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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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Abstract

We conducted a ^{15}N tracer 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, 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. 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.

Keywords *Ntrace* model · Laboratory microcosms · Gross N transformation rates · Plant diversity · The Jena Experiment

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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 quantitatively 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 depend 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 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, USA, 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), and cation exchange (i.e., ad- and desorption) of NH_4^+ on clay minerals (Müller et al. 2007). Moreover, the model simulates the pool sizes of labile and recalcitrant organic N, NH_4^+, and NO_3^-, and adsorbed NH_4^+ (Müller et al. 2007). The objective of this study was 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 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. This includes possible changes in the N availability for plant growth and increasing N leaking in gaseous form to the atmosphere or as NO_3^- to surface and groundwaters with their known detrimental effects on climate and water quality (Sutton et al. 2011).

Materials and methods

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 the 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 on 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 4025 bacterial, 23 archaeal, and 826 unclassified OTUs based on the amplification 16S rRNA gene fragments and 431 fungi, 174 protists, 9 plants, 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).

^{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 in 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 the 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-NH$_4^+$ ammonium, ^{15}N-NO$_3^-$, or both (98 at%), applied as 0.5 μg ^{15}NH$_4$Cl-N and 0.25 μg K^{15}NO$_3$-N (g dry soil)$^{-1}$. 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 1 h each day. Soil samples were extracted by percolation with 100 mL of a N-free nutrient solution (4 mM CaCl$_2$, 2 mM KH$_2$PO$_4$, 1 mM MgCl$_2$, 1 mM K$_2$SO$_4$, 1 mM MgSO$_4$, 25 μM H$_3$BO$_3$, 2 μM MnSO$_4$, 2 μM ZnSO$_4$, 0.5 μM CuSO$_4$, and 0.5 μM Na$_2$MoO$_4$; Nadelhoffer 1990) 12 h 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. 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$_3^-$ was determined with the bacterial denitrification method, in which *Pseudomonas aureofaciens* is used to convert NO$_3^-$ to N$_2$O, followed by isotope analysis (Sigman et al. 2001; McIlvin and Casciotti 2011). The isotope ratios of the N$_2$O gas were analyzed with a Gas-Bench II pre-concentration unit interfaced with the 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$_2$O by reduction with azide (Zhang et al. 2007). The N$_2$O is then purified and analyzed as described above for NO$_3^-$-derived N$_2$O.

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 dry soil]$^{-1}$ h^{-1}) was calculated as the mean of the O$_2$ consumption rates determined between 14 and 24 h 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 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$_2$SO$_4$ by shaking for 30 min. 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 ratio.

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 the 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 the concentrations of TN 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 eight gross N transformation rates 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. 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 the 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,
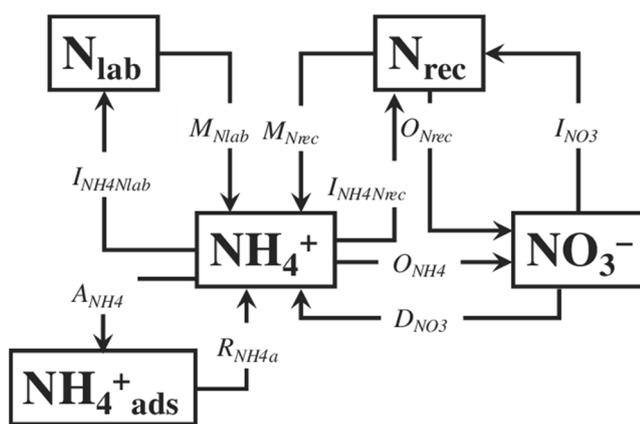
Fig. 1 Schematic representation of the ^{15}N tracing model (Müller et al. 2007). It includes five N pools: dissolved ammonium (NH_4^+), dissolved nitrate (NO_3^-), labile soil organic N (N_{lab}), recalcitrant organic N (N_{rec}), and exchangeable (termed "adsorbed" in previous work; Müller et al. 2004, 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 ($I_{NH4-Nrec}$), immobilization of NH_4^+ to labile organic N ($I_{NH4-Nlab}$), 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}, which was negligible in our study), adsorption of NH_4^+ into the exchangeable NH_4^+ pool (A_{NH4}), release of exchangeable (adsorbed) NH_4^+ to NH_4^+ (R_{NH4a})

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, USA) and the ^{15}N tracing model, that was separately set up, in Simulink 7.4 (The MathWorks Inc.).

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 ($I_{NH4-Nlab} + I_{NH4-Nrec}$). 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 additionally normalized to the TN concentration of the soil solid phase.

Statistical analyses

We used repeated measures and sequential ANOVA (type I sum of squares) to inspect the 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 $I_{NH4-Nlab}$ was log-transformed to improve the 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

Table 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)$^{-1}$ day^{-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
$I_{NH4-Nlab}$	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
$I_{NH4-Nrec}$	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

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 $p < 0.05$.

Results

Pool-size changes of dissolved mineral N during the incubation

The N amendments, which contributed less than 6% 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% 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 from 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. 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)$^{-1}$ on day 1 to 1.18 ± 0.11 μg N (g soil)$^{-1}$ on 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 2).
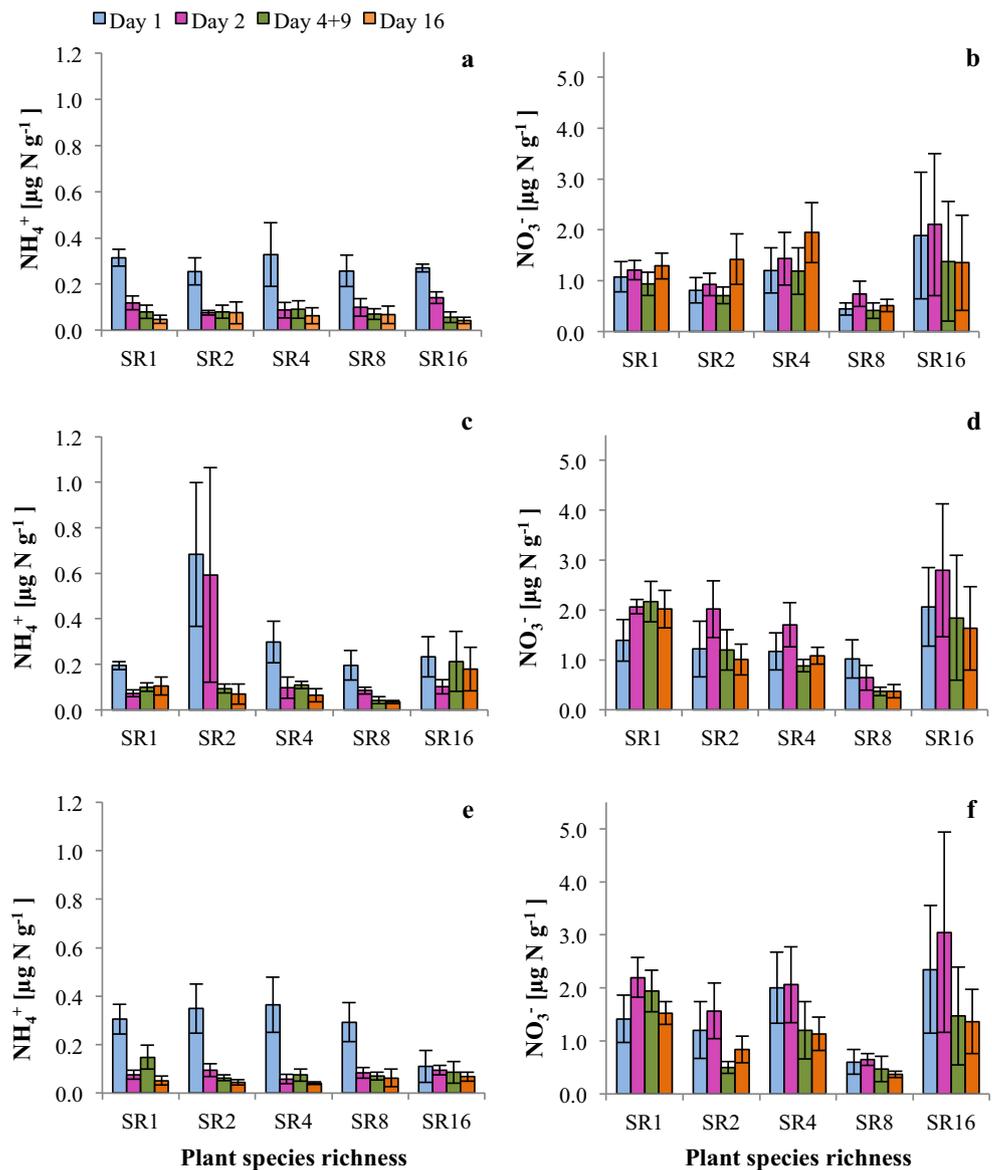
The interaction between day and species richness had a marginally significant influence on the dissolved NO_3^- pool (Table 2). Functional group richness did not show significant effects on the dissolved NH_4^+ and NO_3^- pool sizes (Table 2). The relationship between the presence of grasses and the dissolved NH_4^+ pool size was significantly positive (Table 2; Fig. 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 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 deteced 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 S1). Plant species richness increased both, the initial labile and recalcitrant organic N pools (Table S2; Fig. S1).

Gross NH_4^+ production

Gross N mineralization from labile organic N ranged between 0.01 and 1.94 μg N (g soil)$^{-1}$ day^{-1} and from recalcitrant organic N between 0.006 and 1.35 μg N (g soil)$^{-1}$ day^{-1} (means and standard deviations [SD] are shown in Table 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; Fig. 5a). The presence of legumes or

Fig. 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

Gross NO_3^- production

Gross heterotrophic and autotrophic nitrification rates ranged from 0.05 to 3.66 μg N (g soil)$^{-1}$ day^{-1} and from 0.20 to 3.62 μg N (g soil)$^{-1}$ day^{-1}, respectively (means and SD in Table 1). Neither plant species richness nor functional group richness (Table 4) significantly affected autotrophic nitrification. The presence of legumes significantly increased autotrophic nitrification rates (Table 4; Fig. 6a). We did not detect any significant effects of plant community composition on heterotrophic nitrification from the recalcitrant organic N pool.

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)$^{-1}$ day^{-1}, respectively and the NO_3^- immobilization rates ranged from 0.94 to 6.97 μg N (g soil)$^{-1}$ day^{-1} (means and SD in Table 1). Neither plant species richness nor functional group richness significantly affected the NH_4^+ immobilization into the labile (Table 3) and the recalcitrant organic N pools. Grasses significantly increased the immobilization of NH_4^+ into the labile organic N pool (Table 3; Fig. 6b). There were no significant effects of plant community composition on the immobilization of NO_3^- into the organic N pool. The normalization of N transformation rates and pool sizes to the total N concentrations did not change the overall results (Tables S3–S7).

grasses had a significant positive effect on the N mineralization rate from labile organic N (Table 3; Fig. 5b, c).

Table 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

Source	Dissolved NH_4^+ pool				Dissolved NO_3^- pool			
	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*

Bold figures show significance at $p < 0.05$ and figures in italics show marginal significance at $p < 0.1$. Non-significant within-subject effects are excluded from the table. The arrow indicates a positive (↑) effect

## 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. 7a, b). Furthermore, we found a marginally significant positive relationship between basal respiration and immobilization of NH_4^+ into the labile organic N (Fig. 7c).

Discussion

Pool sizes and gross N transformation rates

The drastic dilution of applied ^{15}N-NH_4^+ in the soil extracts during the incubation (Figs. 2a and 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. 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

Fig. 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: double asterisk indicates $p < 0.01$
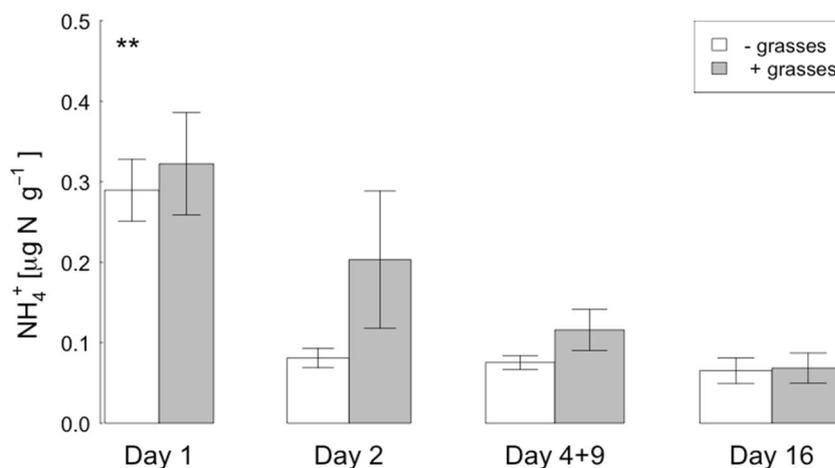
Table 3 Sequential ANOVA results showing the effects of plant species richness, functional group richness and presence (+)/absence (-) of each functional group on gross N mineralization from the labile organic N pool (M_{Nlab}) and on immobilization of NH_4^+ into the labile organic N pool ($I_{NH4-Nlab}$)

Source	M_{Nlab}				$I_{NH4-Nlab}$			
	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

Bold figures show significance at $p < 0.05$ and figures in italics show significance at $p < 0.1$. Arrows indicate positive (↑) and negative (↓) effects

the incubation (Fig. 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. 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)$^{-1}$ day^{-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 ($I_{NH4-Nrec} + I_{NH4-Nlab}$) were also in the range of 0.10–0.88 µg N (g soil)$^{-1}$ day^{-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)$^{-1}$ day^{-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)$^{-1}$ day^{-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)$^{-1}$ day^{-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. 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.

Table 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})

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

Figures in italics show significance at $p < 0.05$. The arrow indicates a positive (↑) effect

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; Fig. 5c). At the same time, the initial

Fig. 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
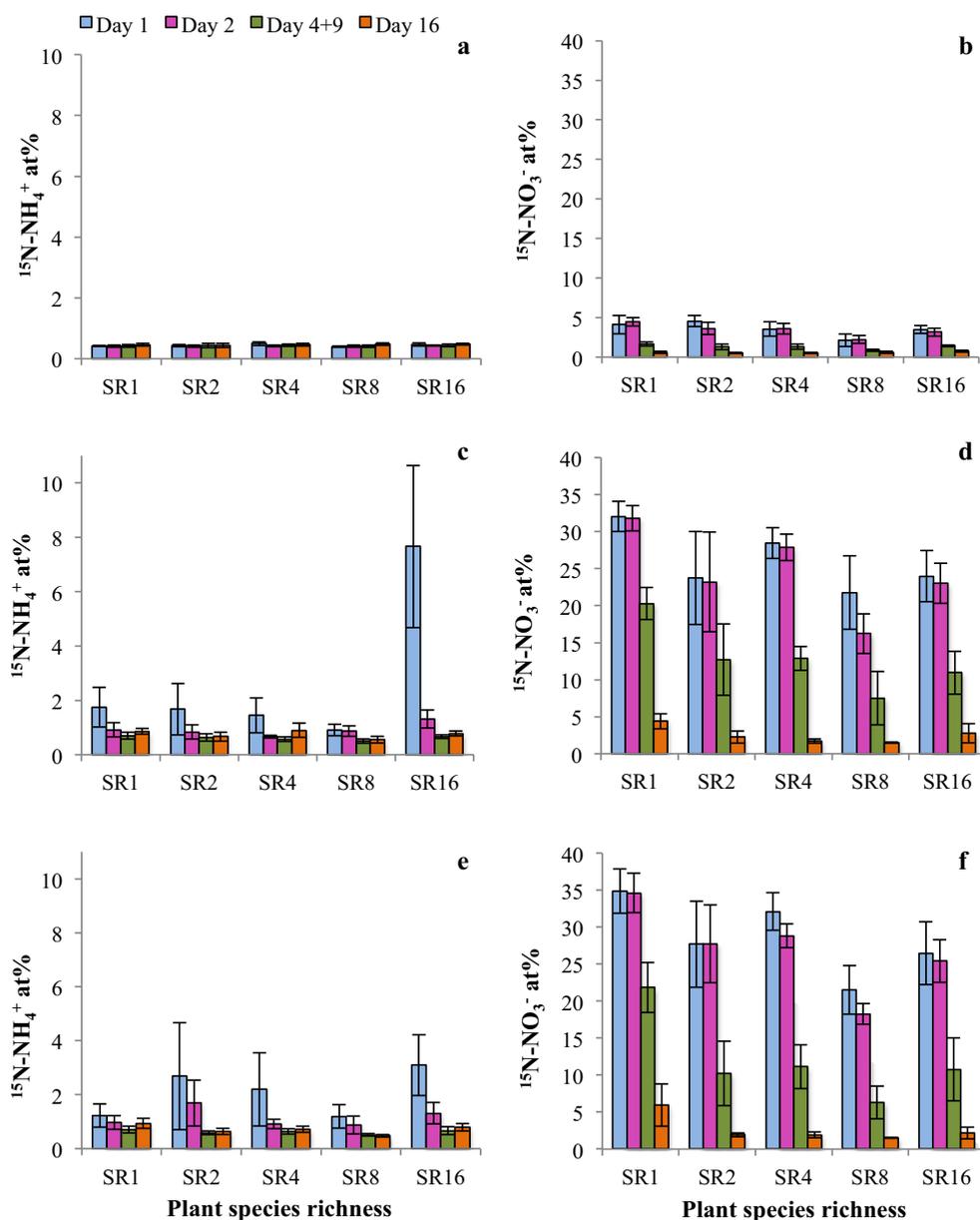
exchangeable (= adsorbed) NH_4^+ pool was marginally significantly lower in the presence of grasses, possibly because of the exhaustive N exploitation of the dense grass roots prior to our experiment without plants (Table S1). This exhaustive N exploitation is also supported by the fact that the presence 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 S2; Fig. S1) 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).

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; Fig. 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, USA, 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
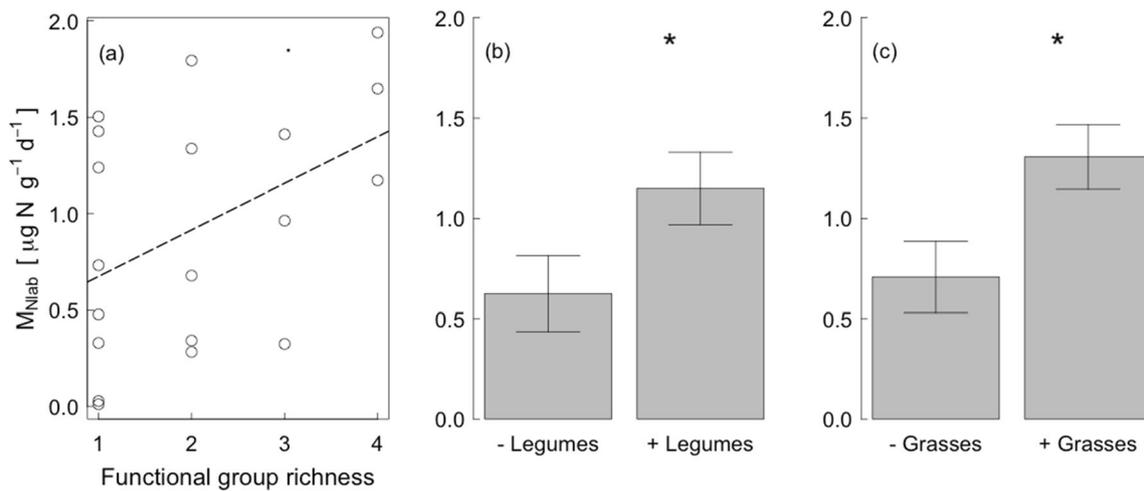
Fig. 5 Relationship between **a** functional group richness and the gross N mineralization from the labile organic N pool (M_{Nlab}) and effects of **b** presence (+)/absence (−) of legumes and (**c**) presence/absence of grasses on the gross N mineralization from the labile organic N pool (M_{Nlab}). The whiskers in Fig. 3b, c represent standard errors. Significance codes: asterisk indicates $p < 0.05$ and solid bullet indicates $p < 0.1$

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. 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 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

Fig. 6 Effects of **a** the presence (+)/absence (−) of legumes on autotrophic nitrification (O_{NH4}) and **b** of presence (+)/absence (−) of grasses on the immobilization of NH_4^+ in the labile organic N pool ($I_{NH4-Nlab}$). Whiskers represent standard errors. Significance code: one asterisk indicates $p < 0.05$, two asterisks indicate $p < 0.01$
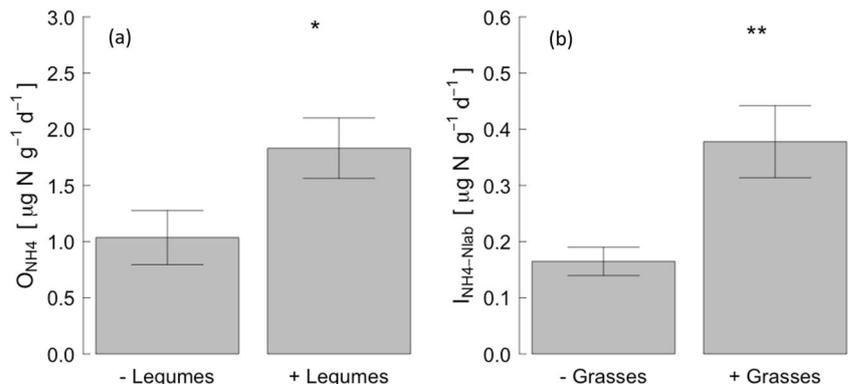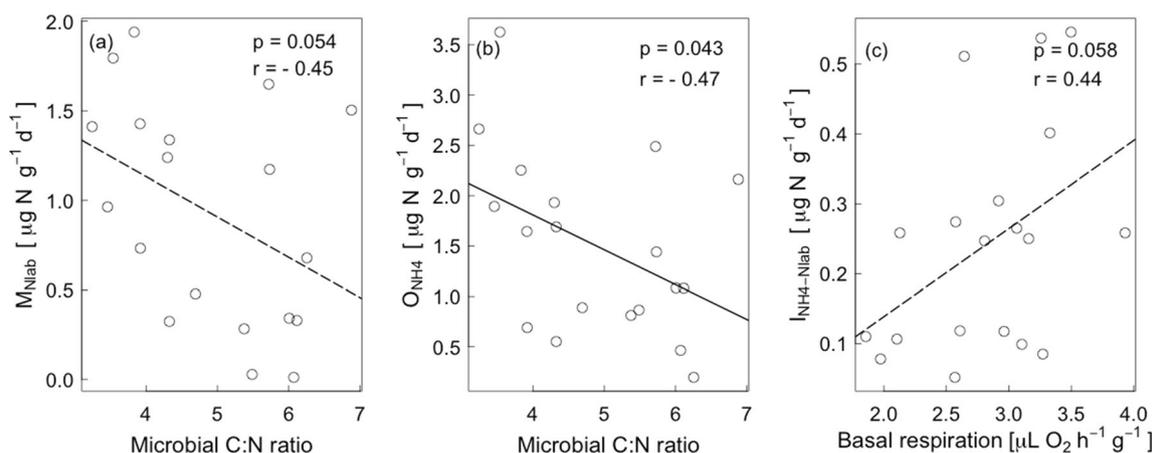
Fig. 7 Relationships between **a** microbial C:N ratio and N mineralization into labile organic N (M_{Nlab}), **b** microbial C:N ratio and autotrophic nitrification (O_{NH4}), and **c** basal respiration and ammonium immobilization from labile organic N ($I_{NH4-Nlab}$). p and r values refer to the 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 differences in y-axis scaling

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; Fig. 5b) is likely related to 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 with that in 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. 6b) might be attributable to an enhanced microbial activity (Fig. 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.

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 4; Fig. 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. 7b). Lower microbial C/N ratios which are associated with a 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.

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.

Data availability The data will be uploaded to the database of the Jena Experiment, which will become public after an embargo time.

Compliance with ethical standards

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