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## **Plant species diversity affects soil–atmosphere fluxes of methane and nitrous oxide**

Niklaus, Pascal A ; Le Roux, Xavier ; Poly, Franck ; Buchmann, Nina ; Scherer-Lorenzen, Michael ; Weigelt, Alexandra ; Barnard, Romain L

**Abstract:** Plant diversity effects on ecosystem functioning can potentially interact with global climate by altering fluxes of the radiatively active trace gases nitrous oxide (N<sub>2</sub>O) and methane (CH<sub>4</sub>). We studied the effects of grassland species richness (1-16) in combination with application of fertilizer (nitrogen:phosphorus:potassium = 100:43.6:83 kg ha<sup>-1</sup> a<sup>-1</sup>) on N<sub>2</sub>O and CH<sub>4</sub> fluxes in a long-term field experiment. Soil N<sub>2</sub>O emissions, measured over 2 years using static chambers, decreased with species richness unless fertilizer was added. N<sub>2</sub>O emissions increased with fertilization and the fraction of legumes in plant communities. Soil CH<sub>4</sub> uptake, a process driven by methanotrophic bacteria, decreased with plant species numbers, irrespective of fertilization. Using structural equation models, we related trace gas fluxes to soil moisture, soil inorganic N concentrations, nitrifying and denitrifying enzyme activity, and the abundance of ammonia oxidizers, nitrite oxidizers, and denitrifiers (quantified by real-time PCR of gene fragments amplified from microbial DNA in soil). These analyses indicated that plant species richness increased soil moisture, which in turn increased N cycling-related activities. Enhanced N cycling increased N<sub>2</sub>O emission and soil CH<sub>4</sub> uptake, with the latter possibly caused by removal of inhibitory ammonium by nitrification. The moisture-related indirect effects were surpassed by direct, moisture-independent effects opposite in direction. Microbial gene abundances responded positively to fertilizer but not to plant species richness. The response patterns we found were statistically robust and highlight the potential of plant biodiversity to interact with climatic change through mechanisms unrelated to carbon storage and associated carbon dioxide removal.

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# Plant species diversity affects soil-atmosphere fluxes of methane and nitrous oxide

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**Author contributions:** PAN and RLB conceived and designed the present study with input from MSL and NB. Trace gas flux measurements and soil analyses were carried out by PAN, MSL and RLB. XLR and FP analyzed functional gene abundances. PAN analyzed the data with input from XLR and RLB. PAN wrote the manuscript with input from all co-authors.

## Abstract

Plant diversity effects on ecosystem functioning can potentially interact with global climate by altering fluxes of the radiatively active trace gases nitrous oxide ( $\text{N}_2\text{O}$ ) and methane ( $\text{CH}_4$ ). We studied effects of grassland species richness (1 to 16) in combination with NPK-fertilizer application ( $\text{N:P:K} = 100:43.6:83 \text{ kg ha}^{-1} \text{ a}^{-1}$ ) on  $\text{N}_2\text{O}$  and  $\text{CH}_4$  fluxes in a long-term field experiment. Soil  $\text{N}_2\text{O}$  emissions, measured over two years using static chambers, decreased with species richness unless fertilizer was added.  $\text{N}_2\text{O}$  emissions increased with fertilization and the fraction of legumes in plant communities. Soil  $\text{CH}_4$  uptake, a process driven by methanotrophic bacteria, decreased with plant species numbers, irrespective of fertilization. Using structural equation models, trace gas fluxes were related to soil moisture, soil inorganic N concentrations, nitrifying (NEA) and denitrifying enzyme activity (DEA), and the abundance of ammonia oxidizers, nitrite oxidizers and denitrifiers (quantified by real-time PCR of gene fragments amplified from soil DNA). These analyses indicated that plant species richness increased soil moisture, which in turn increased N cycling-related activities. Enhanced N cycling increased  $\text{N}_2\text{O}$  emission and soil  $\text{CH}_4$  uptake, with the latter possibly caused by removal of inhibitory  $\text{NH}_4^+$  by nitrification. The moisture-related indirect effects were surpassed by direct, moisture-independent effects opposite in direction. Microbial gene abundances responded positively to fertilizer but not to plant species richness. The response patterns we found were statistically robust and highlight the potential of plant biodiversity to interact with climatic change through mechanisms unrelated to carbon (C) storage and associated  $\text{CO}_2$  removal.

**Keywords:** Functional genes, Jena experiment, microbial activities, nitrification and denitrification, structural equation modelling.

## Introduction

Recent syntheses of experimental research have shown that effects of plant diversity loss on ecosystem functioning can be of similar magnitude as those of other global changes, including warming and drought (Hooper et al. 2012). However, effects of plant diversity interact with other global changes, with mechanisms operating at spatial scales ranging from the plot scale to the globe. For example, global change drivers may affect ecosystem functions directly and indirectly via alterations of plant diversity (Balvanera et al. 2006). Plant diversity changes can alter ecosystem carbon (C) and nutrient cycling, thereby changing the ecosystem-atmosphere exchange rates of greenhouse gases and thus affect climatic drivers at the global scale.

Carbon dioxide ( $\text{CO}_2$ ) is the dominant anthropogenic greenhouse gas, but nitrous oxide ( $\text{N}_2\text{O}$ ) and methane ( $\text{CH}_4$ ) also are important drivers of anthropogenic climate change. Despite their low atmospheric volume mixing ratios, they account for over 25% of the radiative forcing increase since pre-industrial times because of their high warming potential relative to  $\text{CO}_2$  (IPCC 2007). To date, most experimental plant diversity studies have focused on ecosystem functions related to plant primary productivity and the ecosystem's C cycle (e.g., biomass, photosynthesis, leaf area, litter decomposition), which are processes directly relevant for the land-atmosphere exchange of  $\text{CO}_2$ . However, plant diversity effects on land-atmosphere fluxes of  $\text{N}_2\text{O}$  and  $\text{CH}_4$  have only been studied in few mesocosm studies ( $\text{N}_2\text{O}$ : Abalos et al. 2014; Chang et al. 2014; Sun et al. 2013;  $\text{N}_2\text{O}$  and  $\text{CH}_4$ : Niklaus et al. 2006), and relatively little is yet known on responses of the underlying nitrogen (N) cycling processes to plant diversity (Le Roux et al. 2013).

$\text{N}_2\text{O}$  is released from soils as an intermediate or by-product of microbial N cycling (cf. Cabello et al. 2009 for a comprehensive overview of soil N transformations). Under oxic conditions, some  $\text{N}_2\text{O}$  produced as a by-product of nitrification will escape to the atmosphere. Under more anoxic conditions, nitrate is sequentially reduced to  $\text{N}_2\text{O}$ , which generally is lost from soils if it is not further reduced to molecular nitrogen (Firestone and Davidson 1989). Soil  $\text{N}_2\text{O}$  emissions are controlled by many factors (Robertson and Tiedje 1987) including the availability of inorganic N, in particular ammonium and nitrate. Soil moisture also is important because it affects gas diffusivity and oxygen supply. At low water filled pore space, oxygen is readily available and soil organic matter is mineralized, yielding  $\text{NH}_4^+$  and promoting nitrification and associated  $\text{N}_2\text{O}$  emissions. At high water filled pore space, nitrification ceases and nitrate is consumed by denitrification, which is the dominant source of  $\text{N}_2\text{O}$  emissions from soils. At even higher water filled pore space,  $\text{N}_2\text{O}$  emissions drop because most of the  $\text{N}_2\text{O}$  produced is reduced to molecular nitrogen.

60 Soils can be both sources and sinks of atmospheric CH<sub>4</sub>. On an annual basis, most  
61 aerobic soils are a net sink for atmospheric CH<sub>4</sub>, although periodic emissions can occur when  
62 soils are wet. This soil CH<sub>4</sub> sink is essentially driven by the consumption of CH<sub>4</sub> by  
63 methanotrophic bacteria, which use CH<sub>4</sub> as a source of carbon and energy (Dunfield 2007).  
64 At very low redox potential (typically found in wetlands), however, methanogenic archaea  
65 produce CH<sub>4</sub> through several pathways, consuming organic substrates or CO<sub>2</sub> (Conrad 1996).  
66 Non-microbial soil CH<sub>4</sub> emissions have recently been described (Wang et al. 2013), but the  
67 quantitative importance of this process in natural ecosystems remains to be evaluated.  
68 Methanogens and methanotrophs often co-occur in soils, with methanotrophs acting as a  
69 biofilter that oxidises CH<sub>4</sub> produced by methanogens in anoxic soil domains. On the other  
70 hand, occasional methanogenesis can prime methanotrophic activity, leading to increased  
71 uptake of atmospheric CH<sub>4</sub> (West and Schmidt 2002). Several links have been established  
72 between CH<sub>4</sub> oxidation and N transformations, with complex, positive and negative  
73 correlations between the two (Bodelier and Laanbroek 2004). Ammonia has been shown to  
74 inhibit CH<sub>4</sub> oxidation, at least in laboratory cultures (Hanson and Hanson 1996), but on the  
75 other hand inorganic N is an essential nutrient for most methanotrophs. Further, differential  
76 stratification of these processes may occur in the soil, with effects that may be restricted to  
77 some soil layers and therefore not necessarily generate effects on soil-atmosphere CH<sub>4</sub> fluxes  
78 (Stiehl-Braun et al. 2011a).

79 While the fundamental processes involved in soil N<sub>2</sub>O and CH<sub>4</sub> transformations are  
80 reasonably well understood, the ecology of the involved organisms in complex field  
81 ecosystems is far from being as clear (cf. Bodelier and Laanbroek 2004; Robertson 1989).  
82 Predicting N<sub>2</sub>O and CH<sub>4</sub> fluxes is difficult given the large number of factors and organisms  
83 involved, which interact in ways difficult to predict and change in space and time.  
84 Nevertheless, we argue that plant diversity has the potential to alter trace gas fluxes via a  
85 range of mechanisms that can reasonably be predicted and tested. First, more species-rich  
86 plant communities often lead to tighter N cycling, with less inorganic N accumulating (Ewel  
87 et al. 1991; Niklaus et al. 2001; Tilman et al. 1996). This could affect nitrification and  
88 denitrification and associated N<sub>2</sub>O emissions, and possibly also CH<sub>4</sub> dynamics. Second,  
89 increased productivity at higher plant diversity may also translate into an improved supply  
90 of organic substrates for soil microbial communities, which could increase heterotrophic  
91 activity, lead to higher oxygen consumption and increase the amount of soil volume with a  
92 redox potential sufficiently low for denitrification (Sexstone et al. 1985). Third, water use  
93 may increase with plant diversity, resulting in reduced soil moisture and increased soil  
94 diffusivity (Caldeira et al. 2001; Leimer et al. 2014a; Spehn et al. 2000), which may affect both  
95 N transformations and soil CH<sub>4</sub> uptake (Ball et al. 1997). Fourth, altered root densities and  
96 architectures may lead to changes in soil aeration, spatio-temporal organic C deposition or N  
97 use patterns. Fifth, atmospheric N<sub>2</sub> fixation by legumes will have particularly strong impacts

on N cycling, and has been identified as strong determinant of ecosystem responses in many plant diversity experiments (Oelmann et al. 2007; Spehn et al. 2002). With respect to CH<sub>4</sub>, the study by Niklaus et al. (2006) hints at the complexity of interactions at play, with plant diversity effects on CH<sub>4</sub> consumption changing from positive to negative when a soil disturbance treatment was applied in the field.

Here, we present the first study investigating the effects of plant species diversity and fertilization, together with their interaction, on fluxes of N<sub>2</sub>O and CH<sub>4</sub> under field conditions. We investigated the dynamics of these trace gases and the underlying mechanisms at several levels. First, we measured *in situ* flux rates of N<sub>2</sub>O and CH<sub>4</sub>; second, we assessed the enzymatic potential of key N transformations functionally linked to these fluxes; third, we quantified the abundance of nitrifiers and denitrifiers using quantitative PCR of selected functional and ribosomal genes. We further assessed soil environmental conditions (temperature, moisture) and concentrations of inorganic N species. We were interested whether higher plant species richness would lead to more complete N capture and therefore reduced nitrification and denitrification rates and associated N<sub>2</sub>O emissions. We further aimed at testing whether plant diversity effects operated via changes in soil moisture, and whether there was evidence that nitrification promotes soil CH<sub>4</sub> uptake by removal of potentially inhibiting NH<sub>4</sub><sup>+</sup>.

## Materials and Methods

### Study site and experimental design

We studied soil-atmosphere trace gas fluxes in a large grassland biodiversity experiment near Jena, Germany (50°55'N, 11°35'W, 130 m above sea level). In 2002, experimental grassland communities varying in diversity were established on an Eutric Fluvisol with a texture progressing from sandy loam to silty clay with distance from the adjacent Saale river. The present study focused on 78 plots (20 x 20 m) sown with 1, 2, 4, 8 or 16 herbaceous species (see Roscher et al. 2004 for a complete description of the experimental design). Plot community composition was determined by random selection of species from a 60-species pool, with the constraint that species richness and plant functional type richness were as orthogonal as possible. Plant species had previously been assigned to functional types (grasses, small non-legume herbs, tall non-legume herbs, and legumes) based on a cluster analysis combining a large number of morphological and functional traits (Roscher et al. 2004). Starting in 2005, factorial management treatments were established (Weigelt et al. 2009) in subplots (1.6 × 4 m) located along one side of the large plots. In the present study, we analyze data from the main plot ("control" subplots) and the subplot that was managed identically except that fertilizer pellets were added after each mowing ("fertilized" subplots), at a rate of 100kg N ha<sup>-1</sup> a<sup>-1</sup>, 43.6 kg P ha<sup>-1</sup> a<sup>-1</sup> and 83 kg K ha<sup>-1</sup> a<sup>-1</sup>. In total, the study thus presents a split plot design with 70 plots (unit of replication for plant species composition and thus plant species richness) and 2×70=140 subplots (unit of replication for fertilizer application).

### Soil-atmosphere trace gas fluxes

We measured soil-atmosphere fluxes of N<sub>2</sub>O and CH<sub>4</sub> on July 20 and October 17, 2007, and on March 11, April 29, and twice on June 8, 2008 (one day-time and one night-time measurement; Table 1), using static chambers that had been installed in all control and fertilized subplots that were part of this study. The static chambers were 32 cm in diameter and lowered 14 cm into the ground, leaving 11 cm extending above ground. During flux measurements, air-tight lids were fitted and headspace samples collected 5, 20 and 35 min after chamber closure. These samples were injected into pre-evacuated exetainers and analyzed for N<sub>2</sub>O and CH<sub>4</sub> concentrations in the laboratory (Agilent 6890 gas chromatograph equipped with a flame ionization and an electron capture detector, Agilent Technologies Inc., Santa Clara, CA, USA). Concentrations were determined based on calibration against four standard gases with four different concentrations of CH<sub>4</sub> and N<sub>2</sub>O (see Hartmann et al. 2011 for details of the experimental setup). Gas exchange rates were calculated by linear regression of headspace concentrations against time. Concentration changes were linear

with time, and regressions explained >95% of the variation in gas concentration ( $R^2 > 0.95$ ), unless flux rates were very low.

### **Soil sampling**

In October 2007 and again in June 2008, we collected eight 1.5 cm diameter  $\times$  15 cm depth soil cores per subplot within 50 cm of the respective static chamber used for  $N_2O$  and  $CH_4$  flux measurements. Samples were pooled by subplot and sieved (2 mm mesh). A subsample was stored at  $-18^\circ C$  for molecular analyses, while another subsample was stored at  $4^\circ C$  for potential nitrifying and denitrifying activity analysis.

### **Nitrifying and denitrifying enzyme activity**

Nitrifying (NEA) and denitrifying (DEA) enzyme activities were determined in laboratory assays, quantifying transformation rates of  $NH_4^+$  and  $NO_3^-$  under standardized conditions, with neither water availability nor substrate concentration limiting. The goal was to quantify potential activity rates, eliminating limitations by substrate availability or environmental conditions that may have existed in the field.

NEA was measured at soil pH using the method described in Patra et al. (2005). NEA was determined as the difference between soil nitrate concentration before and after a 7-hour incubation of 10 g of soil under conditions favoring nitrification ( $28^\circ C$ , addition of 0.2 mg  $(NH_4)_2SO_4$ -N  $g^{-1}$  dry soil, soil moisture equivalent to 70% water holding capacity).

DEA was assessed over a short time by making all the factors affecting denitrification rate non-limiting (Smith and Tiedje 1979; Tiedje et al. 1989). DEA was determined as the linear production rate of  $N_2O$ , measured by gas chromatography (Agilent P200, Santa Clara, CA) over an 8-hour incubation of 10 g soil under conditions favouring denitrification ( $28^\circ C$ ; 90:10  $He-C_2H_6$  atmosphere providing anaerobic conditions and inhibition of  $N_2O$ -reductase activity; addition of 1 mg glucose-C, 1 mg glutamic acid-C and 0.1 mg  $NO_3^-$ -N per gram dry soil; soil moisture content equivalent to 100% water holding capacity).

### **Nitrifier and denitrifier abundances**

Soil DNA was extracted with the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA). The abundance of beta-proteobacterial ammonia oxidizers, i.e. known ammonia oxidizing bacteria (AOB) in soil, was measured by quantitative PCR (qPCR) according to Le Roux et al. (2008), targeting 16S rRNA gene sequences that are specific for this group (Hermansson and Lindgren 2001).  $5.72 \cdot 10^4$  to  $5.72 \cdot 10^6$  copies of standard DNA (purified AOB 16S rRNA gene PCR product of ATCC19718 *Nitrosomonas europaea*) were used.

The abundance of *nxrA* gene copies from *Nitrobacter*-like nitrite oxidizing bacteria (NOB) was quantified according to Attard et al. (2010). Tenfold standard serial dilutions



ranging from  $10^7$  to  $10^8$  *nirA* copies of genomic DNA from *Nitrobacter hamburgensis* X14 (DSMZ 10229) were used.

Denitrifier abundance was estimated by qPCR targeting fragments of the *nirK* and *nirS* genes encoding the copper and cd1 nitrite reductases, respectively. Quantitative PCR assays were carried out from soil DNA with Lightcycler 480 (Roche Diagnostics, Meylan, France). For *nirK*, thermal cycling and standard curve were carried out according to Henry et al. (2004), except that the last cycle with an annealing temperature of 58 °C was repeated 45 times. *nirS* fragments were amplified as described by Baudoin et al. (2009), using tenfold serial dilutions of genomic DNA of *Pseudomonas aeruginosa* PA14 as standards. We checked melting curves and amplified *nirK* or *nirS* fragment length after running an agarose gel of randomly chosen final PCR products.

For all quantitative PCR assays, measurements were performed in triplicate. Melting curve analysis confirmed the specificity of the amplifications, and possible inhibitory effects of co-extracted humic compounds in soil extracts were checked by dilution series, but no inhibition was observed.

#### **Soil moisture and inorganic N concentrations**

Soil moisture was determined gravimetrically in all fresh samples collected for molecular analysis. Soil ammonium and nitrate concentrations were determined colorimetrically (SAN+, Skalar, Netherlands) after extraction of fresh soil (20 g dry mass equivalent) with 60 mL of 2 M KCl (30 minutes) and filtration.

#### **Data analysis**

All data were analyzed by ANOVA based on mixed-effects models reflecting the experimental design (ASReml 3.0, VSN International, UK). The models included the main design variables plant species richness (as log-linear contrast) and fertilizer application, and their interaction. The specific composition of plant species communities was included as a random term to ensure adequate testing of the species richness effect (note that this random term is equivalent to “plot” since specific plant community compositions are not replicated at the plot level). The Jena Experiment is located on a flood plain adjacent to the Saale river, and spatial, treatment-independent, gradients in soil conditions across the site affected the processes we investigated. We therefore fitted the Cartesian coordinates X and Y indicating the plot’s position, plus their 2<sup>nd</sup> order terms (X·X, Y·Y, X·Y) to account for non-linearity in spatial gradients and for interactions between the two cardinal directions (i.e. allowing for a response surface that may be curved in a direction that does not align with the main coordinate axes). This spatial model explained more variance than fitting the design’s standard blocks which were placed perpendicular to the river.

The different variables necessitated a range of transformations due to their specific distributions. In part, these distributions result from biological processes; however, they also are the result of specific measurement procedures such as the PCR reaction, which is an exponential process and results in a corresponding distribution of measurement errors. All gene abundances were therefore log-transformed. Soil  $\text{CH}_4$  uptake rates as well as nitrification and denitrification enzyme activities were analyzed untransformed. Finally, soil  $\text{N}_2\text{O}$  oxide emissions resembled a log-normal distribution at large values but included very small and negative values due to measurement error, precluding log-transformation (which further would have over-emphasized differences between very small fluxes). We found that taking the fourth square root (with sign reconstruction for negative values), and shifting the abscissa of the first and third quadrant so that the two legs of the function merged with unity slope, resulted in a near-perfect normal distribution of residuals. However, analytical results did not change fundamentally when other transformations were chosen.

After testing for significances of effects of species richness and fertilization, we used structural equation modelling to explore possible causal relationships between trace gas fluxes, soil environmental variables (water content,  $\text{NH}_4^+$  and  $\text{NO}_3^-$  concentrations), potential nitrifying and denitrifying activities, and gene abundances. The goal was to develop an ecologically sensible, parsimonious model for which the model-implied covariance between variables matched the observed covariance structure of the data. These covariance matrices were then compared using  $\chi^2$  tests, with significant results indicating a poor fit. Models were fitted by maximum likelihood using the lavaan software (<http://lavaan.ugent.be>). As with the mixed model analyses, data were first corrected for spatial gradients across the experimental site. Since not all data were present on all dates, these analyses were carried out on temporal averages of all available measurements, the underlying assumption being that effects and correlations were reasonably stable over time. Structural models were constructed under the premises that (1) processes upstream in terms of N transformation influence downstream processes (e.g. nitrification can influence denitrification), but not vice versa; (2) gene abundances can influence microbial community activities, but not vice versa; (3) trace gas fluxes can be influenced by all variables; and (4) the experimental treatments can directly influence all variables. In an educated stepwise process, we then searched for the most parsimonious model that explained the observed covariance structure adequately (insignificant  $\chi^2$  test; P was larger than 0.5 for all corresponding, indicating a very good fit). This was achieved by omitting paths with small, non-significant coefficients.

## Results

### Soil N<sub>2</sub>O emissions

Averaged over all sampling dates, soil N<sub>2</sub>O emissions were left unaffected by plant species richness but increased slightly with fertilizer application ( $P=0.003$ ). When analyzing sampling dates separately, a significant decrease of N<sub>2</sub>O emissions with diversity was found on April 21, 2008 ( $P=0.02$ ), and significant species richness by fertilizer application interactions were found on June 20, 2007 ( $P=0.01$ ) and March 11, 2008 ( $P=0.05$ ), with the same effect being marginally significant on April 21, 2008 ( $P=0.07$ ). These interactions resulted from the fact that the difference in N<sub>2</sub>O emissions between fertilized and control subplots was larger at high than at low plant species richness.

Emissions of N<sub>2</sub>O were strongly controlled by the presence of legumes. We therefore separately analyzed plots that were sown with and without legumes (Fig. 1). In the absence of legumes, species richness slightly reduced N<sub>2</sub>O emissions, while N application slightly increased emissions (both effects marginally significant at  $P=0.06$ ). In plots with legumes, N<sub>2</sub>O emissions were significantly correlated with the fraction of legume biomass recovered in the summer harvest ( $P=0.001$ ) and increased with fertilizer application ( $P=0.02$ ), but were unrelated to plant species richness.

### Soil CH<sub>4</sub> uptake

Averaged over all sampling dates, soil CH<sub>4</sub> uptake rates decreased with increasing plant species richness ( $P=0.02$ ), but remained unaffected by fertilizer application (Fig. 2) and by the abundance of legumes. This effect was remarkably stable over time, with a decrease in CH<sub>4</sub> uptake with species richness detected on October 18, 2007 ( $P=0.05$ ) and June 6, 2008 ( $P=0.002$ ), a marginally significant decrease found on June 20, 2007 ( $P=0.08$ ) and non-significant trends in the same direction on the other sampling dates.

### Nitrifying and denitrifying enzyme activities and functional gene abundances

Nitrifying enzyme activity (NEA) increased with plant species richness ( $P<0.05$ ), irrespective of fertilizer application (Table 2). In contrast, denitrifying enzyme activity (DEA) increased with fertilizer application ( $P<0.001$ ), irrespective of plant species richness (Table 2).

Plant species richness did not affect functional gene abundances, although *nxrA* showed a marginally significant ( $P=0.06$ ) and weak decrease with plant diversity. In contrast, the abundances of AOB, *Nitrobacter*-like NOB and *nirK*- and *nirS*-like denitrifiers increased with fertilizer application (Table 2).

## Structural equation models explaining N<sub>2</sub>O and CH<sub>4</sub> emissions

In the absence of sown legumes, N<sub>2</sub>O emissions were affected negatively by plant species richness, as indicated by a strong direct negative path coefficient (Fig. 3, top). Plant species richness increased soil moisture, resulting in increased potential nitrification (NEA), which in turn stimulated DEA. However, DEA was not significantly linked to N<sub>2</sub>O emissions. When plant communities contained legumes (Fig. 3, bottom), the percentage of legumes (measured as aboveground biomass fraction harvested in June) dominated the effects on N<sub>2</sub>O emissions. Since the fraction of legumes in the plant community was negatively correlated with species richness and fertilizer application, these treatments thus reduced emissions by this pathway. However, plant species richness stimulated N<sub>2</sub>O emissions via increases in soil moisture, which in turn increased NEA (and DEA, but as in the legume-free plots no significant link between DEA and N<sub>2</sub>O fluxes was detected). Soil ammonium or nitrate concentrations had no explanatory power in the analysis and were therefore dropped from all models.

While the net effect of plant species richness on soil CH<sub>4</sub> oxidation was negative, path analysis suggested that a positive indirect effect of richness via increased soil moisture and increased NEA also was at play. However, this positive effect was masked by a larger direct negative effect on CH<sub>4</sub> uptake (Fig. 4). Fertilizer application stimulated AOB abundance, which positively correlated with nitrification activity. Legume abundance also boosted NEA. Ultimately, NEA had a positive effect on CH<sub>4</sub> uptake. Soil ammonium concentration had no explanatory power in this analysis

## Structural equation models linking functional gene abundances with NEA and DEA

Path analysis did not reveal any significant links between potential nitrification and denitrification activities on one hand, and the abundances of AOB, *Nitrobacter*-like NOB or *nirK*-like denitrifiers on the other hand (Fig. 5). Due to limited amounts of extracted soil DNA available, *nirS* abundances could only be determined for both sampling dates in 83 plots, for one sampling date in another 63 plots, and not at all in 10 plots. Analysis of the subset for which both *nirS* and *nirK* abundances were available revealed a similar pattern, i.e. no link between *nirS* and enzyme activities (Online Resource 1).

At the level of enzyme activities, the effects of plant species richness were mediated by soil moisture, while the effects of fertilizer application were mediated by a reduced legume fraction in plant communities. At the level of microbial functional group abundances, N fertilization had a clear positive effect on the abundance of AOB, with cascading effects on the abundance of *Nitrobacter*-like NOB and *nirK*-like denitrifiers (Fig. 5). *nirS*-like denitrifier abundances were affected positively by N fertilization, both directly and indirectly via AOB and *Nitrobacter*-like NOB (Online Resource 1).

## Discussion

Plant species richness decreased soil  $\text{N}_2\text{O}$  emissions—at least in the absence of legumes—and decreased soil  $\text{CH}_4$  uptake. We also detected plant diversity effects on underlying soil microbial processes and the abundances of soil microbial groups related to these processes. However, structural equation modelling suggested that these different effects were linked in a complex way, with net effects on trace gas fluxes emerging from a combination of direct and indirect species richness effects; in this context, “direct” denotes effects not likely related to the dependent variables observed.

Structural equation modelling suggests that the effects on  $\text{N}_2\text{O}$  fluxes were at least in part mediated by positive effects of plant species numbers on soil moisture, which in turn led to accelerated inorganic N cycling, as evidenced by increased NEA and DEA. Positive path coefficients to  $\text{N}_2\text{O}$  fluxes (Fig. 3) suggest that this increase in N transformation capacity indeed stimulated  $\text{N}_2\text{O}$  emissions. At the same time, the structural equation models support a direct negative effect of plant species richness on  $\text{N}_2\text{O}$  emissions, which counteracted the positive effects of increased enzyme activities. A possible mechanism underlying this link may stem from disproportionate effects of species richness on soil inorganic N concentrations and the enzymatic potential (in our study: NEA and DEA) to transform this inorganic N. Many studies have shown more efficient soil inorganic N capture and thus lower soil nitrate concentrations in more species-rich plant communities (Ewel et al. 1991; Niklaus et al. 2001; Tilman et al. 1996). In the Jena experiment, Leimer et al. (2014b) found that soil nitrate concentrations were reduced at high diversity if no or only few legumes were present, which is compatible with the general trend found in other studies. In our analysis, however, soil inorganic N concentrations were unrelated to explanatory variables analyzed. One reason may be that our measurements were not frequent enough to estimate robust average concentrations of these very dynamic parameters. When legumes were present in experimental communities, their fractional contribution to plant community biomass was positively related to nitrification and soil  $\text{N}_2\text{O}$  emissions. In the structural equation models, the negative direct effect from species richness to  $\text{N}_2\text{O}$  emissions that we identified in the absence of legumes vanished (Fig. 3). This observation is compatible with the finding by Leimer et al. (2014b) that soil nitrate concentrations increased with diversity when a large fraction of species were legumes, indicating that a high legume diversity also led to high nitrate concentrations, and that this effect exceeded the higher inorganic N capture efficiency in more diverse plant communities that was otherwise found.

Net soil  $\text{CH}_4$  uptake decreased persistently with plant species numbers. The soil’s  $\text{CH}_4$  balance is determined by the difference between methanogenesis and  $\text{CH}_4$  oxidation, with the latter dominated by methanotrophic bacteria. We did not observe net  $\text{CH}_4$  emissions during our study, indicating that  $\text{CH}_4$  oxidation consistently outweighed methanogenesis; in

fact, we think that methanogenesis did not play an important role in the present system and that the observed patterns were thus solely caused by variation in methanotrophic activity. Soil  $\text{CH}_4$  oxidation is often limited by gas transport rates, which in turn depend on water filled pore space (Ball et al. 1997). Increased soil moisture at high plant species richness could therefore explain the observed pattern. However, structural equation modelling suggested indirect positive effects of moisture on  $\text{CH}_4$  oxidation via increased nitrification (Fig. 4). This mechanism is plausible, given that methanotrophy can be inhibited by high ammonium concentrations; under these conditions, all processes consuming soil ammonium will indirectly promote  $\text{CH}_4$  oxidation. Apart from nitrification, ammonium assimilation by plants and immobilization in microbial biomass would exert such an effect. Path coefficients further suggested a soil moisture-independent direct negative effect of richness on soil  $\text{CH}_4$  uptake. This effect persisted even if a (non-significant) link from soil moisture to  $\text{CH}_4$  uptake was added to the model, suggesting that this effect was unrelated to soil moisture. The potential mechanisms supporting this link remain elusive; however, it also should be noted that path models do not provide strict tests of causalities but rather indicate possible mechanistic links based on correlations. The ecology of soil methanotrophic bacteria consuming atmospheric  $\text{CH}_4$  is only poorly understood to date, in part due to the lack of success in isolating such organisms from soils (Dunfield 2007). However, it is known from experiments with intact soil cores and with available laboratory cultures that  $\text{CH}_4$  oxidation is negatively affected by many chemical compounds, including ethylene (Jäckel et al. 2004), some organic acids (Wieczorek et al. 2011), and terpenes (Amaral et al. 1998); one possibility is that the detrimental effect of these substances results from the lack of specificity of methane-monooxygenase, which leads to co-metabolic activity harmful to the bacteria, e.g. by suicide activation (Mahendra and Alvarez-Cohen 2006; Prior and Dalton 1985). In this light, one possible explanation for the soil-moisture independent reduction of  $\text{CH}_4$  uptake in plant species-rich communities could be an increased production of such compounds inhibiting  $\text{CH}_4$  oxidation.

It is surprising that soil inorganic N concentrations were not significantly related to any other variable measured, whereas species richness, fertilization, and legume presence had significant effects on these parameters. This could be due to the fact that soils are spatially heterogeneous, and so are the biogeochemical transformations we investigated. For example, denitrification often occurs in the micro-anaerobic parts of soil aggregates (Sexstone et al. 1985). On the other hand, recent micro-autoradiographic analyses indicated that  $\text{CH}_4$  assimilation preferentially takes place on aggregate surfaces to which  $\text{CH}_4$  can readily diffuse (Stiehl-Braun et al. 2011a; Stiehl-Braun et al. 2011b). Relating system-level processes such as land-atmosphere trace gas fluxes to bulk soil measures of putative drivers, e.g. to soil inorganic N concentrations or enzymatic activities, neglects the micro-scale character of the interactions involved. When processes respond non-linearly to substrate

availability, different effects will be found when a resource such as nitrate is homogeneously or heterogeneously distributed. Our measurements, as is typical for ecosystem-level field studies, integrate the small-scale heterogeneity and thus are unable to account for small-scale interactions in hot spots and at hot moments, which show episodic disproportionately high biogeochemical transformation rates (McClain et al. 2003). We consider it likely that the marked effect of legumes is related to such heterogeneity. For example, high local concentrations of nitrate can occur in the vicinity of legume nodules, possibly creating hot-spots of denitrification and N<sub>2</sub>O emissions. Efficient capture of inorganic N by neighboring plants will not draw down inorganic N concentrations at these locations, although it will reduce nitrate leaching losses. Such localized high concentrations will be difficult to detect in bulk soil measurements. This reasoning is in-line with a previous investigation in which we found nitrification to be only weakly related to ammonium and denitrification only weakly related to nitrate, whereas legume presence had comparably large effects (Le Roux et al. 2013). With respect to CH<sub>4</sub> fluxes, high bulk soil ammonium concentrations may not affect CH<sub>4</sub> oxidation if they occur in soil space that is distinct from the spatial niche of the active methanotrophs (Hartmann et al. 2011; Stiehl-Braun et al. 2011a).

The quantification of microbial guilds by functional gene abundance promised some insight into the community dynamics underlying N transformations. In our study, different experimental treatments were reflected differently in functional gene abundances and in enzyme activities, and no links between the two could be detected in structural equation models. Soil moisture-mediated effects of plant species could be detected in enzyme activities but not in the community size of the respective microbial guilds. On the other hand, fertilizer effects exerted a cascading effect via increased ammonia oxidizer communities to nitrite oxidizers and denitrifiers. Interestingly, no link from nitrite oxidizer gene abundances to *nirK* gene abundances was detected (Fig. 5), although the latter is downstream of the first in terms of N transformation processes. This relationship can be understood in the light of ammonium (and not nitrite) oxidation being the rate-limiting step of nitrification. Probably reflecting differences in ecology of denitrifier groups (Xie et al. 2014), *nirS* gene abundances were dependent on ammonia oxidizer 16S genes abundances indirectly via nitrite oxidizer genes. Overall, however, ammonia oxidizer community size appeared to control denitrifier community size, mostly directly but to some extent also indirectly via intermediate (in terms of N processing) nitrite oxidizer communities. Environmental controls rather than community size have also been detected in contexts other than species richness (e.g. fertilizer and drought effects: Hartmann et al. 2013; land use change: Attard et al. 2011), underlining that gene abundance data reflect biological activities only under specific conditions.

Our study revealed that many effects of plant species richness on microbial processes were mediated by effects on soil moisture. Reports on species richness effects on soil

moisture are equivocal (Caldeira et al. 2001; Rosenkranz et al. 2012; Spehn et al. 2000); Caldeira et al. (2001) reported positive effects of species richness on grassland top soil moisture but negative effects below 15 cm depth. In the Jena experiment, Rosenkranz et al. (2012) found similar positive effects on top soil moisture as we did, while Leimer et al. (2014a) detected no such effects. In general, patterns in soil moisture seem to have been caused by decreased evaporative losses under higher species richness due to soil surface shading, with concomitant increase in water capture from deeper soil layers due to better soil exploration by roots. If processes are soil moisture driven, the depth at which the respective microbial transformations take place therefore is critical. N<sub>2</sub>O production due to nitrification and denitrification often occur in the top soil; soil CH<sub>4</sub> oxidation generally also dominates in the top soil, whereas methanogenesis often occurs deeper in the soil (Conrad 1996).

The patterns that emerged from our study, in particular from structural equation modelling, were robust and coherent, integrating well into our current understanding of the fundamental ecological processes. With respect to the fluxes of N<sub>2</sub>O and CH<sub>4</sub> per se, one must note that the effects of species richness and fertilization were small compared to the total variation in our data, which commands a cautious interpretation (Low-Décarie et al. 2014). One reason for the large fraction of unexplained variance may be that the between-plant community variation is typically very high in experimental plant diversity experiments, in particular at low species richness. The observed diversity effects thus result from averaging over the large inter-community variation within plant species richness levels. In the Jena experiment, as in many field biodiversity studies, specific species combinations are not replicated in independent plots, so that the diversity-independent composition effect cannot be separated from unrelated (random) variation, with the exception of simple contrasts such as the presence or absence of specific plant functional types. When studying trace gas dynamics and the underlying microbial processes, one further is confronted with large spatial and temporal variation inherent to the processes. Despite this challenging variability, the present field study is the first to present clear and consistent responses of soil trace gas exchange to plant species richness and fertilization. Controlled experimental studies as the present one complement observational research carried out under more natural conditions but in which it is more difficult to isolate effects of plant diversity from potentially confounding factors (e.g. Keil et al. 2015; Sutton-Grier et al. 2011).



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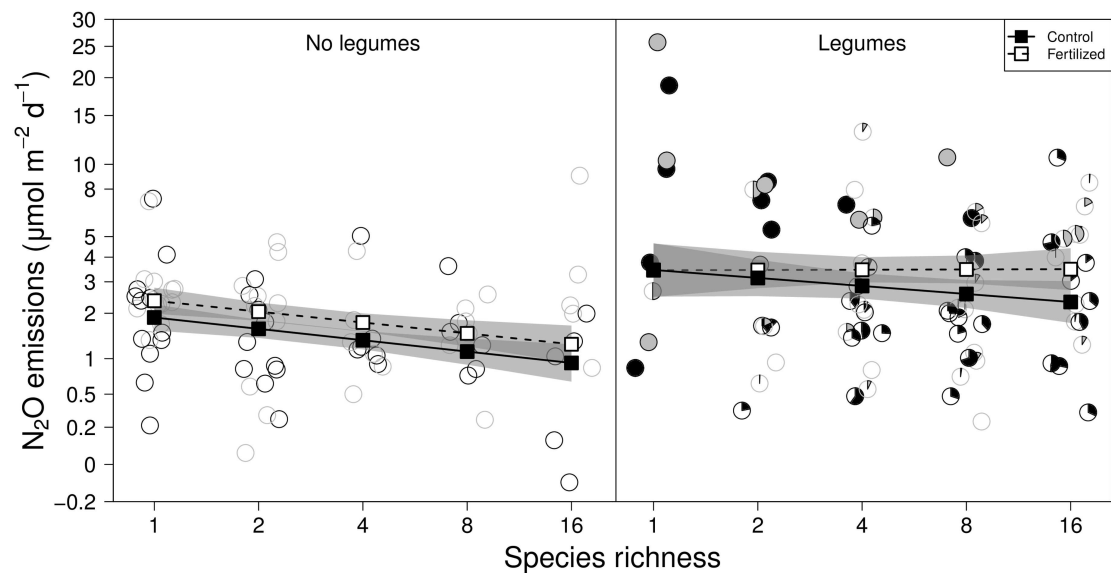
## Tables

Table 1. Environmental and meteorological conditions during the  $\text{N}_2\text{O}$  and  $\text{CH}_4$  flux measurements. Soil temperature and moisture refer to 8 cm depth, and are daily averages. Note that the data for June 8, 2008 refers to one day-time and one night-time measurements.

Date	Soil temperature	Soil moisture	Precipitation	
			past 24h	past week
	(°C)	( $\text{m}^3 \text{H}_2\text{O m}^{-3}$ )	(mm)	(mm)
20. June 2007	21.6	28.0	0.0	1.0
18. Oct 2007	10.1	30.5	0.0	0.0
11. March 2008	6.8	36.7	0.0	0.1
21. April 2008	8.9	35.9	0.0	0.1
6. June 2008	20.6	23.2	0.0	0.7

Table 2. Effects of plant species richness (logSR: log-linear effect) and fertilizer application (Fert) on nitrifying and denitrifying enzyme activities (NEA and DEA) and the abundances of ammonia oxidizing bacteria (AOB), *Nitrobacter*-like nitrite oxidizing bacteria (NOB), and *nirK*-and *nirS*-like denitrifiers (*nirK* and *nirS*). Effect sizes are given for model-predicted changes along the full plant species gradient of 1 to 16 species. Gene abundances are modelled on a log scale, and effect sizes thus are given from the back-transformed model prediction.

Parameter	Significance of effects			Effect sizes	
	logSR	Fert	logSR $\times$ Fert	logSR	Fert
NEA	*	n.s.	n.s.	+10%	
DEA	n.s.	***	n.s.		+7%
AOB	n.s.	***	n.s.		+51%
NOB	(*)	**	n.s.	(-27%)	+47%
<i>nirK</i>	n.s.	*	n.s.		+11%
<i>nirS</i>	n.s.	***	n.s.		+138%



639  
640 Fig. 1. Soil N<sub>2</sub>O emission rates for plots without (left) and with (right) legumes planted.  
641 Individual symbols show values for each plot (dark = control; light = fertilized plots), with  
642 pies inside symbols indicating the fraction of legumes in aboveground plant biomass  
643 harvested in June 2007 and 2008. Square symbols and lines indicate model predicted means  
644 for both fertilizer treatments; shaded areas are corresponding standard errors.  
645



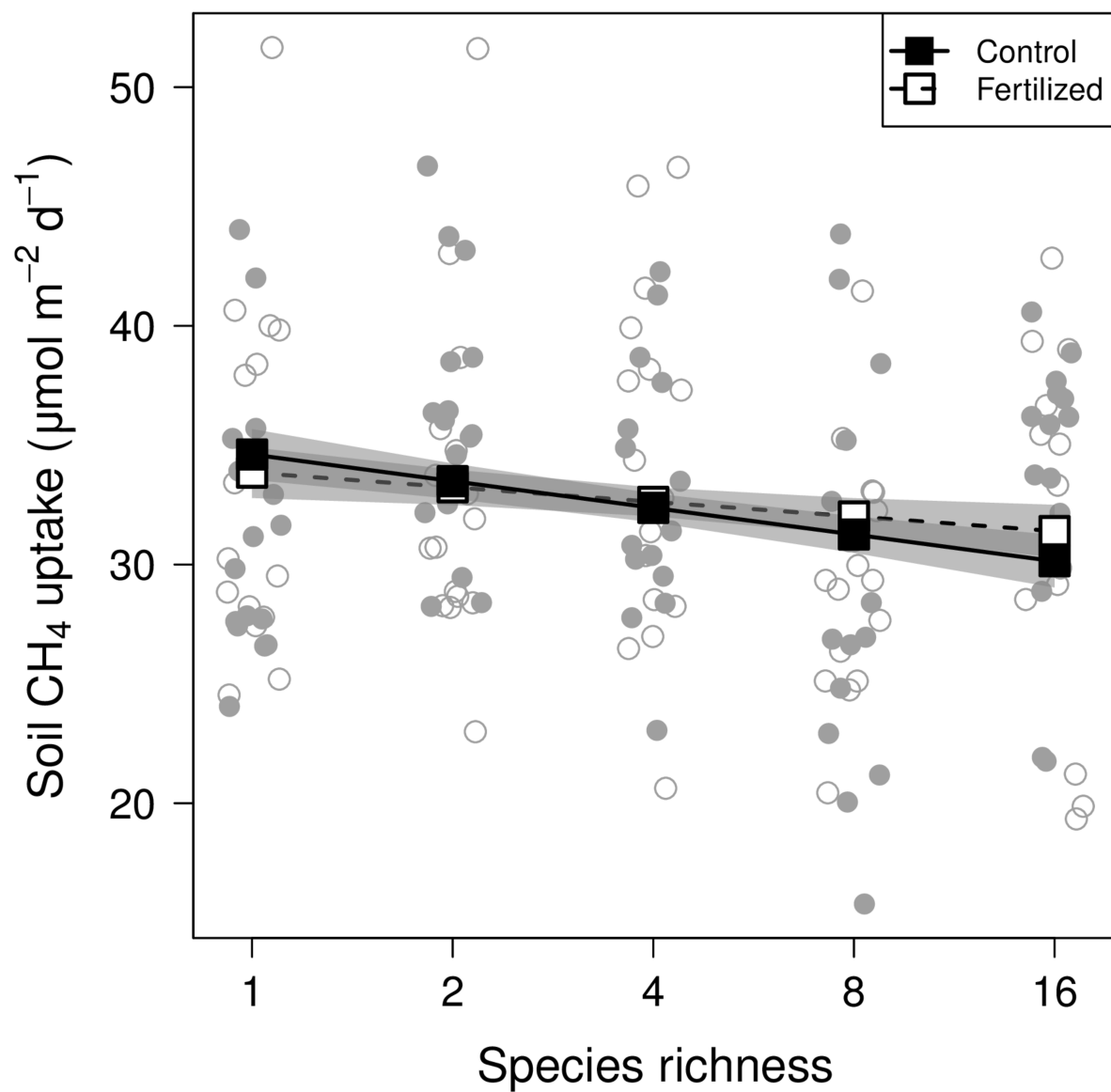


Fig. 2. Net soil CH<sub>4</sub> uptake rate as function of plant species richness and fertilizer application. Closed and open circles indicate values for each control and fertilized subplot, respectively. Squares and lines indicate model-predicted means for both fertilizer treatments; shaded areas are corresponding standard errors.

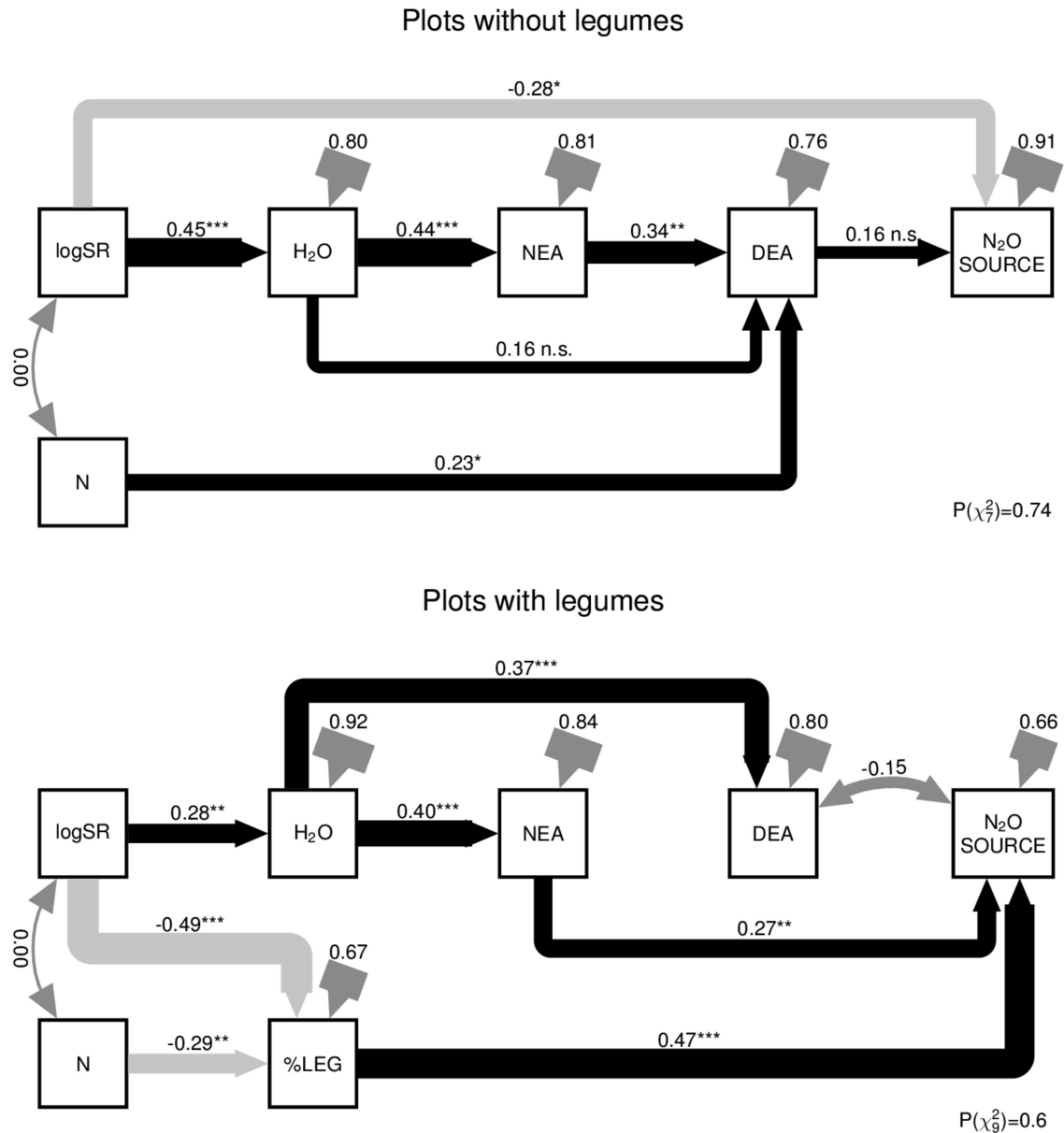
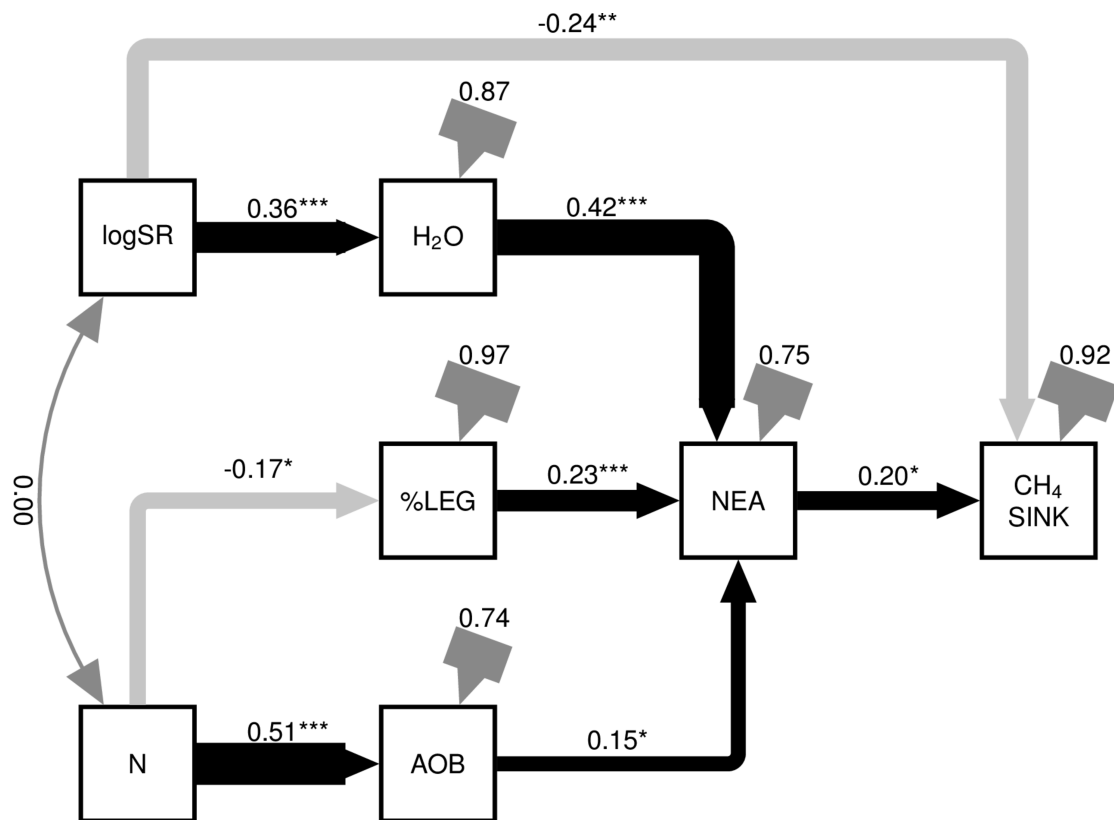


Fig. 3: Path diagram with standardized path coefficients showing effects of plant species richness (log-transformed, "logSR") and fertilizer ("N") on soil moisture ("H<sub>2</sub>O"), soil microbial nitrification and denitrification enzyme activities ("NEA" and "DEA"), and soil N<sub>2</sub>O emissions ("N<sub>2</sub>O SOURCE"), separately for plots without (top) and with (bottom) legumes. Arrows indicate standardized path coefficients (light: negative; dark: positive) together with their significance (\* P<0.05, \*\* P<0.01, \*\*\* P<0.001). "%LEG" indicates the mean fraction of legume aboveground biomass harvested in June 2007 and 2008.  $\chi^2$  statistics indicate no significant deviation of measured and model-implied covariance structure.



$P(\chi^2_{12})=0.9$

Fig. 4. Path diagram showing effects of plant species richness ("logSR") and fertilizer application ("N") on soil moisture ("H<sub>2</sub>O"), nitrification enzyme activity ("NEA"), the abundance of ammonia oxidizing bacteria ("AOB") and soil CH<sub>4</sub> uptake ("CH<sub>4</sub> SINK"). Arrows indicate standardized path coefficients (light: negative; dark: positive) together with their significance (\* P<0.05, \*\* P<0.01, \*\*\* P<0.001). "%LEG" indicates the mean fraction of legume aboveground biomass harvested in June 2007 and 2008.  $\chi^2$  statistics indicate no significant deviation of measured and model-implied covariance structure.

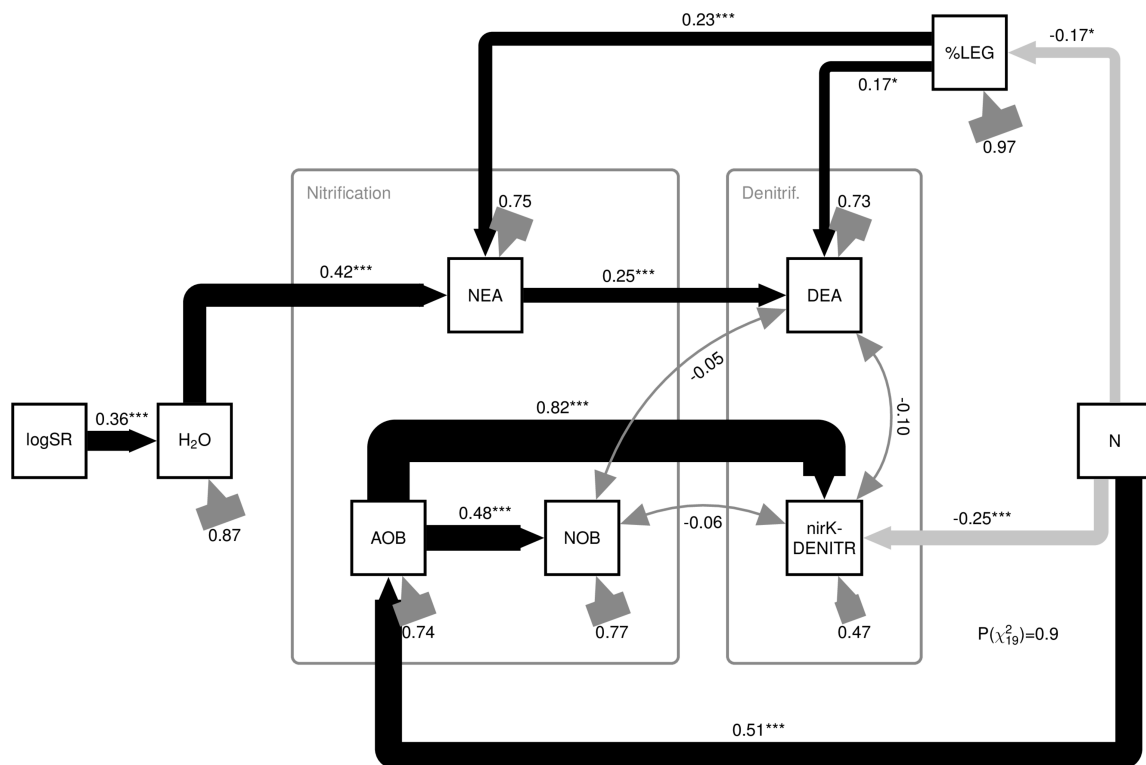


Fig. 5. Path diagram showing relationships between species richness ("logSR"), fertilizer application ("N"), nitrifying and denitrifying enzyme activities ("NEA" and "DEA"), and abundances of key microbial functional groups ("AOB": ammonia oxidizing bacteria, "NOB": *Nitrobacter*-like nitrite oxidisers, "nirK-DENITR": *nirK*-like denitrifiers). Arrows show standardized path coefficients (light: negative; dark: positive) together with their significance (\* P<0.05, \*\* P<0.01, \*\*\* P<0.001). The correlation between the exogenous variables "logSR" and "N" is zero and omitted for clarity.  $\chi^2$  statistics indicate no significant deviation of measured and model-implied covariance structure.