Global Change Biology

Global Change Biology (2013) **19**, 3688–3697, doi: 10.1111/gcb.12348

Nitrogen deposition weakens plant–microbe interactions in grassland ecosystems

CUNZHENG WEI*†, QIANG YU‡§, EDITH BAI‡, XIAOTAO LÜ‡, QI LI‡, JIANYANG XIA¶, PAUL KARDOL¶, WENJU LIANG‡, ZHENGWEN WANG‡ and XINGGUO HAN*‡

State Key Laboratory of Vegetation and Environmental Change, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China, †University of Chinese Academy of Sciences, Beijing 100049, China, ‡State Key Laboratory of Forest and Soil Ecology, Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang 110164, China, §Department of Biology, Graduate Degree Program in Ecology, Colorado State University, Fort Collins, Colorado 80523, USA, ¶Department of Botany and Microbiology, University of Oklahoma, Norman, OK, USA, ¶Department of Forest Ecology and Management, Swedish University of Agricultural Sciences, Umeå 901 83, Sweden

Abstract

Soil carbon (C) and nitrogen (N) stoichiometry is a main driver of ecosystem functioning. Global N enrichment has greatly changed soil C : N ratios, but how altered resource stoichiometry influences the complexity of direct and indirect interactions among plants, soils, and microbial communities has rarely been explored. Here, we investigated the responses of the plant-soil-microbe system to multi-level N additions and the role of dissolved organic carbon (DOC) and inorganic N stoichiometry in regulating microbial biomass in semiarid grassland in northern China. We documented a significant positive correlation between DOC and inorganic N across the N addition gradient, which contradicts the negative nonlinear correlation between nitrate accrual and DOC availability commonly observed in natural ecosystems. Using hierarchical structural equation modeling, we found that soil acidification resulting from N addition, rather than changes in the plant community, was most closely related to shifts in soil microbial community composition and decline of microbial respiration. These findings indicate a down-regulating effect of high N availability on plant–microbe interactions. That is, with the limiting factor for microbial biomass shifting from resource stoichiometry to soil acidity, N enrichment weakens the bottom-up control of soil microorganisms by plant-derived C sources. These results highlight the importance of integratively studying the plant-soil-microbe system in improving our understanding of ecosystem functioning under conditions of global N enrichment.

Keywords: aboveground-belowground linkages, compensatory effects, microbial carbon limitation, N saturation, resource stoichiometry, structural equation modeling

Received 27 April 2013; revised version received 15 July 2013 and accepted 20 July 2013

Introduction

The impact of nitrogen (N) deposition on ecosystem functioning is an important aspect of anthropogenic global change. With the increase in N deposition from fossil fuel combustion and application of artificial fertilizers, effects of N enrichment on ecosystem structure and functioning have been widely studied (e.g., Clark & Tilman, 2008; Galloway *et al*., 2008; Bobbink *et al*., 2010). Although low levels of N addition, particularly in N-limited ecosystems, generally increase biomass production (LeBauer & Treseder, 2008; Xia & Wan, 2008), alleviation of N limitation following N enrichment has been suggested as a main driver of the loss of global plant species diversity (Stevens *et al*., 2004; Clark & Tilman, 2008).

Correspondence: Dr. Xingguo Han, tel. +86 24 83970301, fax +86 24 83970300, e-mail: xghan@ibcas.ac.cn
CZW and QY contributed equally to this paper.

N enrichment can substantially alter the soil physicochemical environment such as lower soil pH (e.g., Guo *et al*., 2010; Phoenix *et al*., 2012). Soil acidification following N enrichment can lead to leaching of calcium and magnesium, and activate mineral-associated aluminum, which may negatively affect plant growth due to magnesium limitation (Lucas *et al*., 2011) or aluminum toxicity (Aber *et al*., 1998). In addition, N enrichment may alter soil microbial community structure and functioning with further consequences for ecosystem processes and productivity. Although discrepancies exist, N enrichment generally reduces the abundance of soil fungi, in particular arbuscular mycorrhizal fungal biomass, but increases or has little effect on bacterial biomass (Treseder, 2008; Gutknecht *et al*., 2012). A variety of mechanisms underlying N effects on soil microbial abundance and biomass have been proposed, including direct effects as well as indirect effects via shifts in soil properties (Treseder, 2008; Ramirez *et al*., 2012). For example, N-induced shifts in plant biomass or

composition can alter the belowground carbon (C) allocation, and hence, influence the amount of C available to microbes (Wallander, 1995). As such, N enrichment has the potential to alter the strength of plant–microbe interactions. Importantly, shifts in microbial abundance and community composition may in turn provide feedback to plant productivity and community composition (Kardol *et al*., 2007).

Changes in above- and belowground ecosystem components following N enrichment do not occur in isolation (Tylianakis *et al*., 2008). The plant-soil-microbe system is tightly linked by C and nutrient cycling, such as the dynamics of available soil C (dissolved organic carbon, DOC) and N (inorganic N). Soil resource stoichiometry influences plants, microbes, and their interactions and feedbacks. Recently, it has been shown that coupled DOC and nitrate cycling result in negative relationships between DOC and nitrate concentrations in soils, lakes, and oceans (Taylor & Townsend, 2010; Weyhenmeyer & Jeppesen, 2010; Heffernan & Fisher, 2012). On the other hand, some studies have shown neutral or positive effects of N enrichment on soil DOC (Aber *et al*., 1998; Liu & Greaver, 2010), indicating that N enrichment may alter soil resource stoichiometry (i.e., moderating the trade-off between DOC and inorganic N accumulation), and hence, may alleviate microbial C limitation. However, most of these previous studies were performed under non-saturated N conditions. While increase in human activity has already resulted in widespread occurrence of N saturation resulting from continuous exposure to high levels of N deposition, we know little about how the integrated plant-soil-microbe system responds when N concentrations reach the point of saturation.

Understanding effects of N enrichment on plant–microbe interactions thus requires a whole-system approach explicitly considering the coupling between C and N cycles. The Eurasian temperate steppe represents the world's largest grassland biome across the Eurasian continent, and has experienced intensifying anthropogenic disturbance, including increasing levels of N deposition. Plant productivity in temperate steppe is generally strongly N-limited. Hence, N enrichment has important consequences for the functioning of these ecosystems (Bai *et al*., 2010; Lan & Bai, 2012). We tested effects of N enrichment on plants, soil properties, and microbial communities in steppe grasslands, using a multi-level N addition experiment. Multi-level experiments are a powerful tool in identifying critical thresholds and the shape of response functions for ecosystem responses to N enrichment and saturation (Bradford *et al*., 2012; Kardol *et al*., 2012). We then used structural equation modeling (SEM) to gain a mechanistic understanding of how soil properties and altered plant community composition mediate effects of N enrichment on soil microbial community composition and respiration. Our aims are as follows: (i) to study effects of N enrichment on the trade-off between inorganic N and DOC accumulation; (ii) to explore effects of N enrichment on plant-soil-microbe systems and interactions among these components using a path-relation network and structural equation models; and (iii) to integratively study soil resource stoichiometry (i.e., DOC and inorganic N correlations) and plant–microbe interactions to gain a mechanistic understanding of N addition effects on above-belowground linkages.

Materials and methods

Site description and experimental design

The field experiment was conducted near the Inner Mongolia Grassland Ecosystem Research Station (IMGERS), which is located in the Xilin River, Inner Mongolia Autonomous Region of China (116°42′E, 43°38′N). The mean annual precipitation of the site is approximately 350 mm; most precipitation falls from June to August. The mean annual air temperature is −0.4 °C, with the mean monthly temperature ranging from −23.0 °C in January to 17.9 °C in July. The growing season is approximately 150 days. The soil is classified as dark chestnut (Calcic Chernozem According to ISSS Working Group RB, 1998). Vegetation is dominated by the perennial rhizomatous grass *Leymus chinensis* and the perennial bunchgrass *Stipa grandis*.

The N addition experiment was conducted in a grassland that has been fenced since 1999 to prevent grazing by large vertebrate herbivores. Forty-two 8 × 8 m plots were laid out in a randomized block design in 2006. Plots were separated by 1 m walkways. The experiment had seven treatments with six replicates each, including N addition at 0, 0.4, 0.8, 1.6, 2.8, and 4.0 mol N m^{-2} yr^{-1} (added as urea), and a control treatment. Hereafter, treatments will be denoted as: Control, N_0, $N_{0.4}$, $N_{0.8}$, $N_{1.6}$, $N_{2.8}$, and $N_{4.0}$. Each plot, except for Control, received 0.05 mol P m^{-2} (as KH_2PO_4) to ensure that N was the only limiting nutrient. The fertilizer was thoroughly mixed with sand and then applied to the plots in May every year since 2006 (see also Yu *et al*., 2010; Wei *et al*., 2012).

Plant biomass

The aboveground vegetation was sampled in mid-August by clipping all plants at the soil surface using a 0.5 × 1.0 m quadrat randomly placed in each plot with the restriction of no spatial overlap of quadrats among years and only data in 2009 were used in the data analyses. All living vascular plants were sorted to species, and all plant materials, including litter and standing dead biomass, were oven-dried at 65 °C for 48 h and weighted. We used the dry mass of all living plants per quadrat averaged over the six replicates for each treatment to estimate aboveground biomass production. We classified the plants into four functional groups (PFGs) based on life form

(as in Bai *et al.*, 2004), including perennial rhizome grasses (PR), perennial bunchgrasses (PB), perennial forbs (PF), and shrubs, and semishrubs (SS). After the aboveground biomass harvest, in each plot, two soil cores with a diameter of 6.5 cm at 0–15 cm depth were sampled to determine root biomass. Root samples were placed in a cooler and transported to the laboratory. In the laboratory, root samples were soaked in deionized water and cleaned from soil residuals over a 0.5 mm sieve.

Soil properties

Soil samples (3 cm diameter) were collected from each plot after the plant biomass harvest in 2009. Each sample comprised five soil cores at a depth of 0–15 cm. The samples were placed in individual plastic bags and then immediately stored at 4 °C. Soil moisture (SM) was determined as mass loss after drying the soil at 105 °C for 24 h. The soil pH was determined with a glass electrode in a 1 : 2.5 soil : water solution (w/v). Nitrate-N (NO_3^--N) and ammonium-N (NH_4^+-N) was extracted with 2 M KCl. NH_4^+ was measured with the salicylate method, while NO_3^- was measured using the cadmium reduction method on a FIAstar 5000 Analyzer (FIAstar 5000 Analyzer; Foss Tecator, Hillerød, Denmark). Dissolved organic carbon (DOC) was extracted by adding 50 ml of 0.5 M potassium sulfate (K_2SO_4) to subsamples of 12.5 g homogenized soil, and by agitating it on an orbital shaker at 120 rpm for 1 h. The filtrate was analyzed using a TOC analyzer (High TOC, Elementar).

Microbial abundance, respiration, and community composition

Soil microbial biomass carbon (MBC) was estimated by using a chloroform fumigation extraction method (Brookes *et al.*, 1985). Microbial respiration was measured as CO_2 evolution of fresh soil samples at 60% of water-holding capacity and incubated in sealed containers for 24 h at 25 °C. The CO_2 efflux from the soil was determined by NaOH absorption followed by titration of the residual OH^- with 0.1 M HCl (Page, 1982). Phospholipid fatty acid (PLFA) analysis was used to evaluate microbial community composition. PLFA was extracted from soil samples and then fractionated and quantified following protocols described by Bossio *et al.* (1998). The extracted fatty acid methyl esters were identified using a MIDI peak identification system (Microbial ID. Inc., Newark, DE, USA). Peak areas were converted to nmol lipid g dry soil^{-1} using internal standards (19:0 nonadecanoic methyl ester). The total nmol lipid g dry soil^{-1} (sum of all lipids with 20 or less carbons) was used as an index of microbial biomass (Frostegård & Bååth, 1996; Gutknecht *et al.*, 2012). Individual lipids were used as biomarkers to indicate broad groups within the microbial community: 16:1ω 5c for arbuscular mycorrhizal fungi (AMF; Balser *et al.*, 2005); 18:2ω 6,9c for general fungi excluding AMF (GF; Balser *et al.*, 2005). The ratio of fungal lipids (average 16:1ω c5, 18:1ω 9c, and 18:2ω 6,9c) to bacterial lipids (average 15:0 iso, 15:0 anteiso, 16:0 2OH, 16:0 iso, 16:1ω 7c, 16:0 10 methyl, 17:0 iso, 17:0 anteiso, 17:0 cyclo, 18:1ω 5c, and 18:1ω 7c) was used to indicate the fungal to bacterial ratio (Frostegård & Bååth, 1996).

Statistical analyses

Analysis of variance (ANOVA) followed by Duncan's post hoc tests was used to test the effects of N addition on total plant and functional group biomass, plant species richness, abiotic soil properties, and microbial parameters. Polynomial and exponential decay regressions were used to test relationships of inorganic N and MBC with DOC. Results from the best-fitting regression models are presented.

SEM is based on a simultaneous solution procedure, where the residual effects of predictors are estimated (partial regressions) once common causes from intercorrelations have been statistically controlled for (Grace, 2006). Prior to the SEM procedure we reduced the number of variables for plant and microbial community composition through Principal Component Analysis (PCA) (Veen *et al.*, 2010). Plant functional group biomass and microbial (bacterial, AMF, and fungal) PLFAs were used as raw data for the PCAs. The first principal components (PC1) were used in the subsequent SEM analysis to represent microbial community composition (PC1 explained 94.5% of the variation, Fig S1), and plant community composition (PC1 explained 87.5% of the variation, Fig S2). We started the SEM procedure with the specification of a conceptual model of hypothetical relationships, based on a priori and theoretical knowledge (Fig. S3). We assumed that N addition alters soil abiotic properties and plant community composition and species richness, which in turn affect microbial community composition and respiration. In the SEM analysis, we compared the model-implied variance–covariance matrix against the observed variance–covariance matrix. Data were fitted to the models using the maximum likelihood estimation method. Adequacy of the models was determined using χ^2 tests, Akaike Information Criteria (AIC), and root square mean errors of approximation (RMSEA) (Wei *et al.*, 2012; Jassey *et al.*, 2013). Adequate model fits are indicated by a nonsignificant χ^2 test ($P > 0.05$), low AIC, and low RMSEA (<0.05) (Grace, 2006). We improved the adequacy of the model by removing relationships between observed variables in the prior models based on Modification Indices (Table S3).

All univariate analyses were performed using SPSS 17.0 (SPSS, Chicago, IL, USA). PCA was performed using CANOCO Version 4.5 (Plant Research International, Wageningen, The Netherlands). SEM analyses were performed using AMOS 7.0 (Amos Development, Spring House, Pennsylvania, USA).

Results

Responses of components of the plant-soil-microbe system to N addition

N addition significantly reduced plant species richness from 7 at N_0 to 4 at N_4 plots ($F_{6,\ 35} = 4.95$, $P < 0.001$; Table 1). Shoot biomass decreased with increasing levels of N addition, although the trend was not statistically significant ($F_{6,\ 35} = 1.338$, $P = 0.267$; Table 1). Root

Table 1 Plant species richness and biomass, soil abiotic parameters, and soil microbial parameters for Control and N addition treatments

N addition rate (mol N m^{-2} yr^{-1})	Control 0	N_0 0	$N_{0.4}$ 0.4	$N_{0.8}$ 0.8	$N_{1.6}$ 1.6	$N_{2.8}$ 2.8	N_4 4.0
Plants							
Richness	6 ± 0.42^{abc}	7 ± 0.67^{a}	6 ± 0.40^{ab}	5 ± 0.17^{bcd}	5 ± 0.31^{bcd}	4 ± 0.49^{d}	4 ± 0.56^{cd}
Shoot biomass	81.07 ± 2.57	81.37 ± 5.00	74.83 ± 10.48	71.77 ± 4.52	72.20 ± 8.55	63.37 ± 8.09	56.37 ± 11.44
Root biomass	326.9 ± 30.0	498.4 ± 135.1	334.3 ± 85.9	323.5 ± 38.1	411.0 ± 44.1	459.9 ± 64.8	396.7 ± 101.3
Soils							
pH	7.48 ± 0.04^{a}	7.49 ± 0.11^{a}	6.96 ± 0.05^{b}	6.70 ± 0.05^{c}	6.19 ± 0.05^{d}	5.76 ± 0.09^{e}	5.76 ± 0.10^{e}
NO_3^--N	16.11 ± 2.86^{e}	22.44 ± 4.03^{e}	17.34 ± 1.74^{cd}	28.06 ± 4.74^{abc}	37.17 ± 6.49^{ab}	34.26 ± 3.80^{ab}	40.81 ± 7.51^{a}
NH_4^+-N	7.45 ± 0.69^{e}	7.72 ± 0.78^{e}	9.97 ± 1.40^{cd}	13.11 ± 1.44^{c}	18.51 ± 1.74^{b}	22.17 ± 2.25^{b}	34.95 ± 1.54^{a}
DOC	93.3 ± 5.07^{c}	92.8 ± 2.99^{c}	92.7 ± 3.98^{c}	91.5 ± 2.12^{c}	112.1 ± 6.03^{b}	121.1 ± 6.86^{ab}	132.5 ± 7.21^{a}
Microbial community							
Respiration	1.86 ± 0.26^{a}	1.93 ± 0.08^{a}	1.57 ± 0.11^{ab}	1.45 ± 0.14^{ab}	1.40 ± 0.18^{ab}	1.47 ± 0.17^{ab}	1.13 ± 0.14^{b}
MBC	281.8 ± 19.9^{a}	261.2 ± 36.8^{ab}	247.7 ± 34.5^{ab}	194.0 ± 27.4^{bc}	137.3 ± 19.6^{cd}	90.19 ± 17.4^{d}	91.3 ± 16.9^{d}
Total PLFA	43.02 ± 1.63^{a}	41.62 ± 2.81^{ab}	36.4 ± 1.27^{bc}	33.39 ± 1.71^{cd}	32.23 ± 1.51^{cde}	28.51 ± 2.38^{de}	26.90 ± 1.54^{d}

Plant biomass (g m^{-2}); NO_3^--N, NH_4^+-N concentration (mg kg^{-1} soil); DOC (mg kg^{-1} dry soil); Respiration (g CO_2-C g^{-1} h^{-1} soil), MBC (mg kg^{-1} dry soil); PLFA (nmol g^{-1} soil).
Data are mean ± SE (N = 6). Different letters denote significant differences among treatments ($P < 0.05$).

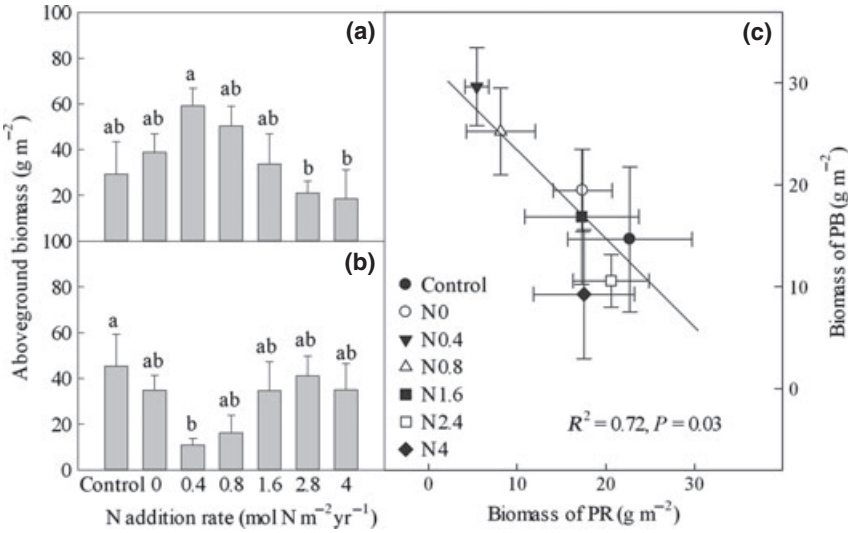

Fig. 1 Aboveground biomass responses of the two dominant plant functional groups to N addition (mean ± SE). (a) Perennial bunchgrasses (PB), (b) Perennial rhizome grasses (PR), (c) Relationship between the biomass of PR and PB across the N addition treatments (mean ± SE).

biomass did not significantly differ between control and N addition treatments ($F_{6,\ 35}$ = 1.572, P = 0.185; Table 1). Plant functional groups varied in their responses to N addition. Biomass of perennial bunch grasses (PB) increased with low levels of N addition, but decreased at high levels of N addition, with a threshold at 0.4 mol N m^{-2} yr^{-1} ($F_{6,35}$ = 2.314, P = 0.074; Fig. 1a). In contrast, biomass of PR showed the exact opposite response (Fig. 1b). Across all treatments, the biomass of PR was negatively correlated with biomass of PB (Fig. 1c). Perennial forbs, shrubs and semishrubs did not show significant responses to N addition (data not shown).

Soil inorganic N concentrations strongly increased across the N gradient (253% and 469% increase at the highest N addition rate for NO_3^--N and NH_4^+-N, respectively; both $P < 0.01$; Table 1). N addition decreased soil pH gradually from 7.5 in the Control to 5.8 in the N_4 treatment ($F_{6,\ 35}$ = 101.73, $P < 0.01$; Table 1). Dissolved organic carbon (DOC) significantly

increased with N addition, but only at levels of 1.6 mol N m^{-2} yr^{-1} and higher ($F_{6,\ 35}$ = 9.68, P < 0.001; Table 1).

Microbial respiration decreased with N addition, but differed significantly from the Control treatment only at the highest level of addition ($F_{6,\ 35}$ = 9.733, P < 0.01; Table 1). Microbial biomass carbon (MBC) decreased with N addition at levels of 0.8 mol N m^{-2} yr^{-1} and higher ($F_{6,\ 35}$ = 9.931, P < 0.001; Table 1). N addition decreased total, bacterial, and fungal (both saprophytic, and arbuscular mycorrhizal) PLFA, as well as the fungal to bacterial ratio (all P < 0.01; Table 1; Fig. 2).

Integrated response of the plant-soil-microbe system to N addition

The final SEM model adequately fitted the data describing interaction pathways among plant, soil, and microbial ecosystem components in response to N addition (χ^2_{13} = 13.473, P = 0.412; standardized path coefficients are given in Fig. 3). The final model explained 82% of the variation in pH, 86% of the variation in NH_4^+-N concentration, 32% of the variation in NO_3^--N concentration, 15% and 47% of the variation in plant community composition and richness, and 57% and 25% of the variation in microbial community composition and respiration, respectively (Fig. 3). Consistent with the ANOVA results (Table 1), N addition decreased soil pH and increased soil inorganic N concentrations (all P < 0.05; Fig. 3). Soil acidification (i.e., lower soil pH) decreased plant species richness (P < 0.001); interestingly, richness was unaffected by direct effects of increased N concentrations (Fig. 3). Soil NH_4^+-N concentration altered plant community composition, while pH altered microbial community composition (P < 0.001). Altered microbial community composition resulted in a decline of microbial respiration (Fig. 3). The relationships between the remaining exogenous and endogenous variables were not significant, but improved the model fit (Table S1).

Soil resource stoichiometry responses to N enrichment

Across treatments, concentrations of soil inorganic N (NO_3^--N and NH_4^+-N) were significantly and positively correlated with DOC (Fig. 4a). A significant negative relationship was found between MBC and DOC (Fig. 4b).

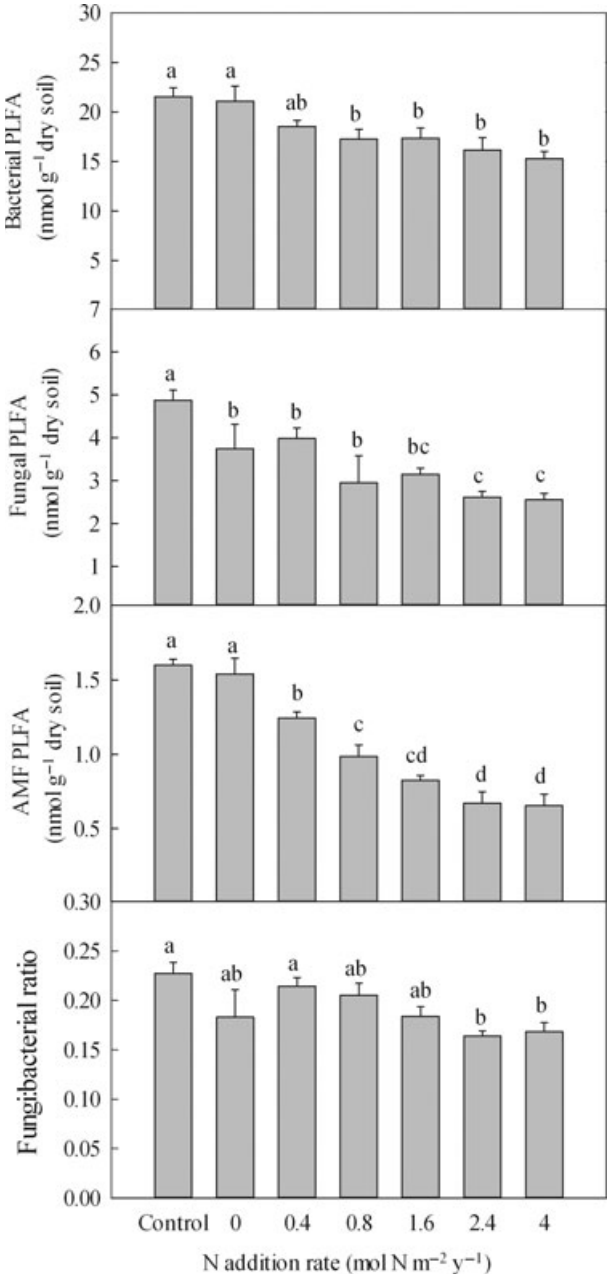

Fig. 2 Phospholipid fatty acid (PLFA) concentrations (mean ± SE) for Control and N additions treatments. Different letters above bars denote significant differences among treatments (P < 0.05).

Discussion

We studied the integrative effects of N addition and saturation on the plant-soil-microbe system, and elucidated the underlying mechanisms using structural equation modeling. Consistent with previous studies (e.g., Sinsabaugh *et al.*, 2004; Liu & Greaver, 2010), N addition increased both dissolved organic carbon (DOC) and inorganic N concentrations. However, we found that soil acidification following N addition, rather than inorganic N, altered soil microbial community composition, decreased soil microbial respiration. Negative correlations between DOC and MBC suggest

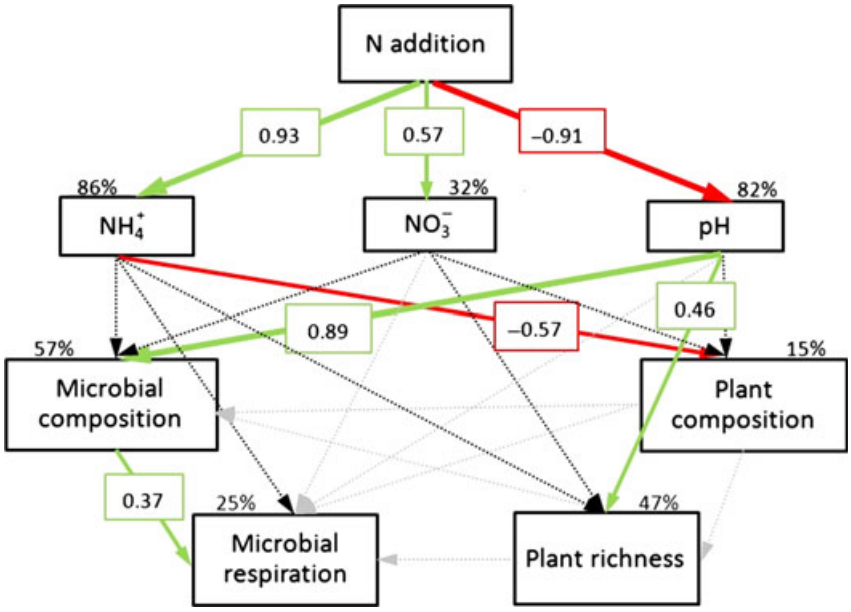

Fig. 3 Structural equation model of N addition effects on the plant-soil-microbe system. Relationships between N addition (exogenous variable) and soil pH, nitrate and ammonium concentration, microbial community composition and respiration, and aboveground plant biomass and richness (endogenous variables). The final model fit the data well: $\chi^2_{13} = 13.473$, $P = 0.412$, Akaike Information Criteria (AIC) = 77.453, RMSEA = 0.03. Numbers at arrows are standardized path coefficients (equivalent to correlation coefficients). Width of the arrows indicates the strength of the relationships. Green arrows indicate significant positive relationships and red arrows indicate significant negative relationships ($P < 0.05$). Black dashed arrows indicate nonsignificant relationships ($P > 0.05$). Gray dashed arrows indicate paths removed to improve model fits (see Methods). Percentages close to endogenous variables indicate the variance explained by the model (R^2).

that under conditions of N enrichment, soil microbes are no longer C-limited. The shift from microbial C limitation to inhibition by soil acidification decoupled C and N cycling, which eliminated the trade-off between accumulation of DOC and inorganic N, and as such weakened the bottom-up control of soil microbes by plant-derived C.

Resource stoichiometry feedbacks to plant–microbe interactions

In contrast to the widespread stoichiometric control of negative DOC-nitrate relationships (Taylor & Townsend, 2010), we found a positive correlation between DOC and inorganic N across our N addition gradient. Microbial-mediated coupling of DOC and nitrate cycling has been shown to contribute to trade-offs between C and N accumulation. For example, the onset of C limitation can drive rapid nitrate accrual, while at low organic carbon : nitrate ratios, denitrification may constrain the extent of nitrate accretion, as has been shown for a variety of ecosystems (Taylor & Townsend, 2010; Weyhenmeyer & Jeppesen, 2010). However, our results indicate that under conditions of N saturation, microbial responses in semiarid grasslands are no longer driven by DOC or inorganic N limitation. Illustratively, the negative correlation between DOC and MBC we found suggests that under N saturation microbial biomass was not limited by DOC. Hierarchical structural equation modeling showed that soil acidification following N enrichment, rather than DOC and inorganic N, affected microbial community composition and respiration. Soil acidification resulting from N addition is well documented for grasslands, forests, and other ecosystems (e.g., Högberg *et al.*, 2006; Guo *et al.*, 2010). We did not test the mechanistic effects of soil acidification on soil microbial communities, but available calcium and magnesium limitation (Lucas *et al.*, 2011) as well as aluminum toxicity (Aber *et al.*, 1998) could have played an important role.

Carbon limitation can shape soil heterotrophic microbial communities (Wardle *et al.*, 2004), and hence, plants and microbes may be strongly linked via plant-derived C compounds. Several studies in semiarid grassland of northern China, as well as in other ecosystems of the world, have documented significant effects of plant productivity, community composition, and richness, on microbial community structure and functioning (e.g., Chen *et al.*, 2009; Jiang *et al.*, 2011). However, we showed how N enrichment can shift microbial

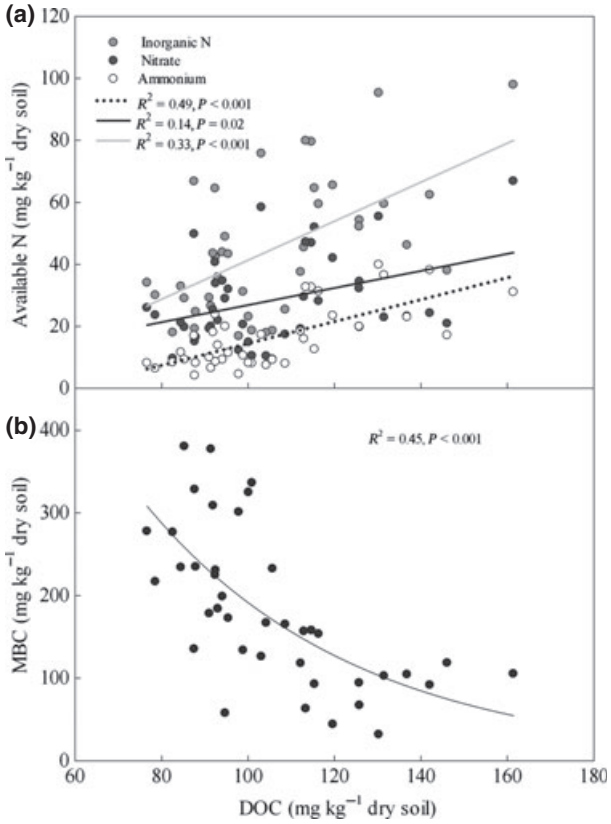

Fig. 4 Relationships between dissolved organic carbon (DOC) concentration and inorganic N concentrations (a), and between DOC concentration and microbial biomass C (MBC) (b). Regression lines in panel a: gray = total inorganic N, solid black = NO_3^--N, dotted black = NH_4^+-N.

limitation from plant-derived C to soil acidification, and as such decoupled the interactions between plants and soil microbial communities.

Responses of the plant-soil-microbe system to N addition

We found a 42% increase in DOC at the highest levels of N addition (4 mol N m^{-2} yr^{-1}). Previous studies also documented increased DOC concentrations under N enrichment, which could result from an increase in aboveground litter input and C content of the organic layer coupled with decreases in microbial decomposition, and might increase the pool of ecosystem C available for leaching (Liu & Greaver, 2010). However, we did not find significant responses of litter fall and soil organic matter accumulation across the N gradient in our experiment (data not shown), suggesting that an increase in plant litter input is not a likely explanation for the increase in DOC. Increased N availability may reduce the need for plants to invest C in nutrient-absorbing systems, and by that induce a shift in C allocation in favor of above-ground tissue production at the expense of root production (Treseder, 2008). As such, increased N availability may have resulted in decreased root biomass and by that root exudation (Pan *et al.* 2011; Currey *et al.*, 2011). We therefore speculate that a decrease in microbial decomposition and utilization of labile C (Marstorp *et al.*, 2000) resulted in an increase in DOC across the N addition gradient, by that resulting in a negative correlation between MBC and DOC (Fig. 4).

We found a strong compensatory shift in abundance between the two dominant plant functional groups (i.e., perennial rhizome grasses and perennial bunchgrasses) across the N addition gradient. A previous study in the same area found similar compensatory effects between these two plant functional groups in responses to fluctuations in precipitation, which resulted in increased temporal ecosystem stability (Bai *et al.*, 2004). Our results indicate that N enrichment can also cause compensatory effects between these two plant functional groups, which to some degree may help maintain ecosystem stability under conditions of N saturation. A critical threshold or tipping point (0.4 mol N m^{-2} yr^{-1}) was found where dominance changed from bunch grasses to rhizome grasses. Rhizome grasses are generally better adapted to high N levels (Bai *et al.*, 2010). Perennial bunch grasses contributed most to the decrease, probably as a consequence of acidification-induced aluminum toxicity or magnesium and calcium limitation (Delhaize & Ryan, 1995; Aber *et al.*, 1998; Roem *et al.*, 2002; Van Den Berg *et al.*, 2005). Therefore, when N deposition would reach the threshold values, as already has been observed in other areas of the world (Galloway *et al.*, 2008), shifts in plant functional group composition may significantly alter the structure of grassland ecosystems (Clark & Tilman, 2008; Bai *et al.*, 2010). Soil acidification following N addition also reduced plant species richness in this study, supporting previous findings that soil acidification is one of the main mechanisms for species loss under N enrichment (Roem *et al.*, 2002; Cleland & Harpole, 2010). However, recent studies in grassland an old-field ecosystems showed that plant species loss under N enrichment could be related to light (Hautier *et al.*, 2009) or competition for resources 'other than light' (Dickson & Foster, 2011). Together these findings indicate that the mechanisms underlying effects of N enrichment on plant species diversity differ among ecosystems; this context-dependency needs further study.

Nitrogen addition negatively affected several components of the microbial community, including bacterial and fungal biomass, as well as microbial respiration rate. Soil pH explained 57% of the variation in the microbial community composition. Similar to plants, in acid soils, microbes may become magnesium- or

calcium-limited or experience aluminum toxicity (Vitousek *et al*., 1997). The decreased fungal to bacterial ratio observed under N additions is somewhat surprising. Several previous studies have shown that soil acidification, as we observed in our N addition experiment favors fungi over bacteria (e.g., Högberg *et al*., 2007; Rousk *et al*., 2009); fungi are generally better adapted to low soil pH than bacteria (Alexander, 1980). However, the fungal to bacterial ratio does not always respond predictably to soil acidification. For example, Bååth & Anderson (2003) showed an increase in the ratio between fungal PLFA and bacterial PLFA with increasing soil pH. Also, when plants invest fewer resources in acquiring nutrients under N enrichment, they likely reduce their dependence on AMF for nutrient acquisition (Aerts & Chapin, 2000; McDowell *et al*., 2002). The drastic decline in AMF PLFA under N additions observed in our study may constitute a major driver that led to the decreases in fungal biomass under N additions. In contrast, under high levels of N addition, bacteria may be affected by soil DOC or shifts in soil quality rather than by plant root production (Aber *et al*., 1998). A recent meta-analysis by Treseder (2008) revealed that bacterial biomass generally does not respond to low levels of N addition. Moreover, other factors, such as shifts in litter quality, may also contribute to bacterial and fungal responses to N enrichment (e.g., Högberg *et al*., 2003). Together, these findings highlight the need for further exploration of differential microbial responses to N inputs in grasslands.

Ecosystem implications of nitrogen saturation

Biological responses to N deposition are nonlinear, and N saturation marks a critical threshold point at which ecosystem functions may shift in unpredicted ways. N saturation occurs when supplies of ammonium and nitrate are in excess of the total combined plant and microbial demand. Hence, N saturation alleviates N limitation of plant productivity and microbial activity, with important consequences for the biogeochemical dynamics of ecosystems (e.g., Aber *et al*., 1998). Multilevel experiments are necessary to reveal such nonlinear responses of ecosystem dynamics to global changes, and to identify potential threshold levels (Kardol *et al*., 2012). We showed that microbial biomass was significantly reduced beyond the rate of 0.8 mol N m^{-2} yr^{-1}, while the two dominant plant functional groups showed a divergent response to N addition at a critical threshold of 0.4 mol N m^{-2} yr^{-1}. Soil pH also changed significantly at N addition rates of 0.4 mol m^{-2} yr^{-1} or more. These findings suggest that the N saturation levels may be different for plants and soil microbes. Differential N saturation levels for different ecosystem components may contribute to the decoupling of plant-soil-microbe interactions under N enrichment.

Interestingly, the N saturation thresholds for plant functional groups, soil microbes, and soil pH for our grassland ecosystem were all lower than the 10.5 g N m^{-2} yr^{-1} saturation level for plant productivity found by Bai *et al.* (2010), but much higher than the documented critical thresholds for European and American forests (from 0.3–0.8 to 1.9 g N m^{-2} yr^{-1}) (Reynolds *et al*., 1998; Fenn *et al*., 2010). High N saturation levels as observed in this study could indicate that N limitation is more prominent in semiarid grassland than in temperate forests. However, care should be taken when comparing N saturation levels across ecosystems. N availability for plant and microbes may not be a linear function of N addition rate (Wei *et al*., 2012), and may vary with climate. In arid ecosystems, such as Inner Mongolian grasslands, with hot and dry conditions during the growing season, part of the added urea-N may be lost to the air. Volatilization loss from fertilizer application is favored by high soil temperature and moist conditions followed by rapid drying (Bouwman *et al*., 2002). The actual N saturation levels might therefore be somewhat lower than what we showed in this study.

In conclusion, the current N deposition rate in Inner Mongolia is still relatively low (0.4–6 g N m^{-2} yr^{-1}; Liu *et al*., 2011) compared to the saturation levels we found. However, a drastic increase in N deposition resulting from anthropogenic disturbances (e.g., N fertilization, coal mining) is predicted for the near future (He *et al*., 2007; Galloway *et al*., 2008; Liu *et al*., 2011). Given the relatively low threshold values for plants and soil microbes, effects of N saturation on the plant-soil-microbe system are therefore important to be considered in further developing terrestrial models for global change studies, especially given the important consequences for resource stoichiometry.

Acknowledgements

We are grateful to the Inner Mongolia Grassland Ecosystem Research Station (IMGERS) for providing the experimental sites and elemental analyses. This work was supported by the National Natural Science Foundation of China (30830026, 31270476), and the Key Program of the Chinese Academy of Sciences Project (KZCX2-YW-T06, KZCX2-YWBR-20). We thank Nianpeng He, Haiyang Zhang, and Jianjun Chen for field assistance, and two anonymous reviewers for constructive comments on an earlier version of this manuscript. The authors have declared no conflict of interest.

References

Aber J, Mcdowell W, Nadelhoffer K *et al.* (1998) Nitrogen saturation in temperate forest ecosystems. *BioScience*, **48**, 921–934.

Aerts R, Chapin F III (2000) The mineral nutrition of wild plants revisited: a re-evaluation of processes and patterns. *Advances in Ecological Research*, **30**, 1–67.

Alexander M (1980) Effects of acidity on microorganisms and microbial processes in soil. In: *Effects of Acid Precipitation of Terrestrial Ecosystems* (eds Hutchinson TC, Havas M), pp. 363–374. Plenum Publishing Corp., New York.

Bååth E, Anderson TH (2003) Comparison of soil fungal/bacterial ratios in a pH gradient using physiological and PLFA-based techniques. *Soil Biology & Biochemistry*, **35**, 955–963.

Bai YF, Han XG, Wu JG, Chen ZZ, Li HL (2004) Ecosystem stability and compensatory effects in the Inner Mongolia grassland. *Nature*, **431**, 181–184.

Bai YF, Wu JG, Clark CM *et al.* (2010) Tradeoffs and thresholds in the effects of nitrogen addition on biodiversity and ecosystem functioning: evidence from inner Mongolia Grasslands. *Global Change Biology*, **16**, 358–372.

Balser T, Treseder K, Ekenler M (2005) Using lipid analysis and hyphal length to quantify AM and saprotrophic fungal abundance along a soil chronosequence. *Soil Biology and Biochemistry*, **37**, 601–604.

Bobbink R, Hicks K, Galloway J *et al.* (2010) Global assessment of nitrogen deposition effects on terrestrial plant diversity: a synthesis. *Ecological. Applications*, **20**, 30–59.

Bossio D, Scow K, Gunapala N, Graham K (1998) Determinants of soil microbial communities: effects of agricultural management, season, and soil type on phospholipid fatty acid profiles. *Microbial Ecology*, **36**, 1–12.

Bouwman AF, Boumans LJM, Batjes NK (2002) Estimation of global NH_3 volatilization loss from synthetic fertilizers and animal manure applied to arable lands and grasslands. *Global Biogeochemical Cycles*, **16**, 8–14.

Bradford MA, Wood SA, Maestre FT, Reynolds JF, Warren RJ (2012) Contingency in ecosystem but not plant community response to multiple global change factors. *New Phytologist*, **196**, 462–471.

Brookes P, Landman A, Pruden G, Jenkinson D (1985) Chloroform fumigation and the release of soil nitrogen: a rapid direct extraction method to measure microbial biomass nitrogen in soil. *Soil Biology Biochemistry*, **17**, 837–842.

Chen SP, Lin GH, Huang JH, Jenerette GD (2009) Dependence of carbon sequestration on the differential responses of ecosystem photosynthesis and respiration to rain pulses in a semiarid steppe. *Global Change Biology*, **15**, 2450–2461.

Clark CM, Tilman D (2008) Loss of plant species after chronic low-level nitrogen deposition to prairie grasslands. *Nature*, **451**, 712–715.

Cleland EE, Harpole WS (2010) *Nitrogen Enrichment and Plant Communities. 5 Year in Ecology and Conservation Biology*. Blackwell Publishing, Oxford.

Currey PM, Johnson D, Dawson LA *et al.* (2011) Five years of simulated atmospheric nitrogen deposition have only subtle effects on the fate of newly synthesized carbon in Calluna vulgaris and Eriophorum vaginatum. *Soil Biology and Biochemistry*, **43**, 495–502.

Delhaize E, Ryan PR (1995) Aluminum toxicity and tolerance in plants. *Plant Physiology*, **107**, 315–321.

Dickson TL, Foster BL (2011) Fertilization decreases plant biodiversity even when light is not limiting. *Ecology Letters*, **14**, 380–388.

Fenn ME, Allen EB, Weiss SB *et al.* (2010) Nitrogen critical loads and management alternatives for N-impacted ecosystems in California. *Journal of Environmental Management*, **91**, 2404–2423.

Frostegård A, Bååth E (1996) The use of fatty acid analysis to estimate bacterial and fungal biomass in soil. *Biology and Fertility of Soils*, **22**, 59–65.

Galloway JN, Townsend AR, Erisman JW *et al.* (2008) Transformation of the nitrogen cycle: recent trends, questions, and potential solutions. *Science*, **320**, 889–892.

Grace JB (2006) *Structural Equation Modeling and Natural Systems*. Cambridge University Press, Cambridge, UK, New York.

Guo JH, Liu XJ, Zhang Y *et al.* (2010) Significant acidification in major Chinese croplands. *Science*, **327**, 1008–1010.

Gutknecht JL, Field CB, Balser TC (2012) Microbial communities and their responses to simulated global change fluctuate greatly over multiple years. *Global Change Biology*, **18**, 2256–2269.

Hautier Y, Niklaus PA, Hector A (2009) Competition for light causes plant biodiversity loss after eutrophication. *Science*, **324**, 636–638.

He CE, Liu XJ, Fangmeier A, Zhang FS (2007) Quantifying the total airborne nitrogen input into agroecosystems in the North China Plain. *Agriculture, Ecosystems & Environment*, **121**, 395–400.

Heffernan JB, Fisher SG (2012) Plant–microbe interactions and nitrogen dynamics during wetland establishment in a desert stream. *Biogeochemistry*, **107**, 379–391.

Högberg MN, Bååth E, Nordgren A, Arnebrant K, Högberg P (2003) Contrasting effects of nitrogen availability on plant carbon supply to mycorrhizal fungi and saprotrophs – a hypothesis based on field observations in boreal forest. *New Phytologist*, **160**, 225–238.

Högberg P, Fan H, Quist M, Binkley D, Tamm CO (2006) Tree growth and soil acidification in response to 30 years of experimental nitrogen loading on boreal forest. *Global Change Biology*, **12**, 489–499.

Högberg MN, Högberg P, Myrold DD (2007) Is microbial community composition in boreal forest soils determined by pH, C-to-N ratio, the trees, or all three? *Oecologia*, **150**, 590–601.

Jassey VEJ, Chiapusio G, Binet P *et al.* (2013) Above- and belowground linkages in Sphagnum peatland: climate warming affects plant-microbial interactions. *Global Change Biology*, **19**, 811–823.

Jiang LL, Han XG, Dong N, Wang YF, Kardol P (2011) Plant species effects on soil carbon and nitrogen dynamics in a temperate steppe of northern China. *Plant and Soil*, **346**, 331–347.

Kardol P, Cornips NJ, Van Kempen MML, Bakx-Schotman JMT, Van der Putten WH (2007) Microbe-mediated plant-soil feedback in pioneer stages of secondary succession causes long-lasting historical contingency effects in plant community composition. *Ecological Monographs*, **77**, 147–162.

Kardol P, De Long JR, Sundqvist MK (2012) Crossing the threshold: the power of multi-level experiments in identifying global change responses. *New Phytologist*, **196**, 323–326.

Lan ZC, Bai YF (2012) Testing mechanisms of N-enrichment induced species loss in a semiarid Inner Mongolia grassland: critical thresholds and implications for long-term ecosystem responses. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **367**, 3125–3134.

LeBauer DS, Treseder KK (2008) Nitrogen limitation of net primary productivity in terrestrial ecosystems is globally distributed. *Ecology*, **89**, 371–379.

Liu LL, Greaver TL (2010) A global perspective on belowground carbon dynamics under nitrogen enrichment. *Ecology Letters*, **13**, 819–828.

Liu X, Duan L, Mo J *et al.* (2011) Nitrogen deposition and its ecological impact in China: an overview. *Environmental Pollution*, **159**, 2251–2264.

Lucas RW, Klaminder J, Futter MN, Bishop KH, Egnell G, Hogberg P (2011) A meta-analysis of the effects of nitrogen addition on base cations: implications for plants, soils, and streams. *Forest Ecology and Management*, **262**, 95–104.

Marstorp H, Guan X, Gong P (2000) Relationship between dsDNA, chloroform labile C and ergosterol in soils of different organic matter contents and pH. *Soil Biology & Biochemistry*, **32**, 879–882.

McDowell RW, Brookes PC, Mahieu N, Poulton PR, Johnston AE, Sharpley AN (2002) The effect of soil acidity on potentially mobile phosphorus in a grassland soil. *Journal of Agricultural Science*, **139**, 27–36.

Page AL, Miller RH, Keeney DR (eds.) (1982) *Methods of Soil Analysis, Part 2: Chemical and Microbiological Properties*. American Society of Agronomy, Madison, WI, USA.

Pan QM, Bai YF, Wu JG, Han XG (2011) Hierarchical plant responses and diversity loss after nitrogen addition: testing three functionally-based hypotheses in the inner Mongolia Grassland. *PLoS One*, **6**, e20078.

Phoenix GK, Emmett BA, Britton AJ *et al.* (2012) Impacts of atmospheric nitrogen deposition: responses of multiple plant and soil parameters across contrasting ecosystems in long-term field experiments. *Global Change Biology*, **18**, 1197–1215.

Ramirez KS, Craine JM, Fierer N (2012) Consistent effects of nitrogen amendments on soil microbial communities and processes across biomes. *Global Change Biology*, **18**, 1918–1927.

Reynolds B, Wilson E, Emmett B (1998) Evaluating critical loads of nutrient nitrogen and acidity for terrestrial systems using ecosystem-scale experiments (NITREX). *Forest Ecology and Management*, **101**, 81–94.

Roem W, Klees H, Berendse F (2002) Effects of nutrient addition and acidification on plant species diversity and seed germination in heathland. *Journal of Applied Ecology*, **39**, 937–948.

Rousk J, Brookes PC, Bååth E (2009) Contrasting soil pH effects on fungal and bacterial growth suggest functional redundancy in carbon mineralization. *Appied and Environmental Microbiology*, **149**, 1589–1596.

Sinsabaugh RL, Zak DR, Gallo M, Lauber C, Amonette R (2004) Nitrogen deposition and dissolved organic carbon production in northern temperate forests. *Soil Biology Biochemistry*, **36**, 1509–1515.

Stevens CJ, Dise NB, Mountford JO, Gowing DJ (2004) Impact of nitrogen deposition on the species richness of grasslands. *Science*, **303**, 1876–1879.

Taylor PG, Townsend AR (2010) Stoichiometric control of organic carbon–nitrate relationships from soils to the sea. *Nature*, **464**, 1178–1181.

Treseder KK (2008) Nitrogen additions and microbial biomass: a meta-analysis of ecosystem studies. *Ecology Letters*, **11**, 1111–1120.

Tylianakis JM, Didham RK, Bascompte J, Wardle DA (2008) Global change and species interactions in terrestrial ecosystems. *Ecology Letters*, **11**, 1351–1363.

Van Den Berg LJ, Dorland E, Vergeer P, Hart MA, Bobbink R, Roelofs JG (2005) Decline of acid-sensitive plant species in heathland can be attributed to ammonium toxicity in combination with low pH. *New Phytologist*, **166**, 551–564.

Veen GF, Olff H, Duyts H, Van Der Putten WH (2010) Vertebrate herbivores influence soil nematodes by modifying plant communities. *Ecology*, **91**, 828–835.

Vitousek PM, Aber JD, Howarth RW *et al*. (1997) Human alteration of the global nitrogen cycle: sources and consequences. *Ecological Application*, **7**, 737–750.

Wallander H (1995) A new hypothesis to explain allocation of dry matter between mycorrhizal fungi and pine seedlings in relation to nuitrient supply. *Plant and Soil*, **169**, 243–248.

Wardle DA, Bardgett RD, Klironomos JN, Setälä H, Van Der Putten WH, Wall DH (2004) Ecological linkages between aboveground and belowground biota. *Science*, **304**, 1629–1633.

Wei CZ, Zheng HF, Li Q *et al*. (2012) Nitrogen addition regulates soil nematode community composition through ammonium suppression. *PLoS ONE*, **7**, e43384.

Weyhenmeyer GA, Jeppesen E (2010) Nitrogen deposition induced changes in DOC: NO3–N ratios determine the efficiency of nitrate removal from freshwaters. *Global Change Biology*, **16**, 2358–2365.

Xia JY, Wan SQ (2008) Global response patterns of terrestrial plant species to nitrogen addition. *New Phytologist*, **179**, 428–439.

Yu Q, Chen QS, Elser J *et al*. (2010) Linking stoichiometric homeostasis with ecosystem structure, functioning, and stability. *Ecology Letters*, **13**, 1390–1399.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Species-sample biplot of Principal Component Analysis (PCA) of plant community composition.
Figure S2. Species-sample biplot of Principal Component Analysis (PCA) of microbial phospholipid fatty acids (PLFA).
Figure S3. Illustration of all plausible interaction pathways in the studied plant-soil-microbe system.
Table S1. Results of structural equation modeling of N addition effects on the plant-soil-microbe system as illustrated in Fig. 3.