Nitrogen-15 tracing to elucidate the biodiversity-N cycle relationship in a grassland experiment

Zur Erlangung des akademischen Grades einer
DOKTORIN DER NATURWISSENSCHAFTEN (Dr. rer. nat.)
von der KIT Fakultät für
Bauingenieur-, Geo- und Umweltwissenschaften
des Karlsruher Instituts für Technologie (KIT)

genehmigte

DISSERTATION

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Tag der mündlichen Prüfung: 7. Oktober 2020
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Karlsruhe (2020)

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Acknowledgements

It's hard to put into words how much I have learnt during my PhD journey. I feel like I've grown as a scientist and as an individual. It is the people I have met, who have helped me in so many ways, that I need to thank for.

First, I would like to thank my supervisors Prof. Dr. Wolfgang Wilcke and Prof. Dr. Yvonne Oelmann for their valuable insight, direction and guidance throughout these years. Thank you for your constructive comments, sound feedback and useful discussion.

I would also like to thank all the members of Soil Science group for creating a work environment that was motivational and, perhaps more importantly, very warm and welcoming. Thanks to Sophia Leimer for always helping me with the R statistics and also helping me with the translation for the bureaucratic work during the initial phase of my PhD. Furthermore, I would like to thank Martin Kull for helping me with the setup of the laboratory analysis and Benjamin Bandowe for the constant motivation. I further like to acknowledge Prof. Dr. Moritz Lehman and Thomas Kuhn from the University of Basel for the collaboration to measure my samples for isotopic analysis and and Prof. Dr Christoph Müller from the University of Giessen for the *Ntrace* modelling. I am particularly grateful to those who contributed various plant and soil data for my results evaluation and interpretation.

I would like to acknowledge the Deutsche Forschungsgemeinschaft (DFG, FOR 1451, Wi 1601/4) and the Swiss National Science Foundation (SNSF, 200021E-131195/1) for funding this project, which received additional support from the Friedrich Schiller University Jena and the Max Planck Society. Furthermore, I thank the German Academic Exchange Service (DAAD) for the STIBET scholarship and the Bayer Foundation for the Bayer fellowship who supported me in the later stage of my PhD study.

Last but not the least, I am indebted to my family and friends especially my parents for believing in me and for their constant inspiration, understanding and support. They have always encouraged me to follow my own path and I couldn't have walked it without them. A special thanks to Puja Shrestha for the constant support, patience and encouragement.

Summary

In the recent years, biodiversity has been declining at an alarming rate mainly due to human activities. Several biodiversity studies have shown that plant diversity plays an important role in ecosystem functioning.

The objectives of my study were (i) to adapt and test two methods to determine the ^{15}N concentration in ammonium and to assess the effects of plant species richness (1- 16), functional group richness (1-4) and presence/absence of particular functional groups (legumes, grasses, tall herbs, small herbs) on nitrogen pool sizes and transformation rates in The Jena Experiment, a grassland biodiversity experiment with the help of (ii) a laboratory experiment to parameterize the ^{15}N-tracing model *Ntrace* and (iii) a ^{15}N-tracer experiment in the field. To determine the ^{15}N concentration in ammonium extracted from soil, I tested two methods: microdiffusion and hypobromite-azide. Furthermore, I conducted a ^{15}N-tracer experiment in the laboratory to determine the nitrogen exchange between five soil nitrogen pools (labile and recalcitrant organic nitrogen, dissolved ammonium and nitrate in soil solution, and exchangeable ammonium) and eight N transformations (gross N mineralization from labile and recalcitrant organic nitrogen, ammonium immobilization into labile and recalcitrant organic nitrogen, autotrophic nitrification, heterotrophic nitrification, nitrate immobilization, adsorption of ammonium) using the *Ntrace* model. Moreover, a ^{15}N pool-dilution experiment was conducted in the field to investigate if and how plant diversity affects the gross rates of N mineralization, microbial ammonium consumption and inorganic nitrogen immobilization.

My results demonstrated that (i) the two methods to measure ^{15}N isotope composition of ammonium in soil extracts worked well and showed that the microdiffusion method is best suited for high N masses, while the hypobromite-azide method is suitable for low N masses. (ii) In the microcosm experiment, gross N mineralization and autotrophic nitrification increased significantly in the presence of legumes because legumes have the ability to fix atmospheric nitrogen which resulted in higher soil nitrogen concentrations in legume-containing plots. Similarly, the presence of grasses significantly increased the soil ammonium pool, N mineralization and ammonium immobilization, likely because of enhanced microbial activity by providing large amounts of rhizodeposits through their dense root systems. (iii) In the field experiment, plant species richness showed an unexpected negative effect on gross nitrogen mineralization and microbial ammonium consumption, while the gross inorganic nitrogen immobilization did not show any relationship with plant species richness. Structural equation modeling showed that increasing plant species richness significantly decreased gross N mineralization and microbial ammonium consumption rates via increased root C/N ratios. Root C/N ratios increased, because of the replacement of legumes by small herbs with increasing species richness. The presence of legumes also increased gross N mineralization, microbial ammonium consumption and gross inorganic N immobilization rates because of improved N supply by N fixation. In the presence of small herbs, microbial NH_4^+ consumption and gross inorganic N immobilization rates increased which I attributed to their increased rhizodeposition, stimulating microbial growth.

I conclude that plant community composition is a significant control of nitrogen processes in soil which influences the nitrogen bioavailability and nitrogen leaking of grassland ecosystems into the atmosphere and surface and groundwater.

Zusammenfassung

In den letzten Jahren ist die Biodiversität in besorgniserregendem Maß zurückgegangen, hauptsächlich aufgrund menschlicher Aktivität. Mehrere Biodiversitätsstudien haben gezeigt, dass Pflanzenvielfalt eine wichtige Rolle für das Funktionieren von Ökosystemen spielt.

Die Ziele meiner Arbeit waren (i) zwei Methoden zur Analyse des ^{15}N-Gehalts von Ammonium anzupassen und zu überprüfen und den Einfluss der Pflanzenartenzahl (1-16), der Zahl der funktionellen Gruppen (1-4) und der Präsenz/Absenz einzelner funktioneller Gruppen (Leguminosen, Gräser, große Kräuter, kleine Kräuter) auf Stickstoffpools und -tranformationsraten im Jena-Experiment, einem Grünland-Biodiversitätsexperiment, mithilfe (ii) eines Laborexperimentes zur Parametrisierung des ^{15}N Tracingmodells *Ntrace* und (iii) eines Feldexperimentes zu bestimmen. Zur Bestimmung des ^{15}N-Gehaltes in aus dem Boden extrahiertem Ammonium prüfte ich zwei Methoden: die Mikrodiffusion und Hypobromit-Azid. Außerdem führte ich ein ^{15}N-Tracerexperiment im Labor durch, um den Austausch zwischen fünf Stickstoffpools im Boden (labiler und rekalzitranter organischer Stickstoff, gelöstes Ammonium und Nitrat in der Bodenlösung und austauschbares Ammonium sowie acht N-Transformationen (Brutto-N-Mineralisierung aus labilem und rekalzitrantem organischem Stickstoff, Ammoniumimmobilisierung in labilen und rekalzitranten organischen Stickstoff, autotrophe Nitrifikation, heterotrophe Nitrifikation, Nitratimmobilisierung, Adsorption von Ammonium) mithilfe des Modells *Ntrace* zu bestimmen. Im Feld wurde ein ^{15}N-Poolverdünnungsexperiment durchgeführt, um zu untersuchen, ob und wie sich die Pflanzenvielfalt auf die Bruttoraten der N-Mineralisierung, den mikrobiellen Ammoniumverbrauch und die Immobilisierung von anorganischem Stickstoff auswirkt.

(i) Die zwei Methoden zur Messung des ^{15}N-Gehalts von Ammonium funktionierten gut und zeigten, dass die Mikrodiffusion für hohe N-Massen und die Hypobromit-Azid für niedrige N-Massen am besten geeignet sind. (ii) Das Laborexperiment zeigte, dass Brutto-N-Mineralisierung- und autotrophe Nitrifikationsraten in Anwesenheit von Leguminosen erhöht waren, da Leguminosen atmosphärischen Stickstoff binden können, was zu höheren Stickstoff-Konzentrationen im Boden unter Leguminosen-haltigen Pflanzenmischungen führte. In ähnlicher Weise vergrößerte das Vorhandensein von Gräsern den Ammonium-Pool, und erhöhte die N-Mineralisierungs- und Ammoniumimmobilisierungsraten signifikant, was wahrscheinlich auf eine gesteigerte mikrobielle Aktivität zurückzuführen ist. Letztere wird vermutlich durch große Mengen an organischen Wurzeleinträgen aufgrund der dichten Gras-Wurzelsysteme stimuliert. (iii) Im Feldexperiment wirkte sich eine zunehmende Pflanzenartenzahl unerwartet negativ auf die Brutto-Stickstoffmineralisierung und den mikrobiellen Ammoniumverbrauch aus, während die Brutto-Immobilisierung von anorganischem Stickstoff keinen Zusammenhang mit der Pflanzenartenzahl aufwies. Strukturgleichungs-Modellierung zeigte, dass eine zunehmende Pflanzenartenzahl die Brutto-N-Mineralisierung und den Verbrauch von mikrobiellem Ammonium über erhöhte Wurzel-C/N-Verhältnisse signifikant verringerte. Die Wurzel-C/N-Verhältnisse nahmen zu, da mit zunehmender Artenzahl Leguminosen durch kleine Kräuter ersetzt wurden. Das Vorhandensein von Leguminosen erhöhte auch die Brutto-N-Mineralisierung, den mikrobiellen Ammoniumverbrauch und die anorganischen N-Immobilisierungsraten aufgrund der verbesserten N-Versorgung durch die Stickstoff-Fixierung. In Gegenwart kleiner Kräuter nahmen der mikrobielle NH_4^+ -Verbrauch und die anorganischen N-Immobilisierung zu, was ich auf die Stimulation der Mikroorganismen durch hohe organischen Wurzeleinträge zurückführe.

Meine Ergebnisse zeigen, dass die Zusammensetzung der Pflanzengemeinschaft Stickstoffprozesse im Boden beeinflusst, die die Bioverfügbarkeit von Stickstoff und die Freisetzung von Stickstoff in die Atmosphäre und in Oberflächen- und Grundwasser steuern.

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List of Abbreviations

AIC	Akaike Information Criteria
AMF	arbuscular mycorrhizal fungi
A_{NH4}	adsorption of ammonium
ANOVA	analysis of variance
$CaCl_2$	calcium chloride
CH_3COOH	acetic acid
CFA	continuous flow analyzer
$CuSO_4$	copper sulfate
DON	dissolved organic nitrogen
EA-IRMS	elemental analyzer-isotope ratio mass spectrometer
GFI	goodness of fit index
HCl	hydrochloric acid
H_3BO_3	boric acid
H_2SO_4	sulfuric acid
IAEA	International Atomic Energy Agency
$I_{NH4\text{-}Nlab}$	immobilization of ammonium to labile organic nitrogen
$I_{NH4\text{-}Nrec}$	immobilization of ammonium to recalcitrant organic nitrogen
I_{NO3}	immobilization of nitrate
KCl	potassium chloride
KH_2PO_4	potassium dihydrogenphosphate
K_2SO_4	potassium sulfate
$MgCl_2$	magnesium chloride
MIRR	maximum initial respiratory response
MgO	magnesium oxide
$MgSO_4$	magnesium sulfate
M_{Nlab}	mineralization of labile organic nitrogen
M_{Nrec}	mineralization of recalcitrant organic nitrogen
$MnSO_4$	manganese sulfate
$NaAsO_4$	sodium arsenite
NaN_3	sodium azide

$NaHSO_4$	ammonium hydrogen sulfate
Na_2MoO_4	sodium molybdate
NaOH	ammonium hydroxide
NH_4^+	ammonium
$NH_4^+{}_{ads}$	adsorbed ammonium
NH_4Cl	ammonium chloride
$(NH_4)_2SO_4$	ammonium sulfate
N_{lab}	labile organic nitrogen
N_2O	nitrous oxide
NO_3^-	nitrate
N_{rec}	recalcitrant organic nitrogen
O_{NH4}	oxidation of ammonium
O_{Nrec}	oxidation of recalcitrant organic nitrogen
PTFE	polytetrafluoroethylene
SE	standard error
SEM	structural equation model
TOC	total organic carbon
TDN	total dissolved nitrogen
TN	total nitrogen
USGS	United States Geological Survey
$ZnSO_4$	zinc sulfate
χ^2	chi square

1. Summarizing overview

1.1 Introduction

Biodiversity loss has become a global concern. The interest in this issue has grown from concerns about the potential ecological consequences of the loss of biodiversity caused by the increased impact of anthropogenic activities on natural and managed ecosystems (Loreau 2001, Spehn et al. 2002, Weisser et al. 2017). Biodiversity experiments have mainly reported increased community biomass productivity with increasing plant diversity (Tilman et al. 2001; Spehn et al. 2005; Marquard et al. 2009), because of complementarity effects in species-rich mixtures (Hooper and Vitousek 1998; Fargione et al. 2007; Reich et al. 2012). Complementarity effects occur when more-diverse communities increase their performance above the expected performance of monocultures through acquiring more nutrients, light and space (Hooper and Vitousek 1997; Naeem et al. 2002). Complementarity also includes the process of facilitation, for example by legumes, which increase the nutrient availability for neighboring plants via nitrogen fixation (Fargione et al. 2007).

Nitrogen (N) is the most important nutrient in regulating primary productivity in many ecosystems (Elser et al. 2007; Fay et al. 2015). No other nutrient essential for life takes as many chemical forms in soil as N which undergoes complex microbially mediated transformations that are related to the quantity and quality of soil organic matter (Wedin and Pastor 1993; Benbi and Richter 2002; Booth et al. 2005; Fornara et al. 2011; Lang et al. 2015). The quality and quantity of organic matter in grassland soils is influenced by the plant diversity responsible for differences in litterfall, root turnover, and root exudates (Allan et al. 2013; Solly et al. 2013). Plant litter varies in chemical composition; therefore, changes in plant communities could alter the production and types of organic compounds in soil, thereby controlling the composition and function of microbial communities (Zak et al. 2003). Moreover, environmental conditions, such as soil pH, soil moisture, soil temperature, and soil texture influence gross N transformations by changing microbial biomass or activity associated with substrate availability (Booth et al. 2005).

The study of N and its transformations has received a great deal of attention because of the importance of N in all ecosystems and the marked impact of human activities on the N cycle. The N transformation processes that are most important for plants are those associated with the mineralization-immobilization turnover of ammonium (NH_4^+) and nitrate (NO_3^-), because these

two inorganic N species represent the major forms of bioavailable N taken up by plants (Marschner 2012). Gross N mineralization includes the release of organic N as NH_4^+ which can also serve as a substrate for nitrification. Nitrification is another key process in which NO_3^- is produced by the autotrophic conversion of NH_4^+ to NO_3^- or the heterotrophic oxidation of organic N in soil. Next to plant uptake, nitrification represents the second largest sink of NH_4^+ (Marschner 2012). Nitrogen immobilization is the conversion of mineral N to organic N by microorganisms, which compete with plants for organic N. Gross nitrogen mineralization and nitrification rates in soil are primarily controlled by the microbial activity, as well as environmental factors, such as substrate availability, quality, and quantity, soil moisture and temperature (Booth et al. 2005). For grasslands, previous work has suggested that the nitrification to microbial immobilization ratio is an important factor controlling NO_3^- leaching (Stockdale et al. 2002).

Plant available mineral N concentrations (NH_4^+ and NO_3^-) in soil depend on the relation between mineralization (ammonification and nitrification), uptake by plants and soil organisms, N_2 fixation, denitrification, volatilization and leaching (Corre et al. 2002, Schimel and Bennett 2004). Biodiversity experiments revealed that plant diversity influences pool sizes of N in soil. It has been reported that NO_3^- concentrations in soil solution and in KCl extracts decreased with increasing plant species richness because of the complementary and more exhaustive resource use of different plant species (Hooper and Vitousek 1998; Scherer-Lorenzen et al. 2003; Oelmann et al. 2007; Leimer et al. 2014). However, Leimer et al. 2014 found that this negative relationship reversed if more than 25% of legume species were included in the mixture. The KCl-extractable NO_3^- concentrations were higher on mixtures with legumes than on mixtures without legumes (Oelmann et al. 2007). Presence of legumes increased, and presence of grasses decreased NO_3^- concentrations both in soil KCl-extracts and soil solution (Hooper and Vitousek 1998; Scherer-Lorenzen 2003; Oelmann 2007; Leimer et al. 2014). Previous studies have shown that the NH_4^+ concentrations in soil solution were frequently not detectable, therefore the effects on plant diversity and NH_4^+ concentrations were not analyzed (Hooper and Vitousek 1998; Niklaus et al. 2001; Oelmann et al. 2007; Dijkstra et al. 2007). However, the KCl-extractable NH_4^+ concentrations were higher in the presence of legumes (Hooper and Vitousek 1998). Similar to NO_3^- concentrations, dissolved organic nitrogen (DON) and total dissolved nitrogen (TDN) in soil solution showed the same effects of species richness, presence of legumes and presence of grasses (Oelmann et al. 2007).

Plant diversity also influences several N transformations processes in soil via plant uptake of N and modifications of ecosystem properties like the size and composition of the microbial community or biomass production (Hooper and Vitousek 1998; Spehn et al. 2005). Despite the importance of N cycling, it is still unclear how plant diversity affects the complex N transformation rates in soil (Fornara et al. 2011). Most studies on biodiversity-N cycle relationship have focused on net N turnover rates (Accoe et al. 2004; Fornara and Tilman 2009; Fornara et al. 2011; Rosenkranz et al. 2012; Mueller et al. 2013). However, net N turnover rates do not provide an understanding about the rates of all the individual processes involved in N cycling (Hart et al. 1994; Verchot et al. 2002; Cheng et al., 2013). Gross N transformation rates associated with individual soil N pools can provide a better elucidation of the mechanisms and processes involved in the N cycle (Bedard-Haughn et al. 2006; Cheng et al. 2014; Zhang et al. 2016). Previous biodiversity studies in grassland reported that increasing species richness increased net N mineralization rates (Rosenkranz et al. 2012; Mueller et al. 2013), as well as net nitrification rates (Scherer-Lorenzen et al. 2003; Mueller et al. 2013). There are currently only few studies that have evaluated the relationship between biodiversity and gross N transformation rates. Zak et al. (2003) and West et al. (2006) reported a positive biodiversity-gross N mineralization relationship in nutrient-poor, sandy soils from Minnesota, USA in laboratory experiments under optimum conditions for microbial activity. To understand the consequences of biodiversity loss including possible changes in plant N availability and gaseous N leaking to the atmosphere or nitrate leaching to surface and groundwaters, a more profound understanding of the biodiversity – N cycle relationship is necessary (Sutton et al. 2011).

In addition to plant species richness, certain plant functional groups can also have large effects on N cycling in grassland ecosystems (Scherer-Lorenzen et al. 2003; Oelmann et al. 2007; Dybzinski et al. 2008; Fornara and Tilman 2009; Fornara et al. 2011; Leimer et al. 2015). Legumes can fix atmospheric nitrogen by their symbiosis with Rhizobia and convert it to plant-available inorganic forms. There is abundant evidence that such fixation can facilitate the input of substantial amounts of N to soil, which also increases N availability for other species (Mulder et al. 2002; Spehn et al. 2002; Spehn et al. 2005). Besides legumes, grasses influenced N pools and transformations in soil. Nitrate leaching might decrease in the presence of grasses due to their extensive rooting system (Hooper and Vitousek 1998). Oelmann et al. (2007) reported that the presence of grasses decreased mineral N pools in soil compared to plant communities

without grass species because of their dense and extensive root system. This extensive rooting system is efficient in taking up soil N and thus can reduce mineral N pools in soil (Oelmann et al. 2007).

The analysis of the N stable isotope composition of NH_4^+ and NO_3^- is increasingly used in biogeochemical and ecological studies to better understand processes involved in N cycling (Sebilo et al. 2004). In order to measure ^{15}N of NH_4^+ and NO_3^-, the NH_4^+ and NO_3^- should be separated or converted to N species (N_2 or N_2O) that can be introduced into a mass spectrometer (Homes et al. 1998). The most frequently used methods to determine the N isotopic composition of NH_4^+ include the microdiffusion (Brookes et al. 1989; Stark and Hart 1996) and hypobromite-azide methods (Zhang et al. 2007). The methods used to analyze ^{15}N of NO_3^- include the microdiffusion (Sørensen and Jensen 1991, Stark and Hart 1996), bacterial denitrification (Sigman et al. 2001) and chemical denitrification (McIlvin and Altabet 2005, Lachouani et al. 2010).

Nitrogen-15 pool dilution is an extensively used technique to quantify gross rates of mineralization, nitrification, and microbial immobilization. The principle of this technique is based on labeling of one pool with ^{15}N and the subsequent monitoring of the dilution of this pool (Hart et al. 1994, Booth et al. 2005). However, this technique only allows to quantify gross N transformation rates over short period of time (up to 24 h), it is not possible to get reliable results over longer time periods or to estimate other important mechanisms. To overcome this obstacle, a numerical data analysis based on a ^{15}N tracing model (*Ntrace*) was used to quantify gross N transformation rates in a laboratory incubation experiment. The advantage of using the *Ntrace* model is that it quantifies several simultaneously occurring gross N transformation rates (Müller et al. 2007).

The overall objective of my study was to elucidate the biodiversity – N cycle relationship by considering as many pools and gross and net N turnover rates as possible to go beyond the existing knowledge restricted to a few selected pools and processes. To reach this objective, I used ^{15}N tracer experiments, which also required to adapt and improve existing measurement methods of ^{15}N concentrations in NH_4^+. In detail, I followed three objectives:

1) To adapt and test two methods of analyzing ^{15}N isotopic composition of NH_4^+ in soil extracts **(Chapter 2)**

2) To apply the *Ntrace* model, a complex N turnover model considering five soil N pools and eight N transformation processes, to data obtained from laboratory incubation experiment in microcosms without plants and to evaluate the legacy effects of plant community composition (species richness, functional group richness, presence/absence of four functional groups - legumes, grasses, tall herbs, and small herbs) on the N pools sizes and gross N transformation rates in grassland soils **(Chapter 3)**

3) To investigate in a ^{15}N pool-dilution experiment in the field if plant species richness, functional group richness and presence/absence of individual functional groups affect the rates of gross N mineralization, microbial NH_4^+ consumption and gross N immobilization and to determine the underlying controls responsible for the potential relationships **(Chapter 4)**

1.2 Materials and methods

1.2.1 Study site

A long-term grassland diversity experiment "the Jena Experiment" (www.the-jena-experiment.de) was established in 2002 (Roscher et al. 2004; Weisser et al. 2017). The experimental site is located on the floodplain of the river Saale in Jena, Germany (50°55' N, 11°35' E; 130 m above sea level). The site had been used as arable land for at least 40 years before the establishment of the Jena Experiment. Mean annual air temperature is 9.9°C, and mean annual precipitation amounts to 610 mm (1980-2010, Hoffmann et al. 2014). The soil at the site is classified as Eutric Fluvisol developed from 2-m thick loamy fluvial sediments (IUSS Working Group WRB, 2014). The soil texture ranges from sandy loam close to the river to silty loam with increasing distance from the river. The experimental site is mown twice and weeded three times a year to maintain the designed diversity levels. The biomass is removed after mowing/weeding. A major aim of its establishment is to explore the effect of biodiversity on nutrient cycling and trophic interactions.

A detailed description of the experimental design is provided in Roscher et al. (2004). The main experiment consists of 82 plots (20 m × 20 m) in four blocks with different levels of plant species richness (1, 2, 4, 8, 16, and 60) and 1-4 functional groups (grasses, legumes, small herbs,

and tall herbs). The mixtures were randomly drawn from a pool of 60 species representing typical Central European mesophilic grasslands. Each level of species richness was replicated on 16 plots except for the 16 and 60 species richness levels, which were only replicated on 14 and 4 plots, respectively. For the microcosm experiment **(Chapter 3)**, I included one block (Block 2) with 19 plots. The field experiment **(Chapter 4)** was conducted on 78 plots of all four blocks. The 60-species mixtures were excluded in my study, because of their low number of replicates.

Fig. 1.1: Aerial view of the Jena Experiment (Photo courtesy: Alexandra Weigelt)

1.2.2 Testing of two methods for ^{15}N-NH_4^+ analysis

I tested and adapted two methods (i) microdiffusion and (ii) hypobromite-azide to measure ^{15}N of NH_4^+ in KCl extracts and soil solutions. The microdiffusion method is based on releasing NH_4^+ from soil extracts in the form of ammonia (NH_3) by increasing the pH to > 9.5 with magnesium oxide (MgO). The released NH_3 is then collected on acidified filter discs enclosed in a polytetrafluroethylene (PTFE) envelope, where it is again protonated to NH_4^+ (Stark and Hart 1996). The hypobromite-azide method is based on conversion of NH_4^+ to N_2O. First, NH_4^+ is oxidized to nitrite (NO_2^-) using BrO^-. Using a sodium azide buffer solution, NO_2^- is then converted to N_2O under acidic conditions (Zhang et al. 2007).

1.2.3 ^{15}N tracing experiment in microcosms in the laboratory

Soil samples were collected from Block 2 of the experimental site in October 2014. Approximately 400 g of field-fresh soil was sampled from each plot by combining 15 soil cores (Ø=1 cm, depth=15 cm). The soil samples were sieved (< 2 mm) in field-fresh state, and from each soil sample three replicates of 100 g of soil were produced. Soil sample replicates were amended with ^{15}N NH_4^+, NO_3^- or both (98 at%). After the ^{15}N-label addition, samples were mixed thoroughly to ensure a homogeneous ^{15}N distribution and were placed in incubation vessels with ceramic filter (pore diameter of 0.4 µm). Fig. 1.2 A shows the incubation vessels where I put soil samples and inserted glass wool above and below the soil samples to prevent dispersion during rinsing. Inside the grey box shown in Fig. 1.2 A, 200 ml measuring jars/containers can be seen (Fig. 1.2 B) where the soil solution is collected through the ceramic filter after applying a vacuum. The vacuum is applied through the small valve that is visible on the right side of the Fig. 1.2 A and on the downward side of the Fig. 1.2 B. Fig. 1.2 C is the schematic representation of incubation vessels shown in Fig. 1.2 A, B. Finally, all the incubation vessels containing the soil samples were sealed with rubber stoppers and incubated for 16 days at a constant temperature of 20 ± 1 °C (Fig. 1.2). The soil samples were aerated by removing the rubber stoppers for one hour each day to maintain aerobic conditions inside the incubation vessels. Soil samples were extracted by percolation with 100 mL of a N-free nutrient solution (4 mM $CaCl_2$, 2 mM KH_2PO_4, 1 mM $MgCl_2$, 1 mM K_2SO_4, 1 mM $MgSO_4$, 25 µM H_3BO_3, 2 µM $MnSO_4$, 2 µM $ZnSO_4$, 0.5 µM $CuSO_4$ and 0.5 µM Na_2MoO_4; Nadelhoffer 1990) 12 hours and 2, 4, 9 and 16 days after the ^{15}N application. The nutrient concentrations were adjusted to optimize conditions for soil microorganisms (Nadelhoffer 1990). Percolation leaching was facilitated by applying a vacuum of 20 kPa for half an hour. To reduce the analytical load, samples of Days 4 and 9 were combined to yield a single composite sample **(Chapter 3)**.

Concentrations of NH_4-N and NO_3-N in the extracts were measured with a continuous flow analyzer (SAN++, Skalar, Breda, The Netherlands). The ^{15}N isotopic compositions of NO_3^- were determined by using the bacterial denitrification method, in which *Pseudomonas aureofaciens* is used to convert NO_3^- to N_2O, followed by isotopic analysis (Sigman et al. 2001,

McIlvin and Casciotti 2011). The isotope ratios of the N_2O gas were analyzed with a Gas-Bench II pre-concentration unit interfaced with Delta V Plus isotope ratio mass spectrometer (Thermo Fischer Scientific, Bremen, Germany). The N isotope ratios in NH_4^+ were determined using the hypobromite-azide method, in which NH_4^+ is first converted to NO_2^-, and further to N_2O by reduction with azide (Zhang et al. 2007). The N_2O is then purified and analyzed as described above for NO_3^--derived N_2O **(Chapter 3)**.

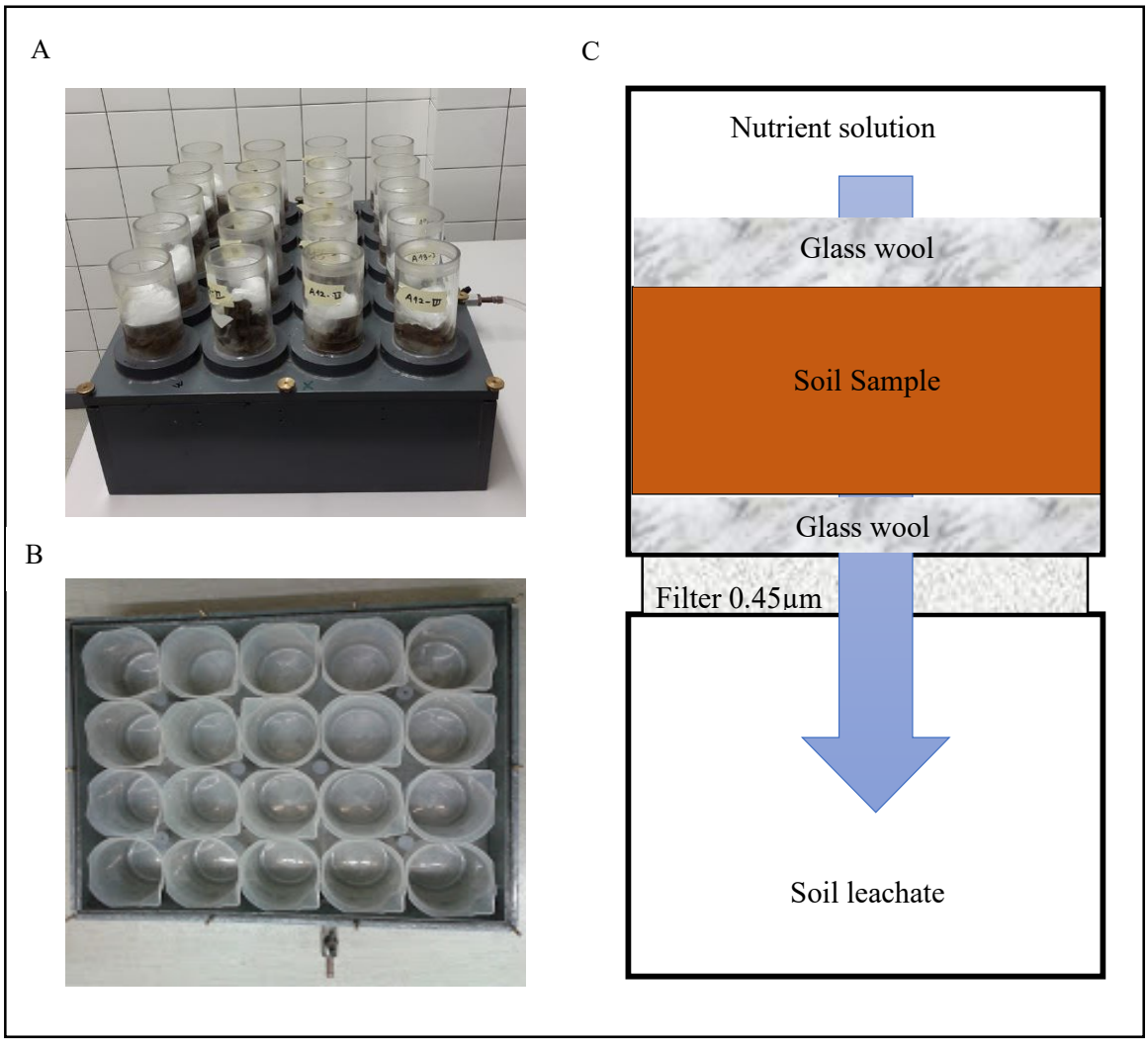

Fig. 1.2: Pictures (A, B) and schematic diagram (C) showing the incubation set-up used in the laboratory incubation experiment.

1.2.4 Isotope pool-dilution experiment in the field

The isotope pool-dilution method (Davidson et al. 1991) was conducted in the field to determine gross N mineralization, microbial NH_4^+ consumption and gross inorganic N immobilization rates in soil. The soil NH_4^+ pool was labeled with ^{15}N-NH_4Cl at 98 at% excess.

The ^{15}N enrichment of the NH_4^+ pool is diluted and decreased because the unlabeled N from the organic pool gets mineralized to NH_4^+ by microorganisms.

The field incubation experiment was carried out in April 2011 in all four blocks of the study site. Two pairs of stainless steel cores (Ø = 56 mm, h = 41 mm, V = 100 cm^3) were taken from the first 5 cm of the soil of each plot (one pair for each time step, t1 and t2), closed at the bottom side with a polyethylene lid to prevent leaching losses and immediately reburied. A disturbed soil sample was taken to determine the natural ^{15}N abundance and mineral N concentrations on each plot before the soil samples were enriched with ^{15}N. The soil samples in the cores were labeled with a NH_4Cl solution (5 mg L^{-1} N, 98 at% ^{15}N) using a high-precision, digital dispenser (Brand, Wertheim, Germany) coupled to a side-port needle, which injected the solution horizontally to ensure a homogeneous distribution of the 5-mL label within the cores. For every core, the injections were uniformly distributed at five points, each point receiving 1 mL of the tracer solution. In total, 25 µg N (98 at% ^{15}N) were added to each core.

One pair of the soil cores was removed from the soil after 15 minutes (t1) to account for N fixation by illites and calculate the ^{15}N tracer recoveries. Then, the soil samples were shaken with 1 M KCl solution for one hour to extract NH_4^+ and NO_3^-. The same procedure was followed for the remaining soil cores that were collected after 24 hours (t2). The extracts were immediately frozen at –20 °C and transported in frozen state to the laboratory for further chemical analyses.

The concentrations of NH_4-N and NO_3-N in the soil extracts were measured by high-resolution colorimetric detection using a continuous flow analyzer (CFA Autoanalyzer 3 HR, Seal Analytical GmbH, Norderstedt, Germany). I used the microdiffusion method (Stark and Hart 1996) to determine the $^{15}N/^{14}N$ isotope ratios of NH_4^+ in the soil extracts. In the microdiffusion method, NH_4^+ is volatilized as NH_3 by increasing the pH to > 9.5 with MgO. The released NH_3 was then collected on an acidified (2.5 M $NaHSO_4$) filter disk enclosed in a polytetrafluoroethylene (PTFE) envelope, where it reacted back to NH_4^+. The N isotope ratios were determined with an Elemental Analyzer (EA 1110, Carlo Erba Instruments, Milan, Italy) coupled to an isotope-ratio mass spectrometer (MAT Delta Plus, Thermo Finnigan, Bremen, Germany) at the Stable Isotope Center, University of Göttingen **(Chapter 4)**.

1.2.5 Soil and plant community properties

For the interpretation of my results in Chapters 3 and 4, I included data of soil and plant variables from other groups working in The Jena Experiment, the same site as this study.

For the analysis of aboveground biomass, plants were clipped at 3 cm above ground level within the harvesting area of two replicate 20 cm × 50 cm subplots per plot. Plant material was sorted into sown species, weeds, and dead aboveground biomass. Biomass of each sown species was determined after drying at 70°C for at least 48 h (Weigelt et al. 2010). For shoot C/N ratio analysis, all the plant material from one plot was pooled together to obtain a representative value for the plant community of the respective plot. A small subsample of this material was milled to fine powder using a ball mill (MM 400, Retsch GmbH, Haan, Germany) and up to 5 mg from each plot was used for C and N analysis (Flash EA 112, Thermo Fisher, Milan, Italy).

Community roots were collected per plot for the root C/N ratio analysis. Two cuboid soil cores from 20 cm × 10 cm to 40 cm × 15 cm with a depth of 20 cm were excavated and washed. To reduce disturbance to the experimental plots, the sampling sizes of the soil cores were varied to collect enough root material. Sampling depth was 20 cm throughout and covered the main rooting horizon where on average 90% of community standing root biomass in the Jena Experiment plots can be found. Roots were collected, cleaned, and sorted to fine (< 2 mm) and coarse roots after washing. Fine roots were oven-dried at 65°C and ground with a ball mill (MM 400, Retsch GmbH, Germany) and analyzed for total C and N concentrations using an elemental analyzer (Flash 2000, ThermoFisher Scientific Inc, Waltham, MA, USA).

To determine the concentrations of organic C and total N in soil, five soil samples per plot (0-5 cm) were taken. All replicates were combined and homogenized. Soil samples were dried at 40 °C and sieved (< 2 mm). The dried samples were ground using a ball mill. An aliquot of these samples was analyzed for total C and N concentrations by an elemental analyzer (vario Max CN, Elementar Analysensysteme GmbH, Langenselbold, Germany). Inorganic C concentrations were determined by elemental analysis after burning the organic carbon at 450 °C in a muffle furnace. Organic C concentrations were calculated by subtracting inorganic C concentrations from total C concentrations. The same is true for calculating organic N concentrations.

For the measurement of soil microbial biomass and basal respiration, soil samples were taken with a steel corer (5 cores per plot, depth 5 cm, diameter 5 cm) and sieved. Microbial biomass C and microbial respiration was measured using an O_2 micro-compensation apparatus (Scheu 1992). O_2 consumption of soil microorganisms in 5 g of fresh soil was measured at 22°C over a period of 24 h. Basal respiration [µl O_2 g^{-1} dry soil h^{-1}] was calculated as the mean of the O_2 consumption rates determined between 14 to 24 hours after the start of the measurements. Substrate-induced respiration was calculated from the respiratory response to D-glucose for 10 h at 22°C (Anderson and Domsch 1978). Glucose was added according to preliminary studies to saturate the catabolic enzymes of microorganisms (4 mg g^{-1} dry weight solved in 400 µL deionized water). The mean of the lowest three readings of O_2-consumption values within the first 10 h was taken as maximum initial respiratory response (MIRR; [µL O_2 g^{-1} dry soil h^{-1}]) and microbial biomass (µg C g^{-1} dry soil) was calculated as 38 × MIRR (maximum initial respiratory response) (Eisenhauer et al. 2010).

The microbial C/N ratio of 38 plots (Blocks 1 and 2 only) was determined from the data of microbial biomass C and N, which was measured using chloroform fumigation extraction. Two samples of 7 g soil were taken from each plot, one was fumigated with chloroform vapor for 24 h and the other was not fumigated. Both, the fumigated and non-fumigated samples were extracted with 40 mL 0.5 M K_2SO_4 by shaking for 30 minutes. Total C and N concentrations in the extracts were analyzed by dry combustion in a DIMA-TOC 100 Analyzer (Dimatec, Essen, Germany). Microbial biomass C was calculated as (total C in fumigated soil – total C in non-fumigated soil)/0.45 (Wu et al. 1990). Likewise, microbial biomass N was calculated as (total N in fumigated soil – total N in non-fumigated soil)/0.54 (Brookes and Landman 1985).

1.2.6 Quantification of gross N transformation rates

The rates of eight gross N transformations were determined by integrating the experimental data i.e. pool sizes and ^{15}N enrichment in various N pools with time in the *Ntrace* model (Müller et al. 2007). The measured NH_4^+ and NO_3^- concentrations and ^{15}N enrichment values were supplied to the model and gross N transformation rates were calculated using zero-order or first-order kinetics. The best fit between modeled and observed data was determined based on Akaike Information Criterion (AIC) by stepwise modification of the parameters included in the optimization routine and their respective kinetic settings (Table 3.1). Based on the kinetic settings and the final parameters, gross N transformation rates were calculated by integrating

the rates over the 16-day period divided by the total time. The *Ntrace* model was programmed in the software MatLab 7.9 (The MathWorks Inc., Natick, MA, U.S.A.) and the ^{15}N tracing model that was separately set up in Simulink 7.4 (The MathWorks Inc.) **(Chapter 3)**.

To evaluate the field experiment, I used analytical equations to calculate the rates of gross N mineralization, microbial NH_4^+ consumption, gross inorganic N immobilization, net N mineralization and its components net ammonification and net nitrification using Eqs. 1 to 6, respectively. Eqs. 1-4 and 6 are from Hart et al. (1994) and Eq. 5 is from Rosenkranz et al. (2012) **(Chapter 4)**.

$$m = \frac{[NH_4^+]_{t1} - [NH_4^+]_{t2}}{t} * \frac{\log\left(\frac{APE_{t1}}{APE_{t2}}\right)}{\log\left(\frac{[NH_4^+]_{t1}}{[NH_4^+]_{t2}}\right)} \quad \text{Eq. 1}$$

$$c = m - \frac{[NH_4^+]_{t2} - [NH_4^+]_{t1}}{t} \quad \text{Eq. 2}$$

$$i = m - nm \quad \text{Eq. 3}$$

$$nm = \frac{[NH_4^+ + NO_3^-]_{t2} - [NH_4^+ + NO_3^-]_{t1}}{t} \quad \text{Eq. 4}$$

$$na = \frac{[NH_4^+]_{t2} - [NH_4^+]_{t1}}{t} \quad \text{Eq. 5}$$

$$nn = \frac{[NO_3^-]_{t2} - [NO_3^-]_{t1}}{t} \quad \text{Eq. 6}$$

where m = gross N mineralization rate [µg N (g dry soil)$^{-1}$ day^{-1}]

c = microbial NH_4^+ consumption rate [µg N (g dry soil)$^{-1}$ day^{-1}]

i = gross inorganic N immobilization rate [µg N (g dry soil)$^{-1}$ day^{-1}]

nm = net N mineralization rate [µg N (g dry soil)$^{-1}$ day^{-1}]

na = net ammonification rate ([µg N (g dry soil)$^{-1}$ day^{-1}]

nn = net nitrification [µg N (g dry soil)$^{-1}$ day^{-1}]

$[NH_4^+]_{t1}$ = KCl-extractable NH_4^+ concentration at t1 [µg N (g dry soil)$^{-1}$]

$[NH_4^+]_{t2}$ = KCl-extractable NH_4^+ concentration at t2 [µg N (g dry soil)$^{-1}$]

APE_{t1} = at% ^{15}N excess of the NH_4^+ pool at t1

APE_{t2} = at% ^{15}N excess of the NH_4^+ pool at t2

t = time difference between t1 and t2 [day]

1.2.7 Statistical analysis

To test for the effect of response variables with time (day), repeated measures and sequential ANOVA (type I sum of squares) were performed with plant species richness, functional group richness, and presence/absence of each functional group as between-subject factors and time (day) as the within-subject factor **(Chapter 3)**. A hierarchical ANOVA (type I sum of squares) was used to test for the effects of plant species richness and functional group composition on studied gross and net transformation rates **(Chapter 3 and 4)**.

Lilliefors normality test and histograms were used to check for the normal distribution of residuals. The residuals vs. fitted and Q-Q plots were also used to check the assumption of homoscedasticity and normality of the residuals **(Chapter 3 and 4)**. NH_4^+ and NO_3^- pools were log-transformed; M_{Nlab} and O_{NH4} were square root-transformed; and $I_{NH4\text{-}Nlab}$ was log-transformed to improve normal distribution of the residuals **(Chapter 3)**. Gross N mineralization and microbial NH_4^+ consumption rates were square root-transformed; and net nitrification rates were box-cox power transformed ($\lambda = 1.1$) after removing the outliers to approximate normal distribution. For net N mineralization and net nitrification data, extreme outliers were removed if they deviated by more than two standard deviations from the mean (6 outliers removed from each net rates). The ANOVA was performed with block, plant species richness and presence/absence of each functional group as explanatory variables to analyze the effect of plant species richness and presence/absence of each functional group on mineral N pools and gross N transformations. The functional groups were fitted in the following order: legumes, grasses, tall herbs, and small herbs **(Chapter 3 and 4)**. The lab incubation experiment was carried out only in Block 2, therefore the block effect was removed from the ANOVA **(Chapter 3)**. A separate model was set up to test the effect of functional group richness on N pools and gross N transformation rates. The interactions between plant species richness and presence/absence of functional groups were not significant and therefore were not included in the final models. Correlations between the selected variables were analyzed using Pearson's correlations test. All the statistical analyses were conducted in R studio (R Studio, Version 1.1.456, R Studio Inc., Boston, MA USA) with the free statistical software R 3.5.1 (R Core

Team 2016). The ANOVAs were performed with the function *aov()* and Pearson's correlation with the function *cor.test()* **(Chapter 3 and 4)**.

To explain the species richness and functional groups effects that were detected in the ANOVAs, I first ran Pearson correlations between all potential explaining variables and the three considered gross N turnover rates - gross N mineralization, microbial NH_4^+ consumption and gross inorganic N immobilization and then applied Structural Equation Modeling (SEM). According to the ANOVAs, plant species richness, legumes and small herbs were included as the exogenous variables in the SEM. Since gross inorganic N immobilization was not significantly related with species or functional group richness, gross N mineralization and microbial NH_4^+ consumption rates were only considered in the SEM. The result of SEM did not show an adequate model fit (Fig. S4.1, Table S4.2) even after including all the potential variables (total organic carbon, aboveground and belowground community biomass, soil moisture, root C/N, microbial biomass. Therefore, the potentially mediating variables in the SEM were chosen on the basis of literature knowledge and the results of Pearson's correlations (Table S4.1). I included root C/N ratio and microbial biomass C as potential mediators of the effect of plant species richness and functional groups (legumes, small herbs) on gross N mineralization and microbial NH_4^+ consumption rates. Furthermore, I included a path between gross N mineralization and microbial NH_4^+ consumption rates to determine if microbial NH_4^+ processing depends on the amount of NH_4^+ produced. Based on the p values, the non-significant paths in the SEMs were removed from the final model. I used the χ^2 test (> 0.05), P value (> 0.05), goodness of fit index (GFI > 0.9), comparative fit index (CFI > 0.9) and normed fit index (NFI > 0.9) to evaluate the model fit (Tables S2-S4). SEM was conducted using the R package "lavaan" (Rosseel 2012) **(Chapter 4)**.

1.3 Results and discussion

1.3.1 Testing two methods to determine the nitrogen isotopic composition of ammonium in soil extracts (Chapter 2)

I successfully established two different chemical methods, namely the microdiffusion and hypobromite-azide methods to determine the ^{15}N-enriched N-isotopic composition of NH_4^+ in soil extracts. The microdiffusion method involves the release of NH_4^+ from solution as ammonia (NH_3) under alkaline conditions produced by using magnesium oxide. The NH_3 is trapped into an acidified filter disc as NH_4^+ and then ^{15}N isotopic composition of NH_4^+ was analyzed in an Elemental Analyzer-Isotope Ratio Mass Spectrometer This method is the most

commonly used one. However, it requires a large sample volume (50 mL) and comparatively high amount of dissolved NH_4^+ (~20 µg N). The recovery for this method was 98.3% (n = 8, SD = ±2.6%). In the hypobromite-azide method, NH_4^+ is first oxidized to NO_2^- by hypobromite and then to N_2O using a sodium azide and acetic acid buffer solution. The produced gaseous N_2O is then analyzed for its N isotopic composition with a Gas Bench-Isotope Ratio Mass Spectrometer. The recovery was 99.6% (n = 8, SD = ±2.8%). The latter method is ideal for for the samples with low NH_4^+ content (~0.2 µg N) and sample volume (5 mL). It requires less time for preparation than the microdiffusion method.

1.3.2 The biodiversity-N cycle relationship: A ^{15}N tracer experiment with soil from plant mixtures of varying diversity to model N pool sizes and transformation rates (Chapter 3)

To evaluate the effects of plant diversity on five N pools and eight N transformation rates, I conducted a ^{15}N tracing experiment in laboratory microcosms with field-fresh soil samples from a grassland biodiversity experiment. The increase in the pool sizes of the initial labile and recalcitrant organic matter with increasing species richness can be attributed to the positive effect of increasing species richness on organic matter and total N accumulation at the study site as a consequence of the positive species richness-biomass production relationship. In the presence of legumes, gross N mineralization and autotrophic nitrification increased significantly because of higher soil N concentrations in legume-containing plots and high microbial activity. Similarly, the presence of grasses significantly increased the soil NH_4^+ pool, gross N mineralization, and NH_4^+ immobilization, likely because of enhanced microbial biomass and activity by providing large amounts of rhizodeposits through their dense root systems. The increased microbial activity accelerated the decomposition of soil organic matter, which is also reflected by the positive effect of grasses on gross N mineralization rates. Furthermore, this study revealed that heterotrophic nitrification of organic N is an important process of NO_3^- production in the studied grassland soils, because heterotrophic nitrification rates were similar to the rates of autotrophic nitrification and also because heterotrophic nitrification is the other direct way of producing mineral N from organic N. In our experiment, previously reported plant species richness effects on the N cycle, observed in a larger-scale field experiment within the Jena Experiment, were not seen. However, specific plant functional groups had a significant positive impact on the N cycling in the incubated soils.

1.3.3 Plant diversity influenced gross nitrogen mineralization, microbial ammonium consumption and gross inorganic N immobilization in a grassland experiment (Chapter 4)

I investigated whether and how plant diversity affects gross N mineralization, microbial ammonium (NH_4^+) consumption and gross inorganic N immobilization in grasslands via isotopic pool dilution. The gross N mineralization and microbial NH_4^+ consumption rates decreased with increasing species richness, while the gross inorganic N immobilization rate was not related with species richness. Structural equation modeling (SEM) showed that increasing plant species richness significantly decreased gross N mineralization and microbial NH_4^+ consumption rates via increased root C/N ratios. Root C/N ratios increased, because of the replacement of legumes (low C/N ratios) by small herbs (high C/N ratios) and because of increasing competition for light which resulted in a higher shoot height associated with lower C/N ratios of the above than belowground biomass, because of an increased N-use efficiency. However, in the SEM remained an unexplained direct negative path from species richness to two of the three studied gross N turnover rates. Therefore, there must be additional, still unidentified processes behind the species richness effect potentially including changed microbial community composition. The presence of legumes increased gross N mineralization, microbial NH_4^+ consumption, and gross inorganic N immobilization rates likely because of improved N supply by N fixation. The positive effect of small herbs on microbial NH_4^+ consumption and gross inorganic N immobilization could be attributed to their increased rhizodeposition, stimulating microbial growth.

1.3.4 Do laboratory and field experiments reveal the same relationships between biodiversity and components of the N cycle?

I determined gross N transformation rates in a laboratory incubation experiment using the N-cycle model *Ntrace* (Müller et al. 2007) **(Chapter 3)** and in a field incubation experiment using the analytical equations (Hart et al. 1994; Rosenkranz et al. 2012) **(Chapter 4)**. Although the focus of my study was on the gross N turnover rates, I additionally calculated the rates of net N mineralization and its components net ammonification and net nitrification and analyzed their relationships with plant diversity in the field experiment. Furthermore, I determined the effects of plant diversity on KCl-extractable mineral N (NH_4^+ and NO_3^-) concentrations measured shortly before the ^{15}N pool dilution experiment in the field.

Tables 1.1 and 1.2 show an overview of the mineral N pools and all measured N transformation rates in the laboratory incubation and field incubation experiments, respectively. The range of total N mineralization rates (M_{Nlab} + M_{Nrec}) determined in the laboratory experiment (0.40 - 4.07 µg N g^{-1} d^{-1}) was comparable to the gross N mineralization rates calculated in the field experiment (0.04 - 6.20 µg N g $soil^{-1}$ d^{-1}). Similarly, the total inorganic N immobilization rates ($I_{NH4\text{-}Nlab}$ + $I_{NH4\text{-}Nrec}$ + I_{NO3}) calculated in the laboratory experiment (1.07 - 7.30 µg N g^{-1} d^{-1}) were in a comparable range to the rates of gross inorganic N immobilization calculated in the field experiment (-3.27 - 8.51 µg N g^{-1} d^{-1}).

Table 1.1: Overview of the effects plant species richness (SR), functional group richness (FGR), presence (+)/absence (-) of legumes (Leg), grasses (Gr), tall herbs (Th) and small herbs (Sh) on mineral N pools gross N transformation rates in a laboratory incubation experiment. Arrows indicate positive (↑) or negative (↓) effects. Significance codes: **p<0.01, *p<0.05, •p<0.1

N pools and transformation rates	SR	FGR	Leg	Gr	Th	Sh
Dissolved NH_4^+ pool	NS	NS	NS	** ↑	NS	NS
Dissolved NO_3^- pool	NS	NS	NS	NS	NS	NS
Labile organic N pool (N_{lab})	** ↑	NS	NS	NS	NS	NS
Recalcitrant organic N pool (N_{rec})	** ↑	Ns	NS	NS	NS	NS
Adsorption of NH_4^+ to cation exchangers	NS	NS	NS	NS	NS	NS
Mineralization of N_{lab} to NH_4^+	NS	• ↑	* ↑	* ↑	• ↓	NS
Mineralization of N_{rec} to NH_4^+	NS	NS	NS	NS	NS	NS
Immobilization of NH_4^+ to N_{lab}	NS	NS	NS	** ↑	NS	NS
Immobilization of NH_4^+ to N_{rec}	NS	NS	NS	NS	NS	NS
Oxidation of N_{rec} to NO_3^-	NS	NS	NS	NS	NS	NS
Immobilization of NO_3^-	NS	NS	NS	NS	NS	NS
Oxidation of NH_4^+ to NO_3^-	NS	NS	* ↑	NS	NS	NS

NS: not significant

In the laboratory experiment, the pool sizes of dissolved mineral N remained nearly constant (Fig. 3.2), which showed that the net N transformation rates were similar for all plots. In the field experiment, plant species richness was not related to net N mineralization, net ammonification and net nitrification (Table 1.2). Functional group richness did not have any significant effects on net N mineralization and net nitrification but had a marginally significant

negative relationship with net ammonification (Table 1.2, Fig. 1.5a). Previous studies have mainly reported positive effects of plant species richness and the presence of legumes on net turnover rates (Rosenkranz et al. 2012; Mueller et al. 2013). A study conducted by Rosenkranz et al. (2012) in the year 2006 at the same experimental site as my study stated that the positive relationship between plant species richness and net ammonification rates was related to topsoil water content. However, in 2011 Fischer et al. (2018) found reduced water contents with increased plant species richness which was attributed to the positive effects of soil aggregation that increased soil infiltration rates. Therefore, I assumed that the decreasing soil water contents in higher species-mixture might help in explaining the negative effects on net ammonification rates found in my study. Furthermore, an unexpected significant negative effect of the presence of legumes was found on net ammonification rates (Table 1.2, Fig. 1.5b). This may be attributable to the positive effect of legumes on microbial NH_4^+ consumption (Table 4.2) and gross inorganic N immobilization (Table 4.3), which resulted in a smaller leftover of NH_4^+ in mixtures with legumes than without legumes. As expected, net nitrification correlated significantly positively with soil KCl-extractable NO_3^- concentrations ($r = 0.37$, $p = 0.014$).

Table 1.2: Overview of the effects of plant species richness (SR), functional group richness (FGR), presence (+)/absence (-) of legumes (Leg), grasses (Gr), tall herbs (Th) and small herbs (Sh) on KCl-extractable mineral N pools before the start of the experiment, gross and net N transformation rates on in a field incubation experiment. Arrows indicate positive (↑) or negative (↓) effects. Significance codes: ***p<0.001, **p<0.01, *p<0.05, •p<0.1

N pools and transformation rates	SR	FGR	Leg	Gr	Th	Sh
KCl-extractable NH_4^+	* ↑	** ↑	• ↓	** ↑	NS	NS
KCl-extractable NO_3^-	** ↓	* ↓	* ↑	NS	• ↓	• ↓
Gross N mineralization	* ↓	NS	* ↑	NS	NS	NS
Microbial NH_4^+ consumption	* ↓	NS	*** ↑	NS	NS	* ↑
Gross inorganic N immobilization	NS	NS	** ↑	NS	NS	• ↑
Net N mineralization	NS	NS	NS	NS	NS	NS
Net ammonification	NS	• ↓	* ↓	NS	NS	NS
Net nitrification	NS	NS	NS	NS	NS	NS

NS: not significant

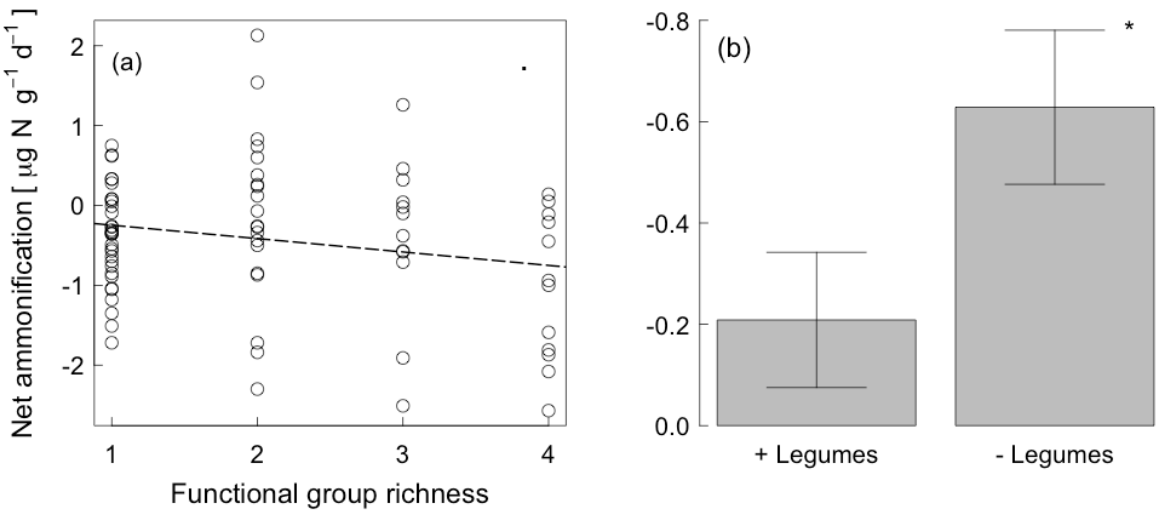

Fig. 1.5: Effects of functional group richness (a) and presence (+)/absence (-) of legumes (b) on net ammonification rates. The dotted line indicates marginal significance at p<0.1. The whiskers in 1.5b represents standard errors. Note the difference in y-axis scaling. Significance codes: *p<0.05 and •p<0.1

In contrast to the laboratory experiment (Table 1.1), in which I did not observe an relationship between plant species richness and gross N mineralization, there was a significant negative plant species richness effect on gross N mineralization rates in the field experiment (Table 1.2) The discrepancy between the laboratory and field experiments might be attributable to the fact that the laboratory experiment was conducted without plants. Moreover, the laboratory experiment was conducted with only one out of four blocks, whereas field experiment was done in all four blocks of the study site, and thus had a lower statistical power. A further difference between the laboratory and field experiments was the fact that the soil samples for laboratory incubations were cold stored until the start of the experiment which is often done. However, cold storage can result in differences in N transformation rates from field conditions (Arnold et al. 2008). In addition, my laboratory incubation was conducted under controlled temperature and/or humidity and optimum nutrient supply (except N) providing optimal growth conditions for microorganisms.

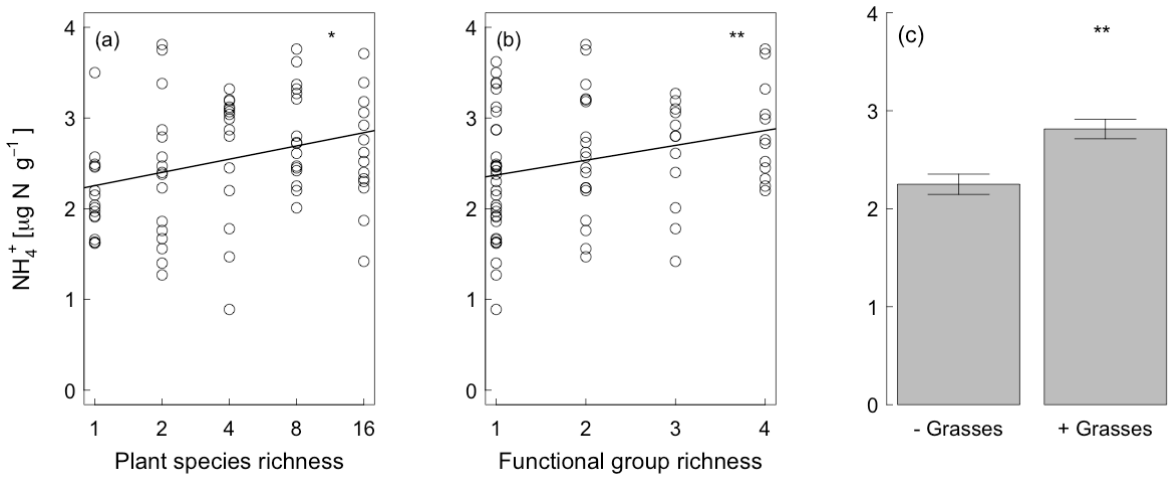

Fig. 1.3: Relationship between KCl-extractable NH_4^+ concentrations and plant species richness (a), functional group richness (b), and presence (+)/absence (-) of grasses (c). The whiskers on 1.3c represents standard errors. The regression lines on 1.3a and b are shown for illustration purpose only. Significance codes: **p<0.01 and *p<0.5

The presence of legumes was found to influence most of the N transformation rates, which I attribute to their ability to fix atmospheric N_2 and increase the N availability of the whole ecosystem. The presence of grasses enhanced NH_4^+ pools in both the laboratory as well as field experiments. This is in line with the earlier findings of Hooper and Vitousek (1998) that nutrient cycling might be more dependent on certain functional groups rather than on species richness. However, later studies have shown that plant species richness significantly influences the N cycle irrespective of the functional group composition of the community (Weisser et al. 2017).

Plant species richness and functional group richness had a significant positive relationship with the KCl-extractable NH_4^+ pool (Table 1.2 and Fig. 1.3a, b) which is in line with the findings from Oelmann et al. (2011) in the Jena Experiment. Presence of grasses increased the NH_4^+ pool in soil KCl-extracts (Table 1.2 and Fig. 1.3c), probably because of their dense rooting system which provides large amount of root exudates thereby increasing the microbial activity (Eisenhauer et al. 2010). Furthermore, I found significant negative effects of plant species richness and functional group richness on KCl-extractable NO_3^- (Table 1.2 and Fig. 1.4a, b) because of more efficient resource use by different communities resulting in the depletion of nutrient concentrations in soil (Hooper and Vitousek 1998; Scherer-Lorenzen et al. 2003; Oelmann et al. 2007; Leimer et al. 2014).

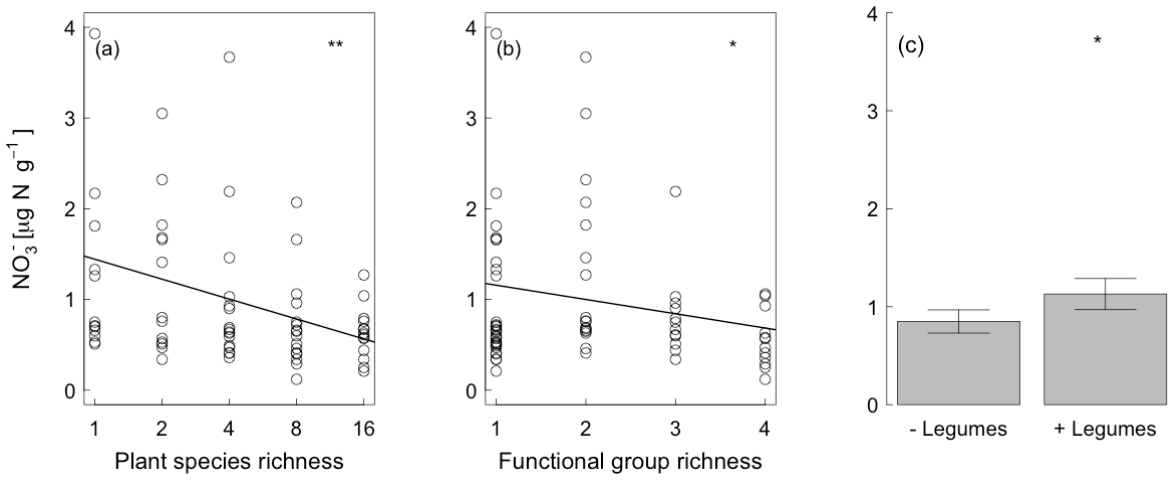

Fig. 1.4: Relationship between KCl-extractable NO_3^- concentrations and plant species richness (a), functional group richness (b) and presence (+)/absence (-) of legumes (c). The whiskers on 1.4c represents standard errors. The regression lines on Fig. 1.4a and b are shown for illustration purpose only. Significance codes: **p<0.01 and *p<0.5

Besides joint positive effects of grasses on dissolved or KCl-extractable NH_4^+ pools and positive effects of legumes on gross N mineralization rates, my laboratory and field experiments revealed some contrasting results for the relationships between plant community composition and components of the N cycle. There can be many reasons for the discrepancies including the lack of living plants, the unrealistic optimum growth conditions for microorganisms, and the low statistical power of the laboratory incubation experiment or the different times of the soil sampling after establishment of the grassland on a former arable land. The field experiment was conducted in 2011 and the laboratory experiment in 2014. My results demonstrate that the findings from laboratory experiments cannot be easily transferred to field conditions and also indicate that the time of the experiment might matter. Future laboratory studies should therefore aim to be as close to the field conditions as possible, i.e. be conducted with plants in growth chambers under temperature and nutrient supply conditions which are similar to the field conditions. Future field studies should be repeated several times after the establishment of the grassland in different seasons and at different times after establishment.

1.4 Error discussion

The laboratory incubation experiment was conducted in the dark and at a constant temperature in a climate chamber for 16 days. I maintained the water content of the incubated

samples by always extracting approximately the same volume as that of the added nutrient solution (100 mL) with the help of a vacuum. Since the incubation vessels were mostly closed

and were opened only for an hour each day to ensure aerobic conditions, the water loss was assumed to be negligible. In this experiment, I included nine blanks to check for any contamination either from the reagents used in nutrient solutions or from the ceramic filters attached to the incubation vessels and found that the inorganic N contents were small (0.02 ± 0.01 µg N). The nutrient solution used for soil percolation was prepared fresh on each day of soil extraction and did not include reagents with any N content. In the field experiment which lasted for a day, the soil extraction was carried out with 1 M KCl solution. Therefore, 1 M KCl blanks were also included and the samples were blank corrected, because there was some N contamination detected in 1 M KCl solution. In the field incubation experiment, two replicates were taken for each time steps (t1, t2) and averaged for better representation of each plot. To avoid chemical or biological changes, the soil extracts from both of the experiments were immediately frozen at -20°C.

Table 1.3: Recoveries of microdiffusion and hypobromite-azide methods to analyze ^{15}N of ammonium in soil extracts

No.	Recovery (%) [Microdiffusion]	Recovery (%) [Hypobromite-azide]
1	95.35	101.51
2	98.47	95.37
3	99.69	97.38
4	101.17	100.44
5	101.47	101.12
6	98.38	102.28
7	97.12	102.61
8	94.34	96.62
Mean	98.25	99.66
SD	2.6	2.79

The detection limit of NH_4-N and NO_3-N measurements with the CFA was 0.02 µg L^{-1}. To reduce the isotopic measurement load for ^{15}N isotopic analysis, I combined the samples from days 4 and 9 to one composite sample. All the vials used for the analysis of mineral N concentrations and ^{15}N measurements were washed in an acid bath (10% HCl) overnight and rinsed afterwards with deionized water. In the microdiffusion method, filter discs were rinsed with deionized water for multiple times and dried in an oven to avoid any contamination.

Table 1.4: Measured versus true values of ^{15}N analysis of in-house ammonium standard [$(NH_4)_2SO_4$] at different ^{15}N enrichments

No.	Measured values (at%)	True values (at%)	Measured values (at%)	True values (at%)	Measured values (at%)	True values (at%)
1	1.464	1.489	3.765	3.736	5.938	5.983
2	1.462	1.489	3.767	3.736	5.967	5.983
3	1.462	1.489	3.758	3.736	5.947	5.983
4	1.466	1.489	3.797	3.736	5.947	5.983
5	1.467	1.489	3.771	3.736	5.970	5.983
6	1.466	1.489	3.756	3.736	5.962	5.983
7	1.461	1.489	3.768	3.736	5.966	5.983
8	1.465	1.489	3.755	3.736	5.970	5.983
9	1.464	1.489	3.761	3.736	5.957	5.983
10	1.467	1.489	3.769	3.736	5.958	5.983
Mean	1.464		3.767		5.958	
SD	0.002		0.012		0.012	
Accuracy (%)	98.4		100.8		99.59	

It is important to have a complete recovery of N present in the samples for ^{15}N isotopic analysis. Incomplete recovery might result in misleading analytical values. Therefore, I was aware that until maximum recovery (>95%) was achieved, the methods adapted for analyzing ^{15}N from NH_4^+ were not applied to the samples from the laboratory incubation and field experiments. Recoveries were >96% for both methods to analyze ^{15}N from NH_4^+ in soil extracts (Table 1.3). Therefore, possible errors due to incomplete recovery were small. The samples were enriched with ^{15}N-NH_4 and ^{15}N-NO_3 at 98 at% excess, so that the isotope fractionation was negligible. Furthermore, I prepared procedural blanks for each batch of ^{15}N measurements both from NH_4^+ and NO_3^- in soil extracts. The procedural blanks prepared in 1 M KCl resulted in average ± standard deviation of 1.6 ± 0.05 µg N (n = 3) and blanks prepared in nutrient solution were (0.012 ± 0.003 µg N, n = 3) µg N. The target N masses in samples were always ten times larger than the N contamination found in the blanks so the blank interference in the

sample analysis were small or negligible. Helium blanks and standard materials were included after every 10 sample measurements in a batch to avoid ^{15}N carry-over or memory effects.

Table 1.5: Measured $\partial^{15}N$ values of in-house ^{15}N ammonium standard analyzed in two different batches

No.	$\partial^{15}N$-$(NH_4)_2SO_4$
1	-1.55
2	-1.47
3	-1.49
4	-1.51
5	-1.78
6	-1.56
7	-1.53
8	-1.54
9	-1.55
10	-1.59
Mean	-1.56
SD	0.09

The accurate measurement of stable isotope ratios requires the determination of the accuracy and precision of the used methods. Therefore, I conducted a number of analyses to optimize the method with respect to its performance and determined the accuracy and precision of my measurements (Table 1.4). Reproducibility of the ^{15}N analysis from NO_3^- was monitored by using certified reference materials (IAEA N3, USGS32), and from NH_4^+ by using in-house standards [^{15}N-$(NH_4)_2SO_4$: 0.366 at% (natural abundance), 1.49 at%, 3.74 at%, 5.98 at%] and by repeating some selected sample measurements which resulted in the deviation of <0.015 at% (n=3). Ten replicates of each standards with different ^{15}N enrichments were analyzed for ^{15}N isotope values to calculate the accuracy and precision of the sample analysis (Table 1.4). Replicates of each standards were comparable among different batches, indicating that the sample preparation procedures were stable (Table 1.5). The accuracy of the analysis was evaluated by the isotopic difference between measured ^{15}N values and true (assigned) ^{15}N values of different standard materials used during the measurements. The measured ^{15}N isotopic values were comparable to that of the true values of the standards, suggesting a good accuracy

of the measurements (Table 1.4). Precision was evaluated by the repeated measurements of replicates.

Although gross rates of microbial NH_4^+ consumption can be calculated from ^{15}N isotopic dilution data, these rates may be overestimated because it is necessary to add NH_4^+ to the substrate pool in order to estimate their rates. The ^{15}N amendments to the soil NH_4^+ pool was less than 2% of the NH_4-N concentration in soil at the experimental period. The addition of NH_4^+ to the inorganic N pool could artificially stimulate NH_4^+ consumptive process like uptake, nitrification and other gaseous loss (West et al. 2006).

1.5 General conclusions

The results of my research allow to draw the following conclusions:

1) The two methods to measure the ^{15}N isotopic composition of NH_4^+ in soil extracts was successfully tested and applied to the samples from the laboratory and field incubation experiment. I found that the microdiffusion method is best suited for comparatively high N masses (~20 μg), while hypobromite-azide method is more sensitive and best suited for low N masses (~0.2 μg). The mean recoveries ± standard deviations of the microdiffusion and hypobromite-azide methods were 98.3±2.6% and 99.6±2.8%, respectively **(Chapter 2)**.

2) In the absence of plant uptake, almost all the produced NH_4^+ was nitrified to NO_3^-. The initial labile and recalcitrant organic N pools increased with increasing species richness which can be attributed to the positive effect of species richness on organic matter and total N accumulation. The presence of legumes significantly increased gross N mineralization and autotrophic nitrification because legumes generally increase soil N concentrations via atmospheric N_2-fixation. Similarly, the presence of grasses significantly increased the soil NH_4^+ pool, gross N mineralization, and NH_4^+ immobilization, likely because of enhanced microbial biomass and activity by providing large amounts of root exudates through their dense rooting systems **(Chapter 3)**.

3) Gross N mineralization and microbial NH_4^+ consumption rates unexpectedly decreased with increasing species richness, which was mainly driven by the positive relationship between root C/N ratios and species richness. Higher species richness increased root C/N ratios because of the replacement of N-rich legumes by small herbs and also because of the dilution of plant

nutrient concentrations by taller growth in response to light competition. Again unexpectedly, functional group richness had negative effects on net ammonification likely due to the reduced soil moisture in topsoil at higher diversity. In the presence of legumes, gross N mineralization, microbial NH_4^+ consumption and gross inorganic N immobilization rates increased significantly because of higher soil N concentrations in legume-containing plots. The possible explanation for the negative effects of legumes on net ammonification could be the positive effects of legumes on microbial NH_4^+ consumption and gross inorganic N immobilization rates, which resulted in a small leftover of NH_4^+ in mixtures with legumes **(Chapter 4)**.

1.6 Authors contributions

I tested and applied the two methods to measure the ^{15}N concentrations of NH_4^+ in soil extracts **(Chapter 2)** with two different soil extract matrices i.e. nutrient solution and KCl **(Chapter 3 and 4)**. I conducted the ^{15}N tracing microcosm experiment in the laboratory. I prepared all the samples and standards for ^{15}N isotopic analysis from NH_4^+ and NO_3^- **(Chapter 3 and 4)**. Thomas Kuhn and I determined the stable N isotope ratios of NH_4^+ and NO_3^-. I calculated gross and net rates of N transformations using analytical equations **(Chapter 4)** while N pool sizes and gross N rates of the laboratory microcosms were determined by Christoph Müller with the *Ntrace* model **(Chapter 3)**. I performed all the statistical analysis with support of Sophia Leimer. Andre Velescu conducted the ^{15}N pool dilution field experiment and supplied the soil extracts for isotopic analysis and concentration data of inorganic N **(Chapter 4)**. Alexandra Weigelt and Hongmei Chen contributed the plant biomass and root C/N data **(Chapter 3)**. Microbial data was contributed by Nico Eisenhauer, Odette Gonzalez and Stephan Scheu **(Chapter 3 and 4)**.

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2. Testing two methods to determine the nitrogen isotopic composition of ammonium in soil extracts

2.1 Abstract

Here, I describe the adaptation of the microdiffusion and hypobromite-azide methods to measure enriched ^{15}N isotope composition of ammonium in soil extracts. The microdiffusion method involves the release of ammonium (NH_4^+) from solution as gaseous ammonia (NH_3) under alkaline conditions produced by using magnesium oxide. The NH_3 is trapped into an acidified filter disc as NH_4^+ and then combusted to N_2 to determine its N isotope ratio using an Elemental Analyzer-Isotope Ratio Mass Spectrometer. This method is the most commonly used one. However, it requires a large sample volume (50 mL) and comparatively high amount of dissolved NH_4^+ (~20 µg N). The mean recovery ± standard deviation for this method was 98.3 ± 2.6%. In the hypobromite-azide method, NH_4^+ is first oxidized to nitrite by hypobromite and then to nitrous oxide using a sodium azide and acetic acid buffer solution. The produced gaseous nitrous oxide (N_2O) is then analyzed for its N isotopic composition with a Gas Bench-Isotope Ratio Mass Spectrometer. The recovery was 99.6 ± 2.8% SD. The latter method is ideal for samples with low NH_4^+ concentration (~ 0.2 µg N) and small sample volume (5 mL). It requires less time for preparation than the microdiffusion method. The accuracy of microdiffusion and hypobromite-azide methods were 98.4 ± 1.6% and 99.2 ± 0.8% of true values, respectively (n = 10 for each method). The errors of ±1.6% and ±0.8% in average is the deviation from the true values. Ten replicate measurements each for both the methods showed the precision of 0.002 at%.

2.2 Introduction

Ammonium (NH_4^+) is one of the bioavailable chemical nitrogen (N) species for plant and microorganisms. The use of ^{15}N-labeled NH_4^+ can help improving the knowledge of the soil N cycle by allowing for the calculation of gross N transformations (Booth et al. 2005). The analysis of the N stable isotope composition of NH_4^+ is increasingly used in biogeochemical and ecological studies to better understand processes involved in N cycling (Sebilo et al. 2004). To determine gross rates of N transformation, ^{15}N pool dilution assays have been developed (Davidson et al. 1991). In pool dilution assays, a specific N pool is labeled using ^{15}N as a tracer and the dilution of the label is followed over time. In order to measure ^{15}N-NH_4^+, the NH_4^+ should be separated or converted to N species (N_2 or N_2O) that can be introduced into a mass

spectrometer (Homes et al. 1998). The most frequently used methods to determine the N isotopic composition of NH_4^+ include the microdiffusion (Brookes et al. 1989; Stark and Hart 1996) and hypobromite-azide methods (Zhang et al. 2007).

In the microdiffusion method, NH_4^+ is diffused into acidified filter discs under alkaline conditions (Brookes et al. 1989; Stark and Hart 1996). This technique is widely used because it does not require particularly skilled operators or specialized equipment. The main limitation of this method is the lengthy diffusion time and the limitation of the sample volume in the sample bottles (Chen and Dittert 2008). In addition to that, it is time-consuming and not reliable at low concentrations (Liu et al. 2014). This method produces N_2 gas as an end product analyte for Elemental Analyzer Isotope Ratio Mass Spectrometry (EA-IRMS). Therefore, the microdiffusion method requires a high amount of N (>1 μmol N) due to an unavoidable background signal caused by the incidental atmospheric N_2 contamination (Zhang et al. 2007).

The hypobromite-azide method is a technique in which NH_4^+ is first oxidized to NO_2^- by hypobromite (BrO^-) and further reduced to nitrous oxide (N_2O) using sodium azide (NaN_3) and acetic acid buffer solution (Zhang et al. 2007). This method does not require separation of NH_4^+ from sample solutions, thereby simplifying the preparation time. The N_2O is the end product of this method, which is considered as a better analyte than N_2 for stable isotope analysis because it only occurs in traces in the atmosphere. When using N_2O, the N requirement for sample analysis is reduced to 10 nmol (Zhang et al. 2015). Therefore, it is possible to analyze samples with low NH_4^+ concentration and small sample volume.

I conducted ^{15}N labeling experiments in the laboratory (Chapter 3) and in the field (Chapter 4) to quantify gross N transformations in grassland soil. The NH_4^+ concentrations in soil extracts from the laboratory incubation experiment were comparatively lower than those from the field incubation experiment. Therefore, I focused on optimizing methods compatible to measure NH_4^+ samples from both experiments. The main aim of this study was to establish the: (i) microdiffusion method as described by Stark and Hart 1996 and (ii) hypobromite-azide method as described by Zhang et al. (2007) to determine the stable N isotopic composition of NH_4^+ in soil extracts from ^{15}N-labeling experiments. I used the microdiffusion method to the soil extracts of the field experiment and the hypobromite-azide method to the soil solutions of the laboratory experiment to analyze ^{15}N of NH_4^+.

2.3 Materials and methods

2.3.1 Microdiffusion method

In the microdiffusion method, NH_4^+ is released from soil extracts in the form of ammonia (NH_3) by increasing the pH to > 9.5 with magnesium oxide (MgO). The released NH_3 is then collected on acidified filter discs enclosed in a polytetrafluroethylene (PTFE) envelope, where it is again protonated to NH_4^+ (Stark and Hart 1996).

2.3.1.1 Preparation of acid traps

Glass fiber filter papers were cut in round shape with the help of a paper puncher and rinsed multiple times with 1 M potassium chloride (KCl) and then with deionized water. Washed filter discs were dried in an oven at 50°C. These filter discs were stored in a desiccator with silica gel as desiccant.

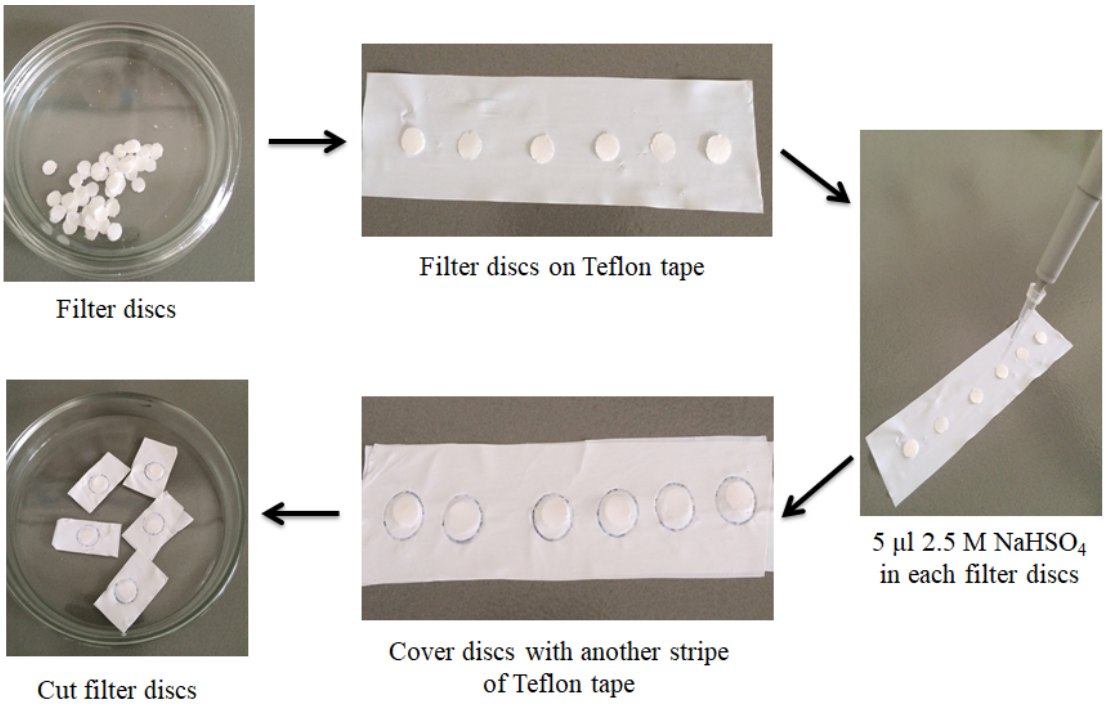

Fig. 2.1: Schematic representation of various steps involved in the preparation of acid traps

A strip of PTFE tape of about 10-15 cm length was cut and the cleaned filter discs were placed on the PTFE tape at ~1 cm distance using tweezers (Fig. 2.1). I added 5 µL of 2.5 M sodium hydrogen sulfate ($NaHSO_4$) on each filter disc and covered it with another strip of PTFE tape on top, and then gently smoothed out with a clean brush to remove wrinkles. The acidified

filter discs were sealed by pressing with an open end of a small tube or pipette tip making a concentric circle around the filter discs (Fig. 2.1). Acidified filter discs were sealed between two strips of PTFE tape because PTFE protects the acid traps from neutralization and is permeable for gases but not liquids. Each acidified filter disc was separated by cutting the PTFE encased acid traps with a clean scissor or a sharp blade. I then placed the prepared acid traps between two petri dishes.

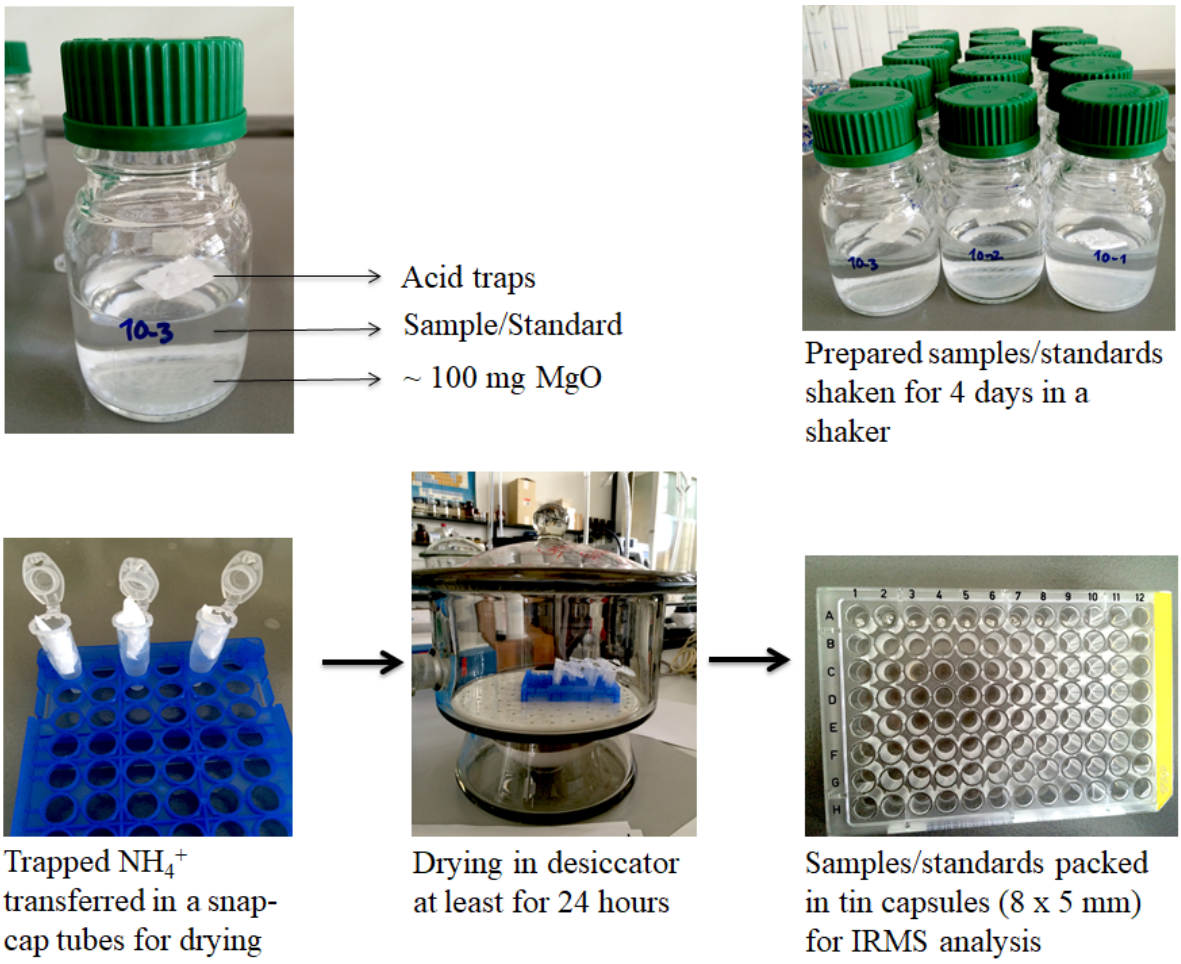

Fig. 2.2: Various steps of sample preparation procedure using the microdiffusion method

2.3.1.2 Ammonium diffusion

Diffusion glass bottles were filled with 50 mL of samples or standards or blanks in which the $^{15}NH_4^+$ concentration was to be measured. The volume of sample solution contained at least 20 µg of NH_4-N because this was the optimal mass of N for EA-IRMS. I added about 100 mg of MgO to the solution and quickly one acid trap per sample bottle. Then, the bottle was closed immediately. The increased pH to > 9.5 reached by the addition of MgO causes the conversion NH_4^+ to NH_3, which is trapped in the acidified filter discs. The prepared sample bottles were

placed in a horizontal shaker at room temperature for the next 2-6 days to trap NH_4^+ into the filter discs. The time to trap NH_4^+ into the acid traps was varied to determine the optimal diffusion time.

2.3.1.3 Drying of acid traps

Sample bottles were opened after the assigned trap time was over and acid traps were removed by using clean tweezers. Each acid trap was rinsed with deionized water to wash out any MgO sticking on the outside of PTFE tape, and then put in 1.5 mL snap cap reaction tube for drying. I placed the tubes in a desiccator for drying which contained a beaker with concentrated sulfuric acid (H_2SO_4) as desiccant. Drying of acid traps was done at least for 24 hours. H_2SO_4 is used for drying acid traps instead of silica gel because it absorbs any atmospheric NH_4^+.

2.3.1.4 ^{15}N isotope analysis

Dried acid traps were opened, and the filter disks were packed into the tin capsules (5x8 mm). The tin capsules were folded and put into micro-titer plates for the isotope analysis. The N isotope ratios were determined with an Elemental Analyzer (EA 1110, Carlo Erba Instruments, Milan, Italy) coupled to an isotope-ratio mass spectrometer (MAT Delta Plus, Thermo Finnigan, Bremen, Germany) at the Stable Isotope Center, University of Göttingen. IAEA N1, IAEA N2, USGS 25 and in-house standard reference material [^{15}N-$(NH_4)_2SO_4$] was used to check the accuracy of the measurements.

2.3.2 Hypobromite-azide method

This method is based on conversion of NH_4^+ to N_2O. First, ammonium is oxidized to nitrite (NO_2^-) using BrO^-. With the help of a sodium azide buffer solution, NO_2^- is then converted to N_2O under acidic conditions (Zhang et al. 2007).

2.3.2.1 Oxidation of NH_4^+ to NO_2^-

The first step involves conversion of NH_4^+ to NO_2^- by using hypobromite oxidation (Eq. 2.1):

$$(2.1) \quad 3BrO^- + NH_3 + OH^- \rightarrow NO_2^- + 2H_2O + 3Br^-$$

This reaction occurs under strongly alkaline conditions. First, I prepared bromate/bromide stock solution by mixing 0.6 g of sodium bromate and 5 g of sodium bromide in 250 mL of deionized water. BrO^- working solution was prepared in two steps. Firstly, by adding 1 mL of stock solution to 50 mL of deionized water and then, by adding 3 mL of 6 M hydrochloric acid

(HCl) to produce Br_2. The working solution was left to react in the dark for five minutes. Secondly, 50 mL of 10 M sodium hydroxide (NaOH) was added quickly to produce BrO^- following Eqs. 2.2 and 2.3.

(2.2) $BrO_3 + 5Br^- + 6H^+ \rightarrow 3Br_2 + 3H_2O$

(2.3) $Br_2 + 4OH^- \rightarrow 2BrO^- + 2H_2O$

According to Zhang et al. (2007), the optimal reaction time of the oxidation was 30 minutes considering the amount of BrO^- and NH_4^+ concentration. Sodium arsenite ($NaAsO_2$) was used to remove excess BrO^- after oxidation. This solution is prepared by mixing 5 g of $NaAsO_2$ in 100 mL of deionized water.

In the study of Zhang et al. (2007), 20 mL of samples or standards were placed in 60-mL vials. However, in our study, we used 20 mL headspace crimp top glass vials that were previously acid rinsed and oven dried. I poured 5 or 10 mL of standards in each 20 mL vial with an NH_4^+ concentration of 0.15 and 0.3 µg vice versa. Standards were prepared with the same sample matrix, i.e. N-free nutrient solution (4 mM $CaCl_2$, 2 mM KH_2PO_4, 1 mM $MgCl_2$, 1 mM K_2SO_4, 1 mM $MgSO_4$, 25 µM H_3BO_3, 2 µM $MnSO_4$, 2 µM $ZnSO_4$, 0.5 µM $CuSO_4$ and 0.5 µM Na_2MoO_4). 300 µL of BrO^- working solution was added to each vial and shaken vigorously. After 30 minutes of reaction time, 60 µL of $NaAsO_2$ solution was added to remove excess BrO^-. 250 µL of 6 M HCl was added to make the pH of the samples or standards acidic (pH 4-5) and then, the vials were crimp sealed. The concentration of NO_2^- produced after NH_4^+ oxidation was analyzed colorimetrically.

2.3.2.2 Conversion of NO_2^- to N_2O

After NH_4^+ oxidation to NO_2^-, NO_2^- was converted to N_2O by using sodium azide buffer solution (Eq. 2.4). The buffer solution was prepared by mixing 2 M sodium azide (NaN_3) solution with 20% acetic acid (CH_3COOH) in a ratio of 1:1. Then, it was purged with helium (He) for two hours to remove any N_2O produced from the reagents. 270 µL of the NaN_3 buffer solution was added to each sample or standard that was previously converted to NO_2^- by using a 1-mL syringe. NaN_3 buffer solution was daily prepared fresh. NaN_3 is highly toxic, so it was always handled under a fume hood. After injecting the buffer solution, vials were shaken and incubated at 30°C for an hour. 170 µL of 10 M sodium hydroxide (NaOH) was added to alkalize the solution and stop the reaction.

(2.4) $HNO_2^- + HN_3^- \rightarrow N_2O + H_2O + N_2$

The isotope ratios of the N_2O gas from the headspace of the crimp top vials were analyzed with a Gas-Bench II pre-concentration unit interfaced with Delta V Plus isotope ratio mass spectrometer (Thermo Fischer Scientific, Bremen, Germany) at the Basel Stable Isotope and Biogeochemistry Laboratory, University of Basel. The isotopic ratio of N_2O was normalized to N_2O reference gas to eliminate instrumental drift. In-house standard reference material [^{15}N-$(NH_4)_2SO_4$] was used to check the accuracy of our measurements. To calibrate the ^{15}N enriched isotopic values, I prepared in-house $^{15}NH_4^+$ standards at different enrichments: 1.49 at%, 3.74 at% and 5.98 at% by diluting $(^{15}NH_4)_2SO_4$ having a known value of 11.6 at%.

2.4 Results and discussion

2.4.1 Microdiffusion method

Different diffusion times were applied for trapping NH_4^+ to determine the duration of the NH_4^+ diffusion time for maximum recovery. Standard solutions prepared in deionized water were kept to diffuse NH_4^+ for 2-6 days. I found that the diffusion times of two (Fig 2.3a) and three days (Fig. 2.3b) did not show good results ($R^2 = 0.91$). However, the standards trapped for four (Fig. 2.3c) and six days (Fig. 2.3d) gave better results, showing similar rates of recovery of more than 96%. I found that there were no differences between the two latter trapping times ($R^2 = 0.99$).

First, I applied the microdiffusion method in standards prepared with deionized water and then, I prepared standards with 1 M KCl because our soil samples from the field experiment were extracted with 1 M KCl. I diffused the standard solution prepared in 1 M KCl only for four (Fig. 2.4a) and six days (Fig. 2.4b). Again, I found that there were no differences when standards were diffused for four or six days. I therefore chose four days as an optimal diffusion time for further analysis of NH_4^+ in KCl extracts. I compared the difference between diffused and non-diffused standards to calculate recoveries. The diffusion time of four days showed recoveries of 98.3% (n = 8, SD ± 2.6%). I compared the contamination of NH_4-N in blanks

prepared in two different matrices (1 M KCl and deionized water) through the ^{15}N signal intensity measured as peak area by EA-IRMS and found that the standards prepared in 1 M KCl showed higher NH_4-N contamination than those prepared in deionized water. To avoid N contamination in filter paper, I washed them multiple times with deionized water and then oven-dried them prior to making the filter discs. However, washing and drying of filter discs did not help to get rid of blanks. Conway (1957) argued that the KCl reagent might contain small amount of N contamination, which probably produced higher blank areas. The rate of diffusion should be checked whenever the sample matrix is changed because the matrix can have a large effect on diffusion rates (Conway 1957). However, the blank effect for the isotopic analysis was small due to the fact that all the samples were ^{15}N-labeled.

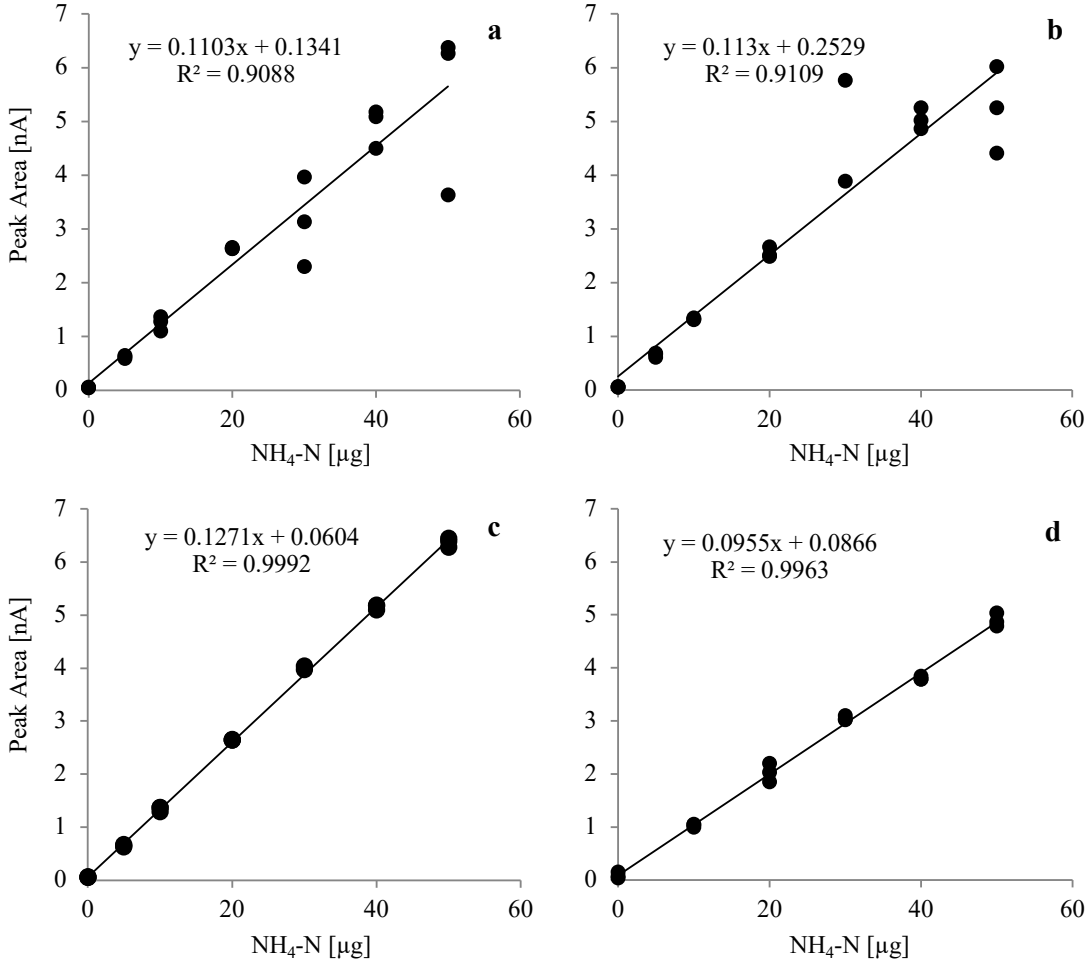

Fig. 2.3: Relationships between standards prepared at different NH_4-N concentrations and signal intensity (N_2 peak area, nA) using microdiffusion method with two days (a), three days (b), four days (c) and six days (d) of diffusion time

Acid traps containing $NaHSO_4$ are more effective than H_2SO_4 in absorbing NH_3. H_2SO_4 contains more moisture and there is a risk of acid dropping into the sample (Brookes et al. 1989), which affects the diffusion process. In addition, H_2SO_4 corrodes tin capsules in which samples are packed for ^{15}N analysis (Brookes et al. 1989).

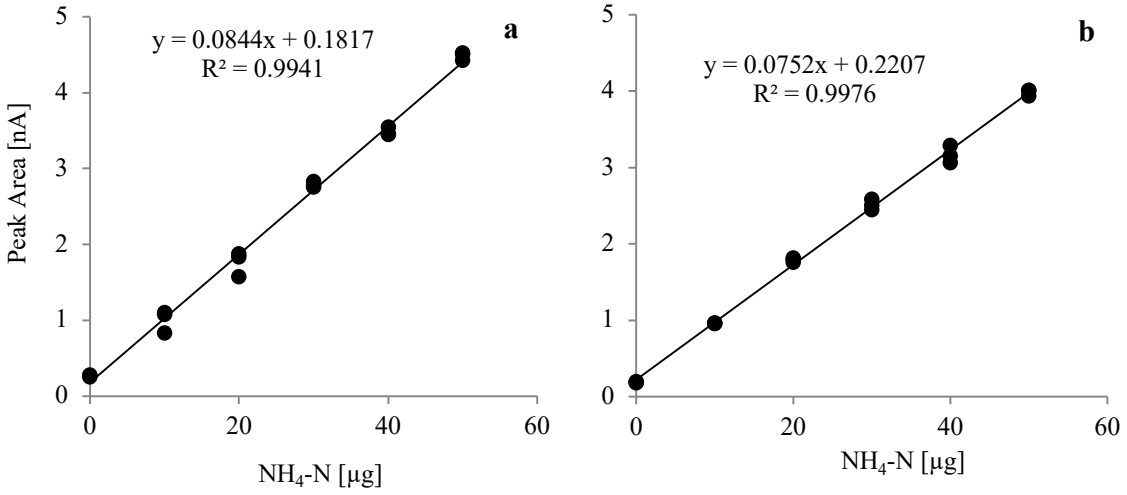

Fig. 2.4: Relationships between standards prepared at different NH_4-N concentrations and signal intensity (N_2 peak area, Vs) using microdiffusion method with four days trapping time in deionized water (a) and 1 M KCl (b)

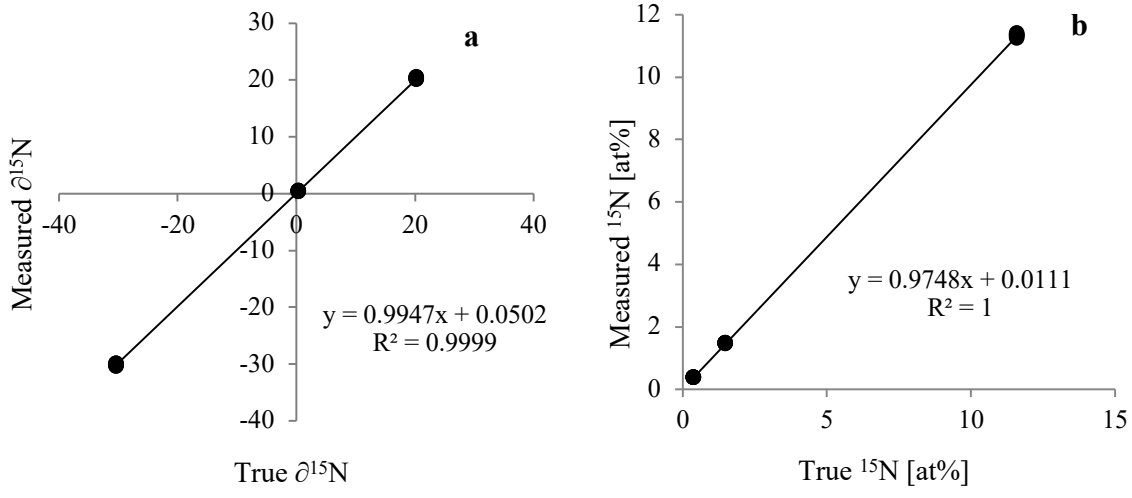

Fig. 2.5: Relationships between true ^{15}N values versus measured ^{15}N values for three certified standards (True values ± standard deviation: USGS 25 = -30.4 ± 0.4, IAEA N1 = 0.4 ± 0.2, IAEA N2 = 20.3 ± 0.2) at ^{15}N natural abundance (a) and for three in-house standards with different ^{15}N enrichment (True values: $(^{15}NH_4)_2SO_4$ = 0.366 at%, 1.49 at%, 11.6 at%)

We analyzed international reference materials (USGS 25, IAEA N1, IAEA N2) to check if the method serves for isotopic values as well (Fig. 2.5a). I measured USGS 25, IAEA N1 and IAEA at -30.17 ± 0.24‰, 0.4 ± 0.07‰ and 20.27 ± 0.16‰, respectively (n=6 for each certified reference materials) which compares well with the certified values of -30.4 ± 0.4‰, 0.4 ± 0.2‰ and 20.3 ± 0.2‰. As the aim of the method adaptation was to measure enriched ^{15}N-NH_4^+ in soil extracts, I tested the method also in in-house NH_4^+ standards with known ^{15}N enrichment of 0.366 at%, 1.49 at% and 11.6 at% (Fig. 2.5b). I plotted measured ^{15}N isotopic values (mean ± standard deviation: 0.367 ± 0.0004 at%, 1.46 ± 0.002 at% and 11.32 ± 0.054 at%) against the true (known) values of standards, which showed R^2=0.99. Ten replicate measurements of in-house standard reference material [^{15}N-$(NH_4)_2SO_4$] resulted, on average, in 98.4±1.6% of the true value, indicating a high accuracy of the measurements. The error of ±1.6% is the average deviation from the true value. Precision of the ^{15}N measurements was ±0.002 at% (n=10). This depicts that the method is fully optimized and ready to be applied further to the soil samples.

2.4.2 Hypobromite-azide method

The hypobromite-azide method was used to determine the isotopic composition of NH_4^+ and NO_3^- in solutions collected from the laboratory incubation experiment in which soil samples were extracted with N-free nutrient solution. Therefore, the standards were prepared in the same N-free nutrient solution as was used for the incubation experiment. The hypobromite oxidation is an important step because it determines the performance of the method. Therefore, the oxidation yield of NH_4^+ to NO_2^- was checked with a photometer. To check the efficiency of the method, I prepared standards with sample volumes of 5 mL (Fig. 2.5a) and 10 mL (Fig. 2 5b).

The result demonstrated that the sample volume of 10 mL produced an approximately twice as high peak area of N_2O than the sample volume of 5 mL (Table 2.1), which means that the method is working properly. Based on these results, I used a sample volume of 5 mL for my analyses. I set all samples to a NH_4^+-N mass of 0.2 µg by dilution if necessary. Furthermore, I checked the recovery of NH_4^+ by comparing the peak size of samples to that of known concentration of standards and observed a mean recovery of 99.6% (n = 8, SD = ±2.8%).

Table 2.1: Blanks and standards prepared at different NH_4^+ masses (0.1 and 0.2 µg N) at 5 mL and 10 ml sample volume versus respective signal intensity (peak area, Vs)

Samples	NH_4^+ (µg N)	Sample volume (mL)	Signal intensity (N_2O peak area, Vs)	Mean signal intensity (N_2O peak area, Vs)
Blank-5-1	0.0	5	1.84	1.83
Blank-5-2	0.0	5	1.81	
Blank-5-3	0.0	5	1.83	
0.1 N-5-1	0.1	5	13.79	14.67
0.1 N-5-2	0.1	5	13.86	
0.1 N-5-3	0.1	5	16.36	
0.2 N-5-1	0.2	5	28.48	28.03
0.2 N-5-2	0.2	5	28.26	
0.2 N-5-3	0.2	5	27.34	
Blank-10-1	0.0	10	3.59	3.60
Blank-10-2	0.0	10	3.49	
Blank-10-3	0.0	10	3.71	
0.1 N-10-1	0.1	10	31.36	31.19
0.1 N-10-2	0.1	10	31.27	
0.1 N-10-3	0.1	10	30.95	
0.2 N-10-1	0.2	10	61.05	60.30
0.2 N-10-2	0.2	10	60.01	
0.2 N-10-3	0.2	10	59.86	

Blank sizes for this method were small (~ 0.012 µg N) and the contribution of those blanks was probably from the reagents or N_2O inside the glass vials. I purged a few blank samples with He to remove any N_2O inside the vial, but this did not reduce blank size. The peak areas of He-purged standards (1.35 ± 0.15 Vs) were not very different from the ones which were not purged with He (1.74 ± 0.11 Vs). So, I assumed that the blank source might be from the reagents used in the preparation.

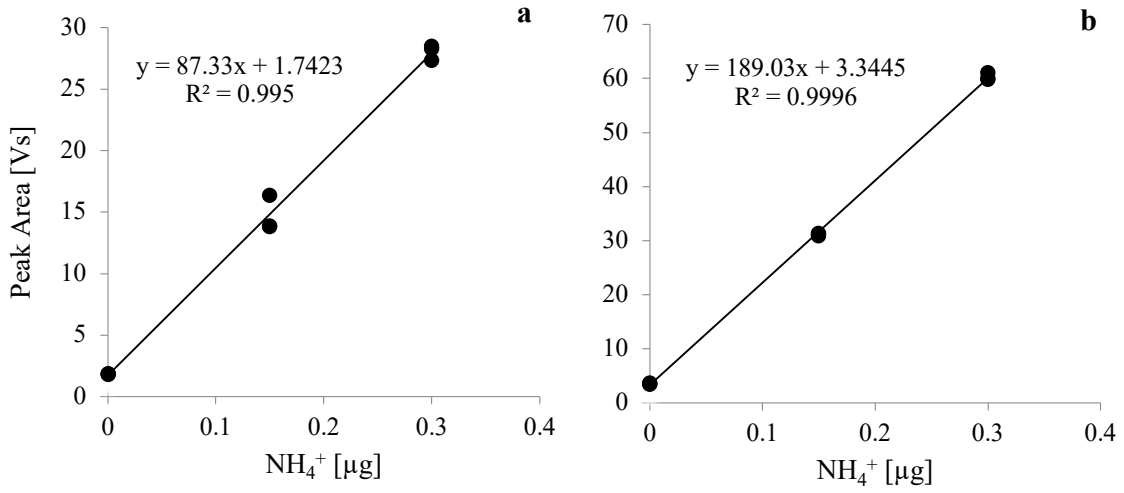

Fig. 2.6: Relationships between different NH_4-N masses and signal intensity (N_2O peak area, Vs) for standards prepared with 5 mL (a) and 10 mL (b) of sample volume. Note the difference in y-axis scaling

As my samples were enrichned with ^{15}N, I used in-house standards with a known ^{15}N enrichment of $(^{15}NH_4)_2SO_4$ for the calibration of isotopic values. The ^{15}N enrichment used were: natural abundance (0.366 at%), 1.49 at%, 3.74 at% and 5.98 at%. Reproducibility of the ^{15}N at% among replicate measurements was excellent with a maximum standard deviation of 0.01 at% (n = 6, for each ^{15}N enrichment). The correlation between the true and measured ^{15}N values showed R^2=0.99, suggesting that the measurements had a very good accuracy. Ten replicate measurements of in-house standard reference material [^{15}N-$(NH_4)_2SO_4$] resulted, on average, in 100.4 ± 0.4% of the true value, indicating a high accuracy of the measurements. The error of ±0.4% is the average deviation from the true value. Precision of the ^{15}N measurements was ±0.002 at% (n=10). As reported by McIlvin and Altabet (2005), the theoretical value of the slope is 0.5 because of 1:1 contribution of N atoms from NH_4^+ and NaN_3 in the produced N_2O. The slope of the linear regression of true ^{15}N values on measured ^{15}N values was 0.49 (Fig. 2 6a) which is close to the expected value of 0.5. The excellent relationship of the true and measured ^{15}N demonstrated that the method was fully established and ready for the further application to the soil extracts.

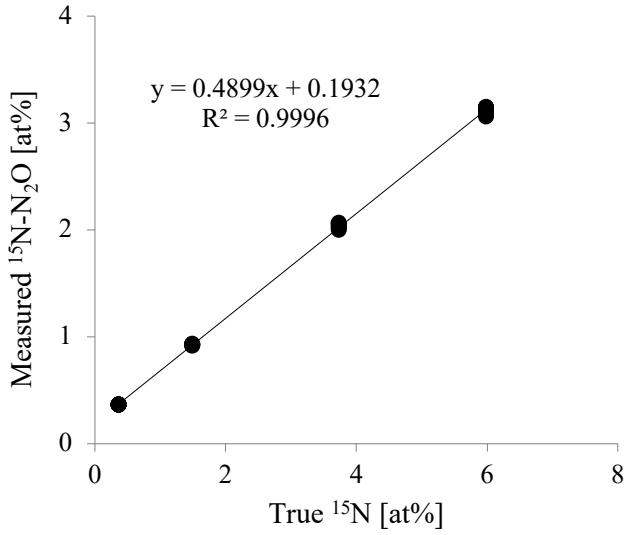

Fig. 2.7: Relationship between the true and measured ^{15}N concentrations of in-house lab standards [True values: $(^{15}NH_4)_2SO_4$ = 0.366 at%, 1.49 at%, 3.74 at%, 5.98 at%].

2.5 Conclusions

I successfully established two different chemical methods to determine enriched ^{15}N concentrations of NH_4^+ in soil extracts. I demonstrated that the microdiffusion method works well when the diffusion time is four days. However, this method is not reliable in case of samples with low N masses and low volumes of sample solutions. Therefore, the hypobromite-azide method was additionally established which is more suitable for samples with low N masses. It is applicable for samples with NH_4^+ content as low as 0.1 µg N and sample volumes of 5 mL. I observed accurate measurements with both the methods, showing on average 98.4 ± 1.6% and 99.2 ± 0.8% of the target values for the microdiffusion and hypobromite-azide methods, respectively; and precise measurements with standard deviation of 0.002 at%. Overall, I conclude that both of the methods have been established and are ready to be used for ^{15}N isotopic analysis in KCl extracts and soil solutions in our lab. I used the microdiffusion method to analyze the KCl extracts of the field experiment and the hypobromite-azide method to analyze soil solutions of the laboratory experiment.

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3. The biodiversity-N cycle relationship: A ^{15}N tracer experiment with soil from plant mixtures of varying diversity to model N pool sizes and transformation rates

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Chapter 3 is published in Biology and Fertility of Soils (2020) 56:1047-1061, DOI https://doi.org/10.1007/s00374-020-01480-x

3.1 Abstract

We conducted a ^{15}N tracing experiment in laboratory microcosms with field-fresh soil samples from a biodiversity experiment to evaluate the relationship between grassland biodiversity and N cycling. To embrace the complexity of the N cycle, we determined N exchange between five soil N pools (labile and recalcitrant organic N, dissolved NH_4^+ and NO_3^- in soil solution, and exchangeable NH_4^+) and eight N transformations (gross N mineralization from labile and recalcitrant organic N, NH_4^+ immobilization into labile and recalcitrant organic N, autotrophic nitrification, heterotrophic nitrification, NO_3^- immobilization, adsorption of NH_4^+) expected in aerobic soils with the help of the N-cycle model *Ntrace*. We used grassland soil of the Jena Experiment, which includes plant mixtures with 1 to 60 species and 1 to 4 functional groups (legumes, grasses, tall herbs, small herbs). The 19 soil samples of one block of the Jena Experiment were labeled with either $^{15}NH_4^+$ or $^{15}NO_3^+$, or both. In the presence of legumes, gross N mineralization and autotrophic nitrification increased significantly because of higher soil N concentrations in legume-containing plots and high microbial activity. Similarly, the presence of grasses significantly increased the soil NH_4^+ pool, N mineralization, and NH_4^+ immobilization, likely because of enhanced microbial biomass and activity by providing large amounts of rhizodeposits through their dense root systems. In our experiment, previously reported plant species richness effects on the N cycle, observed in a larger-scale field experiment within the Jena Experiment, were not seen. However, specific plant functional groups had a significant positive impact on the N cycling in the incubated soil samples.

3.2 Introduction

Anthropogenic activities have resulted in the loss of biodiversity, which can alter ecosystem functions including biomass productivity, organic matter decomposition rates, and nutrient cycling (Loreau et al. 2001; Hooper et al. 2005; Weisser et al. 2017). Nitrogen (N) is the most important nutrient limiting primary productivity in many ecosystems (Elser et al. 2007; Fay et al. 2015). Therefore, knowledge of biodiversity-N cycle relationships is necessary to understand the consequences of biodiversity loss for the N supply of plants and N leaking into the atmosphere and surface and groundwaters. Nitrogen undergoes complex microbially mediated transformations in soil that are related to the quantity and quality of soil organic matter (Wedin and Pastor 1993; Benbi and Richter 2002; Booth et al. 2005; Fornara et al. 2011; Lang

et al. 2015). The quality and quantity of organic matter in grassland soils largely depends on the plant species and functional group richness responsible for differences in litterfall, root turnover, and root exudates (Allan et al. 2013; Solly et al. 2013).

The N transformation processes that are most important for plants and microorganisms are those associated with the depolymerization of organic N into amino acids and mineralization-immobilization turnover of ammonium (NH_4^+) and nitrate (NO_3^-), because these N species represent the major forms of bioavailable N taken up by plants and microorganisms (Davidson et al. 1990; Corre et al. 2002; Schimel and Bennett 2004; Zhang et al. 2016). Depolymerization of organic matter is the process by which proteins in organic matter are broken down into smaller, N-containing fragments, the amino acids which thereby become accessible for plants and microorganisms (Schimel and Bennett 2004; Wild et al. 2015). Gross N mineralization includes the release of amino groups as NH_4^+ which can also serve as a substrate for nitrification. By the mechanism of N immobilization, the mineral N is assimilated by microorganisms, which compete with plants for fixed N. Nitrogen mineralization and nitrification rates are primarily controlled by soil microbial activity, as well as environmental factors, such as the availability, quality and quantity of the microbial C source and mineral nutrients, soil moisture, and temperature (Booth et al. 2005). For grasslands, previous work has suggested that the nitrification to microbial immobilization ratio is an important factor controlling NO_3^- leaching (Stockdale et al. 2002). With regard to controls of the availability of NH_4^+ and subsequent nitrification in an ecosystem, immobilization of NH_4^+, and fixation and release of NH_4^+ by specific clay minerals (illites and interlayer minerals containing illite layers) may also play an important role (Brady and Weil 2002).

Most studies on the biodiversity-mineralization relationship have focused on net N mineralization and/or nitrification rates (Accoe et al. 2004; Fornara and Tilman 2009; Fornara et al. 2011; Rosenkranz et al. 2012; Mueller et al. 2013). However, net rates alone do not provide a process-based understanding of the N cycle (Hart et al. 1994; Verchot et al. 2002; Cheng et al. 2013), which requires the assessment of simultaneously occurring gross N transformations (Hatch et al. 2000, Paterson 2003, Bedard-Haughn et al. 2006, Müller et al. 2007, Cheng et al. 2014). Previous studies reported that increasing species richness increased net N mineralization rates (Rosenkranz et al. 2012; Mueller et al. 2013), as well as net nitrification rates (Scherer-Lorenzen et al. 2003; Mueller et al. 2013). However, there are currently only few studies that have evaluated the relationship between biodiversity and gross

N transformation rates, with contrasting results. Zak et al. (2003) and West et al. (2006) reported for nutrient-poor, sandy soils from Minnesota, U.S.A. a positive biodiversity-gross N mineralization relationship in laboratory incubations, whereas Lama et al. (2020) found the opposite relationship in the Jena Experiment based on a 24 h-^{15}N pool dilution approach where the 0-5 cm surface soil layer was labeled with $^{15}NH_4Cl$ in the field to determine the rates of gross N mineralization, microbial assimilation of NH_4^+, and gross inorganic N immobilization at 76 plots with varying plant mixtures. Lama et al. (2020) attributed their finding to the mechanisms that increase the N-use efficiencies of plants with increasing plant species richness, which slowed down the N cycle, mainly because of increased C/N ratios of the roots.

One possible approach to simultaneously assess co-occurring transformation rates in soil involves the use of ^{15}N-labeled substrates. Müller et al. (2007) developed a ^{15}N tracing model (*Ntrace*) to quantify gross N transformations in soils. The model integrates pathways of N mineralization and immobilization of NH_4^+ and NO_3^- into labile and recalcitrant organic pools, nitrification of NH_4^+ to NO_3^- and from organic N to NO_3^-, dissimilatory nitrate reduction to ammonium (DNRA) (under anaerobic conditions), cation exchange (i.e. ad- and desorption) of NH_4^+ from clay minerals (Müller et al. 2007). Moreover, the model simulates the pool sizes of labile and recalcitrant organic N, NH_4^+, NO_3^-, and adsorbed NH_4^+ (Müller et al. 2007). The objectives of this study were to apply the *Ntrace* model to data obtained from laboratory incubations of field-fresh soil from the Jena Experiment without plants to evaluate the legacy effects of plant community composition (species richness, functional group richness, presence and absence of four functional groups - legumes, grasses, tall herbs, and small herbs) on the N pool size and gross N transformation rates in grassland soils. In line with previous applications of *Ntrace*, we incubated soil without plants so that the plant diversity effect originates from the previous plant effects on the microbial community. In the Jena Experiment, it has been shown that the different mixtures of root deposits into the soil released by the differently diverse plant communities communities and the effects of the plant community composition on abiotic conditions including soil moisture and nutrient availability shape the microbial community composition (Lange et al. 2014; Dassen et al. 2017; Weisser et al. 2017) A better understanding of the relationship between biodiversity and the complex N cycle will improve our prediction of possible biogeochemical consequences arising from the expected loss of biodiversity and changing plant community composition including possible changes in the N availability for

plant growth and increasing N leaking in gaseous form to the atmosphere or as nitrate to surface and groundwaters with their known detrimental effects on climate and water quality (Sutton et al. 2011).

3.3 Materials and methods

3.3.1 Study site

Our study contributed to the Jena Experiment (www.the-jena-experiment.de), a long-term grassland biodiversity experiment established in 2002 (Roscher et al. 2004; Weisser et al. 2017). The site had been used as arable land for at least 40 years before the initiation of the Jena Experiment. The experimental site is located on the floodplain of the river Saale in Jena, Germany (50°55' N, 11°35' E; 130 m above sea level). The mean annual air temperature at the site is 9.9°C, and mean annual precipitation amounts to 610 mm (Hoffmann et al. 2014). The soil at the site is classified as Eutric Fluvisol developed from 2 m thick loamy fluvial sediments (IUSS Working Group WRB 2014). The soil texture ranges from sandy loam close to the river to silty loam with increasing distance from the river. The experimental site is mown twice mimicking the locally common land use as a low-intensity hay meadow and weeded three times per year to maintain the designed diversity levels. The major aim of its establishment was to explore the effect of biodiversity on nutrient cycling and trophic interactions (Roscher et al. 2004).

The detailed description of the experimental design can be found in Roscher et al. (2004), and major results are reviewed in Weisser et al. (2017). The main field experiment consists of 82 plots in four blocks to account for the systematic change in soil texture perpendicular to the river, with a factorial design of different levels of plant species richness (SR: 1, 2, 4, 8, 16, and 60) and 1 to 4 functional groups (grasses, legumes, small herbs, and tall herbs). The mixtures were randomly drawn from a pool of 60 species representing a typical Central European mesophilic grassland. Each level of species richness was replicated on 16 plots, except for the 16 and 60 species richness levels, which are replicated only in 14 and 4 plots, respectively. Only Block 2 (n = 19 plots) was considered for this study, which included all the levels of plant species richness from 1-16 species in fourfold replication, except for the 16-species mixture, for which only three replicates existed.

Because the characterization of the soil microbial community composition might help in the interpretation of our results but was beyond the scope of our study, we refer to two studies from the same soils of the Jena Experiment (Lange et al. 2014; Dassen et al. 2017). Lange et al. (2014) reported for the year 2007 based on phospholipid fatty acid patterns that the fungal-to-bacterial biomass ratio was positively affected by plant functional group richness and negatively by the presence of legumes. Bacteria were more closely related to abiotic differences caused by plant diversity such as soil moisture, while fungi were more affected by plant-derived organic matter inputs defined by the composition of functional groups. Dassen et al. (2017) determined the composition of the fungi, bacteria, archaea, and protists community in the year 2010 based on 454-pyrosequencing. They found 4,025 bacterial, 23 archaeal, and 826 unclassified OTUs based on the amplification 16S rRNA gene fragments and 431 fungal, 174 protist, 9 plant, and 374 unclassified OTUs based on the amplification of eukaryotic 18S rRNA fragments. The most dominant taxonomic group of bacteria was the Chloroflexi. The most diverse bacterial groups were Proteobacteria and Planctomycetes. A total of 19 putative rhizobial OTUs were recovered across the experimental fields. The most dominant taxonomic group of eukaryotes was Ascomycota, which was also the most diverse fungal group. In total, 19 arbuscular mycorrhiza fungi (AMF) OTUs (phylum Glomeromycota) were recovered across all plant communities. Of the main protist supergroups, Rhizaria were well represented. Although protists represent a relatively small proportion (< 2%) of the total eukaryotic community, their diversity was considerable, with 174 detected OTUs. The main findings with respect to the relationship between plant community composition and soil organisms were that plant and functional group richness had little influence on the soil microbial community composition, which was more driven by the presence of legumes and by the small-scale abiotic variation at the field site (Dassen et al. 2017).

3.3.2 ^{15}N tracing experiment and sample analysis

To assess the importance of NH_4^+ fixation by clay minerals such as illites in the study soils, we conducted a sorption experiment in the context of our field ^{15}N tracer experiment reported Lama et al. (2020). We added 25 µg N (98 at% ^{15}N) as NH_4Cl to a 100 cm^3 stainless steel core inserted in the 0-5 cm soil layer and determined the recovery of the applied NH_4^+ by extraction with 1 M KCl 15 min after the application. Our mean recovery (± standard deviation) was 98±1.4%, from which we infer that NH_4^+ fixation is negligible in our study soils.

Soil samples were collected from Block 2 of the experimental site in October 2014, i.e. 12 years after the establishment of the vegetation mixtures. Approximately 400 g of field-fresh soil was sampled from each plot by combining 15 soil cores (Ø=1 cm, depth=15 cm). The soil samples were sieved (< 2 mm) in field-fresh state, and from each soil sample three replicates of 100 g of soil were produced. These field-fresh soil sample replicates were amended with $^{15}N\text{-}NH_4^+$, $^{15}N\text{-}NO_3^-$ or both (98 at%), applied as 0.5 µg $^{15}NH_4Cl\text{-}N\ g^{-1}$ and 0.25 µg $K^{15}NO_3\text{-}N\ g^{-1}$ dry soil. After the ^{15}N-label addition, samples were mixed thoroughly to ensure a homogeneous ^{15}N distribution and placed in incubation vessels with a ceramic filter (pore diameter of 0.4 µm). Above and below the soil samples, glass wool was inserted to prevent dispersion during rinsing. Finally, all the incubation vessels containing the soil samples were sealed with rubber stoppers and incubated for 16 days in the dark at a constant temperature of 20 ± 1 °C. To maintain aerobic conditions inside the incubation vessels, the soil samples were aerated by removing the rubber stoppers for one hour each day. Soil samples were extracted by percolation with 100 mL of a N-free nutrient solution (4 mM $CaCl_2$, 2 mM KH_2PO_4, 1 mM $MgCl_2$, 1 mM K_2SO_4, 1 mM $MgSO_4$, 25 µM H_3BO_3, 2 µM $MnSO_4$, 2 µM $ZnSO_4$, 0.5 µM $CuSO_4$ and 0.5 µM Na_2MoO_4; Nadelhoffer 1990) before ^{15}N labeling and at 12 hours and 2, 4, 9 and 16 days after the ^{15}N application. The nutrient concentrations were adjusted to optimize conditions for soil microorganisms (Nadelhoffer 1990). Percolation leaching was facilitated by applying a 200 kPa vacuum for half an hour. We conducted the experiment with field-fresh soils and determined the initial water content, which ranged from 26.7 to 31.4 vol%, by drying subsamples at 105°C. We maintained the initial water content by always carefully extracting the full volume of the added nutrient solution with the help of a vacuum. Because the microcosms were closed except during the short openings for aeration, we assumed that the evaporation loss was negligible. Consequently, the water content should have remained stable. To reduce the analytical load, samples of days 4 and 9 were combined to yield a single composite sample.

The pH of the soil solutions was measured with a glass electrode (Orion U402-S7, Thermo Fisher Scientific, Waltham, MA, USA). Concentrations of NH_4-N and NO_3-N in the extracts were measured colorimetrically with a continuous flow analyzer (SAN++, Skalar, Breda, The Netherlands). The ^{15}N isotopic composition of NO_3^- were determined by using the bacterial denitrification method, in which *Pseudomonas aureofaciens* is used to convert NO_3^- to N_2O, followed by isotopic analysis (Sigman et al. 2001; McIlvin and Casciotti et al. 2011). The

isotope ratios of the N_2O gas were analyzed with a Gas-Bench II pre-concentration unit interfaced with Delta V Plus isotope ratio mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) at the Basel Stable Isotope and Biogeochemistry Laboratory, University of Basel. The N isotope ratios in NH_4^+ were determined using the hypobromite-azide method, in which NH_4^+ is first converted to NO_2^-, and further to N_2O by reduction with azide (Zhang et al. 2007). The N_2O is then purified and analyzed as described above for NO_3^--derived N_2O.

To determine the concentrations of total N (TN), aliquots of the soil samples were dried and sieved (2 mm mesh), and the dried samples were then ground using a ball mill. TN concentrations were determined with an elemental analyzer (Elementaranalysator vario Max CN, Elementar Analysensysteme GmbH, Hanau, Germany).

Microbial respiration was measured using an electrolytic O_2 micro-compensation apparatus (Scheu 1992). O_2 consumption of soil microorganisms in 5 g of fresh soil was measured at 22°C over a period of 24 h. Basal respiration [µl O_2 g^{-1} dry soil h^{-1}] was calculated as the mean of the O_2 consumption rates determined between 14 to 24 hours after the start of the measurements. The measurement only started after 14 h, because initially, the O_2 consumption showed strong variations which are caused by the soil disturbance and only after 14 h, the respiration rates stabilized.

The microbial C/N ratio from a comparable sampling in 2008 was determined from the data of microbial biomass C and N, which was measured using chloroform fumigation extraction (Brookes and Landman 1985). Two samples of 7 g soil were taken from each plot, one was fumigated with chloroform vapor for 24 h, and the other was not fumigated. Both, the fumigated and non-fumigated samples were extracted with 40 mL 0.5 M K_2SO_4 by shaking for 30 minutes. Total C and N concentrations in the extracts were analyzed by dry combustion in a DIMA-TOC 100 Analyzer (Dimatec, Essen, Germany). Microbial biomass C was calculated as (total C in fumigated soil – total C in non-fumigated soil)/0.45 (Wu et al. 1990). Likewise, microbial biomass N was calculated as (total N in fumigated soil – total N in non-fumigated soil)/0.54 (Brookes and Landman 1985). The microbial C/N data were only available for the year 2008. However, Strecker et al. (2016) showed that both, the basal respiration and the microbial biomass C had similar sizes and similar significant relationships with plant species richness in 2008 and 2014. Thus, the size and activity of the microbial community were similar in 2008 and 2014, from which we inferred that it is likely that this is also true for the microbial C/N.

3.3.3 Quantification of N pools and gross transformation rates

The initial pool size of the exchangeable (= adsorbed) NH_4^+ pool, which represents the NH_4^+ retention by the soil shortly after addition of the $^{15}NH_4^+$, was calculated as the difference between applied NH_4^+ and initial dissolved NH_4^+ (on day 0). Because the first measurement of dissolved NH_4^+ only occurred after 12 h, we inferred the initial dissolved NH_4^+ concentration by back-extrapolation of those measured on days 1 and 2 (Müller et al. 2004). The start values of the exchangeable (= adsorbed) NH_4^+ pool ranged 0.174-0.180 µg N (g soil)$^{-1}$ (mean: 0.177 ± standard error 0.0002 µg N (g soil)$^{-1}$). The initial pool size of soil organic N was calculated from the difference between total soil N and the sum of 1 M KCl-extractable N (NH_4^+-N and NO_3^-N, see Oelmann et al. 2011 for a detailed description of the 1 M KCl extract). Soil organic N was divided into two pools, labile organic N (N_{lab}) and recalcitrant organic N (N_{rec}). In the absence of measured start values of labile and recalcitrant organic N concentrations, we used the model default values of 1% labile and 99% recalcitrant N as start values in line with previous studies in which the same model (*Ntrace*) was applied (Müller et al. 2004; 2007; Huygens et al. 2007). The estimate of 1% labile organic N is based on a study of Causarano et al. (2008). The start values of N_{lab} ranged 21.0-31.0 µg N (g soil)$^{-1}$ (26.4 ± 0.71 µg N (g soil)$^{-1}$) and of N_{rec} 2080-3065 µg N (g soil)$^{-1}$ (2610 ± 70.7 µg N (g soil)$^{-1}$). The changes of the pool sizes of exchangeable (= adsorbed) NH_4^+ and the two organic N pools during our 16-day incubation experiment were minor and therefore we only evaluated the influence of plant community composition on the start values of these pools.

We determined the rates of eight gross N transformation pathways by integrating the experimental data (i.e. pool sizes and ^{15}N enrichment in various N pools with time) in the *Ntrace* model (Müller et al. 2007; Fig. 3. 1). The measured NH_4^+ and NO_3^- concentrations and ^{15}N enrichment values were supplied to the model and gross N transformation rates were calculated using zero-order or first-order kinetics. The best fit between modeled and observed data was determined based on Akaike Information Criterion (AIC) by stepwise modification of the parameters included in the optimization routine and their respective kinetic settings (Table 1). Based on the kinetic settings and the final parameters, gross N transformation rates were calculated by integrating the rates over the 16-day period divided by the total time. The *Ntrace* model was programmed in the software MatLab 7.9 (The MathWorks Inc., Natick, MA, U.S.A.) and the ^{15}N tracing model that was separately set up in Simulink 7.4 (The MathWorks Inc.).

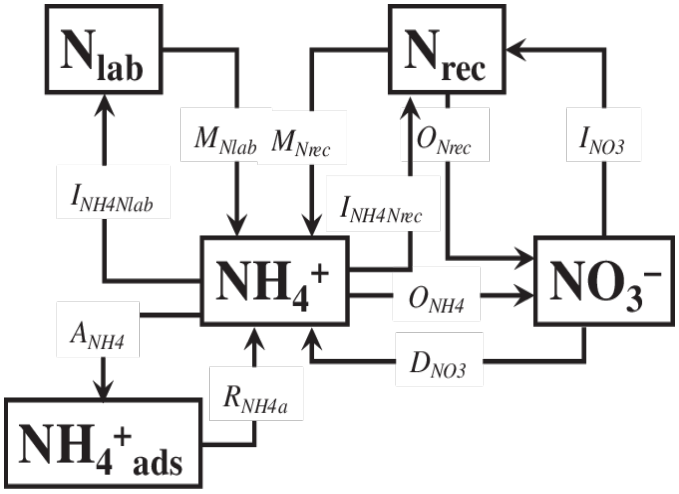

Fig. 3.1: Schematic representation of the ^{15}N tracing model (Müller et al. 2007). It includes five N pools: dissolved ammonium (NH_4^+), dissolved nitrate (NO_3^-), labile soil organic N (N_{lab}), recalcitrant organic N (N_{rec}), and exchangeable (termed "adsorbed" in previous work; Müller et al. 2004; 2007; Huygens et al. 2007) NH_4^+ ($NH_4^+{}_{ads}$) and ten gross N transformation rates: mineralization of recalcitrant organic N (M_{Nrec}), mineralization of labile organic N (M_{Nlab}), immobilization of NH_4^+ to recalcitrant organic N ($I_{NH4\text{-}Nrec}$), immobilization of NH_4^+ to labile organic N ($I_{NH4\text{-}Nlab}$), oxidation of recalcitrant organic N to NO_3^- (O_{Nrec}), oxidation of NH_4^+ to NO_3^- (O_{NH4}), immobilization of NO_3^- to recalcitrant organic N (I_{NO3}), dissimilatory NO_3^- reduced to NH_4^+ (D_{NO3}), adsorption of NH_4^+ into the exchangeable NH_4^+ pool (A_{NH4}), release of exchangeable (adsorbed) NH_4^+ to NH_4^+ (R_{NH4a}).

Total mineralization rates were calculated by summing up mineralization rates from both, the labile and recalcitrant organic N pools (M_{Nlab} + M_{Nrec}). Total NH_4^+ immobilization rates were calculated by summing up NH_4^+ immobilization rates from both NH_4^+ immobilization rates of labile and recalcitrant organic N pools ($I_{NH4\text{-}Nlab}$ + $I_{NH4\text{-}Nrec}$). Total nitrification rates were calculated by summing up the rate of NH_4^+ oxidation and organic N oxidation (O_{NH4} + O_{Nrec}). Since the dissimilatory nitrate reduction to ammonium (DNRA, D_{NO3}) and the desorption of NH_4^+ (R_{NH4a}) were negligible in our experiment at the given conditions, we excluded these two transformation rates from further data analysis. All N transformation rates and N pools were normalized to the TN concentration of the soil solid phase.

3.3.4 Statistical analyses

We used repeated measures and sequential ANOVA (type I sum of squares) to inspect effects of plant species richness, functional group richness, and presence/absence of each functional group on the two dissolved mineral N pools of different days, the initial exchangeable (= adsorbed) NH_4^+ and the total organic N pools and for the eight different gross N transformations. Lilliefors normality test and histograms were used to check for the normal

distribution of residuals. The residuals vs. fitted and Q-Q plots were also used to check the assumption of homoscedasticity and normality of the residuals. NH_4^+ and NO_3^- pools were log-transformed; M_{Nlab} and O_{NH4} were square root-transformed; and $I_{NH4\text{-}Nlab}$ was log-transformed to improve normal distribution of the residuals. The ANOVA was performed with plant species richness and presence/absence of each functional group as explanatory variables to analyze the effect of plant species richness and presence/absence of each functional group on mineral N pools and gross N transformations. The functional groups were fitted in the following order: legumes, grasses, tall herbs, and small herbs. Because we assumed that legumes have the strongest effect on the N cycle as a consequence of their N_2 fixing ability, we fitted legumes before other functional groups. Grasses also significantly impacted N transformations, while small herbs were shown to have the least or no effect (Oelmann et al. 2007; Eisenhauer et al. 2010). The interactions between plant species richness and presence/absence of functional groups were not significant and therefore were not considered in the final models. To avoid the collinearity between functional group richness and each functional group, a separate model was set up to test the effect of functional group richness on N pools and gross N transformation rates. All the statistical analyses were conducted in R studio (R Studio, Version 1.1.456, R Studio Inc., Boston, MA USA) with the free statistical software R 3.5.1 (R Core Team 2016). The type I error rate for all statistical analyses was $p < 0.05$.

3.4 Results

3.4.1 Pool size changes of dissolved mineral N during the incubation

The N amendments, which contributed less than 6 per cent of the existing mineral N pool at the time of the experiment, did not markedly affect the total amount of mineral N in the soil samples (as extracted with 1 M KCl). About 97-99 per cent of the added ^{15}N enrichments were recovered until the end of the experiment (day 16) in the solutions indicating that there were no or negligible gaseous losses by denitrification and/or ammonia volatilization. The pH of the soil solutions ranged 7.6 to 8.2.

Both, the dissolved NH_4^+-N and NO_3^--N concentrations showed parallel temporal courses irrespective of the kind of labeling and the species richness (Fig. 3.2). Across ^{15}N treatments and plots, the average concentrations of NH_4^+ declined from 0.30 ± 0.03 µg N(g soil)$^{-1}$ measured on the first day of incubation to 0.07 ± 0.01 µg N (g soil)$^{-1}$ on Day 16. In contrast, NO_3^- concentrations changed only from 1.28 ± 0.14 µg N (g soil)$^{-1}$ to 1.18 ± 0.11 µg N (g soil)$^{-1}$

analyzed on Day 1 and Day 16 of the incubation experiment, respectively. Differences in the pool sizes of both dissolved NH_4-N and NO_3-N at least between some incubation days were significant as reflected by the significant effect of time (day) on these pools (Table 3.2).

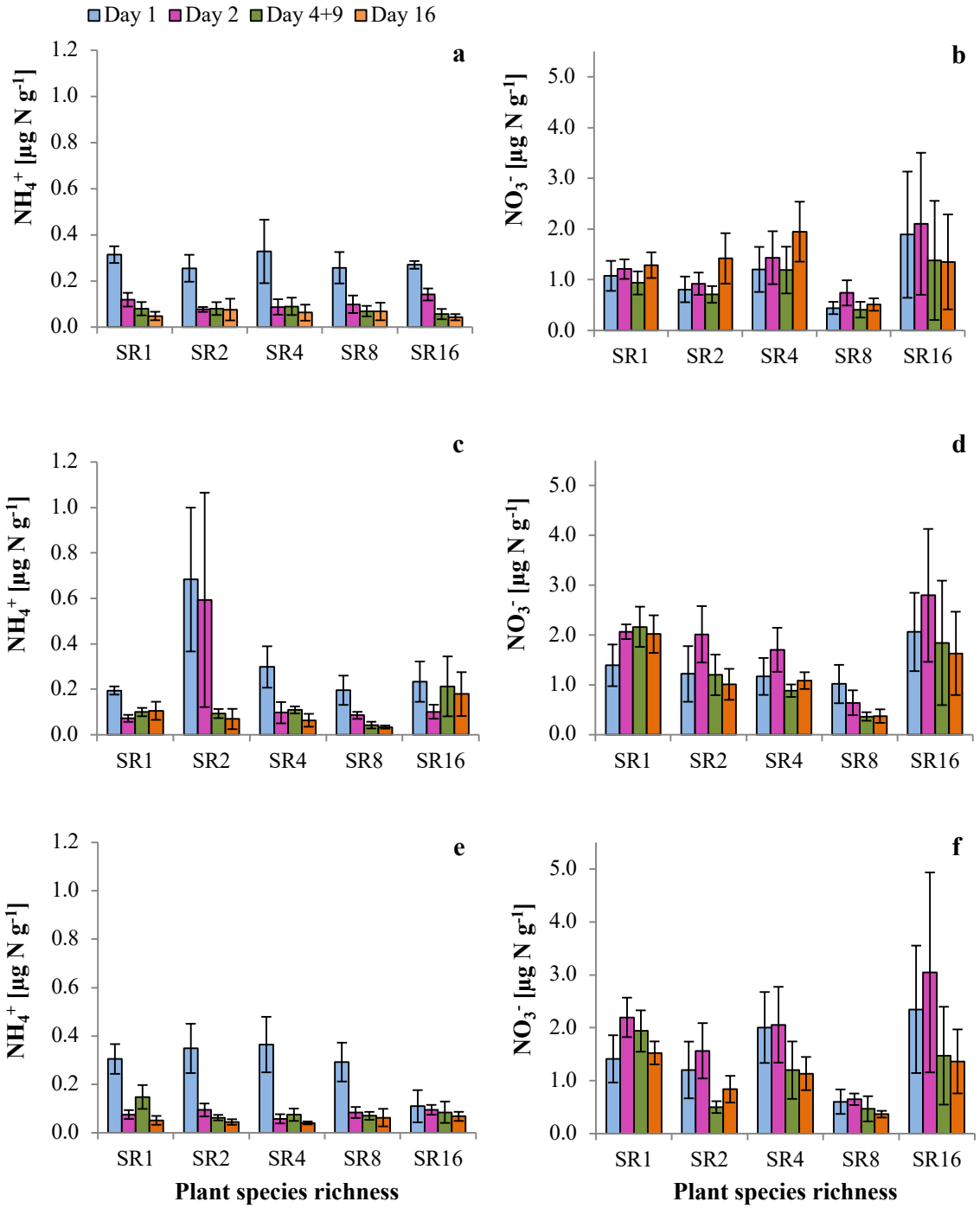

Fig. 3.2: Relationship between plant species richness and inorganic N pools (NH_4^+, NO_3^-) differentiated according to the labeling of ^{15}N-NH_4Cl (a, b), ^{15}N-KNO_3 (c, d) and combination of ^{15}N-NH_4Cl + ^{15}N-KNO_3 (e, f). Data are presented as mean ± standard error (SE). Note the differences in y-axis scaling

The interaction between day and species richness had a marginally significant influence on the dissolved NO_3^- pool (Table 3.2). Functional group richness did not show significant effects on the dissolved NH_4^+ and NO_3^- pool sizes (Table 3.2). The relationship between the presence of grasses and the dissolved NH_4^+ pool size was significantly positive (Table 3.2, Fig. 3.3). The effect of legumes on the dissolved NH_4^+ pool changed over time as reflected by the significant interaction between day and presence of legumes (Table 3.2). The concentration of ^{15}N in dissolved NH_4^+ and NO_3^- remained constant (Fig. 4a) or decreased during the incubation (Fig. 4b-f). Interestingly, ^{15}N was detected in NH_4^+, when only ^{15}N-NO_3^- was applied (Fig. 4c).

The presence of grasses had a marginally significant negative effect on the initial exchangeable (= adsorbed) NH_4^+ pool (Table S3.1). Plant species richness increased both, the initial labile and recalcitrant organic N pools (Table S3.2, Fig. S3.1).

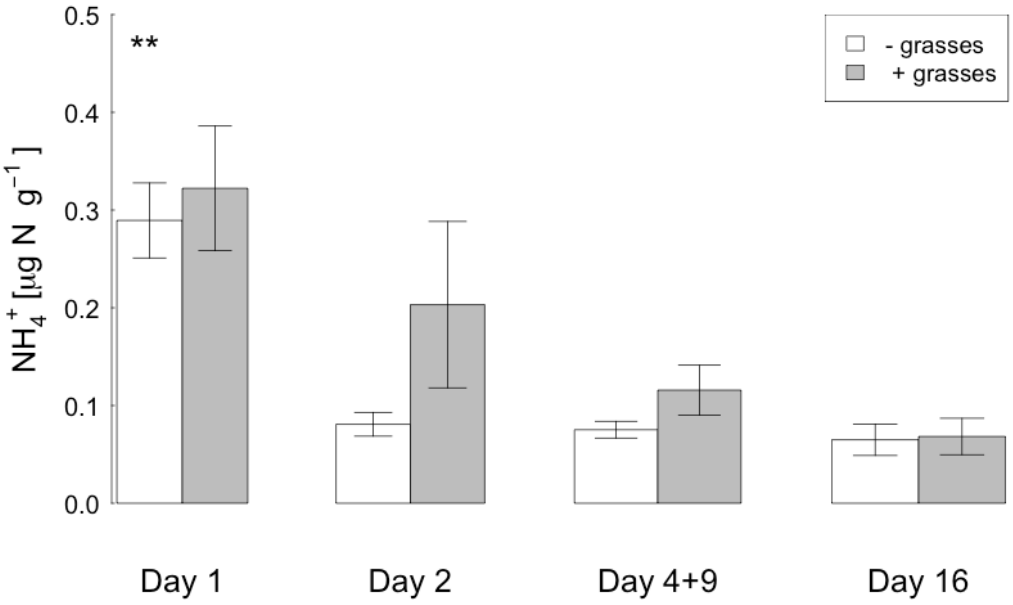

Fig. 3.3: Effect of grasses on the NH_4^+ pool over different days of incubation. Data are presented as mean ± standard error (SE). Grey and white bars represent presence (+) and absence (-) of grasses respectively. Significance code: **p<0.01

3.4.2 Gross NH_4^+ production

Gross N mineralization from labile organic N ranged between 0.01 and 1.94 µg N (g soil) $^{-1}$ d^{-1} and from recalcitrant organic N between 0.006 and 1.35 µg N (g soil)$^{-1}$ d^{-1} (means and standard deviations [SD] are shown in Table 3.1). We did not find any significant relationship between plant diversity and N mineralization from recalcitrant organic N. The positive effect of functional group richness on N mineralization from the labile organic N pool was only

marginally significant (Table 3.3, Fig. 3.5a). The presence of legumes or grasses had a significant positive effect on the N mineralization rate from labile organic N (Table 3.3, Fig. 3.5b, c).

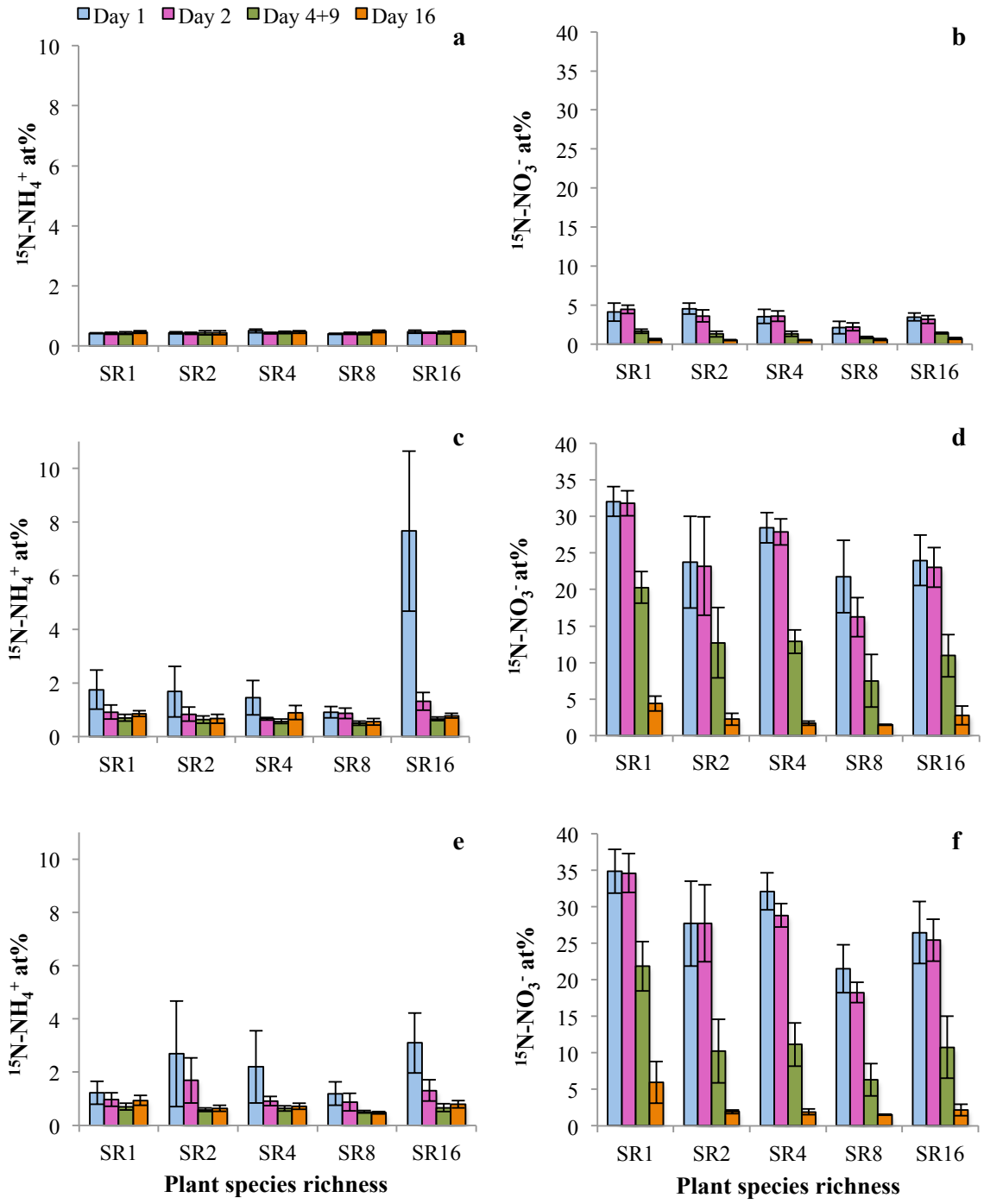

Fig. 3.4: Relationship between plant species richness and ^{15}N enrichment ($^{15}NH_4^+$, $^{15}NO_3^-$) differentiated according to the labeling of ^{15}N-NH_4Cl (a, b), ^{15}N-KNO_3 (c, d) and combination of ^{15}N-NH_4Cl + ^{15}N-KNO_3 (e, f). Data are presented as mean ± standard error (SE). Note the differences in y-axis scaling

3.4.3 Gross NO_3^- production

Gross heterotrophic and autotrophic nitrification rates ranged from 0.05 to 3.66 µg N (g soil) $^{-1}$ d^{-1} and from 0.20 to 3.62 µg N (g soil) $^{-1}$ d^{-1}, respectively (means and SD in Table 3.1). Neither plant species richness nor functional group richness (Table 3.4) significantly affected autotrophic nitrification. The presence of legumes significantly increased autotrophic nitrification rates (Table 3.4, Fig. 3.6a). We did not detect any significant effects of plant community composition on heterotrophic nitrification from the recalcitrant organic N pool.

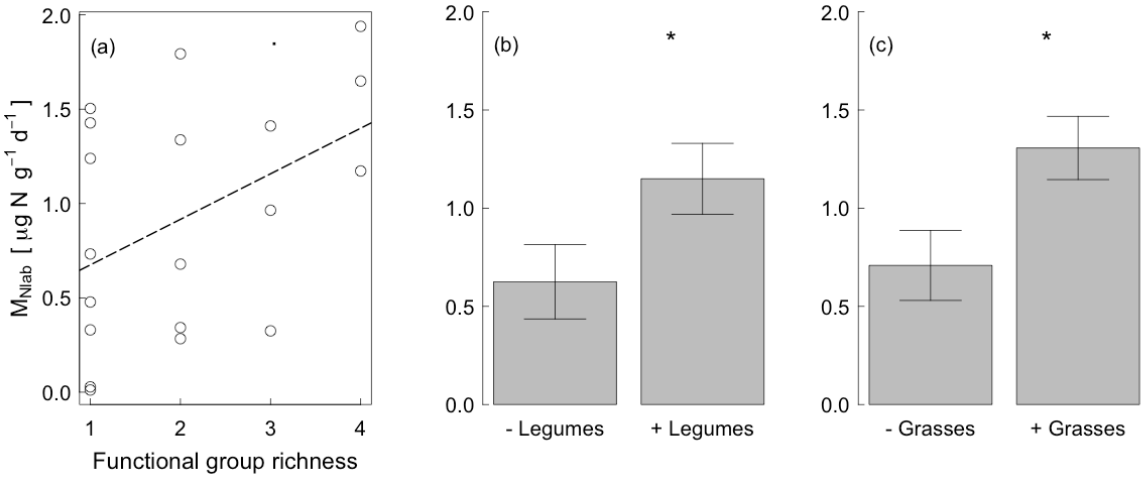

Fig. 3.5: Relationship between functional group richness and the gross N mineralization from the labile organic N pool (M_{Nlab}) (a) and effects of presence (+)/ absence (-) of legumes (b), and presence/absence of grasses (c) on the gross N mineralization from the labile organic N pool (M_{Nlab}). The whiskers on Fig. 3.5b, c represent standard errors. Significance codes: *p<0.05 and •p<0.1

Table 3.1: Description of model parameters and average gross N transformation rates (mean and standard deviation) estimated with the *Ntrace* model reported for each plant species richness (SR) level: 1, 2, 4, 8 and 16 (SR1, SR2, SR4, SR8 and SR16) in a grassland soil.

Parameters	Description	Kinetics *	N transformation rates (μg N (g soil)$^{-1}$ d^{-1})									
			SR1		SR2		SR4		SR8		SR16	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
M_{Nlab}	Mineralization of N_{lab} to NH_4^+	1	0.76	0.68	1.08	0.69	0.58	0.40	1.10	0.74	1.19	0.66
M_{Nrec}	Mineralization of N_{rec} to NH_4^+	0	0.65	0.43	0.67	0.55	0.36	0.32	0.69	0.28	0.45	0.42
$I_{NH4\text{-}Nlab}$	Immobilization of NH_4^+ to N_{lab}	1	0.22	0.14	0.28	0.18	0.24	0.22	0.18	0.09	0.31	0.22
$I_{NH4\text{-}Nrec}$	Immobilization of NH_4^+ to N_{rec}	1	0.03	0.004	0.03	0.01	0.02	0.01	0.02	0.01	0.03	0.003
O_{Nrec}	Oxidation of N_{rec} to NO_3^-	0	1.58	1.37	1.59	0.90	2.57	0.98	1.24	0.95	1.51	1.90
I_{NO3}	Immobilization of NO_3^-	1	2.96	1.97	3.89	2.12	4.09	1.54	3.47	1.31	3.72	2.87
O_{NH4}	Oxidation of NH_4^+ to NO_3^-	1	1.38	0.49	1.82	1.37	0.99	0.37	1.83	1.00	1.45	1.10
A_{NH4}	Adsorption of NH_4^+	1	0.03	0.01	0.03	0.01	0.02	0.01	0.02	0.01	0.03	0.01

*Kinetics: 0 = zero-order, 1 = first-order

Table 3.2: Results of repeated measure ANOVA showing the effects of plant species richness, functional group richness and presence (+)/absence (-) of each functional group on ammonium (NH_4^+) and nitrate (NO_3^-) pools measured on different days of incubation. Bold letters show significance at $p < 0.05$ and italics letters show marginal significance at $p < 0.1$. Non-significant within subject effects are excluded from the table. Arrows indicate positive (↑) effect.

	Dissolved NH_4^+ pool				**Dissolved NO_3^- pool**			
Source	Df	SS	F	P	Df	SS	F	P
Between subject effects								
Plant species richness	1	0.01	0.02	0.885	1	0.18	0.08	0.778
Functional group richness	1	0.00	0.00	0.991	1	0.08	0.04	0.845
Presence of legumes	1	0.28	0.93	0.354	1	0.65	0.29	0.598
Presence of grasses	**1**	**2.78**	**9.25**	**0.009** ↑	1	1.99	0.89	0.362
Presence of tall herbs	1	0.16	0.52	0.484	1	0.12	0.05	0.823
Presence of small herbs	1	0.43	1.44	0.252	1	0.69	0.31	0.586
Within subject effects								
Day	**3**	**29.14**	**28.81**	**<0.001**	**3**	**2.38**	**7.09**	**<0.001**
Day * Presence of legumes	**3**	**2.93**	**2.90**	**0.047**	-	-	-	-
Day * species richness	-	-	-	-	*3*	*0.88*	*2.62*	*0.065*

Table 3.3: Sequential ANOVA results showing the effects of plant species richness, functional group richness and presence (+) /absence (-) of each functional group on gross N mineralization from the labile organic N pool (M_{Nlab}) and on immobilization of NH_4^+ into the labile organic N pool ($I_{NH4\text{-}Nlab}$). Bold letters show significance at p < 0.05 and italics letters show significant at p < 0.1. Arrows indicate positive (↑) and negative (↓) effects.

	M_{Nlab}				$I_{NH4\text{-}Nlab}$			
Source	Df	SS	F	P	Df	SS	F	P
Species richness	1	0.13	1.41	0.256	1	0.06	0.15	0.707
Functional group richness	*1*	*0.52*	*4.06*	*0.060* ↑	1	0.54	1.07	0.315
Presence of legumes	**1**	**0.43**	**4.85**	**0.046** ↑	1	0.13	0.35	0.566
Presence of grasses	**1**	**0.68**	**7.70**	**0.016** ↑	**1**	**3.58**	**9.65**	**0.008** ↑
Presence of tall herbs	*1*	*0.29*	*3.24*	*0.095* ↓	1	0.03	0.07	0.792
Presence of small herbs	1	0.00	0.01	0.907	1	0.56	1.51	0.241

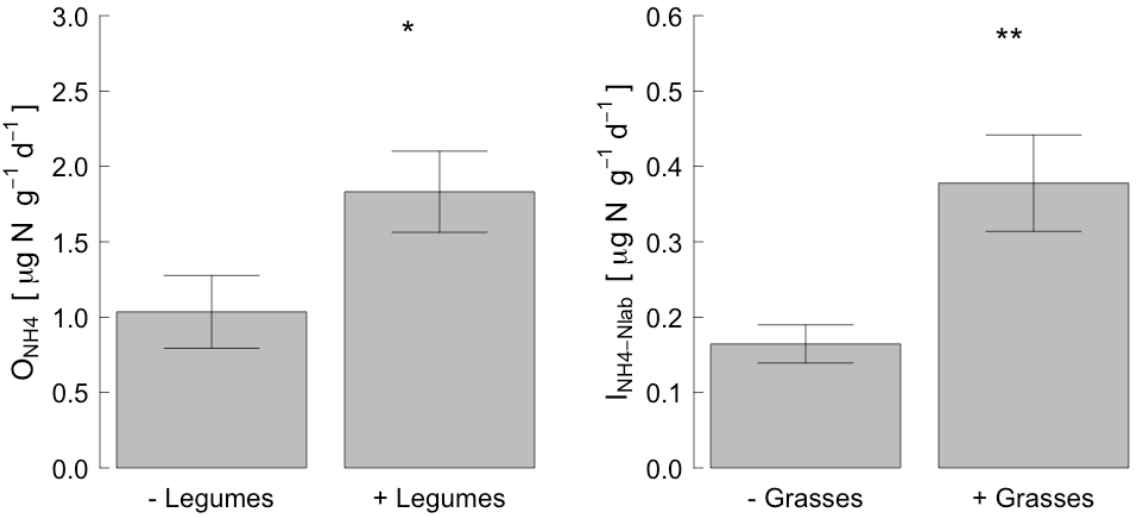

Fig. 3.6: Effects of the presence (+)/absence (-) of legumes on autotrophic nitrification (O_{NH4}) (a) and presence (+)/absence (-) of grasses on the immobilization of NH_4^+ in the labile organic N pool ($I_{NH4-Nlab}$) (b). Whiskers represent standard errors. Significance code: *p<0.05

3.4.4 Gross NH_4^+ and NO_3^- immobilization

The NH_4^+ immobilization rates into the labile and recalcitrant organic N pools ranged from 0.05 to 0.55 and from 0.003 to 0.04 µg N (g soil)$^{-1}$ d^{-1}, respectively and the NO_3^- immobilization rates ranged from 0.94 to 6.97 µg N (g soil)$^{-1}$ d^{-1} (means and SD in Table 3.1). Neither plant species richness nor functional group richness significantly affected the NH_4^+ immobilization into the labile (Table 3.3) and the recalcitrant organic N pools. Grasses significantly increased the immobilization of NH_4^+ into the labile organic N pool (Table 3.3, Fig. 3.6b). There were no significant effects of plant community composition on the immobilization of NO_3^- into the organic N pool.

3.4.5 Microbial properties versus gross N transformation rates

Given their known role as drivers of N transformations (Fornara et al. 2011), microbial activity likely is a principal factor that needs to be considered when trying to explain the observed differences in gross N transformation rates (Booth et al. 2005). Microbial C/N ratios showed a marginally significant negative correlation with N mineralization from labile organic N, and a significant negative correlation with autotrophic nitrification (Fig. 3.7a, b). Furthermore, we found a marginally significant positive relationship between basal respiration and immobilization of NH_4^+ into the labile organic N (Fig. 3.7c).

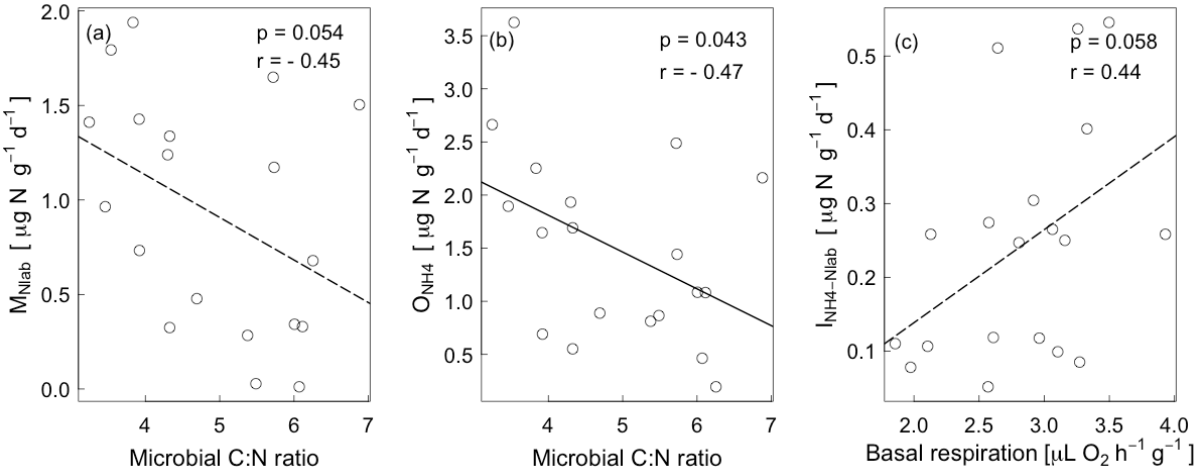

Fig. 3. 7: Relationships between microbial C/N ratio and N mineralization into labile organic N (M_{Nlab}) (a), microbial C/N ratio and autotrophic nitrification (O_{NH4}) (b) and basal respiration and ammonium immobilization from labile organic N ($I_{NH4-Nlab}$) (c). P and r-values refer to the results from Pearson's correlation tests. Solid line shows significance at p<0.05 and dotted lines show significance at p<0.1. Note the difference in y-axis scaling

Table 3.4: Sequential ANOVA results showing the effects of plant species richness, functional group richness and presence (+) /absence (-) of each functional group on autotrophic nitrification (O_{NH4}). Bold letters show significance at p < 0.05. Arrows indicate positive (↑) effects.

Source	Df	SS	F	P
Species richness	1	0.00	0.02	0.906
Functional group richness	1	0.29	2.20	0.157
Presence of legumes	**1**	**0.61**	**5.12**	**0.041** ↑
Presence of grasses	1	0.32	2.74	0.122
Presence of tall herbs	1	0.04	0.37	0.554
Presence of small herbs	1	0.01	0.07	0.790

3.5 Discussion

3.5.1 Pool sizes and gross N transformation rates

The drastic dilution of applied ^{15}N-NH_4^+ in the soil extracts during the incubation (Fig. 3.2a, 4a) indicated that there was a rapid release of unlabeled NH_4^+ from the organic matter into the ^{15}N labeled NH_4^+ pool. Huygens et al. (2007) suggested that the rapid disappearance of labeled NH_4^+ might be attributable to the exchange of the labeled NH_4^+ by adsorbed NH_4^+ on clay minerals or other cation-exchanger sites. The increase in the ^{15}N enrichment of the NH_4^+ pool in the $^{15}NO_3^-$ labeled treatments (Fig. 3.4c) can be attributed to the re-mineralization of recently immobilized $^{15}NO_3^-$. The gradual decline of $^{15}NO_3^-$ concentrations in the soil extracts during the incubation (Fig. 3.4d) demonstrated that NO_3^- at natural abundance entered into the ^{15}N-labeled NO_3^- pool via autotrophic or heterotrophic nitrification. Throughout the incubation, the pool sizes of dissolved mineral N (i.e. the sum of the NH_4-N and NO_3-N concentrations) remained nearly constant (Fig. 3.2), which showed that the net N transformation rates were similar for all plots. The study conducted by Huygens et al. (2007) in unpolluted South Chilean forests also found almost constant pool sizes at low net mineralization and nitrification rates.

The rate of gross N mineralization ($M_{Nrec} + M_{Nlab}$) in our experiment fell within the range of 0.40 – 4.07 µg N (g soil)$^{-1}$ d^{-1} reported in the literature for grasslands (Jamieson et al. 1999; Accoe et al. 2004; Müller et al. 2004; McKinley et al. 2008; Müller et al. 2014). The measured total NH_4^+ immobilization rates ($I_{NH4\text{-}Nrec} + I_{NH4\text{-}Nlab}$) were also in the range of 0.10 – 0.88 µg N (g soil)$^{-1}$ d^{-1} reported by other grassland studies (Hungate et al. 1997; Verchot et al. 2002; Müller et al. 2011). The measured rates of heterotrophic nitrification in this study were similar to or higher than the range of 0.07 – 1.41 µg N (g soil)$^{-1}$ d^{-1} reported in other studies in grassland soils (Müller et al. 2004; 2009; Laughlin et al. 2009). The autotrophic nitrification rates determined in this study are in the range of 0.10 – 2.88 µg N (g soil)$^{-1}$ d^{-1} reported for other grassland studies (Zaman et al. 1999; Accoe et al. 2004; Müller et al. 2009; Demey et al. 2014).

The NO_3^- immobilization rates were similar to or higher than the range of 0.81 – 3.84 µg N (g soil)$^{-1}$ d^{-1} reported in the literature for grasslands (Davidson et al. 1990; Watson et al. 2000; Corre et al. 2002). The NO_3^- immobilization rates in our study were comparable to the total nitrification rates, which showed that the NO_3^- produced via nitrification was completely assimilated by microorganisms, leaving little space for NO_3^- leaching or denitrification. Aber et al. (1989) and Huygens et al. (2007) suggested that N losses via leaching or denitrification

may not occur if N inputs do not exceed plant or microbial N demand. The occurrence of high NO_3^- immobilization is also attributable to the insufficient availability of NH_4^+ in soil (Fig. 3.2) to meet the microbial demand for N (Rice and Tiedje 1989; Corre et al. 2002). However, both nitrification and NO_3^- immobilization rates were higher than under field conditions, because our microcosm experiment did not include plants and thus excluded plant uptake of NH_4^+. This is in line with the suggestion of Kammann et al. (1998) that the increased NO_3^- concentrations observed in laboratory experiments are not likely to occur in the field, because plant uptake and leaching would decrease the NO_3^- concentration in soil.

3.5.2 Plant diversity effects on N pool sizes

The presence of grasses significantly increased the dissolved NH_4^+ pool, probably because of their dense rooting system (Oelmann et al. 2007; Bessler et al. 2009; Ravenek et al. 2014). The dead roots along with their exudates, which remained in our sample, likely increased microbial activity (Van der Krift et al. 2001; Lange et al. 2015; Eisenhauer et al. 2017). The increased microbial activity accelerated the decomposition of soil organic matter, which is also reflected by the positive effect of grasses on the gross mineralization rate (Table 3.3; Fig. 3.5c). At the same time the initial exchangeable (= adsorbed) NH_4^+ pool was marginally significantly lower in the presence of grasses, possibly because of the exhaustive N exploitation of the dense grass roots prior to our experiment without plants (Table S3.1). This exhaustive N exploitation is also supported by the fact that the presence of grasses reduced NO_3^--N and total dissolved N leaching in the Jena Experiment (Leimer et al. 2016). The increase in the pool sizes of the initial labile and recalcitrant organic matter with increasing species richness (Table S3.2, Fig. S3.1) can be attributed to the positive effect of increasing species richness on organic matter and total N accumulation at the study sites of the Jena Experiment as a consequence of the positive species richness-biomass production relationship (Weisser et al. 2017).

3.5.3 Plant diversity effects on NH_4^+ production and immobilization processes

We observed that functional group richness had a marginally significant positive effect on gross N mineralization from the labile organic N pool (Table 3.3, Fig. 3.5a). A similar positive effect of plant species richness on gross N mineralization was reported by Zak et al. (2003) and West et al. (2006) in laboratory incubation experiments with soils of the Cedar Creek biodiversity experiments in Minnesota, U.S.A., where sandy, nutrient-poor soils prevail. In both studies the range of species richness was the same as in our study (i.e., 1 to 16 species, but the

plant community composition was different). However, both studies did not distinguish between mineralization from the labile and recalcitrant organic N pools. Wedin and Pastor (1993) have previously reported that labile organic N is important for the N supply of plants in grassland, while the recalcitrant organic N is responsible for longer-term N storage. Zak et al. (2003) and West et al. (2006) attributed the significant positive relationship between plant species richness and gross N mineralization to the high plant productivity resulting in high organic inputs to soil, which would have remained in the incubated samples of our experiment. Furthermore, we found a marginally significant positive effect of the microbial C/N ratio on gross N mineralization rates (Fig. 3.7a). The microbial C/N ratio is also considered one of the potential variables influencing the rate of N mineralization, because inorganic N production increases when microbial activity increases (Booth et al. 2005).

Most of the studies on the biodiversity-N cycle relationship reported a positive effect of legumes on N pools and transformations. To test if the functional group richness on gross N mineralization was mostly driven by legumes, we ran a separate ANOVA by fitting "presence of legumes" before "functional group richness". We found that functional group richness explained 19.3% of the total variance, of which 19.2% was explained by the presence of legumes alone. This suggests that the presence of legumes indeed explained the functional group richness effect. This is in line with earlier findings of Hooper and Vitousek (1998) that nutrient cycling is more dependent on certain functional groups rather than on species richness. However, later studies have shown that plant species richness significantly influences the N cycle irrespective of the functional group composition of the community (Weisser et al., 2017). We cannot rule out that the failure to see a species richness effect in our experiment is attributable to the comparatively low statistical power of our experiment, which only included the soil samples from one out of four blocks of the Jena Experiment, and also to the fact that the incubation experiment did not include living plants.

In a field experiment at the same study site, Lama et al. (2020) observed a significant negative relationship between species richness and gross N mineralization, which was mainly driven by the increasing root C/N ratios with increasing species richness. Higher species richness increased root C/N ratios via the dilution of plant nutrient concentrations, because of the greater height of plants in species-rich mixtures as a consequence of the competition for light. We can only speculate that under the optimum decomposition conditions of our

incubation experiment, and in the absence of active plants, the negative effect of the increasingly smaller C/N ratios in roots with increasing species richness was overprinted.

The positive influence of legumes on gross N mineralization rates from the labile organic N pool (Table 3.3, Fig. 3.5b) is likely related to the of the fact that legumes generally increase N concentrations in soils (Oelmann et al. 2007; Fornara and Tilman 2008). This results from atmospheric N_2-fixation (Ledgard 2001; Spehn et al. 2002) or the generally higher N concentrations in legumes (Marschner 2012) which will also result in the return of more N to the soil. The legume-derived more readily degradable organic matter is introduced into the soil via rhizodeposition and aboveground litterfall (Read 1996). The N accumulation in soil in the presence of legumes resulted in a higher aboveground biomass in the legume-containing plots of the Jena Experiment compared to the legume-free plots (Marquard et al. 2009) further increasing the available C pool in soil. Moreover, soil microbial biomass C increases in the presence of legumes (Eisenhauer et al. 2010; but see Strecker et al. 2016 for changing legume effects over time), and this might have further enhanced gross N mineralization.

The positive relationship between NH_4^+ immobilized from labile organic N and the presence of grasses (Fig. 3.6b) might be attributable to an enhanced microbial activity (Fig. 3.7c; Eisenhauer et al. 2010). Grasses are characterized by dense fibrous roots with a high length (Weigelt et al. 2008). Therefore, grasses likely enhanced microbial biomass and activity by providing large amounts of root exudates (Van der Krift et al. 2001; Eisenhauer et al. 2010), and this grass effect might have persisted in our experiment without plants.

3.5.4 Plant diversity effects on NO_3^- production and immobilization processes

Our study indicates that heterotrophic nitrification of organic N is an important process of NO_3^- production in the studied grassland soils, because heterotrophic nitrification rates were similar to the rates of autotrophic nitrification and also because heterotrophic nitrification is the other direct way of producing mineral N from organic N. The study by Müller et al. (2004) regarded heterotrophic nitrification as the predominant pathway for NO_3^- production in soils at high recalcitrant organic C in a grassland ecosystem. The NH_4^+ produced by mineralization, which is not taken up by plants or immobilized by microbes, is oxidized by nitrifiers and results in elevated soil NO_3^- concentrations. This assumption is corroborated by the positive correlation between mineralization and nitrification rates reported in the review of Booth et al. (2005). Our

incubation experiment did not include plants and therefore, the produced NH_4^+ was not taken up by plants offering more substrate for the nitrification to NO_3^- than under field conditions with plants. Furthermore, we observed an increasing rate of autotrophic nitrification in the presence of legumes (Table 3.4, Fig. 3.6a), because of the higher N concentrations in the legume-containing plots of the Jena Experiment (Oelmann et al. 2007). In addition, autotrophic nitrification exhibited a significant negative relationship with the microbial C/N ratio (Fig. 3.7b). Lower microbial C/N ratios which are associated with substrate of high quality (Hart et al. 1994) increase microbial activity, thereby enhancing autotrophic nitrification (Booth et al. 2005; Inselsbacher et al. 2013). Previous studies in the Jena Experiment have shown elevated net nitrification (Scherer-Lorenzen et al. 2003) and increased KCl-extractable soil NO_3^- concentrations (Oelmann et al. 2011; Leimer et al. 2014) in the presence of legumes. However, Hooper and Vitousek (1997) and Niklaus et al. (2006) found no effects of plant diversity on nitrification.

3.6 Conclusions

Our study demonstrated that in the absence of plant uptake, almost all the produced NH_4^+ was converted into NO_3^-. We observed a strong legacy effect of legumes for gross N transformations. Legumes particularly had a positive effect on gross N mineralization and autotrophic nitrification. Grasses also increased the dissolved NH_4^+ pool, gross N mineralization, and NH_4^+ immobilization. Heterotrophic nitrification was found to play a vital role in soil N cycling. Consequently, future studies should focus on identifying the controlling factors of heterotrophic nitrification in grassland soils.

The fact that we conducted our experiment without plants as is commonly done to collect the data needed by the used N cycling model *Ntrace* limited the transferability of the results to the field. Therefore, future studies should include plants and be conducted in growth chambers or Ecotrons. Given the partly small effect sizes of plant community composition on several elements of the N cycle, it would also be desirable to increase the statistical power of such experiments by including more replicates of the various species mixtures than we were able to include.

Our results indicate that changing contributions of legumes and grasses in response to environmental and land-use change will markedly influence the N availability for the plant community and possibly also the N leaking into atmosphere and water. However, we could not confirm that species or functional group richness tighten the N cycle and deplete mineral N concentrations in soil, possibly because of a limited statistical power of our experiment.

3.7 Acknowledgments

We thank everyone who helped with the management of the experiment and in particular the initiators, E.-D. Schulze, B. Schmid, and W.W. Weisser, as well as the scientific coordinators C. Roscher, A. Weigelt, and A. Ebeling. Thanks also to all those who assisted us during the weeding campaigns. The Jena Experiment was funded by the Deutsche Forschungsgemeinschaft (DFG, FOR 456 and 1451, Wi 1601/4) and the Swiss National Science Foundation (SNSF, 200021E-131195/1), with additional support from the Friedrich Schiller University Jena and the Max Planck Society. The ^{15}N tracing analysis was carried out in close collaboration with the DFG-funded research unit DASIM (FOR2337).

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3.9 Supplementary materials

Table S3.1: Sequential ANOVA results showing the effects of plant species richness, functional group richness and presence (+) /absence (-) of each functional group on the initial exchangeable (=adsorbed) ammonium pool ($NH_4^+{}_{ads}$). Italics show significance at p < 0.1. The arrow (↓) indicates a negative effect

Source	Df	SS	F	P
Species richness	1	5.640e-07	0.39	0.543
Functional group richness	1	1.810e-07	0.12	0.733
Presence of legumes	1	2.000e-09	0.00	0.968
Presence of grasses	*1*	*6.016e-06*	*4.16*	*0.062* ↓
Presence of tall herbs	1	1.180e-07	0.08	0.780
Presence of small herbs	1	1.400e-07	0.09	0.761

Table S3.2: Sequential ANOVA results showing the effects of plant species richness, functional group richness and presence (+) /absence (-) of each functional group on the initial labile organic N pool (N_{lab}) and on the initial recalcitrant organic N pool (N_{rec}). Bold letters show significance at $p < 0.01$. Arrows (↑) indicate positive effects

	N_{lab}				N_{rec}			
Source	Df	SS	F	P	Df	SS	F	P
Species richness	**1**	**60.22**	**10.04**	**0.007** ↑	**1**	**590869**	**10.05**	**0.007** ↑
Functional group richness	1	18.44	2.01	0.175	1	180791	2.01	0.175
Presence of legumes	1	10.15	1.69	0.216	1	99369	1.69	0.216
Presence of grasses	1	11.82	1.97	0.184	1	115724	1.97	0.184
Presence of tall herbs	1	14.18	2.37	0.148	1	139064	2.36	0.148
Presence of small herbs	1	0.17	0.03	0.867	1	1704	0.03	0.867

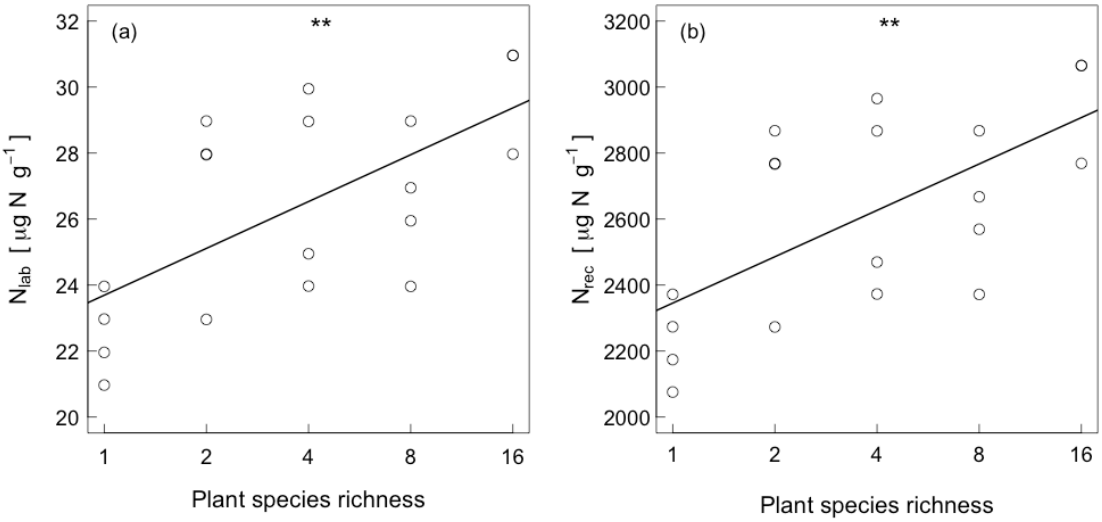

Fig. S3.1: Effect of plant species richness on the labile organic N pool (N_{lab}) (a), and the recalcitrant organic N pool (N_{rec}) (b). Significance codes: **p<0.01. Note the difference in y-axis scaling

4. Plant diversity influenced gross nitrogen mineralization, microbial ammonium consumption and gross inorganic N immobilization in a grassland experiment

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Chapter 4 is published in Oecologia (2020) 193:731-748, DOI https://doi.org/10.1007/s00442-020-04717-6

4.1 Abstract

Gross rates of nitrogen (N) turnover inform about the total N release and consumption. We investigated how plant diversity affects gross N mineralization, microbial ammonium (NH_4^+) consumption and gross inorganic N immobilization in grasslands via isotopic pool dilution. The field experiment included 74 plots with 1-16 plant species and 1-4 plant functional groups (legumes, grasses, tall herbs, small herbs). We determined soil pH, shoot height, root, shoot and microbial biomass, and C and N concentrations in soil, microbial biomass, roots and shoots. Structural equation modeling (SEM) showed that increasing plant species richness significantly decreased gross N mineralization and microbial NH_4^+ consumption rates via increased root C/N ratios. Root C/N ratios increased, because of the replacement of legumes (low C/N ratios) by small herbs (high C/N ratios) and an increasing shoot height, which was positively related with root C/N ratios, with increasing species richness. However, in our SEM remained an unexplained direct negative path from species richness to both N turnover rates. The presence of legumes increased gross N mineralization, microbial NH_4^+ consumption and gross inorganic N immobilization rates likely because of improved N supply by N fixation. The positive effect of small herbs on microbial NH_4^+ consumption and gross inorganic N immobilization could be attributed to their increased rhizodeposition, stimulating microbial growth. Our results demonstrate that increasing root C/N ratios with increasing species richness slow down the N cycle but also that there must be additional, still unidentified processes behind the species richness effect potentially including changed microbial community composition.

4.2 Introduction

Biodiversity loss has raised concern over the consequences for ecosystem functioning (Isbell et al. 2011, Cardinale et al. 2012, Meyer et al. 2016; Weisser et al. 2017). Plant diversity is essential for maintaining a variety of ecosystem functions (Hector et al. 1999; Loreau et al. 2001; Tilman et al. 2001; Roscher et al. 2005; Cardinale et al. 2012), including nitrogen (N) cycling (Spehn et al. 2005; Fornara and Tilman 2009; Oelmann et al. 2011; Reich et al. 2012; Rosenkranz et al. 2012). Biodiversity experiments have mainly reported increased community productivity with increasing plant diversity (Tilman et al. 2001; Spehn et al. 2005; Marquard et al. 2009). A potential reason for the positive species richness-biomass production relationship might be complementarity effects in species-rich mixtures (Hooper and Vitousek 1998;

Fargione et al. 2007; Reich et al. 2012). Complementarity effects occur when more-diverse communities increase their performance above the expected performance of monocultures through acquiring more nutrients and using available light and space more exhaustively (Hooper and Vitousek 1997; Naeem et al. 2002). Complementarity also includes the process of facilitation, for example by legumes, which increase the nutrient availability for neighboring plants via N fixation (Fargione et al. 2007). However, in about 1/3 of the reported experiments, complementarity effects did not increase productivity likely because of the selection for more competitive but less productive species at higher diversity (Cardinale et al. 2011). Furthermore, sampling effects can arise in biodiversity experiments, if the probability of sampling dominant species increases in high diversity levels (Huston 1997; Loreau et al. 2001). To prevent such sampling effects and to be able to detect other mechanisms behind biodiversity-ecosystem functioning relationships, biodiversity experiments need to be carefully designed (Loreau et al. 2001; Roscher et al. 2004). Increasing plant diversity modifies resource availability for soil microbial communities (Zak et al. 2003), which mineralize organic matter and enhance nutrient release by litter decomposition. Plant species differ in their biochemical composition providing an incentive for microbes to derive different resources from different litter types (Gartner and Cardon 2004). This might result in altering overall decomposition rates of mixtures relative to the cumulative composition of individual litter species (Gessner et al. 2010). Jewel et al. (2015) reported a faster decomposition rate of monospecific litter in its environment of origin but not of mixed litter. Although it has been reported that complementarity can result in high plant productivity and N uptake, it is uncertain if the changes in plant diversity affect microbial N dynamics.

Plants play a vital role in ecosystem N cycling because plants assimilate this essential nutrient to produce biomass, which is returned as aboveground and belowground litter to soil where it is decomposed, thereby releasing the N back into the soil solution (Knops et al. 2002; Vitousek et al. 2002). Individual plant species can positively affect the N cycle in soil by the activity of plant roots (e.g., fine root turnover, root exudation; Clarholm 1985; Cadisch and Giller 1997) and by regulating the quality of plant litter (measured as C/N ratios, Aerts et al. 1992; Van Vuuren et al. 1993; Abbas et al. 2013; Guiz et al. 2015). Plant species that host N_2-fixing bacteria can change N cycling by improving the N availability to other co-occurring species (Mulder et al. 2002; Spehn et al. 2005). Another way in which plant species may affect

rates of N cycling is through their association with mycorrhizal fungi, which enhance the ability of plants to acquire nutrients (Hobbie 1992).

Because of the importance of N in all ecosystems and the marked impact of human activities on the N cycle, N and its transformations have received a great deal of attention. The supply rate of N to the plant and microbe community depends largely on gross N mineralization, which is described as the total N transformed from organic N to mineral N forms (NH_4^+, NO_3^-) by microorganisms in soil over a period of time that can be readily taken up by plants and microbes. Microbial ammonium consumption refers to the microbial assimilation of NH_4^+ plus the gross nitrification. Gross inorganic N immobilization is the process of converting inorganic forms of N by microbes and other soil heterotrophs to organic N forms. Net N mineralization refers to the gross mineralized N minus the quickly microbially consumed N. Net ammonification is the difference between gross N mineralization and microbial NH_4^+ consumption, and net nitrification is that between gross nitrification and nitrate immobilization.

Hobbie (1992) reported that the strong relationship between litter quality and gross N mineralization rates might indicate that gross N mineralization rates are determined by the quality of litter input. This was corroborated by the results of Van der Krift et al. (2001) who reported that the quantity and quality of plant litter determine N release in soil. Because the quantity and quality of soil organic matter results from decomposition of aboveground and belowground biomass and rhizodeposition, there is also a link between soil organic matter quantity and quality and N supply via net N mineralization (Benbi and Richter 2002; Hobbie 2015). Soil microbes mineralize organic matter and release nutrients by litter decomposition. Resource availability for soil microorganisms or microbial uptake is also regulated by litter decomposition (Smith and Paul 1990). Plant litter varies in chemical composition; therefore, changes in plant communities could alter the production and types of organic compounds in soil, thereby controlling the composition and function of microbial communities (Zak et al. 2003). Moreover, environmental conditions, such as soil pH, soil moisture, soil temperature, and soil texture influence gross N mineralization by changing microbial biomass or activity associated with substrate availability (Booth et al. 2005; Wang et al. 2016; Zhang et al. 2016).

In particular, root C/N ratios explained high amounts of variance in gross N mineralization rates in soil (Fornara et al. 2011). Litter with high C/N ratios is considered as low quality, whereas litter with low C/N ratios is considered as high quality. Previous studies showed that high root C/N ratios have a strong negative effect on gross N mineralization (Silver and Miya

2001; Fornara et al. 2011). There is increasing evidence that root decomposition may be more important than aboveground plant biomass decomposition for organic matter formation and the associated N stocks in soil (Rasse et al. 2005; Kramer et al. 2010). The work of Ruppenthal et al. (2015) has even suggested that root litter is the dominant source of soil organic matter. Fornara et al. (2011) reported that gross N mineralization rates are mainly driven by changes in C and N concentrations of soil organic matter. Consequently, root decomposition could be the major source of N released by mineralization in soil. This is further supported by Abbadie et al. (1992), who found indirect evidence that the most assimilated N originated from root decay in African grasslands.

Plant diversity influences several N-transformation processes in soil via plant uptake of N and modifications of ecosystem properties like microbial community or biomass production (Hooper & Vitousek 1998; Spehn et al. 2005; Weisser et al. 2017). Previous biodiversity studies in grasslands have mainly reported positive relationships between plant species richness and both gross and net N mineralization rates (e.g. West et al. 2006; Rosenkranz et al. 2012; Mueller et al. 2013) and net nitrification rates in the presence of legumes (Scherer-Lorenzen et al. 2003). Rosenkranz et al. (2012) found that the increasing topsoil water content with increasing plant species richness was the main factor underlying positive effects of plant species richness on net N mineralization rates in the Jena Experiment, the same experimental site as in this study. Another plant diversity experiment showed that positive effects of plant diversity on net N mineralization rates were driven by increased N concentrations in roots (Mueller et al. 2013). In an isotope dilution experiment in the laboratory using soil samples from the BioCON experiment in the North American prairie, gross N mineralization rates increased with increasing plant species richness because of greater microbial activity (West et al. 2006). In addition, net N mineralization rates decreased, and N immobilization rates increased at higher species diversity (West et al. 2006). However, the incubation experiment was conducted inside a laboratory, which could not necessarily be directly comparable to field conditions (e.g., because of cold storage of the samples before lab incubation, controlled incubation temperature, and optimum nutrient supply; Arnold et al. 2008). To our knowledge, no study has been reported that investigated plant diversity effects on microbial NH_4^+ consumption and on gross inorganic N immobilization rates in situ.

Besides plant species richness, the presence or absence of specific plant functional groups can affect N cycling in grassland ecosystems (Scherer-Lorenzen et al. 2003; Oelmann et al.

2007; Dybzinski et al. 2008; Fornara and Tilman 2009; Fornara et al. 2011; Leimer et al. 2015). Legumes constitute a distinct functional group in grasslands because of their ability to fix atmospheric N via symbiotic root microorganisms (Spehn et al. 2002; Marquard et al. 2009). Mulder et al. (2002) reported that non-leguminous plants depend on N_2 fixed by legumes to counter-balance the declining soil N availability in unfertilized (near-) natural ecosystems. Therefore, many studies concluded that with an increased legume biomass, there is a larger plant-available N pool in the soil (Spehn et al. 2002; Booth et al. 2005; Scherer-Lorenzen 2008). This larger plant-available N pool can originate from increased gross N mineralization of N-rich legume litter. Besides legumes, grasses were also found to influence gross N mineralization. Oelmann et al. (2007) reported that the presence of grasses decreased mineral N pools in soil compared to plant communities without grass species because of their dense and extensive rooting system. This extensive rooting system is efficient in taking up soil N and thus can reduce mineral N pools in soil (Oelmann et al. 2007).

The objectives of our study were (i) to investigate if plant species richness, functional group richness or the presence/absence of individual functional groups (together termed plant diversity) affect gross N mineralization, microbial NH_4^+ consumption and gross inorganic N immobilization rates and (ii) to determine the underlying controls responsible for the potential relationships. We hypothesized that there was a positive effect of plant species richness on gross N mineralization rates because of the known positive relationship between plant species richness and microbial activity in the Jena Experiment (Strecker et al. 2016). Secondly, we expected an increasing microbial NH_4^+ consumption and gross inorganic N immobilization with increasing plant species richness because of the higher N demand and the tighter N cycling in species-rich than in species-poor plant mixtures. Thirdly, we hypothesized that the presence of legumes increased gross N mineralization, microbial NH_4^+ consumption and gross inorganic N immobilization because of the smaller C/N ratio of litter in plant mixtures containing legumes compared to plant mixtures without legumes (Chen et al. 2017). Although our focus was on gross N turnover rates, we additionally calculated the rates of net mineralization and its components net ammonification and net nitrification and analyzed their relationship with plant diversity.

4.3 Materials and methods

4.3.1 Study site

Our study was part of the Jena Experiment (www.the-jena-experiment.de), a long-term grassland diversity experiment established in 2002 (Roscher et al. 2004; Weisser et al. 2017). The site had been used as arable land for at least 40 years before the establishment of the Jena Experiment. The experimental site is located on the floodplain of the river Saale in Jena, Germany (50°55' N, 11°35' E; 130 m above sea level). Mean annual air temperature is 9.9°C, and mean annual precipitation amounts to 610 mm (1980-2010, Hoffmann et al. 2014). The soil at the site is classified as Eutric Fluvisol developed from 2-m thick loamy fluvial sediments (IUSS Working Group WRB 2014). The soil texture ranges from sandy loam close to the river to silty loam with increasing distance from the river. The mean bulk density of the topsoil (0-5 cm) of the experimental plots is 1.18 ± 0.1 g cm^{-3}; varying little from 1.21 ± 0.1 g cm^{-3} in Block I with the lowest clay content to 1.17 ± 0.1 g cm^{-3} in Block IV with the highest clay content. The experimental site is mown twice and weeded three times a year to maintain the designed diversity levels. The biomass was removed after mowing/weeding. This management mimics a typical use of semi-natural species-rich mesophilic grassland as hay meadow (Roscher et al. 2004). A major aim of the Jena Experiment is to explore the effect of biodiversity on nutrient cycling and trophic interactions.

A detailed description of the experimental design is provided in Roscher et al. (2004). The main experiment consists of 82 plots (20 m × 20 m) in four blocks to account for the systematic change in soil texture perpendicular to the river with a factorial design (as far as possible) of different levels of plant species richness (1, 2, 4, 8, 16, and 60) and 1-4 functional groups (grasses, legumes, small herbs, and tall herbs). The mixtures were randomly drawn from a pool of 60 species representing typical Central European mesophilic grasslands. All the 16 species of grasses are perennial except *Bromus hordeaceus* L. Each level of species richness was replicated on 16 plots except for the 16 and 60 species richness levels, which were only replicated on 14 and 4 plots, respectively. Since there were only four replicates of the 60-plant species mixture, we excluded them from our data analyses (which reduced the number of considered plots to 78). Of these 78 plots, we lost two because of errors during the laboratory analyses. Those two plots (B2A08 and B4A02) were sown with a species richness level of 2 and 16 and functional group richness of 2 and 3, respectively. Another two plots (B1A09 and

B4A03), both monocultures, were abandoned due to their poor performance (i.e., extremely low target species cover). Therefore, our final analyses were based on 74 plots.

4.3.2. Isotope pool-dilution experiment

We used the isotope pool-dilution method in a field incubation experiment to determine the rates of gross N mineralization in soil (Davidson et al. 1991). We labeled the soil NH_4^+ pool with 98 at% ^{15}N as NH_4Cl. While unlabeled N from the organic pool is mineralized to NH_4^+ by microorganisms, the ^{15}N enrichment of the NH_4^+ pool is diluted. The method of Davidson et al. (1991) is based on several assumptions which are valid for short incubation periods of up to 24 hours. According to these assumptions, (1) there is no or only negligible isotope discrimination by microorganisms during the incubation period, so that the consumption of NH_4^+ alters the pool size, but not the isotope ratio of the pool; (2) the turnover rates are constant; and (3) no N re-mineralization occurs, so that the assimilated ^{15}N is not returned to the labeled pool.

A disturbed soil sample was taken to determine the natural ^{15}N abundance and 1 M KCl-extractable mineral N (NH_4^+-N and NO_3^- -N) concentrations on each plot before starting the experiment. We performed the field experiment and collected soil samples in April 2011. Two pairs of stainless steel cores (Ø = 56 mm, h = 41 mm, V = 100 cm^3) were taken from within the 0-5 cm layer of the soil of each plot (one pair for each time step, t1 and t2), closed at the bottom side with a polyethylene lid to prevent leaching losses and immediately reinserted. We averaged the two cores for each time step for ^{15}N isotopic analysis to improve plot representativity. The soil samples in the cores were labeled with a NH_4Cl solution (5 mg L^{-1} N, 98 at% ^{15}N) using a high-precision, digital dispenser (Brand, Wertheim, Germany) coupled to a side-port needle, which injected the solution horizontally to ensure a homogeneous distribution of the 5-mL label within the cores. For every core, the injections were uniformly distributed at five points, each point receiving 1 mL of the tracer solution. In total, 25 µg N (98 at% ^{15}N) were added as label to each core, which corresponds to less than 2 percent of the NH_4-N concentration in the soil at the time of the experiment.

To account for abiotic N fixation, ensure the ^{15}N enrichment and calculate tracer recoveries, one pair of the soil cores was removed from the soil after 15 minutes (t1) and the remaining soil cores after 24 hours (t2) to calculate the ^{15}N pool dilution after the field incubation. Soil samples from shortly before the pool dilution experiment and from t1 and t2 of the experiment were shaken with 1 M KCl solution for one hour shortly (<2 h) after sampling next to the field site

to extract NH_4^+ and NO_3^- and then filtered through ash-free paper filters (no. 595, Schleicher & Schuell, Dassel, Germany, pore size 4–7 µm). The extracts were immediately frozen at –20 °C and transported in frozen state to the laboratory for further chemical analyses.

The concentrations of NH_4-N and NO_3-N in the 1 M KCl extracts were measured by high-resolution colorimetric detection using a continuous flow analyzer (CFA Autoanalyzer 3 HR, Seal Analytical GmbH, Norderstedt, Germany). We used the microdiffusion method (Stark and Hart 1996) to determine the $^{15}N/^{14}N$ isotope ratios of NH_4^+ in the soil extracts. In the microdiffusion method, NH_4^+ is volatilized as NH_3 by increasing the pH to > 9.5 with MgO. The released NH_3 was then collected on an acidified (2.5 M $NaHSO_4$) filter disk enclosed in a polytetrafluoroethylene (PTFE) envelope, where it reacted back to NH_4^+. The N isotope ratios were determined with an Elemental Analyzer (EA 1110, Carlo Erba Instruments, Milan, Italy) coupled to an isotope-ratio mass spectrometer (MAT Delta Plus, Thermo Finnigan, Bremen, Germany) at the Stable Isotope Center, University of Göttingen. Ten replicate measurements of in-house standard reference material [^{15}N-$(NH_4)_2SO_4$] resulted, on average, in 98.4±1.6% of the true value, indicating a high accuracy of our measurements. The error of ±1.6% is the average deviation from the true value. Precision of the ^{15}N measurements was ±0.002 at% (n = 10).

4.3.3 Plant community and soil properties

Aboveground (shoot) biomass was harvested in May 2011 prior to mowing. Plants were clipped at 3 cm above ground level within the harvesting area of two replicate 20 cm × 50 cm subplots per plot. Plant material was sorted into sown species, weeds, and dead aboveground biomass. Biomass of each sown species was determined after drying at 70°C for at least 48 h (Weigelt et al. 2010). For shoot C/N ratio analysis, all the plant material from one plot was pooled together to obtain a representative value for the plant community of the respective plot. A small subsample of this material was milled to fine powder using a ball mill (MM 400, Retsch GmbH, Haan, Germany) and up to 5 mg from each plot was used for C and N analysis (Flash EA 112, Thermo Fisher, Milan, Italy). Shoot height (regenerative shoot height, i.e. soil surface to highest flower) was measured on five individual plants (without stretching the plants) every meter along a 5-m transect in the central area of the plots (61 m²) by using a ruler.

For the analysis of the root C/N ratio, community roots were collected in September 2013 per plot. The root C/N data were not available for 2011, so we used the data of the nearest

possible date. Root biomass was sampled originally for a root decomposition experiment where the C/N ratio was used as explanatory variable for litter quality (Chen et al. 2017). To minimize disturbance of the experimental plots, we limited larger soil cores (40x15x20 cm) to plots with low standing root biomass and took smaller soil cores (20x10x20 cm) where standing root biomass was sufficiently high to provide enough fine root material. Sampling depth was always 20 cm covering the main rooting horizon where on average 90% of community standing root biomass in the Jena Experiment plots can be found (Chen et al. 2017). Roots were collected, cleaned and sorted to fine (< 2 mm) and coarse roots after washing. Fine roots were oven-dried at 65°C and ground with a ball mill (MM 400, Retsch GmbH, Germany) and analyzed for total C and N concentrations using an elemental analyzer (Flash 2000, ThermoFisher Scientific Inc, Waltham, MA, USA). Studies have found that fine roots are more active and decompose faster than coarse roots in forest ecosystems (Brunner and Godbold 2007, Lukac 2012, Zhang and Wang 2015). Therefore, we expected similar differences between fine and coarse roots in grasslands. Additionally, although variable among communities, root biomass data at the Jena Experiment showed that fine roots made up on average 84% of the total standing root biomass (0-30 cm).

To determine the concentrations of organic C and total N in soil, five soil samples per plot (0-5 cm) were taken in 2011. All replicates were combined and homogenized. Soil samples were dried at 40 °C and sieved (< 2 mm). The dried samples were ground using a ball mill. An aliquot of these samples was analyzed for total C and N concentrations by an elemental analyzer (vario Max CN, Elementar Analysensysteme GmbH, Langenselbold, Germany). Inorganic C concentrations were determined by elemental analysis after burning the organic carbon at 450 °C in a muffle furnace. Organic C concentrations were calculated by subtracting inorganic C concentrations from total C concentrations.

We used mean microbial biomass C data from the four years prior to our experiment (2007-2010, i.e. Phase 2 in Strecker et al. 2016). Microbial biomass C showed a strong temporal variation in the Jena Experiment depending on the microclimatic conditions, which resulted from weather conditions and related plant growth and thus was aggregated to different phases by Strecker et al. (2016). We used Phase 2 data because we expected it to best represent the microbial biomass conditions that prevailed during our in-situ experiment. For the measurement of soil microbial biomass, soil samples were taken with a steel corer (5 cores per plot, depth 5 cm, diameter 5 cm) and sieved. Microbial biomass C of approximately 5 g soil (fresh weight)

was measured using an O_2-microcompensation apparatus (Scheu 1992). Substrate-induced respiration was calculated from the respiratory response to D-glucose for 10 h at 22°C (Anderson and Domsch 1978). Glucose was added according to preliminary studies to saturate the catabolic enzymes of microorganisms (4 mg g^{-1} dry weight solved in 400 µL deionized water). The mean of the lowest three readings of O_2-consumption values within the first 10 h was taken as maximum initial respiratory response (MIRR; [µL O_2 g^{-1} dry soil h^{-1}]) and microbial biomass (µg C g^{-1} dry soil) was calculated as 38 × MIRR (maximum initial respiratory response, Eisenhauer et al. 2010).

The microbial C/N ratio of 38 plots (Blocks 1 and 2 only) was determined from the data of microbial biomass C and N, which was measured using chloroform fumigation extraction. Two samples of 7 g soil were taken from each plot, one was fumigated with chloroform vapor for 24 h and the other was not fumigated. Both, the fumigated and non-fumigated samples were extracted with 40 mL 0.5 M K_2SO_4 by shaking for 30 minutes. Total C and N concentrations in the extracts were analyzed by dry combustion in a DIMA-TOC 100 Analyzer (Dimatec, Essen, Germany). Microbial biomass C was calculated as (total C in fumigated soil – total C in non-fumigated soil)/0.45 (Wu et al. 1990). Likewise, microbial biomass N was calculated as (total N in fumigated soil – total N in non-fumigated soil)/0.54 (Brookes and Landman 1985).

4.3.4 Calculations and statistical analyses

Rates of gross N mineralization, microbial NH_4^+ consumption, gross inorganic N immobilization, net N mineralization and its components net ammonification and net nitrification were calculated using Eqs. 1 to 6, respectively. Eqs. 1-4 and 6 are from Hart et al. (1994) and Eq. 5 is from Rosenkranz et al. 2012.

$$m = \frac{[NH_4^+]_{t1} - [NH_4^+]_{t2}}{t} * \frac{\log\left(\frac{APE_{t1}}{APE_{t2}}\right)}{\log\left(\frac{[NH_4^+]_{t1}}{[NH_4^+]_{t2}}\right)} \quad \text{Eq. 1}$$

$$c = m - \frac{[NH_4^+]_{t2} - [NH_4^+]_{t1}}{t} \quad \text{Eq. 2}$$

$$i = m - nm \quad \text{Eq. 3}$$

$$nm = \frac{[NH_4^+ + NO_3^-]_{t2} - [NH_4^+ + NO_3^-]_{t1}}{t} \quad \text{Eq. 4}$$

$$na = \frac{[NH_4^+]_{t2} - [NH_4^+]_{t1}}{t} \quad \text{Eq. 5}$$

$$nn = \frac{[NO_3^-]_{t2} - [NO_3^-]_{t1}}{t} \quad \text{Eq. 6}$$

where m = gross N mineralization rate [μg N (g dry soil)$^{-1}$ day^{-1}]

c = microbial NH_4^+ consumption rate [μg N (g dry soil)$^{-1}$ day^{-1}]

i = gross inorganic N immobilization rate [μg N (g dry soil)$^{-1}$ day^{-1}]

nm = net N mineralization rate [μg N (g dry soil)$^{-1}$ day^{-1}]

na = net ammonification rate ([μg N (g dry soil)$^{-1}$ day^{-1}]

nn = net nitrification [μg N (g dry soil)$^{-1}$ day^{-1}]

$[NH_4^+]_{t1}$ = NH_4^+ concentration at t1 [μg N (g dry soil)$^{-1}$]

$[NH_4^+]_{t2}$ = NH_4^+ concentration at t2 [μg N (g dry soil)$^{-1}$]

APE_{t1} = at% ^{15}N excess of NH_4^+ pool at t1

APE_{t2} = at% ^{15}N excess of NH_4^+ pool at t2

t = time difference between t1 and t2 [day]

Microbial NH_4^+ consumption includes microbial NH_4^+ immobilization and gross nitrification. Since gross nitrification was not determined in our study, which would have required labeling with $^{15}NO_3^-$, we could not calculate microbial NH_4^+ immobilization. Instead, we calculated gross inorganic N immobilization rates by using Eq. 3. In our calculations of gross inorganic N immobilization, net mineralization and net nitrification rates we neglected possible denitrification. Moreover, we assumed that our addition of $^{15}NH_4^+$ did not change the size of the NH_4^+ and NO_3^- pools in soil substantially.

We used a hierarchical ANOVA (type I sum of squares) to test for effects of plant species richness and functional group composition on gross N mineralization rates, microbial NH_4^+ consumption, gross inorganic N immobilization, net N mineralization and net nitrification rates. Gross N mineralization and microbial NH_4^+ consumption rates were square root-transformed; and net nitrification rates were box-cox power transformed ($\lambda = 1.1$) after removing the outliers to approximate normal distribution (checked with Lilliefors normality test and histograms). The residuals vs. fitted and Q-Q plots were used to check the assumption of homoscedasticity and normality of the residuals. For net N mineralization and net nitrification data, extreme outliers were removed if they deviated by more than two standard deviations from the mean (6 outliers

removed from each net rates). The ANOVA was performed with block, plant species richness, and the presence/absence of each functional group as explanatory variables. All the interactions between plant species richness and presence/absence of functional groups were non-significant and thus, are not displayed in the results. The functional groups were fitted in the following sequence: legumes, grasses, tall herbs, and small herbs. The reason for fitting legumes first among the functional groups is because legumes frequently have shown the strongest effect on the N cycle. Grasses have also often shown an effect on N transformations. To avoid the collinearity between functional group richness and each functional group, a separate model was set up for functional group richness, fitted after block to test the effect of functional group richness on gross N mineralization, microbial NH_4^+ consumption, gross inorganic N immobilization, net N mineralization and net nitrification rates. Correlations between the selected variables were analyzed using Pearson's correlations test. All the statistical analyses were carried out in R Studio (R Studio, Version 1.1.456, R Studio Inc., Boston, MA USA) with the free statistical software R 3.5.1 (R Core Team 2018).

To explain the species richness and functional groups effects that were detected in the ANOVAs, we first ran Pearson correlations between all potential explaining variables and the three considered gross N turnover rates gross N mineralization, microbial NH_4^+ consumption and gross inorganic N immobilization (Table S4.1) and then applied Structural Equation Modeling (SEM). As the goal of the SEM approach was to identify the potential mechanisms behind the significant species richness and functional group effects on gross N turnover rates according to the ANOVAs, plant species richness, legumes and small herbs were included as the exogenous variables in the SEM and the SEM was focused on gross N mineralization and microbial NH_4^+ consumption, because gross inorganic N immobilization was not significantly related with species or functional group richness. Including all the potential variables (total organic carbon, aboveground and belowground community biomass, soil moisture, root C/N, microbial biomass) into one SEM did not result in adequate model fit (Fig. S4.1, Table S4.2). This was even true after removing the non-significant pathways (Fig. S4.2, Table S4.3). Therefore, according to the literature knowledge and the results of Pearson's correlations (Table S4.1), the potentially mediating variables in the SEMs were chosen. We included root C/N ratio and microbial biomass C as potential mediators of the effect of plant species richness and functional groups (legumes, small herbs) on gross N mineralization and microbial NH_4^+ consumption rates. Root litter quality is also considered an important source for organic matter

input after root turnover. We did not include microbial C/N ratio data, because microbial C/N ratio data were only available for two blocks. According to McCune and Grace (2002), the sample size for SEMs should be at least 50. Therefore, the sample size of microbial C/N data is too small for the application of SEM. Furthermore, we included a path between gross N mineralization and microbial NH_4^+ consumption rates to determine if microbial NH_4^+ processing depends on the amount of NH_4^+ produced. Based on the p values, the non-significant paths in the SEMs were removed from the final model. We used the χ^2 test (> 0.05), P value (> 0.05), goodness of fit index (GFI > 0.9), comparative fit index (CFI > 0.9) and normed fit index (NFI > 0.9) to evaluate the model fit (Tables S4.2-S4.4). SEM was conducted using the R package "lavaan" (Rosseel 2012).

4.4 Results

4.4.1 Effects of plant diversity on gross and net N mineralization, net ammonification and net nitrification

Table 4.1: Maximum, minimum and mean values of gross and net nitrogen transformation rates

	N transformation rates [µg N (g dry soil)$^{-1}$ day^{-1}]		
	Minimum	**Maximum**	**Mean**
Gross N mineralization	0.04	6.20	2.12
Microbial ammonium consumption	-1.81	7.24	2.43
Gross inorganic N immobilization	-3.27	8.51	2.28
Net N mineralization	-4.33	5.72	-0.12
Net ammonification	-2.57	2.13	-0.42
Net nitrification	-2.04	4.97	0.31

Table 1 summarizes the means and ranges of all determined N turnover rates. Block had a significant effect on gross N mineralization (Table 4.2), net N mineralization (Table S4.5) and a marginally significant effect on net ammonification (Table S4.6). Plant species richness showed a significant negative effect on gross N mineralization rates (Table 4.2, Fig. 4.1). The mean gross N mineralization rate in the monocultures was 2.25 μg N (g dry soil) $^{-1}$ day^{-1} and in the sixteen plant species mixtures 1.63 μg N (g dry soil)$^{-1}$ day^{-1}, showing a decrease by 28%, which translates to a slope of a regression line of gross N mineralization rates on species number of -0.05 μg N (g dry soil)$^{-1}$ day^{-1} per additional species. Functional group richness had no significant effect on gross N mineralization rates ($F = 0.13$, $p = 0.719$). The presence of legumes increased gross N mineralization rates significantly (Table 4.2). Plant species richness was unrelated with net N mineralization, net ammonification and net nitrification (Tables S4.5-S4.7). Functional group richness was unrelated with net N mineralization ($F = 2.64$, $p = 0.109$) and net nitrification ($F = 2.29$, $p = 0.135$), but was marginally negatively related with net ammonification ($F = 3.32$, $p = 0.073$). The presence of legumes decreased net ammonification significantly (Table S4.6). Expectedly, net nitrification correlated significantly positively with soil 1 M KCl-extractable NO_3^- concentrations from shortly before the experiment ($r = 0.37$, $p = 0.014$, NO_3^- data log-transformed and 6 outliers removed).

Table 4.2: Hierarchical ANOVA results showing the effects of plant species richness (SR) and presence (+) /absence (-) of each functional group on gross nitrogen mineralization rates. Bold letters show significance at p < 0.05. Arrows indicate positive (↑) or negative (↓) effects

Source	Df	SS	SS (%)	F	P
Block	**3**	**1.45**	**10.89**	**3.15**	**0.031**
SR	**1**	**0.62**	**4.66**	**4.05**	**0.048 ↓**
Legumes	**1**	**0.71**	**5.33**	**4.65**	**0.035 ↑**
Grasses	1	0.00	0.00	0.04	0.845
Tall herbs	1	0.26	1.95	1.68	0.199
Small herbs	1	0.31	2.33	2.05	0.157
Residuals	65	9.96			

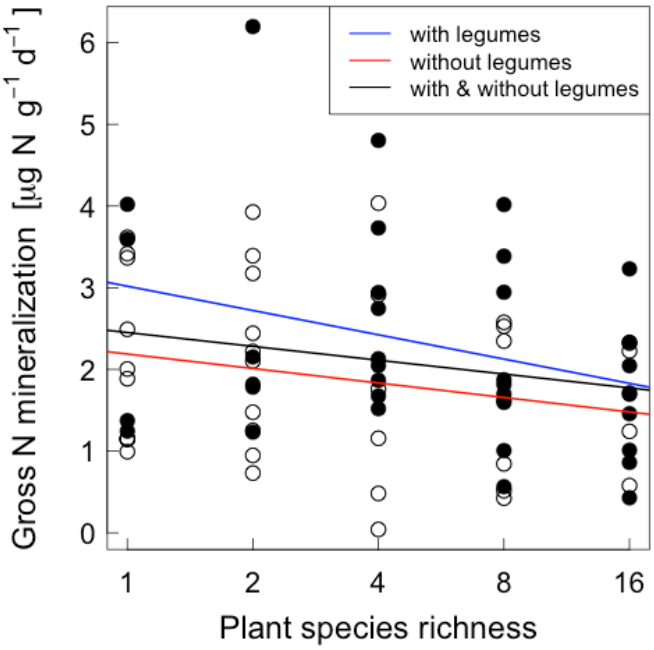

Fig. 4.1: Relationship between plant species richness with/without legumes and gross nitrogen (N) mineralization. Open circles represent plots without legumes and closed circles represent plots with legumes. The regression lines are shown for illustration purpose only

4.4.2 Effects of plant diversity on microbial NH_4^+ consumption and gross inorganic N immobilization

Increasing plant species richness decreased the microbial NH_4^+ consumption rates significantly (Table 4.3, Fig. 4.2). The microbial NH_4^+ consumption rates were on average 2.41 and 1.87 µg N (g dry soil)$^{-1}$ day^{-1} in the plots with one and sixteen plant species, respectively, showing a decrease by 22% that translates into a slope of a regression line of microbial NH_4^+ consumption rates on species number of -0.06 µg N (g dry soil)$^{-1}$ day^{-1} per additional species. Plant species richness was unrelated with gross inorganic N immobilization (Table 4). We did not find a significant effect of functional group richness on microbial NH_4^+ consumption rates ($F = 1.84$, $p = 0.179$) and gross inorganic N immobilization ($F = 2.02$, $p = 0.160$). The presence of legumes and small herbs increased microbial NH_4^+ consumption and gross inorganic N immobilization compared to their absence, although small herbs only had a marginally significant effect on gross inorganic N immobilization (Tables 4.3 and 4.4).

Table 4.3: Hierarchical ANOVA results showing the effects of plant species richness (SR) and presence (+) /absence (-) of each functional group on microbial ammonium consumption rates. Bold letters show significance at p < 0.05. Arrows indicate positive (↑) or negative (↓) effects

Source	Df	SS	SS (%)	F	P
Block	3	0.14	4.52	1.41	0.249
SR	**1**	**0.15**	**4.84**	**4.81**	**0.032 ↓**
Legumes	**1**	**0.50**	**16.13**	**15.64**	**<0.001 ↑**
Grasses	1	0.00	0.00	0.002	0.963
Tall herbs	1	0.04	1.29	1.17	0.283
Small herbs	**1**	**0.19**	**6.13**	**6.02**	**0.017 ↑**
Residuals	65	2.08			

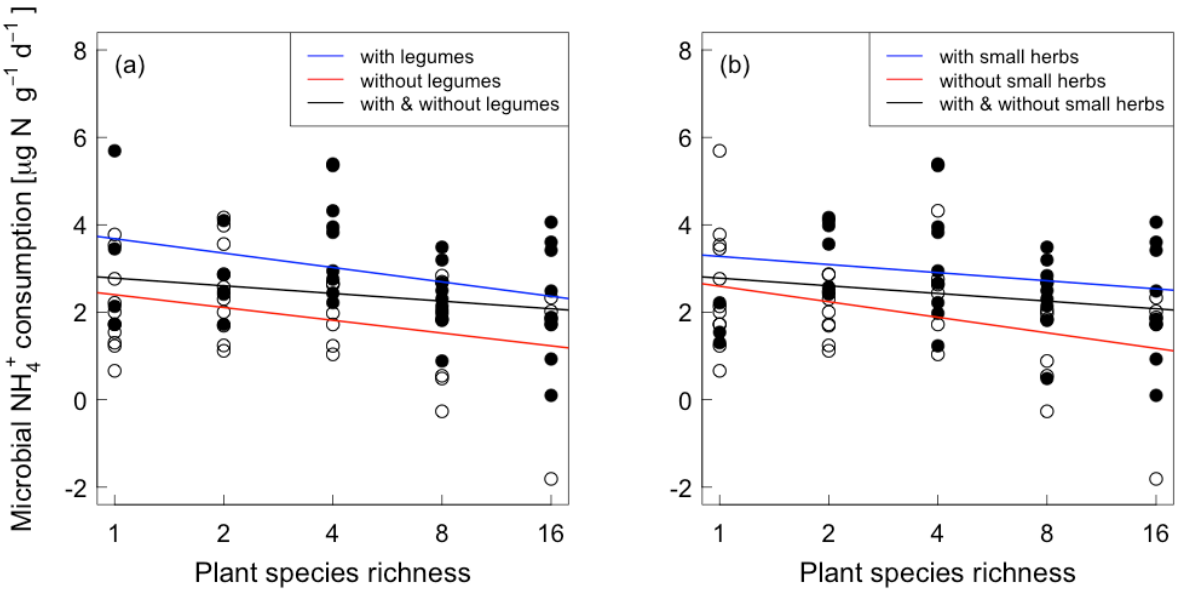

Fig. 4.2: Relationship between plant species richness with/without legumes (a) and plant species richness with/without small herbs (b) and microbial ammonium (NH_4^+) consumption rates. Open circles represent plots without legumes/small herbs and closed circles represent plots with legumes/small herbs. The regression lines are shown for illustration purpose only

Table 4.4: Hierarchical ANOVA results showing the effects of plant species richness (SR) and presence (+) /absence (-) of each functional group on gross inorganic N immobilization rates. Bold letters show significance at p < 0.05 and italics show significance at p < 0.1. Arrows indicate positive (↑) effects

Source	Df	SS	SS (%)	F	P
Block	3	14.59	5.08	1.40	0.250
SR	1	1.64	0.56	0.47	0.494
Legumes	**1**	**26.71**	**9.30**	**7.71**	**0.007** ↑
Grasses	1	0.13	0.05	0.04	0.845
Tall herbs	1	5.62	1.96	1.62	0.207
Small herbs	*1*	*13.35*	*4.65*	*3.86*	*0.054* ↑
Residuals	65	225.07			

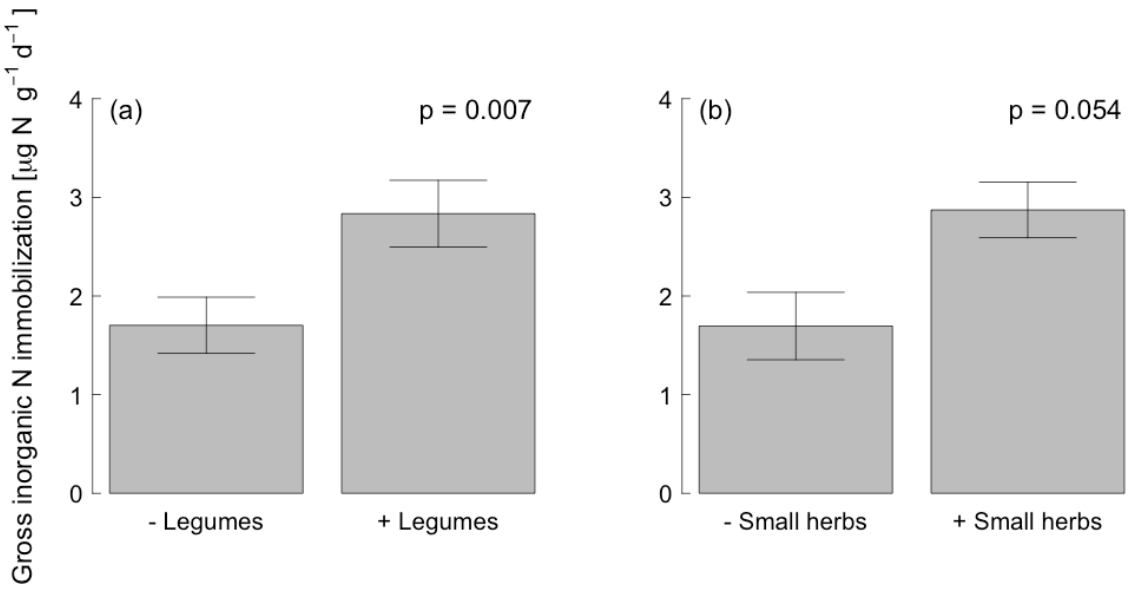

Fig. 4.3: Effects of presence/absence of legumes (a) and small herbs (b) on gross inorganic N immobilization rates. P value is given according to the ANOVA results

4.4.3 Effects of soil and plant community properties on gross N mineralization, microbial NH_4^+ consumption and gross inorganic N immobilization rates

We tested several variables to assess the likelihood that they contributed to mechanisms by which species richness and functional group composition may have influenced gross N mineralization and microbial NH_4^+ consumption rates and to explore which soil and plant community properties drove gross inorganic N immobilization rates (Table S4.1). Soil pH showed a negative correlation with gross N mineralization rates (Fig. 4.4a), reflecting its influence on microbial activity. As expected, microbial biomass C had a positive relationship with microbial NH_4^+ consumption (Fig. 4.5a) and gross inorganic N immobilization rates (Fig. 4.6a). The microbial C/N ratios were negatively correlated with gross N mineralization rates (Fig. 4.4b), gross inorganic N immobilization (Fig. 4.6b) and microbial NH_4^+ consumption rates, although in the latter case only marginally significantly (Fig. 4.5b). We expected that lower litter quality (higher plant and soil C/N ratios) would decrease gross N mineralization and microbial NH_4^+ consumption rates. Supporting this hypothesis, shoot C/N (Fig. 4.4c) and fine root C/N ratios (Fig. 4.4d) had negative relationships with gross N mineralization rates and shoot C/N (Fig. 4.5c) and soil C/N ratios (Fig. 4.5d) had negative relationships with microbial NH_4^+ consumption rates. Furthermore, the total soil N concentrations (Fig. 4.6c) had significant positive and soil organic C concentrations (Fig. 4.6d) had marginally positive relationships with gross inorganic N immobilization rates.

In the SEM set up to find possible explanations of the plant species richness and functional group effects on gross N mineralization and microbial NH_4^+ consumption rates (Fig. 4.7), the effect of plant species richness was mediated by the root C/N ratio. The root C/N ratio was the only variable out of the wealth of available data from the Jena Experiment that contributed significantly to the negative relationship of plant species richness with gross N mineralization and microbial NH_4^+ consumption rates. This negative effect was composed of a significantly positive effect of plant species richness on the root C/N ratio and a further significantly negative effect of the root C/N ratio on gross N mineralization and microbial NH_4^+ consumption rates (Fig. 4.7). The gross N mineralization rates had a significantly positive influence on microbial NH_4^+ consumption rates. The positive effect of the legumes on gross N mineralization and microbial NH_4^+ consumption rates was significantly related with the root C/N ratio and microbial biomass C (Fig. 4.7).

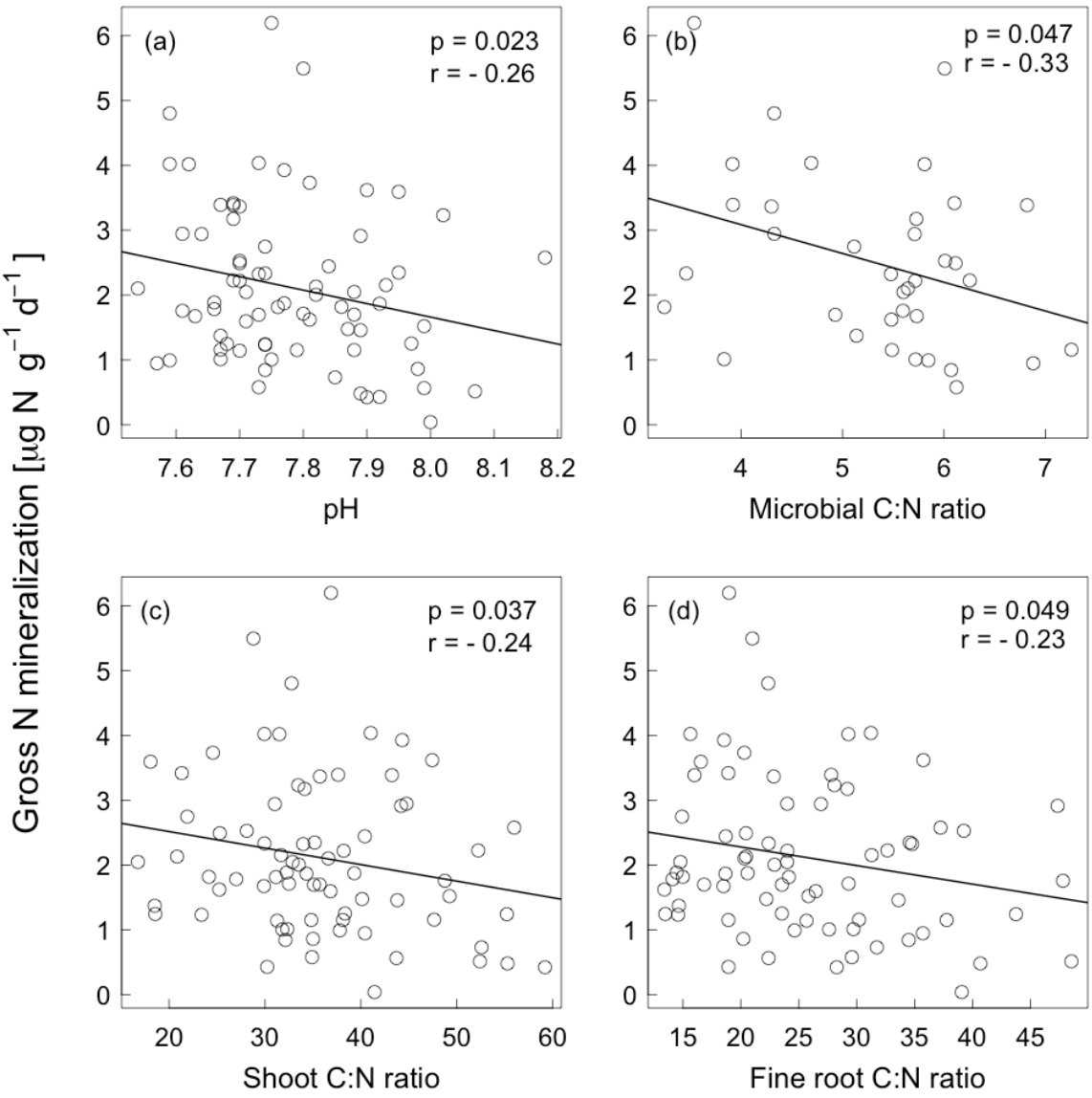

Fig. 4.4: pH (a), microbial carbon to nitrogen (C/N) ratio (b), shoot C/N ratio (c), and fine root C/N ratio (d) versus gross nitrogen mineralization rates. P and r values refer to results from the Pearson's correlation test. The regression lines are shown for illustration purpose only

The presence of small herbs had a positive influence on microbial NH_4^+ consumption rates, which was driven by increased microbial biomass C and increased root C/N ratios. There was also a direct pathway, which described a positive link between plant species richness and gross N mineralization and microbial NH_4^+ consumption rates via microbial biomass C. The direct path relating plant species richness with gross N mineralization and microbial NH_4^+ consumption rates remained significant besides the indirect effects.

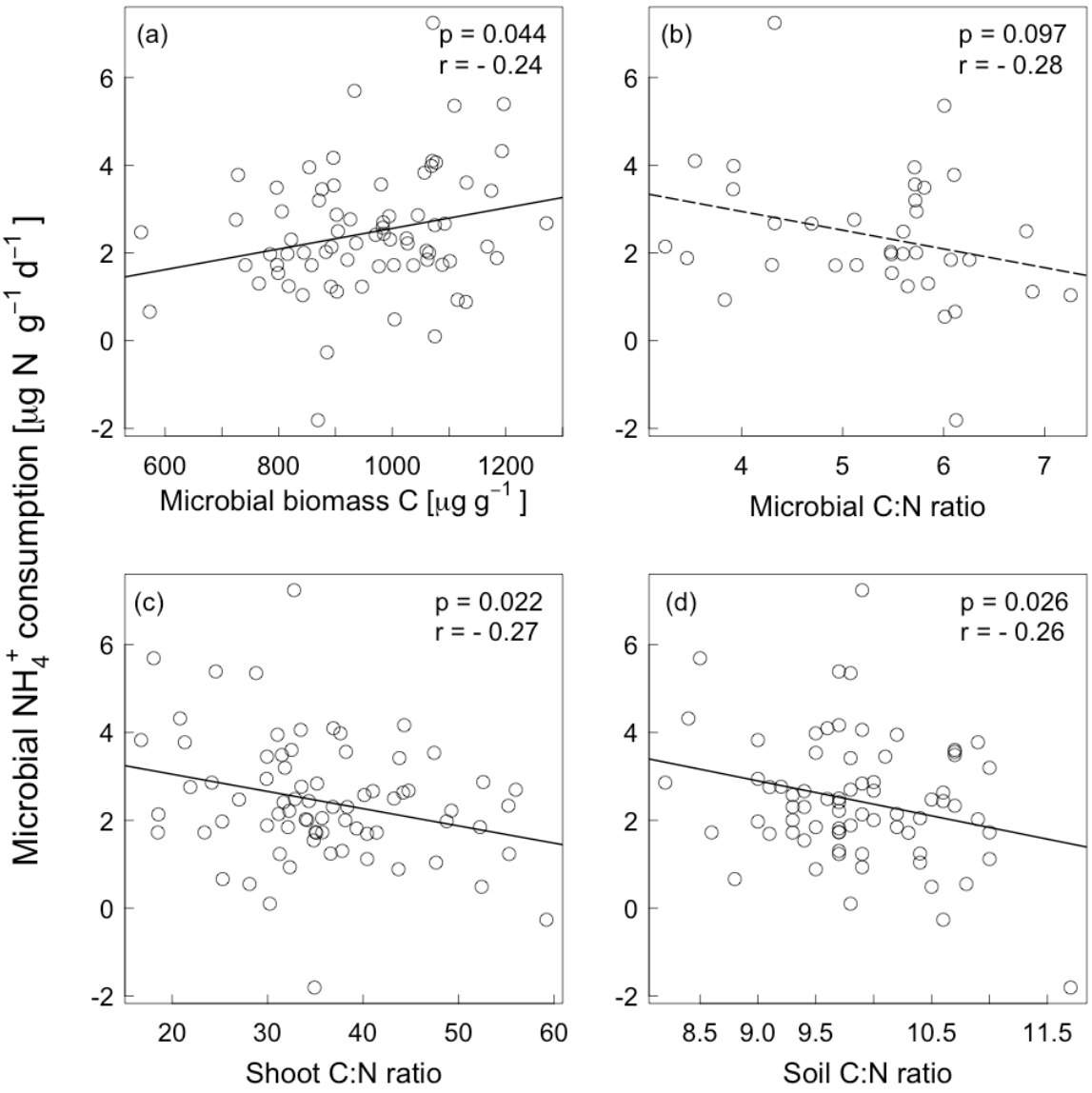

Fig. 4.5: Microbial biomass C (a), microbial carbon to nitrogen (C/N) ratio (b), shoot C/N ratio (c), and soil C/N ratio (d) versus microbial ammonium (NH_4^+) consumption rates. P and r values refer to results from the Pearson's correlation test. The regression lines are shown for illustration purpose only. Solid lines indicate significance at $p < 0.05$ and a dotted line indicates significance at $p < 0.1$

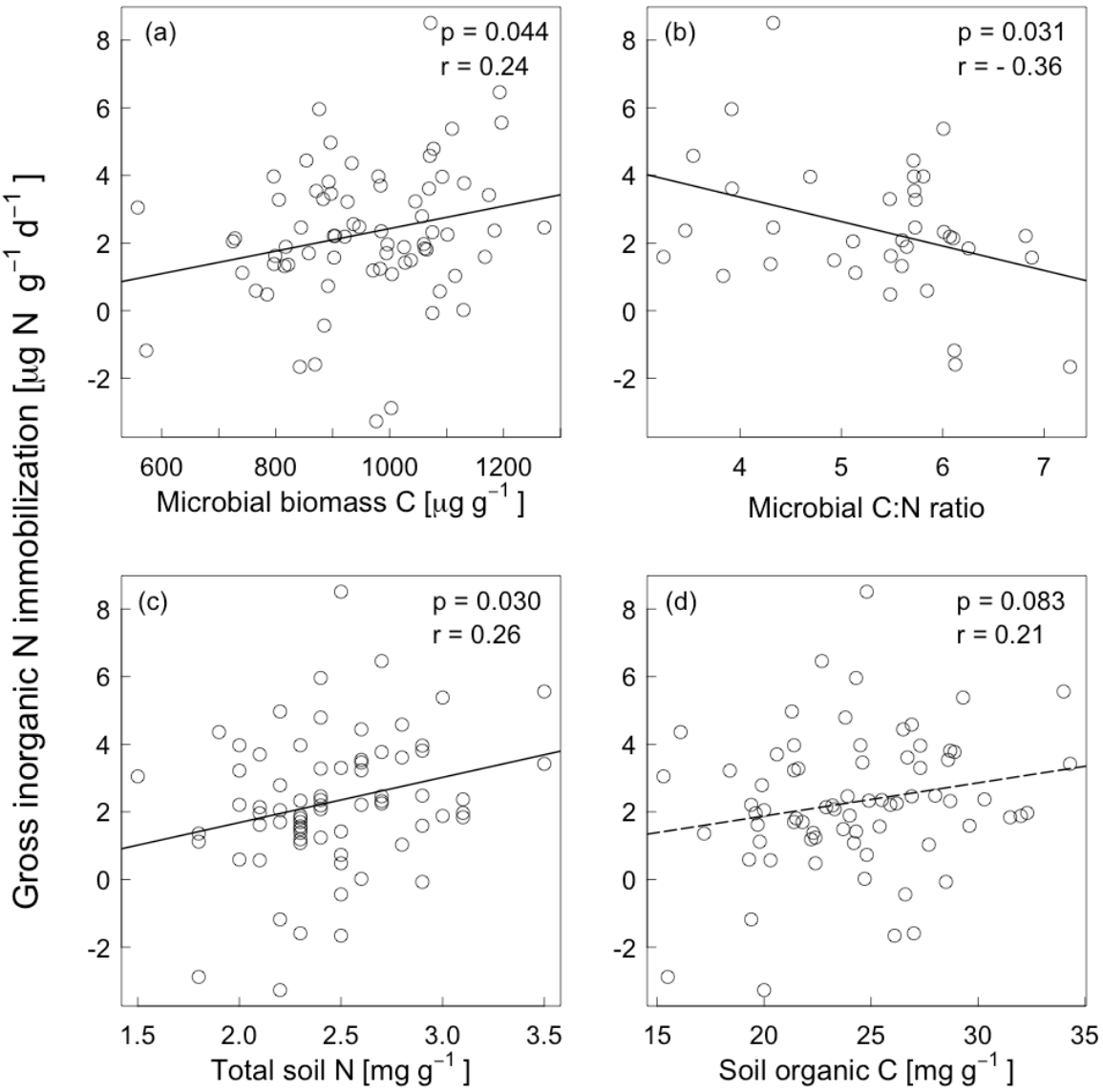

Fig. 4.6: Microbial biomass C (a), microbial carbon to nitrogen (C/N) ratio (b), total soil nitrogen concentrations (c), and soil organic carbon concentrations (d) versus gross inorganic N immobilization rates. P and r values refer to results from the Pearson's correlation test. The regression lines are shown for illustration purpose only. Solid lines indicate significance at $p < 0.05$ and a dotted line indicates significance at $p < 0.1$

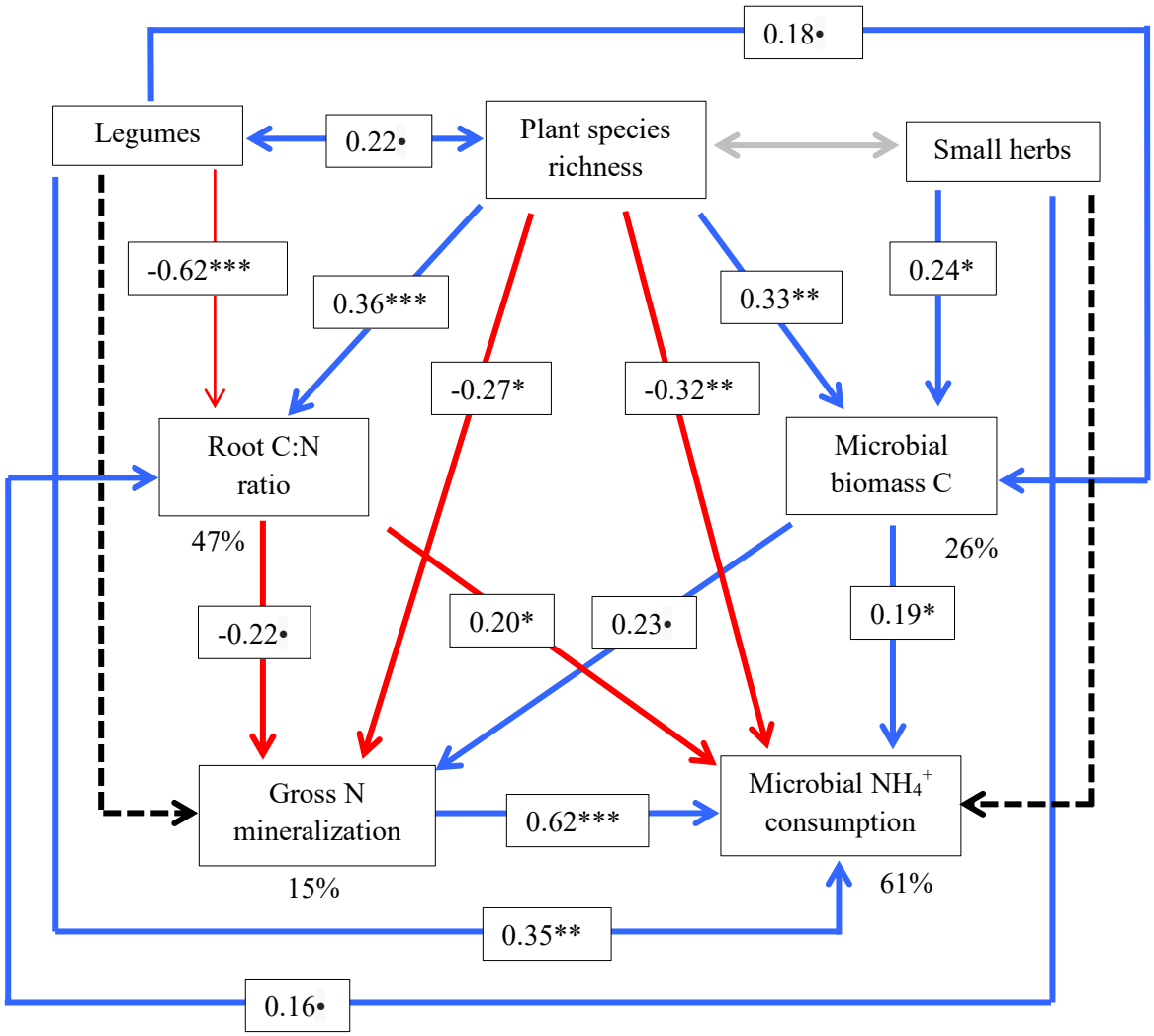

Fig. 4.7: Structural equation model (SEM) to illustrate the underlying paths via which plant species richness and functional groups influenced gross N mineralization and microbial ammonium (NH_4^+) consumption rates. Blue and red arrows represent positive and negative significant relationships, respectively. The grey arrow shows a non-significant pathway. Dotted arrows indicate non-significant pathways that were excluded from the final model. Numbers on the arrows give standardized path coefficients with their significance indicated as ***p < 0.001, **p < 0.01, *p < 0.05, •p < 0.01. Numbers below the variables show the percentage variation explained by corresponding variables (R^2). Fit indices of the model are shown in Table S4.4

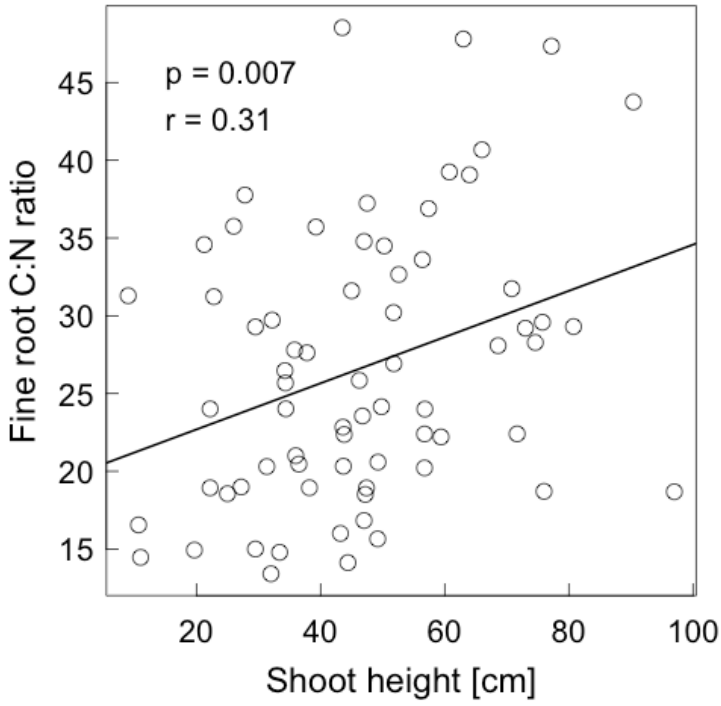

Fig. 4.8:Relationship between mean regenerative shoot height (i.e. soil surface to highest flower) of the vegetation (of the year 2011) and mean fine root C/N ratios (of the year 2013)

4.5 Discussion

4.5.1 Plant species richness negatively affected gross N mineralization rates

The gross N mineralization rates observed in our study fall into the range of 0.32 to 7.09 µg N g^{-1} day^{-1} reported in the literature for comparable grasslands, i.e. natural/semi-natural grasslands with a low use intensity (Table 4.1, Davidson et al. 1991; Jamieson et al. 1999; Hatch et al. 2000; Wang et al. 2016). In their extensive review, Booth et al. (2005) compiled gross N mineralization rates of grasslands showing a wider range from ~1 to ~70 µg N g^{-1} day^{-1} (estimated from a figure) because their data set comprised a wider spectrum of grassland use.

We showed that increasing plant species richness reduced gross N mineralization rates (Table 4.2, Fig. 4.1), which is in contrast to our first hypothesis and the findings of West et al. (2006). Although we detected a significant negative effect of plant species richness on gross N mineralization rates, the effect was small, only explaining 5% of its variance (Table 4.2). Possible reasons for the contrasting results could include differences in soil type or soil pH in the study of West et al. (2006) compared to our study or to the nature of the experiment. The

results of West et al. (2006) originate from a laboratory experiment, while our results were obtained from an in-situ field experiment. Cold storage of the samples before lab incubation, controlled temperature, changed nutrient supply and lack of active plant roots in lab experiments can lead to modifications of N cycling rates relative to field experiments (Arnold et al. 2008). Previous studies from the Jena Experiment have shown a significant positive effect of plant species richness on microbial activity calculated from substrate-induced respiration determined in the laboratory (Strecker et al. 2016), which also led to the expectation of enhanced gross N mineralization in species-rich plant mixtures. Our finding of a negative relationship between plant species richness and gross N mineralization is in line with the fact that plant species richness negatively affected the root decomposition in the Jena Experiment (Chen et al. 2017) and thus likely the N release rate from root turnover.

According to the SEM, the unexpected negative relationship of species richness with gross N mineralization was related with increasing root C/N ratios with higher species richness (Fig. 4.7). Several reasons might explain the increasing root C/N ratios with increasing plant species richness. Guiz et al. (2015) found that N-rich legumes were increasingly replaced by small herbs that have higher root C/N ratios than legumes with increasing species richness. This is in line with reports that legumes contributed increasingly less to total biomass with increasing plant species richness (Gubsch et al. 2011; Roscher et al. 2011). Guiz et al. (2015) further speculated that increasing shoot C/N ratios with increasing plant species richness might be attributable to the dilution of plant nutrient concentrations, because of the higher biomass production in species-rich mixtures, which has frequently been reported for biodiversity experiments including the Jena Experiment (Marquard et al. 2009; Fornara and Tilman 2009; Mueller et al. 2013; Ravenek et al. 2014). In the Jena Experiment, the mean plant height of a plot increased with increasing species richness (Schmidtke et al. 2010), because plants in more species-rich communities have to invest more in shoot structure in response to competition for light resulting in higher C and lower N concentrations because of the higher C/N ratios of stems than of leaves (Abbas et al. 2013; Guiz et al. 2015). Figure 4.8 illustrates that increasing mean shoot height translated into increasing fine root C/N ratios in the Jena Experiment. The negative impact of increasing root C/N ratios on gross N mineralization indicated by the SEM (Fig. 4.7) agrees well with the frequently reported finding that there is a negative relationship between the litter C/N ratio and N mineralization rates (Silver and Miya 2001; Van der Krift et al. 2001; Chen et al. 2017), because a high C/N ratio of plant tissue reflects a low litter quality (Abera et

al. 2014; Zhu et al. 2014). The fact that roots and root exudates play a vital role in regulating N mineralization (Oelmann et al. 2011) through their influence on microbial biomass and activity (Bais et al. 2006; Wang et al. 2018) further supports the important role of root properties in explaining the plant species richness effect on gross N mineralization rates. The SEM also showed another significant pathway which illustrated a positive relationship between plant species richness and gross N mineralization rates via microbial biomass C. Higher plant diversity increased microbial biomass C (Strecker et al. 2016), which further increased gross N mineralization rates (Booth et al. 2005). However, this path is marginally significant and obviously was overwhelmed by the path via the root C/N ratios.

In the Jena Experiment, the C/N ratios of aboveground biomass increased with time between 2003 and 2011. This trend was increasingly pronounced with increasing species richness (Guiz et al. 2015). Because our root C/N ratios originated from a sampling campaign two years after our ^{15}N tracer experiment, the C/N ratios of the roots at the time of our experiment might have been lower and less differentiated between the low and the high species-richness levels. While we cannot control for this effect lacking root data from the time of our experiment, we assume that it was small. The molar C/N ratio of aboveground biomass changed from 24 to 35 (i.e. the mass-related ratio used here from 29 to 41) in eight years, translating into a change rate of 1.45 units yr^{-1}. Provided that the root C/N ratios change in the same way as those of the aboveground biomass, a small change of 2.9 units (< 10% of the aboveground C/N ratio in 2011) could be expected in the two years lag time between our experiment and the measurement time of the root C/N ratios. A change of the root C/N ratios by 2.9 units would translate into a change of 0.09 μg N (g dry soil)$^{-1}$ day^{-1} of the gross N mineralization rate (and of 0.1 μg N (g dry soil)$^{-1}$ day^{-1} of the microbial NH_4^+ consumption rates).

We also considered the possibility that the increasing litter input with increasing species richness, which we infer from the positive plant species richness-biomass relationship, (over-) compensated the decreasing litter quality with increasing species richness. Root biomass as proxy of belowground litter input indeed showed a significant positive correlation with species richness ($p < 0.001$, $r = 0.465$) and microbial biomass ($p = 0.002$, $r = 0.34$). However, neither aboveground nor belowground biomass correlated with gross N mineralization (Table S4.1), suggesting that a higher N flux with increasing litter input did not overrule the effect of the decreasing C/N ratio of both aboveground and belowground biomass. Finally, the significant negative direct path relating species richness with gross N mineralization rates suggests, that

there are unknown drivers underlying this species richness effect, which we were unable to identify in spite of the wealth of available soil and plant properties.

4.5.2 Plant species richness negatively affected microbial NH_4^+ consumption rates and had no effect on gross inorganic N immobilization rates

Microbial NH_4^+ consumption rates in our study fall in the range of 0.8 to 7.2 µg N (g dry soil)$^{-1}$ day^{-1}, earlier reported by various authors in the literature for comparable grasslands (Davidson et al. 1990; Hungate et al. 1997; Hatch et al. 2000). Again, Booth et al. (2005) reported a wider range from ~0.5 to ~80 µg N (g dry soil)$^{-1}$ day^{-1} (estimated from a figure). We observed a negative relationship between plant species richness and microbial NH_4^+ consumption rates (Table 4.3), which is contrary to our second hypothesis. Accordingly, the expected higher N demand and tighter N cycling in species-rich than in species-poor plant mixtures did not lead to increased microbial NH_4^+ consumption with increasing species richness.

According to the SEM, the detected negative effect of species richness on microbial NH_4^+ consumption rates is partially mediated by the root C/N ratio and microbial biomass C (Fig. 4.7). The SEM showed that microbial NH_4^+ consumption rates were also affected by gross N mineralization rates. When less NH_4^+ was released, less NH_4^+ was available for microbial uptake. We assumed that the microbial C/N ratio might also play a role in mediating the effect of plant species richness on microbial NH_4^+ consumption, because of its significant correlation with microbial NH_4^+ consumption (Fig. 4.5b). However, the microbial C/N ratio was only available for a subset of the study plots, which did not allow for including this potential mediator into the SEM. The direct path from species richness to microbial NH_4^+ consumption rates and the indirect one via root C/N ratios showed negative relationships. On the contrary, the indirect path between species richness and microbial NH_4^+ consumption rates via microbial biomass, which increased with species richness mainly because of increasing soil moisture (Lange et al. 2014) showed a positive relationship (Fig. 4.7). An explanation of the different signs of the three detected paths might be a positive correlation between plant species richness and microbial C/N ratio, which in turn would show a negative correlation with the microbial NH_4^+ consumption rates. However, we did not find any effect of plant species richness on the microbial C/N ratio in our restricted data set of two blocks ($r = -0.083$, $p = 0.619$). Instead, we found a marginally significant negative relationship between the microbial C/N ratios and the microbial NH_4^+ consumption rates (Fig. 4.5b). Thus, we cannot support the assumption that the

microbes were increasingly better supplied with N with increasing species richness and therefore reduced their NH_4^+ uptake.

Obviously, the direct and indirect (via root C/N ratios) negative effects of plant species richness on microbial NH_4^+ consumption again overruled its positive indirect effect (via microbial biomass). We can only speculate that the unexpected negative relationship between microbial C/N ratios and microbial NH_4^+ consumption rates in the Jena Experiment is attributable to the changing soil microbial community composition. In the Jena Experiment, the fungi:bacteria ratio increased with increasing species richness (Lange et al. 2014, Eisenhauer et al. 2017). The reduced microbial NH_4^+ consumption rates in spite of the higher microbial C/N ratios could then be attributed to the lower N demand of the fungi relative to the bacteria (Zechmeister-Boltenstern et al. 2015). This assumption is corroborated by findings that plant communities with high litter C/N ratios favor decomposition by fungi, whereas plant communities with low litter C/N ratios favor decomposition by bacteria (Wardle et al. 2004).

We tested the well-known controls of microbial NH_4^+ consumption rates to explain the observed negative effect of plant species richness. However, the species richness effect on microbial NH_4^+ consumption rates could only to a small degree be explained by our SEM (Fig. 4.7). We therefore conclude, that there must again be additional variables responsible for this negative relationship, which have not yet been studied in the Jena Experiment.

Gross inorganic N immobilization rates in our study fall in the range of 0.4 to 10.3 μg N (g dry soil)$^{-1}$ day^{-1}, earlier reported by various authors in the literature for comparable grasslands (Watson et al. 2000; Stockdale et al. 2000; Verchot et al. 2002; Mueller et al. 2004). The comprehensive review of Booth et al. (2005) reported a wider range from ~ 0.1 to ~ 90 μg N g^{-1} day^{-1} (estimated from a figure by combining NH_4^+ and NO_3^- immobilization rates). Plant species richness correlated significantly positively with the 1 M KCl-extractable soil NH_4^+ concentrations from shortly before our pool dilution experiment ($r = 0.30$, $p = 0.008$) and significantly negatively with the 1 M KCl-extractable soil NO_3^- concentrations from shortly before our pool dilution experiment ($r = -0.36$, $p = 0.002$, NO_3^- data log-transformed and 6 outliers removed). The different signs of the latter two correlations might explain that there was no relationship between plant species richness and gross inorganic N immobilization. The opposite relationships might have neutralized each other.

4.5.3 Plant functional group effects on gross N mineralization, NH_4^+ consumption and gross inorganic N immobilization rates

The presence of legumes had a positive effect on gross N mineralization, microbial NH_4^+ consumption and gross inorganic N immobilization rates supporting our third hypothesis (Tables 4.2-4.4). N_2 fixation by legumes may increase soil N availability for other species via the mineralization of N-rich legume litter (Peoples and Craswell 1992; Spehn et al. 2002), and also via rhizodeposition and mycorrhiza (Read 1996). The presence of legumes therefore increased gross N mineralization, microbial NH_4^+ consumption and gross inorganic N immobilization rates because legumes provide high quality litter with a low C/N ratio favoring fast decomposition rates (Abera et al. 2014). Total aboveground biomass usually increases in the presence of legumes (Tilman et al. 2001; Marquard et al. 2009), which is associated with an increased aboveground N storage in the presence of legumes (Spehn et al. 2005; Oelmann et al. 2011). Eisenhauer et al. (2010) also found increased microbial biomass C in the presence of legumes, which likely contributed to increased microbial NH_4^+ consumption and gross inorganic N immobilization rates. Furthermore, our result revealed a positive effect of small herbs on microbial NH_4^+ consumption (Table 4.3) and gross inorganic N immobilization rates (Table 4.4). Strecker et al. (2015) reported increased basal respiration and microbial biomass C in the presence of small herbs (compared to mixtures without small herbs) which increased rhizodeposition, thereby possibly leading to higher microbial NH_4^+ consumption or inorganic N immobilization by microorganisms.

Using plant diversity variables, we were only able to explain 10% of the variance in gross N mineralization, 27% in microbial NH_4^+ consumption and 14% in gross inorganic N immobilization rates (Tables 4.2-4.4). Moreover, the well-known controls of gross N mineralization and NH_4^+ consumption rates (microbial C/N ratio, root C/N ratio, soil C/N ratio, shoot C/N ratio, microbial biomass C, Booth et al. 2005) individually only explained a maximum of 13% of the variance of gross N mineralization, microbial NH_4^+ consumption, and gross inorganic N immobilization rates (Table S4.1). Consequently, there must be additional unidentified controlling factors for the unexpected negative effects of plant species richness on gross N mineralization, microbial NH_4^+ consumption, and gross inorganic N immobilization rates. We speculate that not only the chemical quality of the roots, but also that of rhizodeposits could influence gross N mineralization, microbial NH_4^+ consumption and gross inorganic N

immobilization. In addition to that, the influence of particular species/groups of microorganisms on the N cycle might be more than mass-proportional.

4.5.4 Plant diversity effects on net N mineralization and its components net ammonification and net nitrification

Our finding of negative effects of functional group richness and presence of legumes on net ammonification (Table S4.6) contrasts the literature, which has up to now mainly reported positive plant diversity effects on net turnover rates (Rosenkranz et al. 2012; Mueller et al. 2013). The literature also suggested that the presence of legumes increased the net N release (Scherer-Lorenzen et al. 2003). Rosenkranz et al. (2012) stated that in the year 2006 on the same sites as in our study (The Jena Experiment) the increasing net ammonification rates with increasing species richness were related with increasing topsoil water contents. However, Fischer et al. (2018) showed that in the later course of The Jena Experiment beginning in the year 2010 and particularly 2011, the year of our experiment, the water contents decreased with increasing species richness, which they attributed to the positive effect of species richness on soil aggregation and the subsequently increased water infiltration rates. Thus, the decreasing soil water contents with increasing species richness in the year 2011 might explain the negative effect of functional group richness on net ammonification. Our finding that the presence of legumes decreased net ammonification after the effects of block and species richness had been considered is unexpected (Table S4.6). We attribute this to the positive effect of legumes on microbial NH_4^+ consumption (Table 4.2) and gross inorganic N immobilization (Table 4.3), which resulted in a smaller leftover of NH_4^+ in mixtures with than without legumes.

4.6 Conclusions

Our results demonstrate that both, gross mineralization and microbial NH_4^+ consumption rates determined in the field unexpectedly decreased with increasing species richness, while gross inorganic N immobilization was unrelated with species richness so that we had to reject our first two hypotheses. Again unexpectedly, functional group richness had negative effects on net ammonification rates, which we attribute to the decreasing soil moisture in topsoil with increasing plant diversity in the year of our study (2011). The third hypothesis that the presence of legumes influenced gross mineralization, microbial NH_4^+ consumption and gross inorganic

N immobilization rates positively was, however, supported by our data. This positive effect likely explained the negative effect of the presence of legumes on net ammonification.

Among the wealth of data from the Jena Experiment, only the root C/N ratio was identified to significantly reduce two of the three studied gross N turnover rates but explained a small portion of the total variance in our structural equation model. The root C/N ratio likely increased with increasing species richness because of a species replacement effect from legumes to forbs and because of increasing competition for light which resulted in a higher mean shoot height associated with a lower C/N ratio of the above- and belowground biomass. The negative root C/N ratio effect overwhelmed a positive effect of microbial biomass on gross N mineralization and microbial N consumption. Our results illustrate that the nutrient composition of biomass mediates N turnover processes in the studied grassland ecosystem suggesting that connecting ecological stoichiometry with nutrient fluxes could be a promising avenue to better understanding the biodiversity-nutrient cycling relationship.

The significant direct effect of species richness on gross N mineralization and microbial NH_4^+ consumption rates, which remained in our structural equation model could not be explained based on the available data. We hypothesize that the latter is related with a changing microbial composition with increasing species richness, for which we lack data. Therefore, future experiments should be designed to elucidate the relationships between species richness, microbial community composition and N turnover rates. Generally, relating soil nutrient fluxes with microbial community composition could additionally improve our understanding of the controls of nutrient turnover in soil.

4.7 Acknowledgments

We thank the many people who helped with the management of the experiment and in particular the initiators, E.-D. Schulze, B. Schmid, and W.W. Weisser, as well as the scientific coordinators C. Roscher, A. Weigelt, and A. Ebeling. Thanks also to all the helpers who assisted during the weeding campaigns. The Jena Experiment is funded by the Deutsche Forschungsgemeinschaft (DFG, FOR 456 & 1451, Wi 1601/4) and the Swiss National Science Foundation (SNSF, 200021E-131195/1), with additional support from the Friedrich Schiller University Jena and the Max Planck Society.

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4.9 Supplementary materials

Table S4.1: Pearson correlation coefficients for the relationships of the potential variables with gross N mineralization, microbial NH_4^+ consumption and gross inorganic N immobilization. Significant correlations (p<0.05) are marked in bold and marginal significant correlations (p<0.1) are marked in italics.

Variables	Gross N mineralization		Microbial NH_4^+ consumption		Gross inorganic N immobilization	
	r	p	r	p	r	p
Microbial biomass C	0.094	0.434	**0.238**	**0.044**	**0.238**	**0.044**
Microbial C/N ratio	**-0.326**	**0.047**	*-0.281*	*0.097*	**-0.360**	**0.031**
Soil C/N ratio	-0.091	0.447	**-0.262**	**0.026**	-0.019	0.877
Shoot C/N ratio	**-0.243**	**0.037**	**-0.267**	**0.022**	-0.164	0.162
Fine root C/N ratio	**-0.230**	**0.049**	*-0.210*	*0.072*	-0.036	0.758
Root biomass	-0.113	0.336	-0.031	0.796	0.051	0.665
Shoot biomass	-0.137	0.245	-0.078	0.510	-0.094	0.427
Total N concentration in soil	0.066	0.582	0.126	0.290	**0.256**	**0.030**
Total organic C concentration in soil	0.014	0.907	0.002	0.985	*0.206*	*0.083*
Soil moisture	-0.073	0.542	0.008	0.947	-0.010	0.930
Soil pH	**-0.264**	**0.023**	-0.059	0.616	-0.098	0.407

Table. S4.2: Fit indices for the structural equation model in Fig. S4.1. P = p value, χ^2 = chi squared, GFI = goodness of fit index, CFI = comparative fit index, NFI = normed fit index.

Fit index	Value
P	0.00
χ^2	73.94
GFI	0.85
CFI	0.79
NFI	0.75

Table. S4.3: Fit indices for the structural equation model in Fig. S4.2. P = p value, χ^2 = chi squared, GFI = goodness of fit index, CFI = comparative fit index, NFI = normed fit index.

Fit index	Value
P	0.00
χ^2	47.39
GFI	0.88
CFI	0.86
NFI	0.81

Table. S4.4: Fit indices for the structural equation model in Fig. 4.7. P = p value, χ^2 = chi squared, GFI = goodness of fit index, CFI = comparative fit index, NFI = normed fit index.

Fit index	Value
P	0.07
χ^2	10.2
GFI	0.96
CFI	0.96
NFI	0.04

Table. S4.5: Hierarchical ANOVA results showing the effects of plant species richness (SR) and presence (+) /absence (-) of each functional group on net nitrogen mineralization rates. Bold letters show significance at p < 0.05.

Source	Df	SS	SS (%)	F	P
Block	**3**	**10.00**	**11.50**	**2.83**	**0.046**
SR	1	0.05	0.06	0.05	0.833
Legumes	1	3.26	3.75	2.77	0.101
Grasses	1	0.61	0.70	0.51	0.476
Tall herbs	1	0.50	0.57	0.42	0.518
Small herbs	1	0.73	0.84	0.62	0.433
Residuals	61	71.84			

Table. S4.6: Hierarchical ANOVA results showing the effects of plant species richness (SR) and presence (+) /absence (-) of each functional group on net ammonification rates. Bold letters show significance at p < 0.05 and italics show significance at p < 0.1. Arrows indicate negative (↓) effects

	Df	SS	SS (%)	F	P
Block	*3*	*5.51*	*8.98*	*2.441*	*0.072*
SR	1	0.08	0.13	0.112	0.739
Legumes	**1**	**3.65**	**5.95**	**4.852**	**0.031↓**
Grasses	1	0.15	0.24	0.204	0.653
Tall herbs	1	0.17	0.28	0.222	0.639
Small herbs	1	1.36	2.22	1.802	0.184
Residuals	67	50.45			

Table. S4.7: Hierarchical ANOVA results showing the effects of plant species richness (SR) and presence (+) /absence (-) of each functional group on net nitrification rates.

	Df	SS	SS (%)	F	P
Block	3	1.49	5.16	1.20	0.319
SR	1	0.37	1.28	0.89	0.350
Legumes	1	0.17	0.59	0.40	0.529
Grasses	1	0.61	2.11	1.48	0.229
Tall herbs	1	0.10	0.35	0.24	0.624
Small herbs	1	0.85	2.94	2.06	0.157
Residuals	61	25.29			

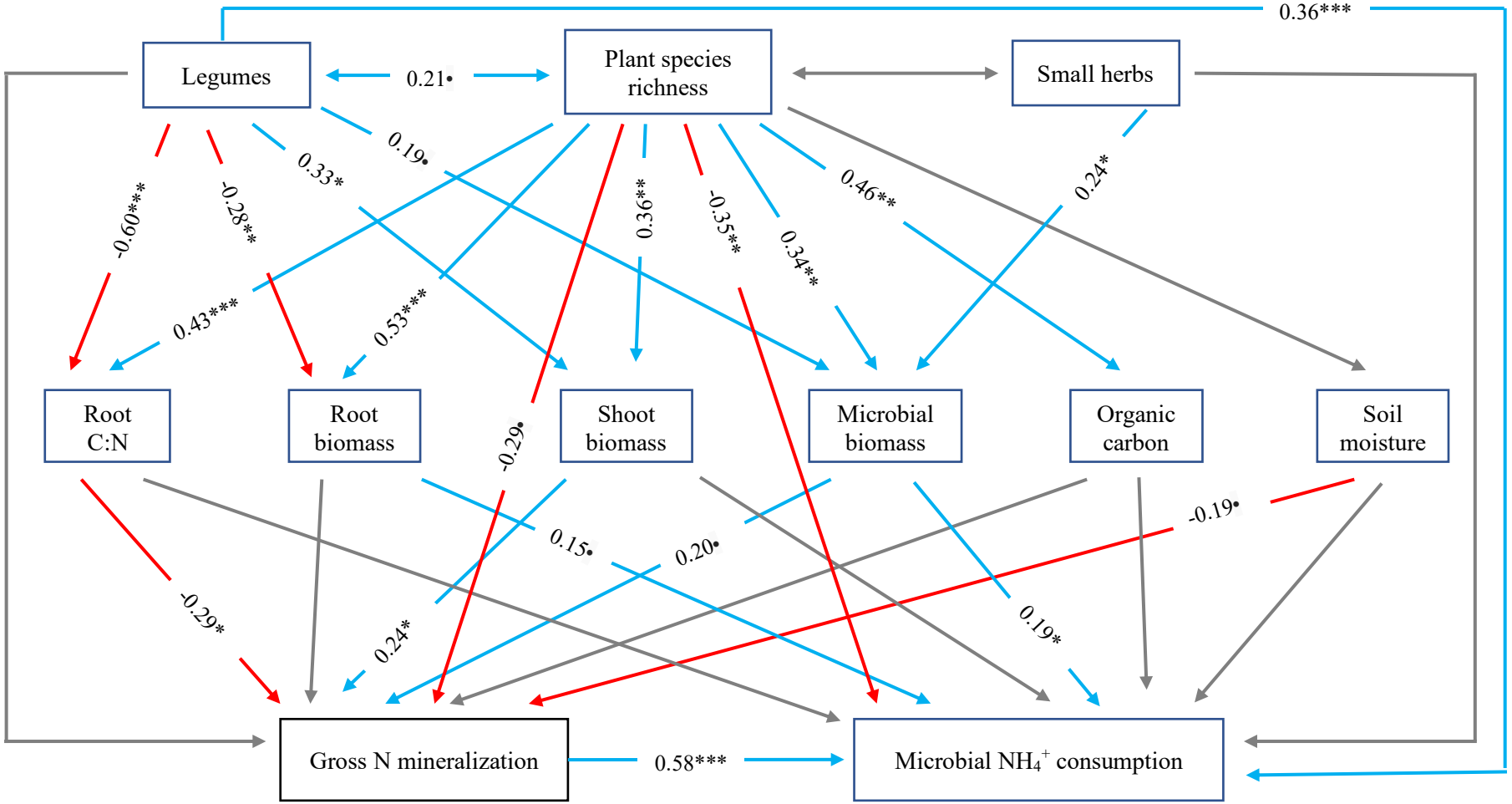

Fig. S4.1: A-priori structural equation model showing the potential causal effects of plant diversity (plant species richness and presence/absence of individual functional groups) on gross N mineralization and microbial NH_4^+ consumption rates. Blue and red arrows represent positive and negative significant relationships, respectively. Grey arrows show non-significant pathways. Numbers on the arrows give unstandardized path coefficients with their significance indicated as ***p < 0.001, **p < 0.01, *p < 0.05, •p < 0.01.

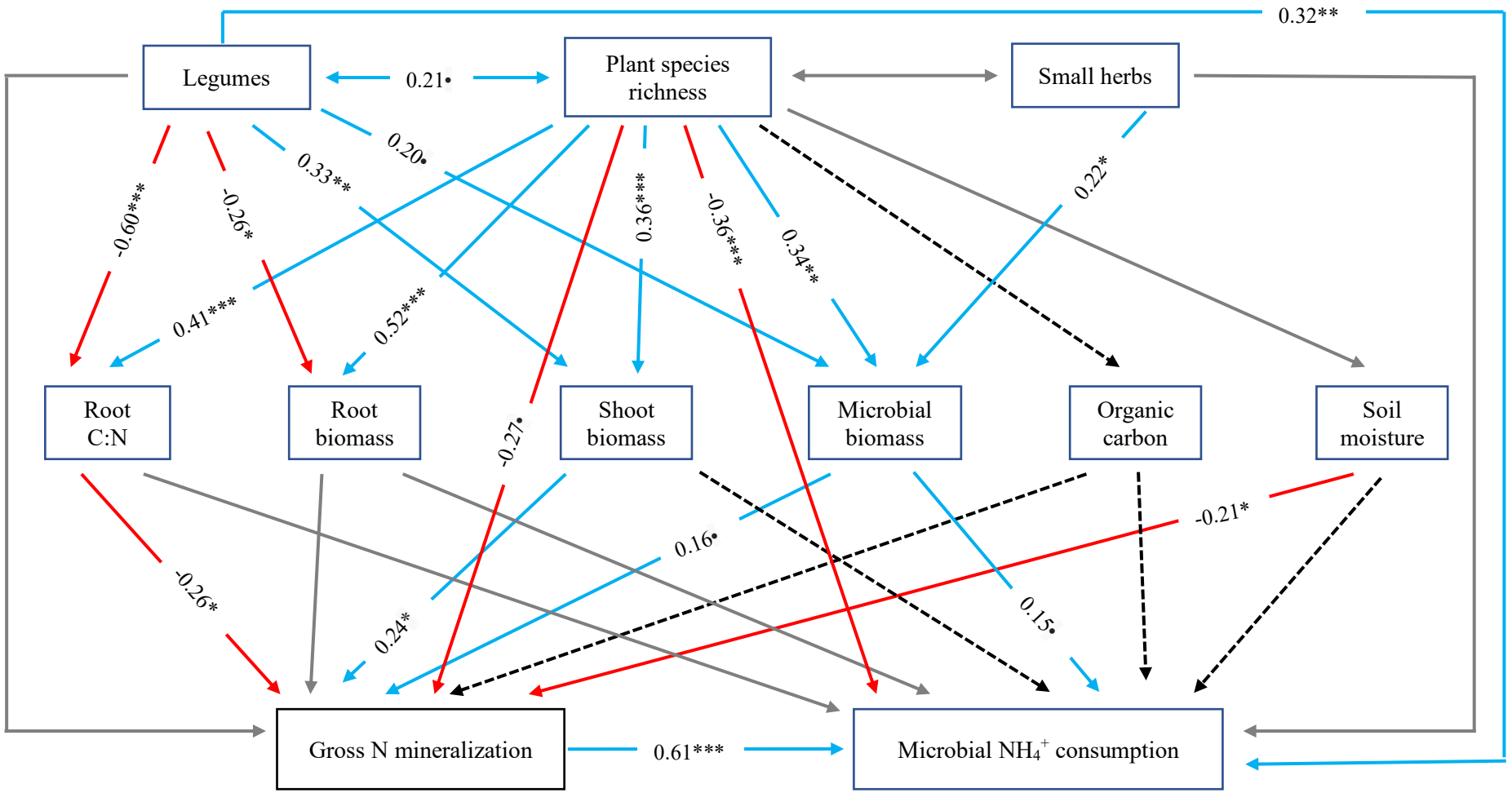

Fig. S4.2: A-priori structural equation model showing the potential causal effects of plant diversity (plant species richness and presence/absence of individual functional groups) on gross N mineralization and microbial NH_4^+ consumption rates. Blue and red arrows represent positive and negative significant relationships, respectively. Grey arrows show non-significant pathway. Dotted arrows indicate non-significant pathways that were sequentially excluded from the final model based on the p values. Numbers on the arrows give standardized path coefficients with their significance indicated as ***p < 0.001, **p < 0.01, *p < 0.05, ˙p < 0.01

Appendix

The appendix is attached as a CD containing the following files:

- Contents (Contents.pdf)
- Dissolved NH_4^+ and NO_3^- pools measured on different incubation days (Day 1, 2, 4+9, 16) from the ^{15}N tracing experiment conducted in the laboratory in Block 2 (01_Npools1_Lab.xlsx)
- Labile organic N (N_{lab}), recalcitrant organic N (N_{rec}) and exchangeable (=adsorbed) NH_4^+ ($NH_4^+{}_{ads}$) pools measured from the ^{15}N tracing experiment conducted in the laboratory in Block 2 (02_Npools2_Lab.xlsx)
- Gross N mineralization rates from the labile organic N (M_{Nlab}) and recalcitrant organic N (M_{Nrec}) calculated from the ^{15}N tracing experiment conducted in the laboratory in Block 2 (03_GrossNmineralization_Lab.xlsx)
- Rates of NH_4^+ immobilization rates into the labile organic N ($I_{NH4\text{-}Nlab}$) and recalcitrant organic N ($I_{NH4\text{-}Nrec}$) calculated from the ^{15}N tracing experiment conducted in the laboratory in Block 2 (04_NH4Immobilization_Lab.xlsx)
- Rates of autotrophic nitrification (O_{NH4}) and heterotrophic nitrification (O_{Nrec}) calculated from the ^{15}N tracing experiment conducted in the laboratory in Block 2 (05_Nitrification_Lab.xlsx)
- Rates of NO_3^- immobilization (I_{NO3}) calculated from the ^{15}N tracing experiment conducted in the laboratory in Block 2 (06_NO3Immobilization_Lab.xlsx)
- Rates of NH_4^+ adsorption into the exchangeable NH_4^+ pool (A_{NH4}) calculated from the ^{15}N tracing experiment conducted in the laboratory in Block 2 (07_NH4Adsorption_Lab.xlsx)
- Concentrations of KCl-extractable NH_4^+ and NO_3^- measured before the start of the ^{15}N pool dilution experiment in the field Blocks 1 to 4 (08_Npools_Field.xlsx)

- Gross N mineralization rates calculated from the ^{15}N pool dilution experiment conducted in the field in Blocks 1 to 4 (09_GrossNmineralization_Field.xlsx)
- Microbial NH_4^+ consumption rates calculated from the ^{15}N pool dilution experiment conducted in the field in Blocks 1 to 4 (10_NH4Consumption_Field.xlsx)
- Gross inorganic N immobilization rates calculated from the ^{15}N pool dilution experiment conducted in the field Blocks 1 to 4 (11_NImmobilization_Field.xlsx)
- Net N mineralization and its components net ammonification and net nitrification rates calculated from KCl-extractable mineral N concentrations measured before the start of the field experiment in Blocks 1 to 4 (12_NetNRates_Field.xlsx)