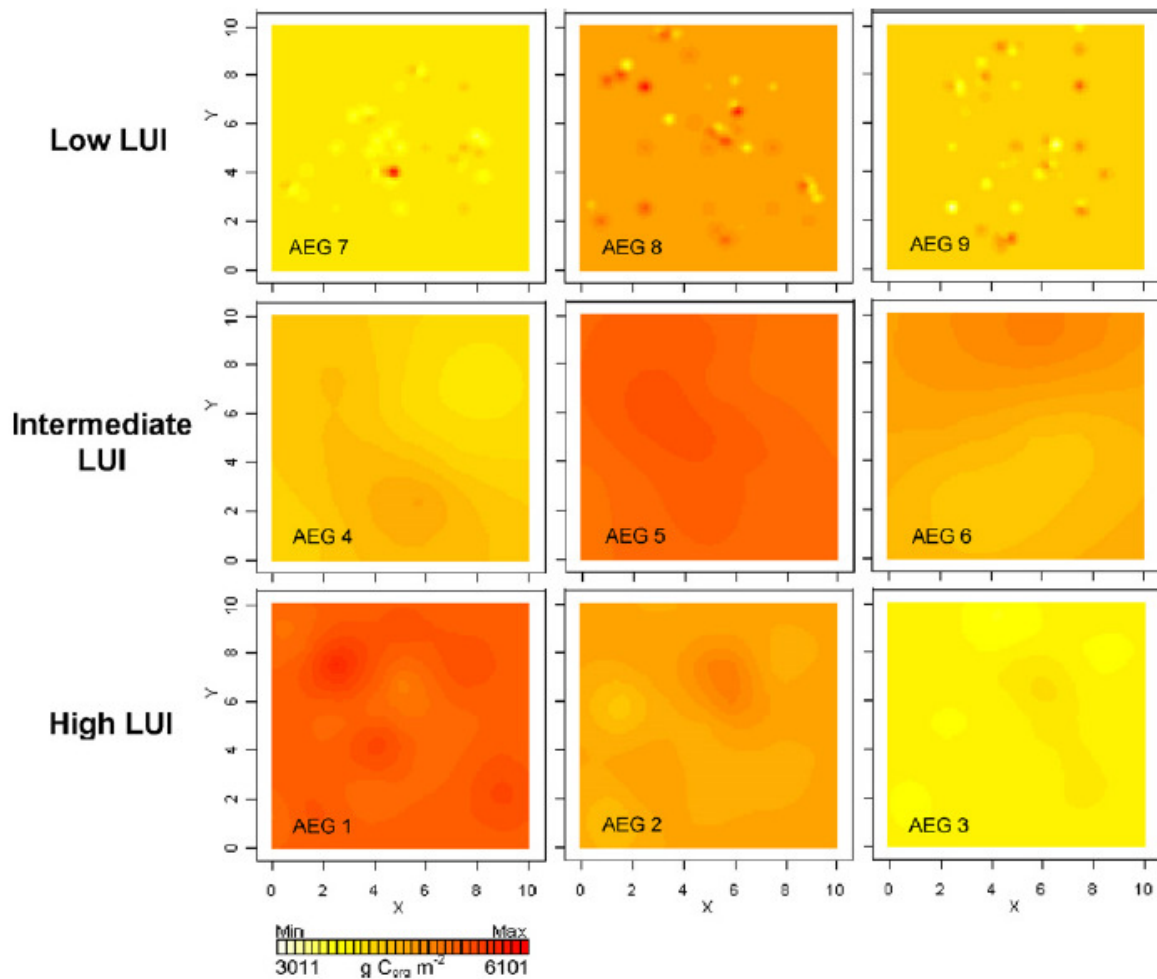


Fig. 4. Kriging maps for the distribution of organic carbon (C_{org}) related to area (m^{-2}) on the nine grassland sites (AEG 1-9).

Microbiological soil properties

Microbial biomass (C_{mic}) and β -glucosidase showed moderate spatial dependencies, but the pRange indicated spatial autocorrelations on scales larger than investigated (> 14 m). The pRange of N_{mic} was much larger in the low (pRange > 14 m) than in the intermediate and high LUIs (< 5.43 m). The spatial autocorrelation for the C_{mic}/N_{mic} ratio and for xylosidase activity was smallest in the low LUI and largest in the intermediate LUI. Ranges of

phosphatase activity showed only slight differences between the LUIs and spatial dependence was more weakly explained in the intermediate than in the other LUIs. Spatial autocorrelation of urease activity was much larger in both low and intermediate than in high LUI (Table 3).

As exemplarily shown for the high LUI site AEG 2 (Fig. 5), the kriged maps indicate similar spatial distribution patterns of different soil chemical properties (C_{org} , N_t , EOC and NH_4^+) as well as microbiological properties N_{mic} and partly C_{mic} . With respect to microbiological properties, distribution patterns of the activities of xylosidase, β -N-acetylglucosaminidase and phosphatase were similar at AEG 2.

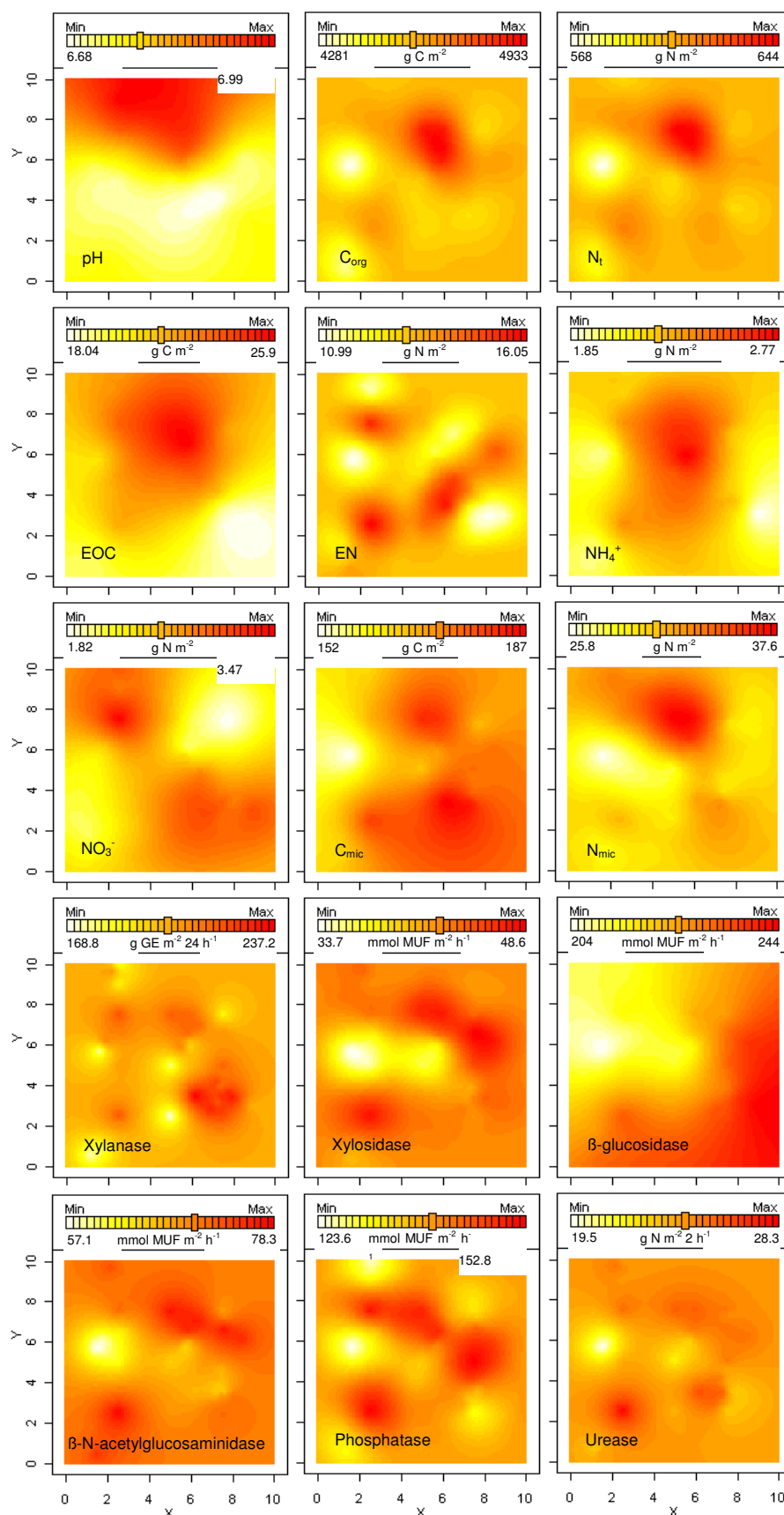


Fig. 5. Kriging maps for all parameters related to area (m^{-2}) for the high land-use intensity grassland site AEG 2.

Correlation between chemical and microbiological soil properties

When all three LUIs were considered together, most of the soil chemical properties were significantly correlated with each other, but high and positive correlation (Spearman's correlation $r_s > 0.7$) was found only between C_{org} and N_t (Table S3). For microbiological properties, C_{mic} , N_{mic} and nitrate were positively and highly correlated with each other. C_{mic} and N_{mic} also showed positive correlations to β -glucosidase, urease, and to a lesser degree to xylosidase activity. The enzyme activities of β -glucosidase, xylosidase and urease were all correlated to each other and also to nitrate. Only weak correlations were found between pH and most of the other properties, whereas a strong negative correlation was found between pH and phosphatase activity.

Correlation analyses performed separately for each LUI indicated that LUI changed the strength of correlation between soil properties (Table S4 a-c). For example, xylanase activity was highly correlated to β -glucosidase and xylosidase activity only in low LUI sites. In addition, high and positive correlations between NH_4^+ and NO_3^- and between EN and the activities of β -glucosidase, xylosidase and xylanase were also found only in the low LUI sites. A positive correlation between microbial biomass (C_{mic} and N_{mic}) and NH_4^+ was seen in the low LUI sites. A high correlation between C_{org} and C_{mic} was found only in the high LUI sites. Fewest strong correlations between microbiological properties were detected for the intermediate LUI sites.

Table S1. Average values (with CV in %) for each of the nine grassland sites with low, intermediate and high land-use-intensity (LUI). Data are related to dry soil (g^{-1}). Physical, chemical and basic microbiological data (C_{mic} , N_{mic} , EOC and EN) for low and high LUI were taken from Keil et al. (2011).

LUI	Low			Intermediate			High		
Plot ID	AEG 7	AEG 8	AEG 9	AEG 4	AEG 5	AEG 6	AEG 1	AEG 2	AEG 3
Bulk density	0.83	0.64	0.74	0.76	0.68	0.80	0.68	0.80	0.82
(g cm^{-3})	(6.34)	(12.69)	(12.06)	(9.02)	(9.44)	(7.80)	(16.95)	(10.50)	(6.89)
pH	7.09	6.94	6.49	5.22	6.23	6.01	6.56	6.80	6.38
	(1.11)	(4.05)	(6.60)	(3.39)	(2.71)	(6.51)	(2.39)	(2.22)	(3.38)
Soil water content (swc)	58.25	76.41	61.52	69.92	74.31	59.01	73.11	63.21	63.07
(% soil dry weight)	(6.87)	(5.98)	(9.43)	(8.20)	(4.22)	8.94	(7.61)	(4.17)	(5.15)
Organic carbon (C_{org})	47.23	72.49	57.69	57.14	76.28	56.49	76.68	57.40	47.83
(mg C g^{-1})	(8.18)	(8.15)	(15.95)	(13.53)	(6.09)	(13.90)	(12.15)	(7.61)	(8.31)
Total nitrogen (N_t)	4.73	7.76	5.13	7.35	8.52	6.12	8.81	7.56	5.70
(mg N g^{-1})	(14.91)	(7.79)	(14.96)	(12.58)	(5.71)	(13.72)	(8.54)	(5.99)	(7.72)
Extractable organic carbon (EOC)	210.04	318.54	277.96	281.33	259.40	217.36	260.23	271.94	177.78
($\mu\text{g C g}^{-1}$)	(26.44)	(19.82)	(16.74)	(18.45)	(10.35)	(11.57)	(19.53)	(14.64)	(16.07)
Extractable nitrogen (EN)	53.54	186.33	105.12	91.65	113.27	111.64	74.08	158.97	116.81
($\mu\text{g N g}^{-1}$)	(23.33)	(22.53)	(19.33)	(11.75)	(10.70)	(13.00)	(18.32)	(14.76)	(22.11)
Ammonium (NH_4^+)	35.38	61.50	21.80	11.69	18.18	27.94	19.29	28.74	35.21
($\mu\text{g N g}^{-1}$)	(21.11)	(28.85)	(43.45)	(26.42)	(29.71)	(25.68)	(60.56)	(30.69)	(29.79)
Nitrate (NO_3^-)	1.34	7.19	0.03	4.13	24.63	14.15	48.31	31.93	15.48
($\mu\text{g N g}^{-1}$)	(74.63)	(56.51)	(641.62)	(65.60)	(36.27)	(48.69)	(26.08)	(36.34)	(37.91)
Microbial biomass carbon (C_{mic})	1442.73	1935.07	1260.40	1493.01	2123.24	1617.44	2694.59	2151.98	1717.25

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($\mu\text{g C g}^{-1}$)	(13.78)	(8.70)	(14.64)	(17.20)	(14.69)	(18.75)	(14.22)	(10.01)	(13.75)
Microbial biomass nitrogen (N_{mic})	222.14	403.09	222.05	244.33	401.17	290.01	430.83	389.14	298.43
($\mu\text{g N g}^{-1}$)	(12.64)	(21.79)	(25.65)	(22.08)	(18.75)	(15.93)	(17.52)	(13.02)	(21.24)
$\text{C}_{\text{mic}}/\text{N}_{\text{mic}}$	6.54	4.90	5.81	6.26	5.40	5.68	6.31	5.58	5.98
	(7.29)	(12.44)	(11.90)	(17.59)	(16.76)	(16.83)	(9.84)	(10.53)	(22.43)
Xylanase	1310.24	3536.32	2314.96	3617.56	2780.93	2253.99	3204.76	2560.19	1880.87
($\mu\text{g GE g}^{-1} 24 \text{ h}^{-1}$)	(28.17)	(30.14)	(30.22)	(29.01)	(26.72)	(26.53)	(25.66)	(24.76)	(19.66)
Xylosidase	127.01	325.51	225.57	365.46	554.92	461.22	585.46	525.40	330.03
($\text{nmol MUF g}^{-1} \text{ h}^{-1}$)	(15.92)	(21.41)	(20.34)	(23.97)	(19.19)	(15.31)	(28.34)	(17.77)	(14.47)
β -glucosidase	671.46	1816.07	1118.23	1742.39	2520.40	1848.43	3377.18	2800.00	1575.58
($\text{nmol MUF g}^{-1} \text{ h}^{-1}$)	(19.93)	(23.74)	(24.30)	(24.89)	(19.74)	(19.15)	(27.71)	(14.83)	(14.14)
β -N-acetylglucosaminidase	416.06	714.80	710.44	593.90	747.90	655.51	888.99	866.77	493.98
($\text{nmol MUF g}^{-1} \text{ h}^{-1}$)	(22.45)	(18.49)	(23.29)	(28.66)	(17.41)	(16.27)	(24.61)	(15.11)	(14.99)
Phosphatase	1130.53	2172.77	1990.83	4365.20	3531.01	3654.76	1891.73	1734.24	1964.04
($\text{nmol MUF g}^{-1} \text{ h}^{-1}$)	(12.63)	(19.53)	(18.21)	(18.46)	(17.42)	(14.98)	(20.68)	(10.40)	(11.67)
Urease	114.95	172.44	88.30	142.80	248.14	167.12	221.51	300.70	154.99
($\mu\text{g N g}^{-1} 2 \text{ h}^{-1}$)	(12.30)	(25.34)	(33.28)	(29.64)	(14.10)	(26.79)	(17.95)	(13.51)	(16.31)

GE, glucose equivalent.

Table S2. Average values (with CV in %) for the nine grassland sites with low, intermediate and high land-use-intensity (LUI). Data are related to area (m^{-2}). Physical, chemical and basic microbiological data (C_{mic} , N_{mic} , EOC and EN) for low and high LUI were taken from Keil et al. (2011).

LUI	Low			Intermediate			High		
Plot ID	AEG 7	AEG 8	AEG 9	AEG 4	AEG 5	AEG 6	AEG 1	AEG 2	AEG 3
Organic carbon (C_{org})	3927.28	4693.68	4228.99	4305.06	5195.28	4512.44	5198.52	4581.06	3901.29
(g C m^{-2})	(10.53)	(12.19)	(13.95)	(11.91)	(9.75)	(10.42)	(18.87)	(13.36)	(11.34)
Total nitrogen (N_{t})	394.23	498.39	377.62	553.11	580.24	488.84	598.10	603.66	465.62
(g N m^{-2})	(17.95)	(13.45)	(12.93)	(10.59)	(9.89)	(10.58)	(16.84)	(12.84)	(10.51)
Extractable organic carbon (EOC)	17.43	20.54	20.61	21.23	17.68	17.45	17.50	21.75	14.51
(g C m^{-2})	(26.34)	(25.27)	(20.01)	(15.93)	(13.13)	(12.47)	(23.67)	(20.66)	(17.00)
Extractable nitrogen (EN)	4.44	12.07	7.71	6.91	7.71	8.95	4.97	12.67	9.57
(g N m^{-2})	(22.56)	(24.65)	(15.89)	(11.21)	(13.00)	(11.25)	(20.89)	(17.46)	(24.81)
Ammonium (NH_4^+)	2.93	3.97	1.57	0.88	1.24	2.25	1.26	2.30	2.88
(g N m^{-2})	(20.04)	(28.87)	(36.78)	(25.45)	(30.75)	(27.89)	(58.50)	(33.48)	(32.53)
Nitrate (NO_3^-)	0.11	0.46	0	0.31	1.66	1.16	3.31	2.54	1.27
(g N m^{-2})	(74.36)	(56.06)	(636.20)	(69.88)	(33.14)	(52.41)	(33.27)	(36.85)	(39.63)
Microbial biomass carbon (C_{mic})	119.78	123.46	92.58	112.21	144.10	130.66	181.77	171.65	140.58
(g C m^{-2})	(9.38)	(14.71)	(17.50)	(15.64)	(14.85)	(18.13)	(20.64)	(14.56)	(16.78)
Microbial biomass nitrogen (N_{mic})	18.43	25.63	16.27	18.50	27.22	23.27	29.12	31.10	24.32
(g N m^{-2})	(12.76)	(20.29)	(24.01)	(22.30)	(18.02)	(16.01)	(24.21)	(17.62)	(21.95)
$C_{\text{mic}}/N_{\text{mic}}$	6.54	4.90	5.81	6.23	5.40	5.68	6.31	5.58	5.98
	(7.29)	(12.44)	(11.90)	(17.52)	(16.76)	(16.83)	(9.84)	(10.53)	(22.43)
Xylanase	108.88	227.33	172.87	271.01	188.33	179.83	215.29	204.59	153.56
($\text{g GE m}^{-2} 24 \text{ h}^{-1}$)	(28.75)	(35.28)	(34.07)	(28.77)	(25.39)	(24.71)	(25.76)	(26.29)	(20.18)

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Xylosidase	10.55	20.85	16.70	27.41	37.73	36.94	39.79	41.78	27.05
(mmol MUF m ⁻² h ⁻¹)	(16.56)	(23.58)	(23.27)	(23.80)	(20.26)	(14.46)	(33.78)	(18.85)	(18.32)
β-glucosidase	55.76	116.01	82.22	130.90	171.49	147.81	228.99	222.77	128.89
(mmol MUF m ⁻² h ⁻¹)	(20.51)	(24.08)	(23.69)	(25.51)	(21.41)	(17.39)	(33.44)	(16.48)	(17.21)
β-N-acetylglucosaminidase	34.49	45.81	52.81	44.60	50.95	52.82	60.10	69.04	40.36
(mmol MUF m ⁻² h ⁻¹)	(21.81)	(22.20)	(26.91)	(27.27)	(19.58)	(17.78)	(28.81)	(17.26)	(17.53)
Phosphatase	93.79	139.57	148.48	328.10	240.93	292.80	127.75	138.24	160.76
(mmol MUF m ⁻² h ⁻¹)	(12.37)	(23.14)	(23.52)	(17.30)	(20.82)	(14.34)	(26.14)	(14.15)	(15.33)
Urease	9.56	11.13	6.43	10.66	16.82	13.30	14.96	24.05	12.67
(g N m ⁻² 2 h ⁻¹)	(14.09)	(25.79)	(29.19)	(27.76)	(12.84)	(23.37)	(23.71)	(18.15)	(18.38)

GE, glucose equivalent.

Table S3. Spearman correlations between the parameters related to area (m²) including all land-use intensities (LUI). Strong correlations are indicated in bold. Physical, chemical and basic microbiological data (C_{mic}, N_{mic}, EOC and EN) for low and high LUI were taken from Keil et al. (2011).

All LUI	pH	SWC	C _{org}	N _t	EOC	EN	NH ₄ ⁺	NO ₃ ⁻	C _{mic}	N _{mic}	C _{mic} /N _{mic}	Xyla	Xylos	β-glu	β-N-ac	Phos
SWC	-0.026 ^{n.s.}															
C _{org}	-0.026 ^{n.s.}	0.538 ***														
N _t	-0.157 ^{n.s.}	0.505 ***	0.781 ***													
EOC	-0.001 ^{n.s.}	0.107 ^{n.s.}	0.348 ***	0.348 ***												
EN	0.043 ^{n.s.}	0.091 ^{n.s.}	0.230 ***	0.265 ***	0.317 ***											
NH ₄ ⁺	0.505 ***	-0.146 ^{n.s.}	-0.105 ^{n.s.}	-0.230 ***	0.051 ^{n.s.}	0.436 ***										
NO ₃ ⁻	-0.053 ^{n.s.}	0.244 ***	0.401 ***	0.633 ***	-0.131 ^{n.s.}	0.198 **	-0.133 ^{n.s.}									
C _{mic}	0.099 ^{n.s.}	0.236 ***	0.441 ***	0.619 ***	0.031 ^{n.s.}	0.191 **	0.080 ^{n.s.}	0.793 ***								
N _{mic}	0.184 **	0.365 ***	0.525 ***	0.629 ***	0.042 ^{n.s.}	0.311 ***	0.112 ^{n.s.}	0.702 ***	0.809 ***							
C _{mic} /N _{mic}	-0.154 ^{n.s.}	-0.255 ***	-0.233 ***	-0.144 ^{n.s.}	0.004 ^{n.s.}	-0.333 ***	-0.111 ^{n.s.}	-0.031 ^{n.s.}	0.058 ^{n.s.}	-0.491 ***						
Xyla	-0.290 ***	0.453 ***	0.434 ***	0.509 ***	0.413 ***	0.275 ***	-0.159 ^{n.s.}	0.202 **	0.229 ***	0.284 ***	-0.141 ^{n.s.}					
Xylos	-0.292 ***	0.266 ***	0.544 ***	0.722 ***	0.046 ^{n.s.}	0.312 ***	-0.275 ***	0.778 ***	0.686 ***	0.654 ***	-0.118 ^{n.s.}	0.417 ***				
β-glu	-0.165 *	0.377 ***	0.557 ***	0.766 ***	0.092 ^{n.s.}	0.309 ***	-0.227 ***	0.821 ***	0.747 ***	0.708 ***	-0.121 ^{n.s.}	0.472 ***	0.922 ***			
β-N-ac	-0.127 ^{n.s.}	0.111 ^{n.s.}	0.466 ***	0.506 ***	0.357 ***	0.338 ***	-0.120 ^{n.s.}	0.394 ***	0.440 ***	0.400 ***	0.000 ^{n.s.}	0.455 ***	0.624 ***	0.622 ***		
Phos	-0.791 ***	0.150 ^{n.s.}	0.304 ***	0.371 ***	0.173 *	0.246 ***	-0.343 ***	0.092 ^{n.s.}	0.013 ^{n.s.}	0.049 ^{n.s.}	-0.088 ^{n.s.}	0.428 ***	0.493 ***	0.333 ***	0.284 ***	
Ureas	0.054 ^{n.s.}	0.295 ***	0.527 ***	0.731 ***	0.045 ^{n.s.}	0.342 ***	-0.057 ^{n.s.}	0.791 ***	0.733 ***	0.772 ***	-0.236 ***	0.307 ***	0.790 ***	0.832 ***	0.407 ***	0.192 **

^{n.s.} P > 0.05; * P < 0.05; ** P < 0.01; *** P < 0.001

Table S4a. Spearman correlations between the parameters related to area (m²) including low land-use intensity (LUI) sites. Strong correlations are indicated in bold. Physical, chemical and basic microbiological data (C_{mic}, N_{mic}, EOC and EN) for low and high LUI were taken from Keil et al. (2011).

Low LUI	pH	SWC	C _{org}	N _t	EOC	EN	NH ₄ ⁺	NO ₃ ⁻	C _{mic}	N _{mic}	C _{mic} /N _{mic}	Xyla	Xylos	β-glu	β-N-ac	Phos
SWC	0.184 ^{n.s.}															
C _{org}	0.037 ^{n.s.}	0.576 ^{***}														
N _t	0.189 ^{n.s.}	0.600 ^{***}	0.805^{***}													
EOC	-0.057 ^{n.s.}	0.139 ^{n.s.}	0.404 ^{***}	0.334 ^{**}												
EN	-0.126 ^{n.s.}	0.744^{***}	0.639 ^{***}	0.608 ^{***}	0.480 ^{***}											
NH ₄ ⁺	0.492 ^{***}	0.554 ^{***}	0.346 ^{**}	0.582 ^{***}	0.086 ^{n.s.}	0.400 ^{***}										
NO ₃ ⁻	0.449 ^{***}	0.545 ^{***}	0.291 [*]	0.578 ^{***}	-0.076 ^{n.s.}	0.349 ^{**}	0.776^{***}									
C _{mic}	0.495 ^{***}	0.332 ^{**}	0.390 ^{***}	0.606 ^{***}	0.114 ^{n.s.}	0.162 ^{n.s.}	0.714^{***}	0.606 ^{***}								
N _{mic}	0.400 ^{***}	0.627 ^{***}	0.529 ^{***}	0.679 ^{***}	0.069 ^{n.s.}	0.484 ^{***}	0.702^{***}	0.666 ^{***}	0.809^{***}							
C _{mic} /N _{mic}	-0.088 ^{n.s.}	-0.690 ^{***}	-0.465 ^{***}	-0.430 ^{***}	-0.017 ^{n.s.}	-0.674 ^{***}	-0.344 ^{**}	-0.383 ^{***}	-0.186 ^{n.s.}	-0.692 ^{***}						
Xyla	-0.288 [*]	0.485 ^{***}	0.490 ^{***}	0.450 ^{***}	0.390 ^{***}	0.736^{***}	0.236 ^{n.s.}	0.215 ^{n.s.}	0.103 ^{n.s.}	0.397 ^{***}	-0.556 ^{***}					
Xylos	-0.205 ^{n.s.}	0.601 ^{***}	0.645 ^{***}	0.552 ^{***}	0.321 ^{**}	0.798^{***}	0.183 ^{n.s.}	0.238 ^{n.s.}	0.158 ^{n.s.}	0.479 ^{***}	-0.656 ^{***}	0.734^{***}				
β-glu	-0.067 ^{n.s.}	0.702^{***}	0.659 ^{***}	0.615 ^{***}	0.316 ^{**}	0.826^{***}	0.330 ^{**}	0.347 ^{**}	0.263 ^{n.s.}	0.577 ^{***}	-0.693 ^{***}	0.730^{***}	0.896^{***}			
β-N-ac	-0.497 ^{***}	0.149 ^{n.s.}	0.364 ^{***}	0.238 ^{n.s.}	0.415 ^{***}	0.464 ^{***}	-0.166 ^{n.s.}	-0.228 ^{n.s.}	-0.136 ^{n.s.}	0.005 ^{n.s.}	-0.188 ^{n.s.}	0.602 ^{***}	0.630 ^{***}	0.570 ^{***}		
Phos	-0.563 ^{***}	0.299 [*]	0.486 ^{***}	0.318 [*]	0.374 ^{***}	0.590 ^{***}	-0.129 ^{n.s.}	-0.113 ^{n.s.}	-0.098 ^{n.s.}	0.124 ^{n.s.}	-0.349 ^{***}	0.662 ^{***}	0.783^{***}	0.662 ^{***}	0.791^{***}	
Ureas	0.606 ^{***}	0.377 ^{***}	0.377 ^{***}	0.589 ^{***}	0.073 ^{n.s.}	0.229 ^{n.s.}	0.741^{***}	0.722^{***}	0.787^{***}	0.741^{***}	-0.316 ^{**}	0.144 ^{n.s.}	0.206 ^{n.s.}	0.319 ^{**}	-0.207 ^{n.s.}	-0.129 ^{n.s.}

^{n.s.} P > 0.05; * P < 0.05; ** P < 0.01; *** P < 0.001

Table S4b. Spearman correlations between the parameters related to area (m^{-2}) including intermediate (intermed.) land-use intensity (LUI) sites. Strong correlations are indicated in bold.

Intermed. LUI	pH	SWC	C _{org}	N _t	EOC	EN	NH ₄ ⁺	NO ₃ ⁻	C _{mic}	N _{mic}	C _{mic} /N _{mic}	Xyla	Xylos	β-glu	β-N-ac	Phos
SWC	0.069 ^{n.s.}															
C _{org}	0.454 ^{***}	0.549 ^{***}														
N _t	0.037 ^{n.s.}	0.685 ^{***}	0.777^{***}													
EOC	-0.560 ^{***}	0.264 ^{n.s.}	0.143 ^{n.s.}	0.479 ^{***}												
EN	0.376 ^{***}	-0.247 ^{n.s.}	0.398 ^{***}	0.040 ^{n.s.}	0.033 ^{n.s.}											
NH ₄ ⁺	0.298 [*]	-0.451 ^{***}	0.127 ^{n.s.}	-0.325 ^{**}	-0.161 ^{n.s.}	0.723^{***}										
NO ₃ ⁻	0.641 ^{***}	0.095 ^{n.s.}	0.479 ^{***}	0.130 ^{n.s.}	-0.341 ^{**}	0.350 ^{***}	0.287 [*]									
C _{mic}	0.258 ^{n.s.}	0.155 ^{n.s.}	0.368 ^{***}	0.172 ^{n.s.}	-0.001 ^{n.s.}	0.331 ^{**}	0.295 [*]	0.646 ^{***}								
N _{mic}	0.583 ^{***}	0.211 ^{n.s.}	0.520 ^{***}	0.270 ^{n.s.}	-0.180 ^{n.s.}	0.206 ^{n.s.}	0.181 ^{n.s.}	0.685 ^{***}	0.683 ^{***}							
C _{mic} /N _{mic}	-0.550 ^{***}	-0.101 ^{n.s.}	-0.279 [*]	-0.126 ^{n.s.}	0.297 [*]	0.030 ^{n.s.}	0.048 ^{n.s.}	-0.260 ^{n.s.}	0.073 ^{n.s.}	-0.609 ^{***}						
Xyla	-0.426 ^{***}	0.299 [*]	0.056 ^{n.s.}	0.320 ^{**}	0.546 ^{***}	-0.030 ^{n.s.}	-0.224 ^{n.s.}	-0.376 ^{***}	-0.121 ^{n.s.}	-0.193 ^{n.s.}	0.117 ^{n.s.}					
Xylos	0.550 ^{***}	0.046 ^{n.s.}	0.478 ^{***}	0.164 ^{n.s.}	-0.153 ^{n.s.}	0.587 ^{***}	0.367 ^{***}	0.563 ^{***}	0.519 ^{***}	0.556 ^{***}	-0.216 ^{n.s.}	-0.108 ^{n.s.}				
β-glu	0.429 ^{***}	0.319 ^{**}	0.603 ^{***}	0.438 ^{***}	0.023 ^{n.s.}	0.457 ^{***}	0.176 ^{n.s.}	0.506 ^{***}	0.485 ^{***}	0.525 ^{***}	-0.233 ^{n.s.}	0.157 ^{n.s.}	0.819^{***}			
β-N-ac	0.090 ^{n.s.}	0.038 ^{n.s.}	0.388 ^{***}	0.222 ^{n.s.}	0.202 ^{n.s.}	0.527 ^{***}	0.411 ^{***}	0.278 ^{n.s.}	0.354 ^{***}	0.252 ^{n.s.}	0.094 ^{n.s.}	0.115 ^{n.s.}	0.555 ^{***}	0.472 ^{***}		
Phos	-0.496 ^{***}	-0.073 ^{n.s.}	-0.004 ^{n.s.}	0.214 ^{n.s.}	0.600 ^{***}	0.243 ^{n.s.}	0.004 ^{n.s.}	-0.432 ^{***}	-0.099 ^{n.s.}	-0.260 ^{n.s.}	0.225 ^{n.s.}	0.503 ^{***}	0.156 ^{n.s.}	0.203 ^{n.s.}	0.354 ^{***}	
Ureas	0.715 ^{***}	0.443 ^{***}	0.722 ^{***}	0.450 ^{***}	-0.229 ^{n.s.}	0.310 [*]	0.131 ^{n.s.}	0.617 ^{***}	0.396 ^{***}	0.664 ^{***}	-0.488 ^{***}	-0.052 ^{n.s.}	0.583 ^{***}	0.717^{***}	0.210 ^{n.s.}	-0.250 ^{n.s.}

^{n.s.} P > 0.05; * P < 0.05; ** P < 0.01; *** P < 0.001

Table S4c. Spearman correlations between the parameters related to area (m^{-2}) including high land-use intensity (LUI) sites. Strong correlations are indicated in bold. Physical, chemical and basic microbiological data (C_{mic} , N_{mic} , EOC and EN) for low and high LUI were taken from Keil et al. (2011).

High LUI	pH	SWC	C_{org}	N_{t}	EOC	EN	NH_4^+	NO_3^-	C_{mic}	N_{mic}	$C_{\text{mic}}/N_{\text{mic}}$	Xyla	Xylos	$\beta\text{-glu}$	$\beta\text{-N-ac}$	Phos
SWC	-0.094 ^{n.s.}															
C_{org}	0.269 ^{n.s.}	0.481 ***														
N_{t}	0.484 ***	0.337 **	0.878 ***													
EOC	0.476 ***	-0.007 ^{n.s.}	0.518 ***	0.690 ***												
EN	0.270 ^{n.s.}	-0.543 ***	-0.138 ^{n.s.}	0.102 ^{n.s.}	0.452 ***											
NH_4^+	-0.145 ^{n.s.}	-0.373 ***	-0.218 ^{n.s.}	-0.165 ^{n.s.}	0.144 ^{n.s.}	0.623 ***										
NO_3^-	0.304 *	0.391 ***	0.626 ***	0.640 ***	0.368 ***	-0.197 ^{n.s.}	-0.521 ***									
C_{mic}	0.249 ^{n.s.}	0.492 ***	0.755 ***	0.824 ***	0.421 ***	-0.035 ^{n.s.}	-0.194 ^{n.s.}	0.670 ***								
N_{mic}	0.421 ***	0.247 ^{n.s.}	0.606 ***	0.738 ***	0.422 ***	0.084 ^{n.s.}	-0.022 ^{n.s.}	0.412 ***	0.756 ***							
$C_{\text{mic}}/N_{\text{mic}}$	-0.341 **	0.311 **	0.051 ^{n.s.}	-0.082 ^{n.s.}	-0.116 ^{n.s.}	-0.171 ^{n.s.}	-0.189 ^{n.s.}	0.222 ^{n.s.}	0.125 ^{n.s.}	-0.500 ***						
Xyla	0.228 ^{n.s.}	0.490 ***	0.591 ***	0.612 ***	0.511 ***	0.010 ^{n.s.}	-0.094 ^{n.s.}	0.444 ***	0.619 ***	0.479 ***	0.061 ^{n.s.}					
Xylos	0.420 ***	0.357 ***	0.684 ***	0.786 ***	0.503 ***	0.111 ^{n.s.}	-0.300 *	0.617 ***	0.784 ***	0.651 ***	-0.002 ^{n.s.}	0.605 ***				
$\beta\text{-glu}$	0.373 ***	0.455 ***	0.684 ***	0.781 ***	0.522 ***	0.008 ^{n.s.}	-0.346 **	0.694 ***	0.802 ***	0.607 ***	0.090 ^{n.s.}	0.635 ***	0.888 ***			
$\beta\text{-N-ac}$	0.494 ***	0.190 ^{n.s.}	0.586 ***	0.742 ***	0.625 ***	0.212 ^{n.s.}	-0.158 ^{n.s.}	0.540 ***	0.686 ***	0.578 ***	0.001 ^{n.s.}	0.549 ***	0.811 ***	0.805 ***		
Phos	-0.308 *	-0.135 ^{n.s.}	0.042 ^{n.s.}	0.050 ^{n.s.}	0.087 ^{n.s.}	0.392 ***	0.425 ***	-0.205 ^{n.s.}	0.122 ^{n.s.}	0.104 ^{n.s.}	0.029 ^{n.s.}	0.104 ^{n.s.}	0.171 ^{n.s.}	0.036 ^{n.s.}	0.075 ^{n.s.}	
Ureas	0.614 ***	0.007 ^{n.s.}	0.471 ***	0.733 ***	0.639 ***	0.461 ***	-0.055 ^{n.s.}	0.468 ***	0.623 ***	0.701 ***	-0.261 ^{n.s.}	0.499 ***	0.766 ***	0.717 ***	0.710 ***	0.104 ^{n.s.}

^{n.s.} $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Discussion

General effects of land-use intensity

The grassland sites were selected to represent three classes of land-use intensity, and classified as sites of low (unfertilized pastures), intermediate (fertilized mown pastures), and high (fertilized mown meadows) LUI. Multivariate analyses (MDS-DFA) including all parameters revealed significant separation only between the low and the other two LUIs, but not between intermediate and high LUI sites. Soil pH and phosphatase activity, together with xylosidase activity, microbial biomass C and N, and nitrate concentration in soil (Fig. 3, Table 2) were found to be the parameters explaining most of the separation of the LUIs. The above mentioned properties are considered as indicators of land-use intensification (Kandeler et al., 1999; Marschner et al., 2003).

Xylosidase and to a lesser degree β -glucosidase, β -N-acetylglucosaminidase and urease showed increasing activities with increasing LUI (Fig. 2). Xylosidase and β -glucosidase are enzymes known to derive mainly from fast-growing bacteria, whereas xylanase and β -N-acetylglucosaminidase are enzymes produced mainly by fungi (Kandeler et al., 1999; Edwards et al., 2011). Since xylanase and β -N-acetylglucosaminidase showed little or no response to the different land use intensities, fertilization might have shifted microbial community structure towards a more bacterially dominated community.

In contrast to the above mentioned enzymes and in contrast to C_{mic} and N_{mic} , which all showed highest values at high LUI, the activity of the phosphatase enzyme was found to be highest in the intermediate LUI and not in high LUI sites. An explanation for this could be that at the fertilized high LUI sites phosphatase activities were restricted by high phosphate concentration in the soil solution acting as an inhibitor of enzyme production and expression (Spiers and McGill, 1979). A close link between phosphatase activity and P mineralization was obtained for the same sites (Alt et al., 2011).

In the different LUI's, soil enzyme production and activity might not only be regulated by the amount, but also by the quality of substrate input due to root exudation of the different plant communities. It was shown for the grassland sites of the Schwäbische Alb that increasing LUI induced a reduction of plant diversity from 94 plant species found in low LUI grasslands to only 56 plant species in high LUI sites (Weiner et al., 2011). The reduction in plant diversity was due mainly to lower species numbers of herbs and bryophytes (S. Socher, personal communication). However, differences in root exudation due to a changed plant community presumably played a minor role at the beginning of the vegetation period in April when the soil for the present study was sampled.

In general, the total pool of microbial biomass carbon and biomass nitrogen increased from low to high LUI, demonstrating that the soil microbial community made use of higher substrate and nutrient availability, as indicated by the greater C_{mic} to C_{org} ratio (data not shown) and the higher nitrate availability in the fertilized high LUI sites (Fig. 2). We assume that in intermediate, and to a greater degree in high LUI sites the turnover of organic matter by microorganisms was higher during the main vegetation period than in low LUI grasslands. It seems that this enhancing effect of fertilization on microbial biomass and enzymatic activity persisted over autumn and winter and we conclude that our results represented the long-term rather than the short-term effect of LUI. Over the long-term, positive effects of increased plant productivity in fertilized grasslands on soil organic carbon were shown to be compensated for by enhanced microbial turnover (Lu et al., 2011). This would lead to almost similar C-stocks in fertilized as compared to non-fertilized grasslands. This is underlined by the C_{org} measured for the grassland soils in the present study, which was found to be similar between the LUIs.

Spatial effects of land-use intensity

Most existing surveys of spatial patterns of soil microorganisms are based on samples collected from one single site or a single land-use type (Savin et al., 2001; Ritz et al., 2004; Franklin and Mills, 2009). This strategy does not allow for a statistical assessment of the specific effects of land-use intensity on microbial biogeography. Using replicated plots at the scale of $10 \times 10 \text{ m}^2$, we were able to test for the first time whether land-use intensity changes the horizontal biogeography of chemical and microbiological soil properties. Applying either an exponential or spherical model to each of the three LUI treatments revealed that most physical, chemical and microbiological properties showed at least a moderate spatial autocorrelation (Table 3). Our results showed that (1) the range of spatial autocorrelation differs between different soil properties, and that (2) land-use intensity affects spatial heterogeneity of soil microbial processes.

Chemical soil properties

In general, chemical soil properties showed spatial autocorrelations within the scale of the study sites ($10 \times 10 \text{ m}^2$). Since soil samples were taken on the upper 10 cm of the soils, it is probable that the patchy distribution of plant communities at the nine sites was the main cause of spatial heterogeneity of soil chemical properties at the plot scale (Ettema and Wardle 2002). The strength of spatial dependence, which is indicated by the $pSill/Sill$ ratios, varied between 13 and 100 % for different chemical soil properties and land-use intensities. High spatial dependence was indicated for C_{org} at small ranges ($pRange < 1 \text{ m}$) for low LUI. This highly local control of C_{org} at a scale of less than 1 m probably indicates the importance

of plant properties (e.g. root architecture), plant density and litter deposition for C input in the top soil of low LUI sites. Increasing land-use intensity reduced the spatial dependence of C_{org} . These differences in spatial dependence between LUIs were obvious for most chemical soil properties (Table 3).

Increasing nutrient input could have distributed nutrient availability more evenly at small-scales, thereby weakening spatial structure at this scale. Furthermore, management practices in high LUI sites increased the quantity of carbon input, as indicated by increased microbial biomass. Together with reduced plant diversity at these sites and subsequent possible changes in the homogeneity/heterogeneity of plant-derived carbon, spatial variability was further reduced. As a consequence, the local environment should have provided lower habitat diversity for the soil biota under high LUI.

Soil microbiological properties

Soil microbiological properties showed greater variation in spatial autocorrelation than soil chemical properties (Table 3). While spatial autocorrelations of soil chemical properties were mostly at the scale covered by the experimental design (< 14 m; the max. lag distance within a 10×10 m² area), microbiological properties exhibited pRanges that were either within this scale or larger. For example, microbial biomass and β -glucosidase activity showed moderate spatial dependencies and pRanges, indicating spatial dependency at larger scales than the plot scale. Since the ratio pSill/Sill of soil microbiological properties was in most cases lower than the ratio for soil chemical properties, it is clear that spatial dependence at the plot scale (10×10 m²) is less important for soil microbiological than for chemical properties. Our findings contrast in part with those of Wallenius et al. (2011) who observed that microbiological variation was mostly seen at scales smaller than 0.5 m. However, spatial distribution of microbial properties can occur at nested scales with, for example, small-scale spatial distribution of soil microorganisms controlled by spatial patterns of rhizodeposits, plot scale spatial distribution controlled by spacing between plants, and regional scale distribution controlled by the topography of the region (Ettema and Wardle, 2002).

Sheep grazing and fertilizer amendments might also contribute to seasonal variation in the biogeography of other soil microbiological properties. For example, an unexpected finding was that in comparison to low LUI, the scale of spatial dependence of most soil microbiological properties changed in the direction of reduced pRanges in high LUI. At our sites, N_{mic} and urease activity showed pRanges of less than 3.3 m in high LUIs, whereas the pRange was larger than 36 m in low LUIs. In low LUIs a higher small-scale heterogeneity was expected because of the unevenly distributed faeces of grazing sheep. This contrasts with the more evenly distributed N fertilizers in high LUI sites. However, the samples were

taken in spring, more than half a year after sheep had grazed the low LUI sites and fertilizer had been applied to the high LUI sites. During this time period differences in small scale heterogeneity could have been diminished by ongoing mineralization, leaching or denitrification of nitrogen.

Interestingly, land-use intensity did not change the spatial pattern of enzymes that are mainly related to fungi (xylanase and β -N-acetylglucosaminidase). The reduced model revealed pRanges lower than 3 m. Therefore, fungal distribution and community structure might be less affected by fertilization but instead governed by plant-soil interactions at fine spatial scales as described for fungi within a mature forest site (Morris, 1999; Burke et al., 2009).

Correlation between soil properties

Using correlation analyses including either all data or data for each LUI separately we were able to clarify the importance of specific environmental properties on abundance and function of soil microorganisms in grasslands of different land-use intensities. The amounts of C_{org} , N_t and NO_3^- in soils correlated significantly with most other properties. The correlation was even stronger when only samples from the high LUI sites were considered. This result was in agreement with other studies showing the importance of C_{org} as a microbial substrate and as habitat for microbial growth and maintenance (Anderson and Domsch, 1989; Steinweg et al., 2008; Wallenius et al., 2011). The stronger correlation of some microbiological properties with N_t than with C_{org} under high LUI may indicate that microbial life in the high LUI grassland sites profits from the higher nitrogen availability.

Soil pH is commonly regarded as an important factor influencing spatial distributions of microorganisms (Fierer and Jackson, 2006; Barnes et al., 2007), but pH effects were found to be more pronounced on bacterial than on fungal community composition (Lauber et al., 2008). In the present study, pH seemed to have less impact on microbial abundance at the different sites than C_{org} . These results might be due to the narrow range of pH values which we observed within each of the nine grassland sites and by the high buffering capacity of Rendzic Leptosols, the dominant soil type of the study sites. Other studies have also shown that small pH gradients did not alter the spatial distribution of microorganisms in soil (Ritz et al., 2004; Drenovsky et al., 2010), supporting our observations. However, we found a strong negative correlation between pH and phosphatase activity. It has been shown that phosphatase activity was mainly due to microbe- and plant-derived acid phosphatases (Wasaki et al., 2005, 2008) and we therefore conclude therefore that a decrease in pH, as observed in the intermediate LUI sites (especially AEG 4) generally increased phosphatase activity.

Xylanase and β -N-acetylglucosaminidase activities were only weakly explained by other properties, indicating that the most important habitat conditions for fungi (e.g. quantity and quality of fungal substrates) were not determined.

Conclusions

In conclusion, we have shown that our approach was useful in characterizing differences in both, spatial patterns of soil microbial communities and their activity due to land-use intensity of grasslands. We could not verify our hypothesis that high land-use intensity decreases the spatial heterogeneity; instead we found a smaller spatial autocorrelation than expected for most of the investigated properties, indicating increased habitat heterogeneity at smaller scales under high LUI. We have no explanation for this finding but possibly the timing of sampling in spring reflected long- rather than short-term effects of land-use intensity on spatial distribution of soil chemical and microbiological properties. Further studies should investigate the potential short-term effects of mowing and fertilization practices and subsequent differences in plant communities on spatial patterns of microbial properties by sampling during the active plant growth period.

However, another important result of our study was the difference in spatial ranges of chemical and microbiological properties: whereas the pRange of chemical soil properties could be explained at the plot scale, soil microbiological properties often showed larger pRanges, providing evidence of spatial heterogeneity at nested scales. Future studies are therefore recommended using nested sampling designs to accommodate multiple scales.

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8 EFFECTS OF WARMING AND DROUGHT ON POTENTIAL N₂O EMISSIONS AND DENITRIFYING BACTERIA ABUNDANCE IN GRASSLANDS WITH DIFFERENT LAND USE

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Running title

Climate change alters potential N₂O emissions in grasslands

One sentence summary: Microorganisms in grassland soils respond to climate change, showing enhanced denitrification potential after experimental warming and drought, but these effects seem to be overruled by site-specific land-use and geographic region.

Abstract

Increased warming in spring and prolonged summer drought may alter soil microbial denitrification. We measured potential denitrification activity and denitrifier marker gene abundances (*nirK*, *nirS*, *nosZ*) in grasslands soils in three geographic regions characterized by site-specific land-use (LUI) after warming in spring, at an intermediate sampling, and after summer drought. Potential denitrification was significantly increased by warming, but did not persist over the intermediate sampling. At the intermediate sampling, the relevance of grassland land-use intensity was reflected by increased potential N₂O production at sites with higher LUI. Abundances of total bacteria did not respond to experimental warming or drought treatments, displaying resilience to minor and short-term effects of climate change. In contrast, *nirS* and *nirK*-type denitrifiers were more influenced by drought in combination with LUI and pH, while the *nosZ* abundance responded to the summer drought manipulation. Land-use was a strong driver for potential denitrification as grasslands with higher LUI also had greater potentials for N₂O emissions. We conclude that both warming and drought affected the denitrifying communities and the potential denitrification in grassland soils. However these effects are overruled by regional and site-specific differences in soil chemical and physical properties which are also related to grassland land-use intensity.

Key words: climate change, microbial community, denitrification, grassland, land-use index, potential N₂O emissions, Biodiversity Exploratories.

9 FINAL CONCLUSIONS AND OUTLOOK

This thesis was conducted to investigate the influence of different types of land-use management on the abundance, activity, spatial distribution, and performance of soil microorganisms and the underlying soil biogeochemical properties in grassland ecosystems.

The study sites investigated in the first study were characterized by the same soil type and climatic conditions, and the management practices were the most likely factor explaining the different spatial distributions of biotic and abiotic properties found between the management types. The data sets were analyzed using geostatistical methods and findings were explained better when calculated at an area basis instead of expressing them per gram dry soil.

Most of the soil biogeochemical properties were defined by a higher range of spatial autocorrelation in high land-use intensity, thus indicating reduced spatial heterogeneity under these management conditions. Interestingly, the ranges of spatial autocorrelation for both AOA and AOB nitrifiers were larger in low land-use intensity than in high land-use intensity. Here, we therefore rejected the hypothesis that high land-use intensity reduces the spatial heterogeneity of habitats for soil microorganisms. Instead, the results indicated increased spatial heterogeneity at high land-use intensity. We discussed a combination of factors, including soil structure, microclimate, and oxygen status being responsible for the spatial patterns of AOA and AOB rather than the soil biogeochemical properties investigated in this study.

Geostatistical analysis and mapping of the spatial distributions further indicated coexistence of AOA and AOB nitrifiers, as well as of *narG*- and *napA*-type nitrifiers, which was further supported by significant positive correlations between the abundances of AOA and AOB, and *narG* and *napA*, respectively. Spatial analysis of *nirK*- and *nirS*-type denitrifiers, in addition with negative Spearman's correlation, indicated niche differentiation of microbes having either of the two functional genes in low land-use intensity.

Concluding the results of the first study, spatial analysis was an appropriate and useful tool to describe the distribution patterns of different microbial guilds involved in the N-cycle with respect to soil biogeochemical properties and land-use management at the plot scale. As spatial heterogeneity decreased with higher land-use intensity for biogeochemical properties, but increased for N-cycling microorganisms, other factors (abiotic and/or biotic) that were not represented in the analyses of the study were driving the microbial distribution in this study. Future research should address for example the role of plant community composition and plant diversity on the spatial distribution and activity of soil microorganisms. The rhizosphere, as a hotspot of microbial activity, is very likely to pose strong control on the

spatial distribution of N-cycling microorganisms through *e.g.* the quality, amount and seasonal variability of root rhizodeposition.

In the second study, a geostatistical model approach was used to clarify whether land-use intensity contributes to spatial variation in microbial abundance and function in grassland ecosystems. In addition to the former study, land-use intensity comprised three management levels: unfertilized pastures (low land-use intensity), fertilized mown pastures (intermediate land-use intensity) or fertilized mown meadows (high land-use intensity). The analyses targeted plot-scale spatial heterogeneity and autocorrelation of soil biogeochemical properties, microbial biomass and enzymes involved in C-, N- and P-cycling.

The measurements of the study fitted best to the geostatistical parameters when data were expressed at an area basis (m^2 in 10 cm soil depth). Geostatistical analysis revealed spatial autocorrelations of chemical soil properties lying within the maximum sampling distance of the plots, whereas a greater variation of *p-Ranges* of soil microbiological properties provided evidence of spatial heterogeneity at multiple scales. An expected decrease in small-scale spatial heterogeneity due to fertilizer amendment and mowing practices in high land-use intensity could not be confirmed for microbiological soil properties. Finding smaller spatial autocorrelations for most of the investigated properties indicated increased habitat heterogeneity at smaller scales under high land-use intensity.

To get a higher resolution of spatial processes of soil microbial activity involved in the C-, N- and P-cycle, future research needs to include assessments of the seasonal variability of plant growth and the effect of temporal peak loads of nutrients, both root exudates and detritus at the interface of rhizosphere and the detritusphere.

In this thesis, it was further investigated the effects of warming in spring and drought in summer on soil properties known to regulate denitrifier gene abundances and potential N_2O emissions in grasslands belonging to three different land-use types along a geographical gradient from Southwest to Northeast German. The study regions (*Schwäbische Alb*, *Hainich*, and *Schorfheide*) each comprised its characteristic soil types, local climate, and moreover local characteristics of land-use indices.

The potential denitrification was significantly increased by warming, but was detectable only for of short period of time. Generally, it was shown that the potential N_2O production increased with enhanced land use, which was expressed as the site-specific land-use index. Both, N_2O emissions and denitrification potential were significantly and positively related with the *nosZ* gene abundance at all samplings, demonstrating the importance of microorganisms in soils to serve as a sink for N_2O budget, as they are capable to perform the full denitrification process under favorable conditions. Overall it could be concluded that

warming induced decreasing O₂ concentrations, resulting in enhanced denitrification, thus being an indirect effect of the increased heterotrophic microbial activity. However, these effects differed between the regions and the LUIs. This outcome is commonly discussed in recent research, and due to the spatial and temporal variability of both, effects of climate change and the performance of soil microorganisms, further research in this field is recommended and necessary. There is abundant data available, obtained from field and laboratory measurements, representing manifold ecosystems, soils, environmental conditions, but also methodological advantages and biases that describe short-term and longer-term N₂O budgets at relatively small scales. Therefore, specially modeling approaches seem to be a promising tool for up-scaling these results on regional or even larger scales. This will be able to deliver a better resolution of the processes that determine the abundance, spatial distribution and function of denitrifying microorganisms that contribute to N₂O emissions into the atmosphere.

To summarize and conclude, this thesis provided novel insights into ecosystem services performed by N-cycling microorganisms in grassland soils. A new setup of geostatistical analyses on replicated grassland sites revealed that soil physico-chemical and microbial properties respond to land-use intensity at different scales. Expressing data on an area basis is appropriate to describe spatial relationships of soil biogeochemical properties and microbial characteristics at the plot scale. Spatial analysis of microbial habitat characteristics, activity, and soil microbial community abundance turned out to be a powerful tool to describe and understand links between environmental drivers and microbial abundance and activity.

Soil microbial abundances were not altered by effects of climate change simulation experiments, but treatments affected the performance of denitrifiers, resulting in increased potential N₂O emissions after warming. Land-use types receiving higher management input generally had higher potentials to N₂O trace gas emissions, while local differences in soil type and microclimate had stronger implications on the performance of the microorganisms than land-use type or experimental treatments.

Drought frequency and intensity, N-deposition by grazing animals and plant nutrient uptake by plants have recently been discussed as potential drivers of N₂O emissions from soil, underpinning the complexity of nitrogen transformations in soil and the consequences for associated N₂O emissions.

Due to the manifold interactions of processes in soils with the abiotic and biotic environment, there are almost infinite possibilities and challenges for future research in the field of soil science and soil biology. Spatial analyses of soil ecological properties usually require large numbers of samples, making traditional analytical procedures costly and time-

consuming. Recently, a promising approach to predict soil biogeochemical and microbiological properties in soils of temperate grasslands has been developed, applying midDRIFTS-based least square regression analysis (see Rasche *et al.*; list of publications). One major advantage of this spectroscopic method is the possibility to measure large sample sets, requiring minimal sample preparation, making this method cost- and time-efficient in comparison to conventional approaches. Beyond such promising new approaches to characterize the spatial variability of microbial communities and their function in soil ecosystems, future studies will have to widen the scope of analyses regarding e.g. soil structure, soil physical status, plant impact (above- and belowground), and seasonal fluctuations influencing soil properties driving the performance of soil microorganisms.

The importance of an integrated assessment of soil microbial processes was also highlighted in a publication that resulted from a close cooperation to this thesis (see Meyer *et al.* 2013, list of publications). We provided *qPCR* data from denitrifiers involved in the ecology of N-cycling communities of grassland ecosystems. To gain additional information and to close another gap of knowledge with respect to nitrogen transformations in soil, these data were additionally linked to aboveground plant biodiversity as well as to water extractable fractions of nitrogen and carbon in soil.

A change of plant community composition was observed and provided evidence of systems dominated by s-strategists in extensively managed grasslands compared to c-strategists in grasslands receiving more intensely management practices. Regarding soil microbial communities, the availability of inorganic nitrogen seemed to regulate the abundance of AOA and AOB. In contrast, the availability of dissolved organic nitrogen determined the abundance of denitrifiers (*nirS* and *nirK*). Nitrogen fixing bacteria (*nifH*) were more abundant in grasslands receiving fertilizer, giving evidence that easily available energy sources outcompete the high availability of inorganic nitrogen at these sites. Altogether, the results indicate that both, the abundance and function of microorganisms driving the nitrogen cycling in soils, might be independently regulated by different abiotic and biotic factors in response to land-use intensity and climate change.

The role of plant community composition is also known to be a determinant for the emission of trace gases from soils and may also influence whether soils act as sink or source of nitrous oxide. Moreover, the microbial community itself was shown to be a mediator of the capacity of soils to serve as a sink for N₂O (Jones *et al.*, 2014) on the one hand, but also that losses of microbial diversity as a consequence of land use and/or climate change affects nitrogen cycling in soils (Philippot *et al.*, 2013), indicating that the buffering capacity (*i.e.* functional redundancy) of soil microbial communities may be over-estimated.

In the light of decreasing above-ground biodiversity as a consequence of land use intensification, climate change and the loss of undisturbed natural soil environments, these effects do proceed in the below-ground environment as well, resulting in manifold interacting feedback mechanisms. Therefore, it still remains a challenging and complex task to unravel the mechanisms that influence the abundance, function and spatial distribution of soil microorganisms. The crucial role that they occupy for the global nitrogen cycle puts denitrifying microorganisms in grassland soils into the focus of further research. This research needs to include a diverse range of research methods, and a combination of classical soil-based analyses, gas measurements, gene abundance and gene expression analyses, spectroscopic forecasting methods and modeling approaches seems to be a promising approach for this challenge.

10 PUBLICATIONS AND PRESENTATIONS

This PhD thesis has been prepared cumulative. Parts of this PhD thesis are published, in preparation to publication, or otherwise presented as follows:

PUBLICATIONS

Daniel Keil, Pascal A. Niklaus, Lars R. von Riedmatten, Runa S. Boeddinghaus, Carsten F. Dormann, Michael Scherer-Lorenzen, Ellen Kandeler, Sven Marhan. (2015): *FEMS Microbiology Ecology*, 91, 2015, fiv066

Effects of warming and drought on potential N₂O emissions and denitrifying bacteria abundance in grasslands with different land use

Annabel Meyer, Andreas Focks, Viviane Radl, Daniel Keil, Gerhard Welzl, Ingo Schöning, Steffen Boch, Sven Marhan, Ellen Kandeler, Michael Schlöter (2013) PLoS ONE 8: e73536.

Different land use intensities in grassland ecosystems drive ecology of microbial communities involved in nitrogen turnover in soil

Frank Rasche, Sven Marhan, Doreen Berner, Daniel Keil, Ellen Kandeler and Georg Cadisch (2013). *Soil Biology & Biochemistry* 57, 504-512.

midDRIFTS-based partial least square regression analysis allows predicting microbial biomass, enzyme activities and 16S rRNA gene abundance in soils of temperate grasslands

Daniel Keil, Annabel Meyer, Doreen Berner, Christian Poll, André Schützenmeister, Hans-Peter Piepho, Anna Vlasenko, Laurent Philippot, Michael Schlöter, Ellen Kandeler, Sven Marhan (2011). *FEMS Microbiology Ecology* 77, 95-106.

Influence of land-use intensity on the spatial distribution of N-cycling microorganisms in grassland soils

Doreen Berner, Sven Marhan, Daniel Keil, Christian Poll, André Schützenmeister, Hans-Peter Piepho, Ellen Kandeler (2011). *Pedobiologia* 54, 341-351.

Land-use intensity modifies spatial distribution and function of soil microorganisms in grasslands

PRESENTATIONS

D. Keil, L. von Riedmatten, A. Vlasenko, P. A. Niklaus, E. Kandeler, S. Marhan (2011). *Effects of Climate Change on Abundance and Activity of Denitrifying Bacteria in Grassland Soils of the Schwäbische Alb*. Best poster award at the General Meeting of the DFG SPP 137 in Bad Blankenburg, Germany.

D. Keil, L. von Riedmatten, A. Vlasenko, P.A. Niklaus, E. Kandeler und S.Marhan (2011). *Influence of warming and reduced precipitation on potential greenhouse gas emissions from denitrification in different grassland land-use types*. Oral presentation, annual meeting of the German Soil Science Society, Berlin, Germany.

D. Keil, A. Meyer, D. Berner, C. Poll, A. Schützenmeister, H-P. Piepho, A. Vlasenko, L. Philippot, M. Schlöter, E. Kandeler und S. Marhan (2010). *Räumliche Heterogenität und Funktion Stickstoff umsetzender Bodenmikroorganismen in Grünlandböden der Schwäbischen Alb*. Oral presentation, meeting of the DBG-Commission III, Frauenchiemsee, Germany.

Marhan, S., Keil, D., Regan, K., Niklaus, P.A., Philippot, L., Poll, C., Kandeler, E. (2011). *The influence of climate change on N-cycling microorganisms in soil*. Poster presentation, meeting "Ecology of soil microorganisms", Prag, Czech Republic.

S.Marhan, D. Berner, D. Keil, A. Schützenmeister, H-P. Piepho, C. Poll, E. Kandeler (2010). *Spatial distribution of soil and microbiological properties in grasslands: a question of land use intensity?* Poster presentation, SOM 2010: Organic matter stabilization and ecosystem functions, Presqu'île de Giens, France.

F. Rasche, D. Berner, D. Keil, S. Becker-Fazekas, S. Marhan, G. Cadisch, and E. Kandeler (2010). *Mid-infrared spectroscopy can predict microbial characteristics of soil microbial communities in different managed grasslands and pastures*. Poster presentation, ISME 2010: 13th International Symposium of Microbial Ecology, Seattle, USA.

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