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Direct and indirect effects of nitrogen enrichment on soil organisms and carbon and nitrogen mineralization in a semi-arid grassland

Correction(s) for this article
Dima Chen
Wen Xing
Zhichun Lan
Muhammad Saleem
Yunqiqige Wu
Shuijin Hu
Yongfei Bai
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Abstract

1. Semi-arid grasslands on the Mongolian Plateau are expected to experience high inputs of anthropogenic reactive nitrogen in this century. It remains unclear, however, how soil organisms and nutrient cycling are directly affected by N enrichment (i.e., without mediation by plant input to soil) vs. indirectly affected via changes in plant-related inputs to soils resulting from N enrichment.
2. To test the direct and indirect effects of N enrichment on soil organisms (bacteria, fungi and nematodes) and their associated C and N mineralization, in 2010, we designated two subplots (with plants and without plants) in every plot of a six-level N-enrichment experiment established in 1999 in a semi-arid grassland.
3. In 2014, 4 years after subplots with and without plant were established, N enrichment had substantially altered the soil bacterial, fungal and nematode community structures due to declines in biomass or abundance whether plants had been removed or not. N enrichment also reduced the diversity of these groups (except for fungi) and the soil C mineralization

rate and induced a hump-shaped response of soil N mineralization. As expected, plant removal decreased the biomass or abundance of soil organisms and C and N mineralization rates due to declines in soil substrates or food resources.
4. Analyses of plant-removal-induced changes (ratios of without- to with-plant subplots) showed that micro-organisms and C and N mineralization rates were not enhanced as N enrichment increased but that nematodes were enhanced as N enrichment increased, indicating that the effects of plant removal on soil organisms and mineralization depended on trophic level and nutrient status.
5. Surprisingly, there was no statistical interaction between N enrichment and plant removal for most variables, indicating that plant-related inputs did not qualitatively change the effects of N enrichment on soil organisms or mineralization. Structural equation modelling confirmed that changes in soil communities and mineralization rates were more affected by the direct effects of N enrichment (via soil acidification and increased N availability) than by plant-related indirect effects. Our results provide insight into how future changes in N deposition and vegetation may modify below-ground communities and processes in grassland ecosystems.

A plain language summary is available for this article.

1 INTRODUCTION

Anthropogenic reactive nitrogen (N) inputs to the terrestrial biosphere have increased three- to fivefold over the past century (Galloway et al., 2008). The N deposition rate is also expected to increase substantially in the semi-arid grasslands on the Mongolian Plateau due to rapid industrialization and urbanization (Hilker, Natsagdorj, Waring, Lyapustin, & Wang, 2014; White, Murray, & Rohweder, 2000). The increases in N inputs greatly affect both above- and below-ground community composition and structure in grasslands (Bai et al., 2010; Chen, Lan, Hu, & Bai, 2015; Kardol & De Long, 2018; Leff et al., 2015) and other terrestrial ecosystems (Bobbink et al., 2010; LeBauer & Treseder, 2008). A growing number of short-term (<8 years) N-enrichment experiments have shown that semi-arid grasslands are extremely sensitive to increases in available N inputs and that the addition of N to semi-arid steppe ecosystems can greatly alter soil organisms (Li et al., 2013; Wei et al., 2012) and C and N cycling (Lu et al., 2011; Niu et al., 2016). Previous studies in Mongolian grasslands have reported that N enrichment causes large shifts in plant community composition and primary production by promoting faster-growing perennial rhizome grasses at the expense of slower-growing perennial bunchgrasses (Bai et al., 2010; Xia & Wan, 2008).

N enrichment can affect soil organisms and C and N cycling by directly altering soil N availability and soil pH (Chen et al., 2015; Niu et al., 2016) and by indirectly altering plant-related inputs to soils (Leff et al., 2015; Manning et al., 2006; Wardle, Gundale, Jäderlund, & Nilsson, 2013). Plant-related inputs are common limiting factors of soil organisms and affect a wide range of soil processes (e.g., nutrient availability, C and N mineralization and fluxes of greenhouse gases; Brant, Myrold, & Sulzman, 2006; Keith et al., 2009; Eisenhauer et al., 2010; Kardol & De Long, 2018). N-enrichment-induced increases in N availability are expected to increase plant growth and the production of root exudates and labile C, alleviating the C limitation for soil organisms (LeBauer & Treseder, 2008; Leff et al., 2015), which rely almost

entirely on plant-derived nutrients in most terrestrial ecosystems (Chen et al., 2016; Keith et al., 2009; Pollierer, Langel, Korner, Maraun, & Scheu, 2007). Previous studies have indicated that N-enrichment-induced changes in plant-derived inputs can have both negative and positive effects on soil C and N cycling (Vourlitis & Zorba, 2007). In addition, N enrichment can also directly alter soil food web and soil processes by increasing N availability and by reducing soil pH. Soil acidification can suppress soil organisms and soil C and N cycling by changing the concentrations of H^+ ions and soil base cations (e.g., Ca^{2+} and Mg^{2+}; Van Den Berg et al., 2005; Rousk et al., 2010). Research has also shown that increases in NH_4^+-N concentration can negatively affect soil organisms and C and N processes (Chen et al., 2015; Niu et al., 2016; Wei et al., 2012). Although the separate effects of N availability versus plant-related inputs on below-ground C dynamics are relatively well understood, we lack direct experimental evidence of the degree to which soil organisms and processes are affected by the direct effects of N enrichment versus the indirect, plant-mediated effects of N enrichment.

Several experiments have determined how N-enrichment treatments in temperate forests and tundra grasslands alter the effects of dominant plant species/functional groups on above- or below-ground properties (Manning et al., 2006; Wardle et al., 2013). Wardle et al. (2013), for example, experimentally manipulated the dominant plant species in a fertilization experiment in a subalpine tundra; they found that removal of dominant plant species greatly increased the biomass of the remaining plant species but had no effect on below-ground variables. Although previous reports have increased our understanding of the effects of N enrichment on soil organisms and C and N cycling, our understanding of how plant-related inputs mediate the effects of N enrichment is still limited. First, the results of these reports have been inconsistent; that is, researchers have found that N-enrichment-induced changes in plant inputs can have negative, positive or no effects on soil micro-organisms and soil C or N mineralization rates (Janssens et al., 2010; Lu et al., 2011; Treseder, 2008). Such inconsistent responses may partly result from the fact that these studies were mostly carried out with a limited range of N levels (e.g., control, moderate or high levels of N enrichment). Second, only a few studies have considered different trophic levels or functional groups of soil organisms in a single experiment (Eisenhauer et al., 2013; Scherber et al., 2010). The inconsistencies in results and the consideration of only a few N levels limit both our understanding of the responses of soil organisms to N enrichment and plant-related inputs and our ability to predict the effect of future anthropogenic stressors on ecosystem functioning and services (Bardgett & Wardle, 2010; Kardol & De Long, 2018; Mariotte et al., 2018).

In the present study, we investigated how changes in plant-related inputs resulting from N enrichment affect soil organisms and C and N mineralization. The study was part of an ongoing, long-term, and multiple-level N-enrichment experiment established in 1999 and located in a typical semi-arid steppe on the Mongolian Plateau. Previous studies from this experiment reported that N enrichment greatly affected above- and below-ground community structure (Bai et al., 2010; Chen et al., 2015). To assess the direct and indirect effects of N enrichment on soil organisms and C and N mineralization, we designated two subplots (one with plants and the other without plants) within each main plot of this long-term N-enrichment experiment in 2010. Here, we report on the effects of plant removal on soil organisms and processes in these plots in 2014, that is, 4 years after initiation of the plant removal. We tested the following hypotheses: (a) N enrichment will negatively affect soil organisms and C and N mineralization rates, and

responses to N enrichment will be weaker for higher trophic groups (nematodes) than for lower trophic groups (micro-organisms; Scherber et al., 2010; Eisenhauer, Cesarz, Koller, Worm, & Reich, 2012); (b) plant removal will negatively affect soil organisms and soil C and N mineralization rates, and these effects will be greater with high than with low levels of N enrichment, which would be consistent with the mass ratio hypothesis (Grime, 1998; Scherber et al., 2010). In other words, the changes in each variable caused by plant removal will be positively related to levels of N enrichment, because ANPP and soil biota abundance will increase as the level of N enrichment increases in subplots with plants (Chen et al., 2016); and (c) the interactive effects between N enrichment and plant removal on soil organisms and C and N mineralization rates will be strong because N enrichment has greatly altered plant community structure and productivity in the long-term N-enrichment experiment at the same site (Bai et al., 2010).

2 MATERIALS AND METHODS

2.1 Study site

This study was conducted at the Inner Mongolia Grassland Ecosystem Research Station (IMGERS, 43°38'N, 116°42'E) of the Chinese Academy of Sciences, which is located in the Xilin River Basin of Inner Mongolia, China, at approximately 1,200 m a.s.l. The semi-arid continental climate is characterized by a mean annual precipitation of 334 mm and a mean annual temperature of 0.9°C (1982–2009). The site has a dark chestnut soil (Calcic Chernozem according to ISSS Working Group RB, 1998), with a loamy-sand texture. Before the long-term N-enrichment experiment began (see next section), the plant community was dominated by *Leymus chinensis* (Trin.) Tzvel., a C_3 perennial rhizomatous grass that is widely distributed on the Eurasian steppe.

2.2 Long-term N-enrichment experiment and vegetation removal treatment

The establishment of the N-enrichment experiment was described by Bai et al. (2010) and is described briefly here. In 1999, a 120-m × 70-m area with uniform vegetation was designated within the permanent research plots of IMGERS. The area was divided into 162 5-m × 5-m plots with 1-m buffers. These plots were laid out in a randomized block design with 18 treatments (including six levels of N enrichment and three N application times) and nine replicate blocks. The six levels of N enrichment were 0, 1.75, 5.25, 10.5, 17.5 and 28.0 g N m^{-2} $year^{-1}$, and the N was applied as commercial NH_4NO_3 fertilizer to the soil surface. The three different N application times were all N applied at the early growing season (May 1–3), all N applied at the middle of the growing season (July 1–5), and 50% of N applied at each time period (Bai et al., 2010). The vegetation removal treatment and sampling in this study were conducted in a subset of the N-enrichment experiment that involved all six levels of N enrichment, one N application time (July 1–5) and five replicates (block 1 to 5), giving a total of 30 plots (Chen et al., 2015).

In April 2010, trenching combined with plant clipping was used to establish one without-plant subplot (0.5-m × 0.5-m) in each N-enrichment plot (5-m × 5-m). Each without-plant subplot was prepared by making vertical cuts in the soil along the subplot boundaries to 50 cm depth such

that all roots crossing the boundaries of the without-plant subplots were severed but not removed. Pieces of 0.3-cm-thick polyethylene board were then inserted into the vertical cuts to a depth of 50 cm in order to prevent roots from growing into the without-plant subplots. Plants in the without-plant subplots were killed by weekly clipping of all the above-ground parts of plants in the subplots at the soil surface, and all of the clipped above-ground parts of plants were moved out of the subplots. One with-plant subplot (the entire plot minus the without-plant subplot) was also designated in each N-enrichment plot. In late August 2012 (about 2 years after trenching + plant clipping), single soil cores (6.5 cm diameter, 0–30 cm depth) were collected from each without-plant subplot to determine the occurrence of dead roots. We found that living root biomass was 86% lower in without-plant subplots than in with-plant subplots.

2.3 Plant and soil sampling and analysis

In late August 2014, above-ground vegetation was sampled in a 0.5-m × 0.5-m quadrat in each with-plant subplot. Living vascular plants were clipped (by species) at the soil surface, oven-dried at 65°C for 48 hr and weighed as above-ground net primary productivity (ANPP). Also, root biomass was sampled in each with-plant subplot by randomly taking three 6.5-cm-diameter soil cores from 0 to 30 cm depth. Roots were cleaned by placing them under running water over a 1-mm screen, and the cleaned roots were oven-dried at 65°C and weighed as root biomass. Four soil cores (2 cm diameter, 0–15 cm depth) were randomly collected from each with- and without-plant subplot. The four soil cores were combined to form one composite soil sample per subplot. After the soil was gently mixed and roots were removed, the moist soil was passed through a 2-mm sieve and separated into two parts. One part was maintained fresh for determination of soil moisture, NH_4^+-N, NO_3^--N, micro-organisms, nematodes and soil C and N mineralization rates. The second part was air-dried for determination of soil pH, soil organic C (SOC), total soil N (TSN) and total soil phosphorus (TSP) as suggested by Sparks et al. (2009).

2.4 Soil C and N mineralization rates

Soil C and N mineralization rates were determined using the aerobic incubation procedure described by Evans, Rimer, Sperry, and Belnap (2001). A 20-g subsample of field-moist soil per sample was incubated in a plastic cap in the dark at 25°C for 21 days. The CO_2 released from the soil was determined after 1, 2, 3, 7, 10, 15 and 21 days of incubation. The soil C mineralization rate for each with- or without-plant subplot was calculated as the average CO_2 released during the 21-day incubation period. The soil N mineralization rate for each with- or without-plant subplot (the 20 g mentioned above) was calculated as the change in total inorganic N content from the start until the end of the incubation.

2.5 Biomass of soil microbial community

The microbial community in soil samples was assessed by analysis of phospholipid fatty acids (PLFAs; Bossio, Scow, Gunapala, & Graham, 1998). The abundance of each individual fatty acid (FA) in a given sample was expressed as FA nmol g^{-1} dry soil against an internal standard (methyl ester C19:0). FAs specific to bacteria (i14:0, a15:0, i15:0, i16:0, a17:0, i17:0, 16:1ω7c, 17:1ω8, 18:1ω9, 18:1ω7c, cy17:0 and cy19:0) and fungi (18:2ω6,9) were used to determine the

abundances of these microbial groups and to calculate the fungi:bacteria ratio (F:B ratio; Frostegård, Tunlid, & Bååth, 2011).

2.6 Diversity and composition of soil bacteria and fungi

Total genomic DNA from 0.5 g of soil was extracted using a FastDNA spin kit from MP Biomedicals (Santa Ana, CA) according to the manufacturer's instructions. The final DNA concentration and purification were determined with a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington), and DNA quality was checked by 1% agarose gel electrophoresis. Bacterial 16S rRNA genes were amplified with PCR primers 338F (5'- ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Fungal internal transcribed spacer (ITS) rRNA genes were amplified with PCR primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3'; Fierer et al., 2012; Leff et al., 2015). The resulting PCR products were extracted from a 2% agarose gel and further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA) and quantified with a QuantiFluor™-ST microfluorometer (Promega). Purified amplicons were pooled in equimolar concentrations and paired-end sequenced for high-throughput 16S rRNA or ITS rRNA gene sequencing on an Illumina MiSeq platform (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). Operational taxonomic units (OTUs) were clustered with 97% similarity cut-off using UPARSE (version 7.1 https://drive5.com/uparse/), and chimeric sequences were identified and removed using UCHIME. Silva and Unite databases were used as references for bacteria and fungi, respectively (Fierer et al., 2012; Leff et al., 2015). Principal component analysis (PCA), based on the relative abundance of each bacterial phylum or fungal class, was separately conducted for with- and without-plant treatments, and the PC1 scores were used as indicators of bacterial or fungal community structure (Supporting Information Table S1).

2.7 Soil nematode communities

Soil nematodes were extracted for 48 hr from 50 g of moist soil per sample by the Baermann funnel method (Barker, 1985). Based on feeding habits and life-history characteristics, all soil nematodes identified to genus level were assigned to four trophic groups (Yeates, Bongers, Goede, Freckman, & Georgieva, 1993): plant-feeders, bacterial-feeders, fungal-feeders and omnivores + carnivores. Because carnivorous nematodes were infrequently found, we included carnivorous nematodes in an omnivorous + carnivorous trophic group. The number of genera was used as an indicator of nematode taxon richness (Bongers, 1990). Like bacterial or fungal community structure, the relative abundance of each of the four trophic groups was assessed using PCA; the PC1 scores of the four trophic groups were used as indicators of nematode community structure (Supporting Information Table S1).

2.8 Statistical analyses

The effects of plant removal and N enrichment on each response variable (soil properties, micro-organisms, nematodes and C and N mineralization rates) were examined using split-plot ANOVA. Plant removal, N enrichment and their interactions were assigned as fixed factors, and

N-enrichment level was nested within block as an error term. The split-plot ANOVAs were performed using the "aov" function in the base package of r. The code used in r was "*aov(Variable~N_Level*Plant_Removal+Error(Block/N_Level)),*" where Variable was the response variable, N_Level was a factor with six levels (Level1 to Level6 for 0, 1.75, 5.25, 10.50, 17.5 and 28.0 g of N m^{-2} $year^{-1}$, respectively), Plant_Removal was binary (0 = with plant, 1 = without plant), and Block was an integer (1–5 for spatial blocks). A lack of statistical interaction between N enrichment and plant removal would indicate that N-enrichment effects on soil properties were dependent on plants and that the N enrichment directly regulated soil properties. The presence of a statistical interaction would indicate that N-enrichment effects on soil properties were independent of plants and that the N enrichment directly regulated soil properties. The data for response variables were transformed by natural logarithms before the analysis to improve normality. Three additional statistical analyses were performed. First, one-way ANOVAs with Duncan's multiple-range tests were performed across all response variables to compare the means among N-enrichment levels for each plant removal treatment or between plant removal treatments for each N-enrichment level. Second, changes in each response variable in response to plant removal along N-enrichment levels were examined using linear or quartic regression with N-enrichment level as a continuous predictor. The Akaike information criterion (AIC) was used to select the most appropriate model. The changes in each variable at each enrichment level caused by plant removal were determined as follows: ln $((1 + \text{Variable}_{\text{without plant}})/(1 + \text{Variable}_{\text{with plant}}))$, except for community structure data (5 + Variable) to avoid negative values. Third, structural equation modelling (SEM) was performed separately for with- and without-plant subplots to analyse hypothetical pathways that may explain how N enrichment affected soil organisms and C and N mineralization rates (Supporting Information Figure S1). All SEM analyses were conducted using the software amos 21.0 (IBM SPSS Inc, Chicago, IL). Other statistical analyses were performed using r version 3.3.2 (R Development Core Team, 2016).

The results of the ANOVA served as the hypothetical base for the initial SEM model. In the initial model, we only used those variables that were significantly affected by N enrichment or plant removal in the ANOVAs (Eisenhauer et al., 2012). To facilitate our SEM analyses and interpretations (Supporting Information Figure S1), we classified some dependent response variables into the following four groups based on prior knowledge: (a) soil N availability (soil NH_4^+-N and NO_3^--N); (b) soil nutrients (SOC, TSN, and TSP); (c) micro-organisms (total biomass, F:B ratio, bacterial and fungal OTU richness, and bacterial and fungal community structure); and (d) soil nematodes (total nematode abundance, nematode taxon richness and nematode community structure). We reduced the number of variables through PCA for each group before SEM analyses were performed (Chen, Lan, et al., 2013a; Veen, Olff, Duyts, & Putten, W.H.v.d., 2010). The first principal component (PC1) explained 41%–90% of the total variance in each group for with- and without-plant subplots (Supporting Information Table S2). The SEMs were implemented using the maximum-likelihood estimation method and were fitted with the χ^2 test. Because of the small number of samples (<5 for each variable) and the substantial complexity of the models evaluated, each pathway in the final model estimated by the maximum-likelihood method was also estimated by the Bayesian method for confirmatory purposes using 95% credibility intervals (Riginos & Grace, 2008). We deleted one pathway from the models with-plant subplots because the Bayesian estimation showed that the pathway overlapped zero with 95% credible intervals.

3 RESULTS

3.1 Responses of soil properties and C and N mineralization to N enrichment and plant-related inputs

Split-plot ANOVA showed that N enrichment increased soil NO_3^--N, NH_4^+-N and TSN but decreased soil pH, soil moisture and soil C mineralization rates in with- and without-plant subplots (Figure 1 and Supporting Information Figure S2). N enrichment did not affect the SOC or C:N ratio in with-plant or without-plant subplots (Figure 1 and Supporting Information Figure S2). N enrichment increased ANPP in with-plant subplots (Supporting Information Figure S2). Soil N mineralization rates were highest at the moderate levels of N enrichment (10.50 and 17.5 g of N m^{-2} $year^{-1}$) in both kinds of subplots (Figure 1 and Supporting Information Table S3). Plant removal increased the soil pH and NH_4^+-N and decreased the SOC, TSN and C and N mineralization rates but did not alter the soil NO_3^--N, C:N ratio or soil moisture (Figure 1 and Supporting Information Figure S2). Only soil NO_3^--N, NH_4^+-N, soil moisture and soil C mineralization rate were affected by the interaction between plant removal and N enrichment (Figure 1 and Supporting Information Figure S2). In addition, plant-removal-induced changes in soil NO_3^--N shifted from negative to positive as the N-enrichment level increased (Figure 5a and Supporting Information Figure S3). The plant-removal-induced positive changes in soil NH_4^+-N and soil pH and negative changes in soil C mineralization rates were lowest at the moderate N-enrichment levels (Figure 5a and Supporting Information Figure S3). The plant-removal-induced negative changes in TSN and positive changes in C:N ratio were amplified as the N-enrichment level increased (Figure 5a and Supporting Information Figure S3).

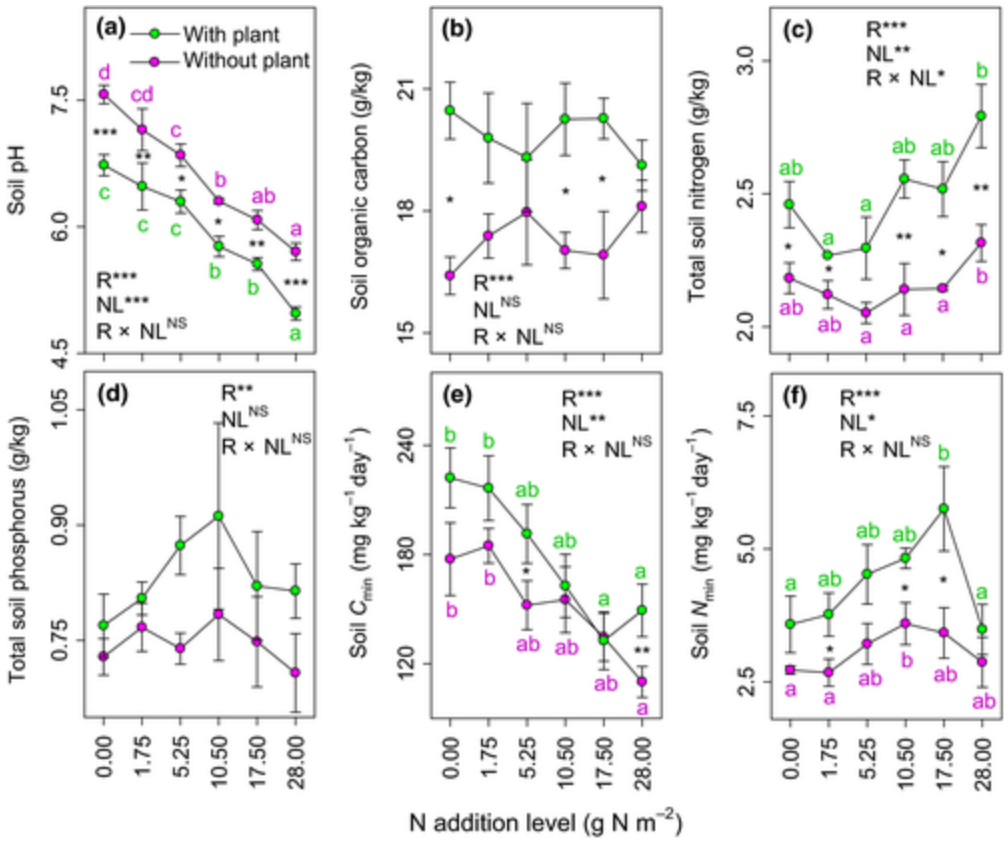

Figure 1

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Responses of soil variables (a–d) and C and N mineralization rates (e–f) to N enrichment in subplots with plants (green points) and without plants (purple points)(a–g). Values are the means (± *SE*) of five replicate subplots. The effects of vegetation removal (R), N enrichment (NL) and their interaction (R × NL) on each response variable were assessed using split-plot one-way ANOVAs (NS, $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Different letters in each vegetation removal treatment indicate significant differences among N-enrichment levels (one-way ANOVA, $p < 0.05$). Asterisks between green and purple points indicate significant differences between with-plant and without-plant treatments for each N-enrichment level (one-way ANOVA; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

3.2 Responses of soil organisms to N enrichment and plant-related inputs

Split-plot ANOVA and regression analyses showed that N enrichment greatly affected soil micro-organisms (Figures 2 and 3; Supporting Information Table S3). N enrichment decreased total microbial biomass, bacterial biomass, fungal biomass and bacterial OTU richness in the with- and without-plant subplots, except that fungal biomass in the without-plant subplots was increased by N enrichment (Figure 2 and Supporting Information Table S3). N enrichment increased the F:B ratio in both kinds of subplots but did not change fungal OTU richness (Figure 2 and Supporting Information Table S3). N enrichment altered the structure of bacterial and fungal communities in both kinds of subplots (Figure 2 and Supporting Information Table S3). For soil bacteria, the N-enrichment-induced changes in community structure were clearly related

to declines in the relative abundance of the dominant *Acidobacteria* and *Chloroflexi* and to increases in the relative abundance of *Firmicutes*, *Proteobacteria* and *Saccharibacteria* (Figure 3 and Supporting Information Figure S4). For soil fungi, the N-enrichment-induced changes in community structure were clearly related to the declines in the relative abundance of the dominant *Zygomycota* and to the increases in the relative abundance of *Ascomycota*, *Eurotiomycetes* and *Sordariomycetes* (Figure 3 and Supporting Information Figure S5).

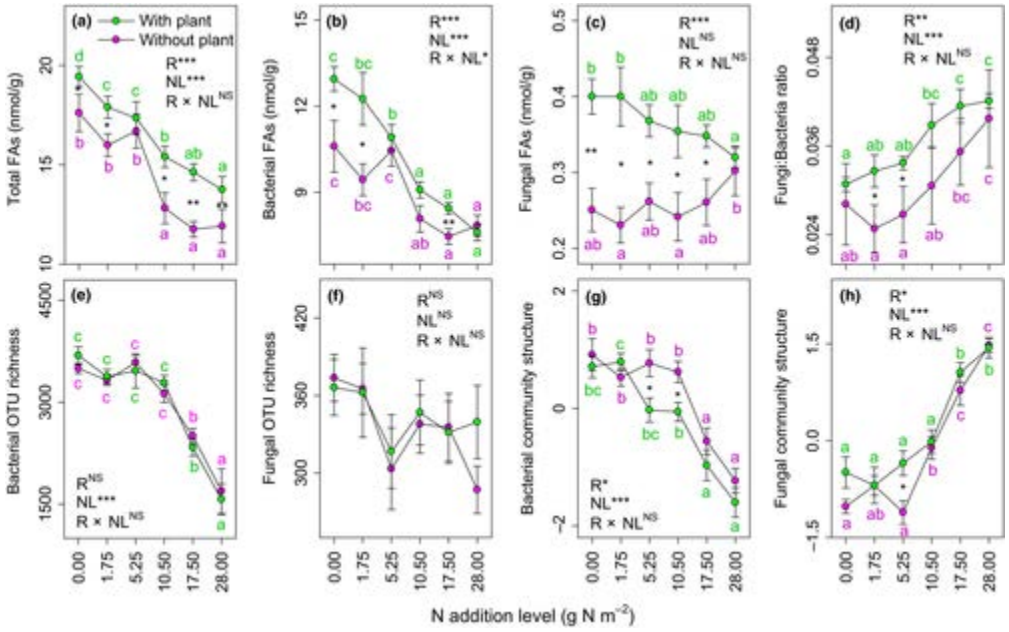

Figure 2

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Responses of soil micro-organisms to N enrichment in subplots with plants (green points) and without plants (purple points) (a–h). Values are the means (±*SE*) of five replicate subplots. Statistical comparisons are described in Figure 1

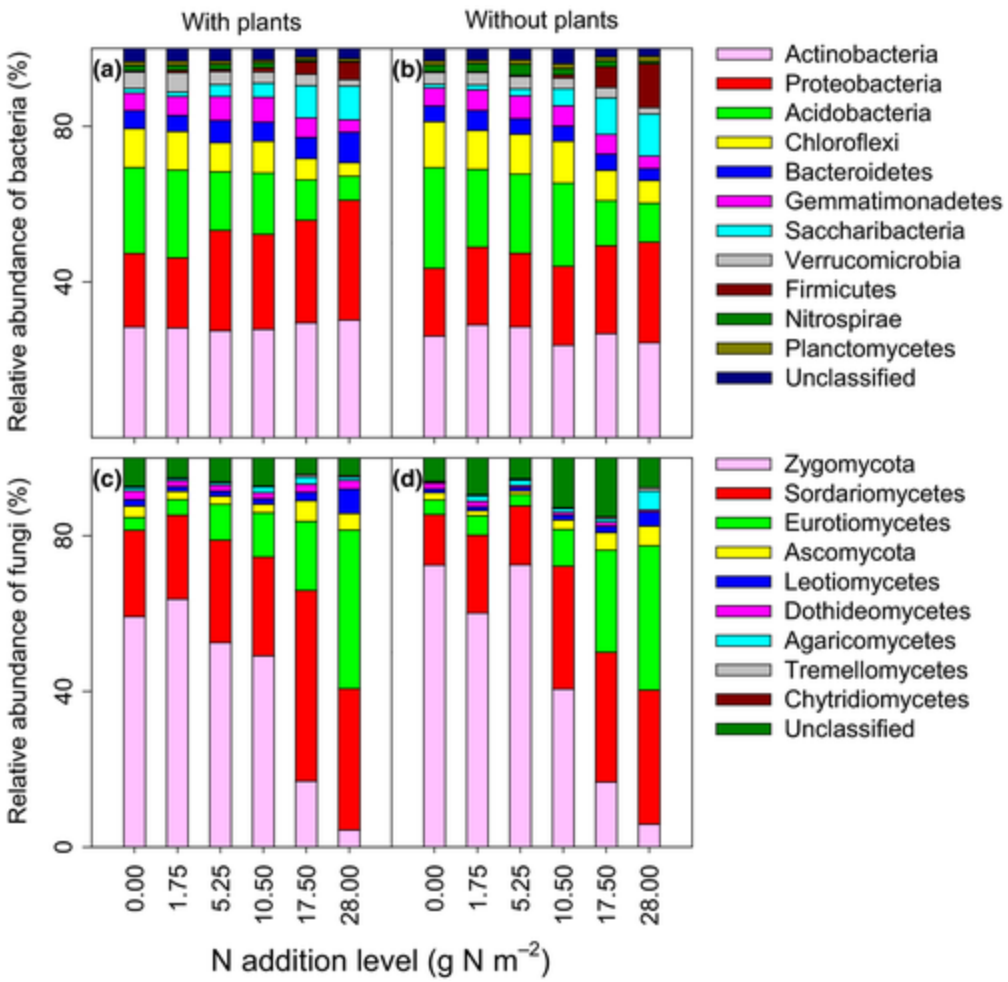

Figure 3

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Responses of relative abundance of dominant phyla/classes (with >1% relative abundance) of bacteria (a, b) and fungi (c, d) to N enrichment in subplots with and without plants. Values are the means of five replicate subplots

Plant removal decreased total microbial biomass, bacterial biomass, fungal biomass and F:B ratio but did not change bacterial or fungal OTU richness (Figure 2 and Supporting Information Table S3). Plant removal altered the bacterial community structure due to the declines in the relative abundance of the dominant *Actinobacteria* and *Proteobacteria* and to the increases in the relative abundance of *Acidobacteria*, *Chloroflexi* and *Firmicutes* (Figure 3 and Supporting Information Figure S4). Plant removal altered the fungal community structure due to the declines in the relative abundance of the dominant *Dothideomycetes* and *Sordariomycetes* and to the increases in the relative abundance of the unclassified (Figure 3 and Supporting Information Figure S5). Surprisingly, there was no statistical interaction between plant removal and N enrichment for most variables concerning the composition and structure of microbial communities (Figure 2 and Supporting Information Figures S4 and S5). In addition, N enrichment weakened the plant-removal-induced negative changes in bacterial biomass and fungal biomass but amplified the negative changes in total microbial biomass and fugal OTU richness and the positive changes in bacterial OTU richness (Figure 5b and Supporting Information Figure S6).

For soil nematodes, split-plot ANOVA and regression analyses showed that N enrichment decreased total abundance due to decreases in the abundance of all trophic groups except plant-feeding nematodes (Figure 4 and Supporting Information Table S3). N enrichment also decreased nematode taxon richness and altered the nematode community structure (Figure 4 and Supporting Information Table S3). Plant removal decreased nematode taxon richness and total nematode abundance due to the decreases in the abundance of bacterial-feeding and plant-feeding nematodes but increased the abundance of fungal-feeding and omnivorous + carnivorous nematodes (Figure 4). The statistical interaction between plant removal and N enrichment was significant only for the abundance of fungal-feeding nematodes and community structure (Figure 4). N enrichment amplified the plant-removal-induced negative effects on total nematode abundance, bacterial-feeding nematode abundance, nematode taxon richness and nematode community structure (Figure 5c and Supporting Information Figure S7). The plant-removal-induced changes in fungal-feeding and omnivorous +carnivorous nematodes shifted from positive to negative as the N-enrichment level increased (Figure 5c and Supporting Information Figure S7).

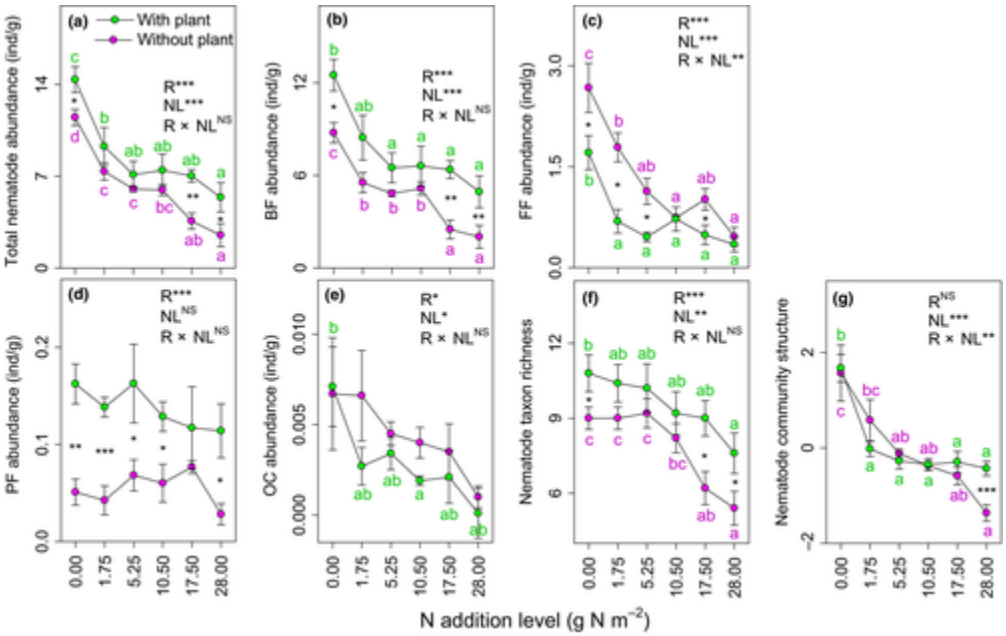

Figure 4

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Responses of soil nematodes to N enrichment in subplots with plants (green points) and without plants (purple points) (a–g). BF: bacterial-feeding; FF: fungal-feeding; PF: plant-feeding; OC: omnivorous and carnivorous. Values are the means (±*SE*) of five replicate subplots. Statistical comparisons are described in Figure 1

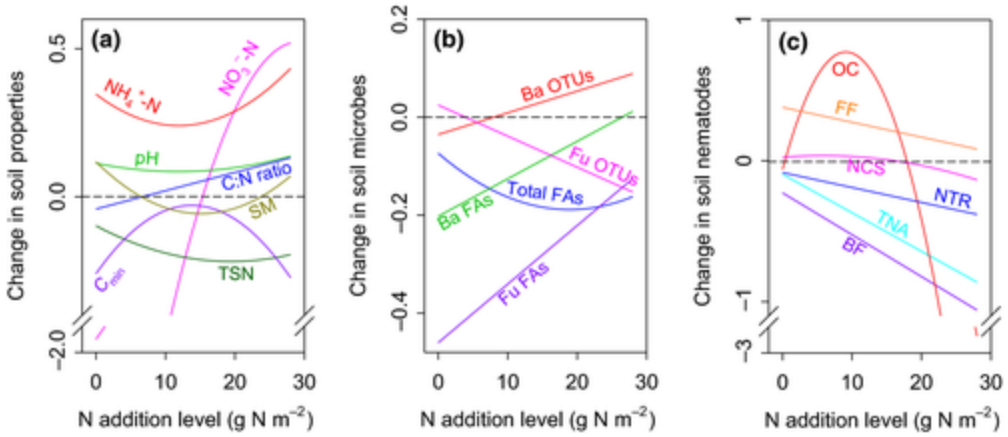

Figure 5

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Changes in soil properties including C and N mineralization rates (a), soil micro-organisms (b) and soil nematodes (c) in response to vegetation removal at the indicated levels of N enrichment. The changes in each variable caused by vegetation removal were determined as ratios of without- to with-plant subplots. SM: soil moisture; TSN: total soil nitrogen; TNA: total nematode abundance; BF: bacterial-feeding; FF: fungal-feeding; OC: omnivorous and carnivorous; NTR: nematode taxon richness; NCS: nematode community structure; Ba, bacterial; Fu, fungal. Regressions lines were estimated using a linear or quadratic model with N-enrichment level as a continuous predictor (n = 30). Only statistically significant regression lines are shown ($p < 0.05$)

3.3 Linking microbial communities to soil nematodes and soil C and N mineralization

Structural equation modelling analyses performed separately for with- and without-plant treatments indicated that N enrichment directly induced increases in soil N availability and soil acidification (Figure 6). The total variations in soil microbe variables (the decrease in total biomass, bacterial OTU richness and PC1 scores for bacterial community structure, and the increase in the F:B ratio and PC1 scores for fungal community structure) were mainly explained (79%–87%) by the increases in soil acidification and soil N availability (Figure 6). The total variation in soil nematode variables (the decrease in total abundance, taxon richness and PC1 scores for community structure) was mainly explained (69%) by soil micro-organisms and soil pH in with-plant subplots (Figure 6a) but was mainly explained (80%) by soil pH and soil N availability in without-plant subplots (Figure 6b). Variation in soil C mineralization rates (48%) was mainly explained by the soil pH, micro-organisms and ANPP in with-plant subplots (Figure 6a) but was mainly explained (45%) by soil pH, micro-organisms and soil N availability in without-plant subplots (Figure 6b). Variation in soil N mineralization rates (43%) was mainly explained by the micro-organisms and ANPP in with-plant subplots but was mainly explained (37%) by soil pH, soil nutrients and micro-organisms in without-plant subplots (Figure 6).

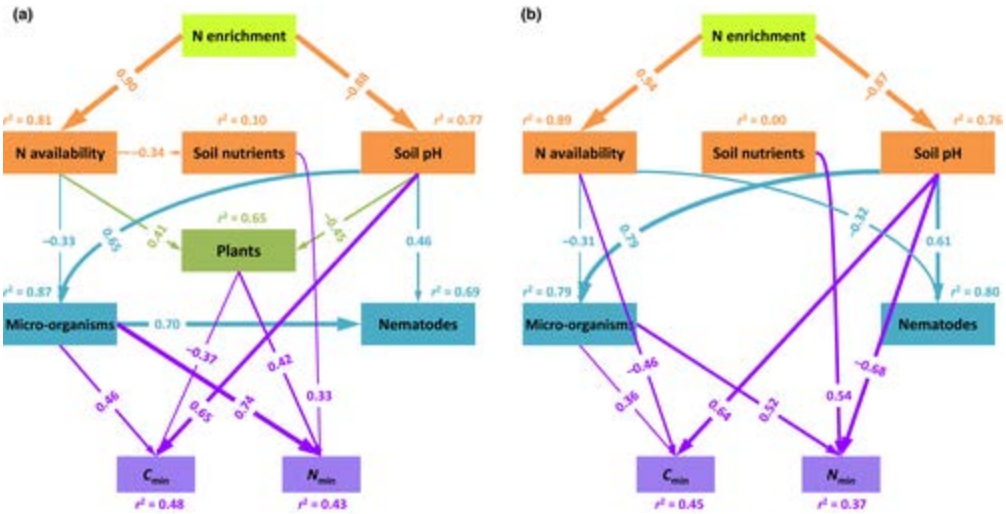

Figure 6

Open in figure viewerPowerPoint

Structural equation model (SEM) analysis of the effects of N enrichment on the soil organisms and C (C_{min}) and N (N_{min}) mineralization rates in subplots with plants (a) and without plants (b). Results of model fitting: (a) $\chi^2 = 16.55$, $p = 0.581$, $df = 20$, $n = 30$; (b) $\chi^2 = 15.40$, $p = 0.522$, $df = 16$, $n = 30$ (a high p value associated with a χ^2 test indicates a good fit of the model to data, that is, no significant discrepancies). Solid arrows indicate significant effects ($p < 0.05$). r^2 values associated with response variables indicate the proportion of variation explained by relationships with other variables. Values associated with solid arrows represent standardized path coefficients

4 DISCUSSION

4.1 Effects of N enrichment on soil organisms and C and N mineralization

Consistent with our first hypothesis, our results showed that the N enrichment reduced microbial biomass, abundance and C mineralization rates. The N-enrichment-induced declines in the biomass or abundance of soil organisms were consistent with previous observations (4 years ago) from the same study site (Chen et al., 2015), although the earlier study did not compare plots with and without plants. In the present study, we further assessed responses of bacteria and fungi based on 16S rRNA (bacteria) and ITS rRNA (fungi) gene sequencing. Our results revealed that N enrichment strongly decreased bacterial OTU richness but did not change fungal OTU richness, which suggests that environmental tolerances are generally wider for fungi than bacteria (Fierer et al., 2012; Rousk et al., 2010). N enrichment altered the bacterial community structure due to declines in the relative abundance of oligotrophic phyla (mainly *Acidobacteria* and *Chloroflex*) and increases in copiotrophic taxa (mainly *Proteobacteria* and *Saccharibacteria*), which was consistent with other reports (Fierer et al., 2012; Leff et al., 2015; Ramirez, Lauber, Knight, Bradford, & Fierer, 2010). Although N enrichment did not alter fungal OTU richness, N enrichment substantially changed the structure of the soil fungal community due to the increases in the relative abundance of wood-decay fungal taxa (*Ascomycota*, *Eurotiomycetes* and *Sordariomycetes*); these changes have often been observed in grassland or farmland soils (He et al., 2016; Zhou et al., 2016). Overall, our study represents one of a few PLFA- and sequence-based assessments of both the bacterial and fungal communities. The

observed changes in the soil microbial communities across the N gradients indicated that N enrichment induced shifts in the predominant microbial life-history strategies, favouring a more copiotrophic bacterial community and a more wood-decaying fungal community.

N enrichment in the current study decreased most variables concerning nematodes, which was consistent with our first hypothesis, but the effects of N on nematodes (higher trophic groups) were not less than those on micro-organisms (lower trophic groups), which was inconsistent with our first hypothesis. Some previous studies reported both negative and positive effects of N enrichment on soil nematodes under different conditions (Eisenhauer et al., 2013; Li et al., 2013; Wei et al., 2012; Zhao et al., 2014). A lack of negative effects might be due to either the ability of nematodes to tolerate different levels of N or to the buffering of the negative effects of N on nematodes by vegetation. Moreover, although low and moderate levels of N enrichment enhanced the N mineralization rate, high levels of N enrichment reduced the N mineralization rates, leading to a hump-shaped relationship between N enrichment and N mineralization rate in subplots with and without plants (Lu et al., 2011; Niu et al., 2016). A hump-shaped curve may result when a large portion of the added N is immobilized in the soil at N-poor sites but is actively recycled in the soil at N-rich sites (Niu et al., 2016). Declines in soil C mineralization rates caused by N enrichment could be linked to a reduction in the microbial substrates (C) and enzymatic activity (Janssens et al., 2010; Ramirez, Craine, & Fierer, 2012).

4.2 Effects of plant removal on soil organisms and C and N mineralization

Plant removal in the current study dramatically decreased the C and N mineralization rates and the biomass or abundance of soil organisms but did not change the overall response patterns of soil organisms or C and N mineralization to N enrichment. These results are consistent with our second hypothesis and with previous findings that the presence of plants enhances soil microbial biomass (Brant et al., 2006; Kaiser et al., 2010; Mariotte et al., 2018). The negative effect of vegetation removal on these organism parameters has been previously associated with the decrease in the plant or litter inputs to the soils (Kardol & De Long, 2018). Kaiser et al. (2010), for example, found that the termination of plant-related inputs in a beech forest decreased the total microbial biomass, bacterial biomass and fungal biomass. Generally, increases in plant-related inputs result in greater bacterial or fungal diversity (Lange et al., 2015; Maestre et al., 2015). Interestingly, we found that soil bacterial or fungal diversity (OTU richness) did not respond to vegetation loss, which is consistent with some previous studies that reported a lack of correlation between plant productivity and bacterial diversity (Schlatter, Bakker, Bradeen, & Kinkel, 2015) or fungal diversity (Waldrop, Zak, Blackwood, Curtis, & Tilman, 2006).

Our results indicated that plant removal also decreased nematode taxon richness and the abundance of total nematodes, bacterial-feeding nematodes and plant-feeding nematodes, but increased the abundance of fungal-feeding and omnivorous + carnivorous nematodes. The decline in the abundance of total nematodes and bacterial-feeding nematodes in response to plant removal likely resulted from declines in soil bacteria, most of which depend on plant inputs as sources of C and energy (Spehn, Joshi, Schmid, Alphei, & Körner, 2000). These results were consistent with previous findings that termination of plant-related inputs decreases the abundance or diversity of soil nematode functional groups (Eisenhauer et al., 2010; Keith et al., 2009; Spehn et al., 2000). Our finding that plant removal increased the abundance of fungal-

feeding and omnivorous + carnivorous nematodes might be associated with the increase in soil pH (Chen, Lan, et al., 2013a; Kuperman & Edwards, 1997). Consistent with our second hypothesis, plant removal caused declines in soil N and C mineralization rates. The declines in C mineralization rates may be associated with the decrease in C substrates entering the soil and with the associated decrease in the biomass/abundance of the soil bacteria (Chen et al., 2016; Janssens et al., 2010). Similar findings from the same grassland showed that plant removal increased the soil inorganic N content due to the reduced consumption of N by plants and microbes (Chen et al., 2016) and that plant removal decreased the N mineralization rate due to the reduction in C inputs to the soils (Kong et al., 2011).

Although vegetation removal changed microbial parameters, C and N mineralization rates did not increase with N addition rate. These results seem to be inconsistent with the mass ratio hypothesis, which states that the controls of ecosystem properties are "in proportion to inputs to primary production" (Grime, 1998). Our results were probably inconsistent with the mass ratio hypothesis because vegetation removal not only altered different components of soil biodiversity and litter input but also altered the edaphic properties (Chen et al., 2016; Wardle et al., 2013). The lack of association between effects of vegetation removal and N enrichment or changes in plant variables (Supporting Information Table S4) was consistent with a previous research (Zhu, Panke-Buisse, & Kao-Kniffin, 2015), which also found that the rhizosphere priming effects on C and N mineralization rates did not affect by N-enrichment levels. Moreover, the vegetation-removal-induced changes in most nematode variables were amplified by N enrichment, thus suggesting a positive correlation between plant biomass and the abundance of soil nematode groups as predicted by the mass ratio hypothesis (Supporting Information Table S4; Chen et al., 2016). It is important to note that our plant removal treatment was initiated in 2010, 11 years after the main experiment (N addition) was initiated; the effects of plant removal on soil properties may have been different if subplots with and without plants had been established 1999. Following 15 years of plant removal, for example, bacterial and fungal OTU richness may have been significantly reduced due to reductions in SOC, TSN and other substrates. Moreover, the soil mineralization rates in present study determined by sieved soils with favourable conditions may be higher than the in-situ rates measured in the field. Overall, these findings provide direct evidence that soil trophic groups differ in their responses to a reduction in the plant-related inputs and that the mass ratio hypothesis does or does not apply depending on trophic level, relative abundance of taxa, life-history strategies and soil nutrient status.

4.3 Interactions between n enrichment and vegetation removal for soil organisms and C and N mineralization

Finally, we tried to determine the interactive effects of N enrichment and vegetation removal on soil organism communities and on C and N mineralization. Our results indicate that soil organisms and mineralization were more affected by the direct effect of N enrichment rather than by the indirect effect mediated by plants. For soil micro-organisms, our SEM results further confirmed that the dramatic changes in soil micro-organisms under N enrichment were largely due to increase in soil acidification (a direct effect) in both with- and without-plant subplots. Soil acidification may affect soil biodiversity and trophic interactions by suppressing bacteria and by stimulating fungi and thus could alter microbially driven ecosystem processes (Chen et al., 2015; Rousk et al., 2010; Treseder, 2008). Although N enrichment increases ANPP and may thereby

increase the supply of C for soil micro-organisms, the positive effect on soil organisms was offset by the negative effect of soil acidification. An increase in the F:B ratio may be due to the fact that fungi are more tolerant than bacteria to H^+ ions (Chen, Lan, et al., 2013a; Fierer et al., 2012; Rousk et al., 2010). Similarly, there was no statistical interaction between N enrichment and vegetation removal on C and N mineralization rates. Our SEM results indicated that N enrichment mainly controlled the changes in C and N mineralization rates by affecting soil acidification, micro-organisms and nutrient contents.

For nematode variables, the statistical interaction between N enrichment and vegetation removal was significant only for the abundance of fungal-feeding nematodes and for nematode community structure. In the subplots with plants, our SEM results indicated that the N-enrichment-induced changes in the soil nematodes were determined by different pathways but were mainly driven by a reduction in their food resources (e.g., bacteria and fungi) and by an increase in soil acidification. In subplots without plants, however, these effects were driven by increases in soil acidification and N availability. The N-enrichment-induced increase in soil acidification will also increase Al^{3+} and NH_4^+ ions, which can reduce taxon richness due to their toxic effects on microbes and nematodes (Chen et al., 2015; Chen, Lan, et al., 2013a; Kuperman & Edwards, 1997). In addition, large organisms in the soil food web are thought to be controlled by bottom-up prey resources such as bacteria and fungi (Bardgett & Wardle, 2010; Pollierer et al., 2007), which could explain why the N-enrichment-induced decrease in soil micro-organisms was strongly associated with the decline in the nematode abundance in subplots with plants. However, this dominant role of prey food resources on soil nematodes was not evident in the subplots without plants. Therefore, vegetation removal is likely to reduce the abundance of micro-organisms in the soil food web and may also weaken the relationships between micro-organisms and nematodes in the grassland ecosystems, indicating that ecosystem health (such as sustainability or resilience) may not be maintained in present scenario of N deposition and biodiversity loss.

Although there was statistical interaction between N enrichment and plant removal on C mineralization rates, our SEM results indicated that the changes in C and N mineralization rates were mainly controlled by the N-enrichment-induced direct effects (mostly via soil acidification, soil micro-organisms or soil nutrients). There was no significant interaction between soil nematodes and soil C or N mineralization rate whether plants were removed or not in our experiment, but such interactions were often documented in previous reports (Bardgett & Wardle, 2010; Ingham, Trofymow, Ingham, & Coleman, 1985). A possible explanation for the lack of these interactions in our study is that, in semi-arid grasslands, soil N mineralization is regulated more by soil nutrients and the soil environment than by soil nematode communities (Chen, Zheng, et al., 2013b). Altogether, our results indicate that plant-related inputs did not qualitatively change the effects of N enrichment on soil organisms or mineralization and that the positive effects of increased plant-related inputs induced by N enrichment did not counteract the negative effects of N enrichment, even though plant biomass increased as N enrichment increased. The N-enrichment-induced changes in soil organisms and processes were more affected by the direct effects of N enrichment (via soil acidification and increased N availability) than by plant-related indirect effects. These results increase our understanding of how future changes in N deposition and vegetation may alter below-ground communities and processes in grassland ecosystems.

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AUTHOR CONTRIBUTIONS

D.C. and Y.B. designed the study. D.C., W.X., Z.L. and Y.W. performed the experiment. D.C. and M.S. compiled and analysed the data. D.C., M.S., S.H. and Y.B. led the writing, with input from all co-authors.

DATA ACCESSIBILITY

Data are available from the Dryad Digital Repository https://doi.org/10.5061/dryad.fs21935 (Chen et al., 2018).