Soil acidification exerts a greater control on soil respiration than soil nitrogen availability in grasslands subjected to long-term nitrogen enrichment

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Summary

1. Terrestrial ecosystems worldwide are receiving increasing amounts of biologically reactive nitrogen (N) as a consequence of anthropogenic activities. This intended or unintended fertilization can have a wide-range of impacts on biotic communities and hence on soil respiration.

2. Reduction in below-ground carbon (C) allocation induced by high N availability has been assumed to be a major mechanism determining the effects of N enrichment on soil respiration. In addition to increasing available N, however, N enrichment causes soil acidification, which may also affect root and microbial activities. The relative importance of increased N availability vs. soil acidification on soil respiration in natural ecosystems experiencing N enrichment is unclear.

3. We conducted a 12-year N enrichment experiment and a 4-year complementary acid addition experiment in a semi-arid Inner Mongolian grassland. We found that N enrichment had contrasting effects on root and microbial respiration. N enrichment significantly increased root biomass, root N content and specific root respiration, thereby promoting root respiration. In contrast, N enrichment significantly suppressed microbial respiration likely by reducing total microbial biomass and changing the microbial community composition.

4. The effect on root activities was due to both soil acidity and increased available N, while the effect on microbes primarily stemmed from soil acidity, which was further confirmed by results from the acid addition experiment. Our results indicate that soil acidification exerts a greater control than soil N availability on soil respiration in grasslands experiencing long-term N enrichment.

5. These findings suggest that N-induced soil acidification should be included in predicting terrestrial ecosystem C balance under future N deposition scenarios.

Key-words: base mineral cations, below-ground carbon allocation, microbial respiration, plant functional group, root nitrogen content, root respiration, root specific respiration, soil microbial community

Introduction

Anthropogenic reactive nitrogen (N) inputs, originated mainly from fossil-fuel burning and artificial fertilizer application, have increased three- to five-fold over the past century (Galloway et al. 2008). In many areas of the globe and especially in Asia, N deposition is expected to continue to increase (Zhao et al. 2009; Bobbink et al. 2010). It is well-established that dramatic increases in N inputs have wide-ranging ecological impacts on the biotic community, biogeochemical cycles and greenhouse gas emissions. Detrimental outcomes include the loss of biological diversity (Bobbink et al. 2010), N saturation and N export (Vitousek et al. 1997), and eutrophication (Stevens et al. 2010). Long-term N deposition can also result in soil acidification, leading to the transport of base cations from soil to groundwater (Bobbink et al. 2010; Guo et al. 2010). N deposition, however, can also have positive effects, such as the stimulation of plant growth and the associated increase in the uptake of atmospheric carbon (C), which would help
mitigate climate change (Pregitzer et al. 2008; Thomas et al. 2010). Despite much research, the underlying mechanisms by which N deposition affects C cycling remain largely unknown and controversial, even though the understanding of such mechanisms is essential for predicting global ecological change (Magnani et al. 2007).

Several recent reviews have showed that the availability of N for primary production primarily affects soil CO$_2$ efflux and soil organic C turnover and therefore the storage of soil organic C (Magnani et al. 2007; Janssens et al. 2010). However, while N deposition increases N availability, it simultaneously causes soil acidification, as a result of several processes: (i) co-occurring deposition of H$^+$ with NO$_3^-$; (ii) release of H$^+$ when NH$_4^+$ is taken up by plants; (iii) release of H$^+$ when microbes oxidize NH$_4^+$ in the soil and (iv) losses of base cations (i.e., Ca$^{2+}$, Mg$^{2+}$, and Na$^+$) (Guo et al. 2010). The soil acidification can also affect C allocation to soils and hence soil CO$_2$ efflux by changing base cations and biotic communities in soil (Oulehle et al. 2011). Yet, few studies have experimentally assessed the relative effects of increased N availability vs. acidification on below-ground C cycling in natural ecosystems.

Soil respiration includes the release of CO$_2$ from soil as a consequence of microbial activities associated with litter decomposition and the respiration of live roots and their symbionts (Kuzyakov 2006). Soil respiration is one of the largest C effluxes between the atmosphere and terrestrial ecosystems and plays a vital role in regulating the atmospheric CO$_2$ concentration and climate on Earth (Davidson et al. 2002). An accurate determination of both soil microbial respiration and root respiration is needed to predict the net ecosystem C balance (Bond-Lamberty, Wang & Gower 2004), since the responses of root and microbial activities to N enrichment often vary among biotic communities and environmental conditions (Kuzyakov 2006). N enrichment is known to affect soil respiration, but the results have been inconsistent and ecosystem dependent (Janssens et al. 2010). One potential reason for this inconsistency may arise from different responses of its two components (soil microbial respiration and root respiration) to N inputs. To date, few studies have directly assessed the effect of long-term N enrichment on root and microbial respiration in the field. This lack of understanding hinders our ability to predict the impact of future N deposition on ecosystem C balance.

Two pathways or mechanisms may primarily drive the N effects on soil respiration and its two components (Fig. 1). In the first pathway, N enrichment directly increases N availability for plants and microbes but its effects may depend on the existing N status in soil. When N is abundant, N inputs may reduce plant below-ground C allocation and suppress root and microbial respiration, because plants may reduce their investment in fine roots and in mycorrhizal symbiosis (Phillips & Fahey 2007; Janssens et al. 2010). In contrast, in highly N-limited environments, this increase in soil N availability could also enhance the below-ground C allocation and thus both microbial and root respiration (LeBauer & Treseder 2007; Liu & Greaver 2010). The increases in quality and quantity of above-ground and below-ground biomass (BB; Sayer, Powers & Tanner 2007; LeBauer & Treseder 2008; Xia & Wan 2008) following N enrichment may not only alleviate C limitation for soil microorganisms but also enhance root respiration. In the second pathway, N enrichment results in soil acidification, thereby decreasing below-ground C allocation and soil respiration (Högberg et al. 2006; Oulehle et al. 2011). Soil acidification may suppress plant and microbial communities by increasing soil H$^+$ and Al$^{3+}$ (Van Den Berg et al. 2005; Rousk, Brookes & Bååth 2009) and enhancing leaching of base mineral cations (e.g., Ca$^{2+}$, Mg$^{2+}$, and Na$^+$). These changes in the soil cations could negatively affect plant biomass and microbial biomass, and thus suppress root and microbial respiration.

![Fig. 1. A conceptual diagram of N enrichment effects on root and microbial respiration.](image-url)
soil acid cations (H\(^+\) and Al\(^{3+}\)), soil base cations (Ca\(^{2+}\), Mg\(^{2+}\), and Na\(^+\)) and N availability (soil NH\(_4\)\(^+\)-N and soil NO\(_3\)\(^-\)-N). We then quantify the effects of N enrichment on total soil respiration and its components (soil microbial respiration and root respiration) via pathways of soil N availability and soil acidification. Finally, we use the results of complementary soil acid-addition experiment to evaluate the relative roles of N availability and soil acidification in determining how N enrichment affects soil respiration.

**Materials and methods**

**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 an altitude of approximately 1200 m a.s.l. The semi-arid continental climate is characterized by a mean annual (1982–2009) precipitation of 334 mm and a mean annual temperature of 0.9 °C. Precipitation mainly falls in the growing season (June–August), which is coincident with high temperatures. The site has a dark chestnut soil (Calcic Chernozem according to ISSS Working Group RB, 1998), with a loamy-sand texture (Bai et al. 2010).

**LONG-TERM NITROGEN ENRICHMENT EXPERIMENT**

The establishment of the N enrichment experiment was described by Bai et al. (2010) and is briefly described here. In 1999, a 120-m × 70-m location with fairly uniform vegetation was designated within the permanent research plots of IMGERS. The location was divided into 162 5-m × 5-m plots with 1-m buffers. These plots were laid out in a randomized block design including nine replicate blocks. Each replicate block included six levels of N enrichment (0, 1.75, 5.25, 10.50, 17.5, and 28.0 g N m\(^{-2}\) year\(^{-1}\)). N was added as commercial pelleted NH\(_4\)NO\(_3\) fertilizer at the middle of the growing season (July 1–5). The low N enrichment level (1.75 g of N m\(^{-2}\) year\(^{-1}\)) was designed to be close to the ambient atmospheric N deposition (1–1.9 g N m\(^{-2}\) year\(^{-1}\)) in this region (Liu et al. 2011) and the high N enrichment levels (17.5 and 28.0 g of N m\(^{-2}\) year\(^{-1}\)) were in the ranges of agricultural N inputs (15–30 g N m\(^{-2}\) year\(^{-1}\)) (Guo et al. 2010).

**ACID ADDITION EXPERIMENT**

To assess the potential effects of the soil acidification pathway on soil respiration and its two components, we established a complementary acid addition experiment near the N addition experimental site to generate comparable degrees of soil acidification resulting from N inputs (see details in Chen et al. 2013). Briefly, a 15-m × 20-m field near the N enrichment experiment was selected within the permanent research plots of IMGERS in 2009, divided into 35 plots (2-m × 2-m). Five replicate plots for each of seven treatments were established in a randomized block design. The treatments included seven rates of addition of a sulphuric acid solution: 0, 2.76, 5.52, 9.28, 11.04, 13.80 and 16.56 mol H\(^+\) m\(^{-2}\). Acid was added at these rates three times (September 2009, June 2010, and September 2010). At each time, each dose of 98% sulphuric acid was diluted in 80 L of well water (water was also added for the rate of 0 mol H\(^+\) m\(^{-2}\)). The resulting soil pH in the acid addition plots (4.1–7.6) were well into the ranges of soil pH in the N enrichment experiment (4.7–7.2), although the levels of acid addition extend well beyond possible inputs (c. 0.2 mol H\(^+\) m\(^{-2}\) year\(^{-1}\)) from atmospheric acid deposition in this steppe (Guo et al. 2010).

**TRENCHING OF SUBPLOTS AND MEASUREMENT OF SOIL CO\(_2\) EFFLUX**

For both experiments, the trenching method was used to estimate total soil CO\(_2\) efflux and to separate its sources into soil microbial and root respiration (Kuzyakov 2006; Sayer & Tanner 2010; Chen et al. 2011). In April 2010, one trenched subplot (0.5-m × 0.5-m) was established in each plot (5-m × 5-m). Each trenched subplot was prepared by making vertical cuts in the soil along the boundaries to 50 cm depth such that all roots crossing the boundaries of the trenched subplots were severed but not removed. Pieces of 0.3-cm-thick polyethylene board were then inserted into the vertical cuts to prevent roots from growing into the subplots. Our previous investigation showed that root biomass of 50–100 cm depth contributes only 8% to total root biomass (0–100 cm depth), indicating that the polyethylene board in to 50 cm depth could prevent 92% of root ingrowth into trenched subplots. Roots in the trenched subplots were killed by weekly cutting of all the above-ground parts of plants in the subplots at the soil surface. In late August 2012 (about 2 years after trenching treatment), single soil cores (5 cm diameter, 0–50 cm depth) were collected from each trenched subplot to examine the occurrence of dead roots. We did not find visual living or dead roots (0–50 cm depth) in any of the trenched subplots from both experiments. We also found there was no difference in soil moisture between trenched and un-trenched subplots (see Fig. S1, Supporting Information).

In June 2012, two steel, square collars (30-cm × 30-cm and 5 cm in height) were placed in each plot, one in the trenched subplot and the other outside the trenched subplot. The steel collars were inserted into the soil to a depth of 3 cm for measurement of soil CO\(_2\) efflux (Chen et al. 2011). To minimize the effect of acid neutralization on soil CO\(_2\) efflux from carbonates in deep soil, we measured soil CO\(_2\) efflux during the growing season (June to August) of 2012 after about 2 years of trenching treatment. Soil CO\(_2\) efflux was measured on three consecutive replicate days for each month (during 20th–29th) using three Li-Cor 840 infrared gas analyzers (IRGAs) (Li-Cor, Inc., Lincoln, Nebraska, USA), each of which was attached to a removable soil CO\(_2\) efflux chamber (30-cm × 30-cm and 30 cm in height) (Niu et al. 2010). To minimize effects of variations in climate factors (e.g. temperature and soil moisture) among treatments, all soil CO\(_2\) efflux measurements were completed within one replicate day between 9:00 and 11:00 a.m. Each measurement usually required 3 min.

**MEASUREMENT OF SPECIFIC ROOT RESPIRATION**

To elucidate the effects of shifts in plant species induced by N enrichment on root respiration, in late August 2012 we measured the specific root respiration (nmol CO\(_2\) g\(^{-1}\) dry root biomass s\(^{-1}\)) of five dominant plant species in each plot using a root excision method modified from Burton & Pregitzer (2002) and Chen et al. (2009) (see Fig. S2). Briefly, composite samples of fine roots (c. 2–3 g fresh weight) were collected from the top 30 cm of soil at locations outside the treatment plots. The fine roots were immediately placed in a 15-cm-long and 3-cm-diameter PVC respiration cuvette attached to a Li-Cor 840 IRGA. Steady respiration rates were achieved within 5 min after samples were placed in the cuvette. The input concentration of CO\(_2\) for the cuvette was maintained at 1000 p.p.m. to approximate surface soil CO\(_2\) concentration. After respiration was measured, the root samples
were oven-dried at 65 °C for 48 h and weighed and then were determined root N content by micro-Kjeldahl digestion method.

PLANT SAMPLING
In late August 2012, above-ground vegetation was sampled in a 0.5-m × 0.5-m quadrant in each plot of the N enrichment experiment; this was done by clipping all plants in the quadrant at the soil surface. To avoid edge effects, the quadrant was placed at least 30 cm inside each plot. Live vascular plants were sorted into species and oven-dried at 65 °C for 48 h and weighed. To assess the effects of shifts in the composition of plant functional groups on soil CO2 efflux, we classified all plants into two functional groups: perennial rhizome species (PRs) and perennial bunchgrasses (P Bs). These two plant functional groups represented 88% of the total plant biomass. After the above-ground biomass (AB) was sampled, three soil cores (6.5 cm diameter and 0–20 cm depth) were collected in each plot to determine plant live BB. Identifiable roots (live or dead, assessed visually based on colour, elasticity and resilience) were rinsed from the soil cores under running water, were collected on a 1-mm screen, oven-dried at 65 °C, and weighed.

SOIL SAMPLING AND ANALYSIS
After harvesting the plant biomass, four soil cores (2 cm diameter, 0–15 cm depth) were randomly collected from each plot of the N enrichment experiments and combined to form one composite soil sample per un-trenched plot or trenched subplot. After the soil was gently mixed and roots were removed, the moist soil was gently mixed and roots were removed, the moist soil was sieved (1-cm mesh) and oven-dried at 65 °C for 48 h and weighed.

Matreya Inc., State College, PA, USA). 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), fungi (18:2ω6ω9) and arbuscular mycorrhiza fungi (AMF) (16:1ω5) were used to determine the abundances of these microbial groups and to calculate fungi/bacteria ratios.

SOIL MICROBIAL COMMUNITY STRUCTURE
The microbial community in soil samples was assessed by analysis of phospholipid fatty acids (PLFAs). PLFAs were extracted from the soil as described by Bostick et al. (1998), and FAs specific to bacteria, fungi (AMF), and arbuscular mycorrhiza fungi (AMF) (16:1ω5) were used to determine the abundances of these microbial groups and to calculate fungi/bacteria ratios.

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RESULTS

RESPONSES OF SOIL ABIOTIC PROPERTIES AND SOIL RESPIRATION
N enrichment significantly enhanced soil N availability, reduced soil pH and altered soil cation content (Fig. 2). Compared to the control, soil NO3-N increased by 94–151% in the treatments of 5–25 g N m−2 year−1 and soil NH4+-N increased by 351–1153% in the plots with N additions of 10.5–28.0 g N m−2 year−1, respectively (Fig. 2a–b). Soil pH decreased across the N enrichment gradient by 0.25–1.76 units (Fig. 2c). N addition increased the content of Al3+ from 40.5 to 55.3 mg kg−1 and decreased the contents of Ca2+, Mg2+ and Na+ (Fig. 2d–g).

Total soil CO2 efflux and its sources (soil microbial and root respiration) were substantially changed by N enrichment, although there was significant difference between months (Fig. 3). For each month or average of 3 months, total soil respiration and soil microbial respiration declined with increasing N enrichment (Fig. 3a–b). In contrast, root respiration increased with N enrichment (Fig. 3c). The complementary acid addition experiment showed that the responses of total soil respiration, microbial respiration and root respiration to acid addition levels had similar trends with N enrichment experiments (Fig. 3d–f). Specifically, the total soil respiration and soil microbial

respiration increased with soil pH while root respiration declined with soil pH for both experiments, although the three soil CO$_2$ efflux rates were different between N enrichment and acid addition experiments (Fig. 3g–i).

RESOURCES OF SOIL MICROBIAL AND PLANT COMMUNITIES

ANOVA analyses indicated that N enrichment substantially altered the soil microbial community (Table 1). N addition significantly decreased total FAs by up to 27%, bacterial FAs by up to 28%, fungal FAs by up to 20%, and AMF FAs by up to 57% relative to control plots. The fungi/bacteria ratio increased with increasing N enrichment rate (Table 1). N enrichment substantially altered the plant community (Table 1). N addition significantly increased the AB by up to 173%, BB by up to 36%, and PR biomass by up to 1320%. N addition significantly decreased the PB biomass by up to 97% and BB/AB by up to 50% (Table 1).

PATHWAYS DETERMINING SOIL CO$_2$ EFFLUX

Most variables or categories examined in this study were correlated with one another, making this data set appropriate for SEM analysis (see Fig. S4). SEM analyses indicated that N enrichment directly induced changes in soil N availability and soil acidification, and that N enrichment directly explained 80% of the total variance in soil N availability and 74% of the total variance in soil acidification (Fig. 4). The pathway of soil acidification directly altered soil base cations (Al$^{3+}$, Ca$^{2+}$, Mg$^{2+}$ and Na$^+$), plant communities and microbial communities. The soil acidification pathway directly explained 68% of the total variance in soil base cation concentrations, and the increases in contents of H$^+$ were associated with decreases in Ca$^{2+}$, Mg$^{2+}$ and Na$^+$ and an increase in Al$^{3+}$. The observed changes in soil acidification, soil base cations, and soil N availability explained about 62% of the total variance in plants (biomass and composition). The increase in AB and BB and decrease in BB/AB were mainly attributed to the increase in soil N availability (Fig. 4). The changes in plant community components in PR and PB were mainly attributed to soil acidification. The changes in the soil microorganisms (decreases in bacterial FAs, fungal FAs and AMF FAs and increases in fungi/bacteria ratio) were apparently affected by both soil acidification and changes in soil base cations (Fig. 4). Surprisingly, soil N availability and plants (biomass and composition) appeared to lack significant direct effect on soil microbial community (Fig. 4).

The different responses of root respiration and soil microbial respiration to N enrichment could be explained by differences in the responses of the plant community and microbial community. The plant community pathway alone...
explained 61% of the total variance in root respiration (Fig. 4). The increases in AB and BB and shifts of plant community composition were associated with increases in root respiration (Fig. 4). Linear regression showed that BB and PR explained 25 and 39% of the variation in root respiration, respectively (Fig. 5a–b). In addition, specific root respiration and root N content of the five dominant species increased with increasing N enrichment level (Fig. 5c–d). We also found that both specific root respiration and root N content were higher for PR species (*Leymus chinensis* and *Carex korshinskii*) than for PB species (*Achnatherum sibiricum* and *Stipa grandis*) (Fig. 5c–d).
Table 1. Responses of plant and soil microbial community variables to N enrichment

<table>
<thead>
<tr>
<th>Level of N enrichment (g N m⁻² year⁻¹)</th>
<th>Response variable</th>
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<tr>
<td></td>
<td>0.00</td>
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<tr>
<td>Plant</td>
<td></td>
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<tr>
<td>AB (g m⁻²)</td>
<td>116 (7)</td>
</tr>
<tr>
<td>BB (g m⁻²)</td>
<td>1199 (44)</td>
</tr>
<tr>
<td>PR (g m⁻²)</td>
<td>15.8 (2.4)</td>
</tr>
<tr>
<td>PB (g m⁻²)</td>
<td>4.7 (4.7)</td>
</tr>
<tr>
<td>BB/AB</td>
<td>10.15 (5.56)</td>
</tr>
<tr>
<td>Soil microorganisms</td>
<td></td>
</tr>
<tr>
<td>Total FAs (nmol g⁻¹)</td>
<td>19.4 (0.5)</td>
</tr>
<tr>
<td>Ba FAs (nmol g⁻¹)</td>
<td>12.2 (0.4)</td>
</tr>
<tr>
<td>Fu FAs (nmol g⁻¹)</td>
<td>0.399 (0.022)</td>
</tr>
<tr>
<td>AMF FAs (nmol g⁻¹)</td>
<td>0.750 (0.022)</td>
</tr>
<tr>
<td>F/B</td>
<td>0.031 (0.001)</td>
</tr>
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</table>

Values are means (SE) of five replicate plots for each level of N enrichment. Different letters indicate significant differences among the levels of N enrichment (one-way ANOVA, P < 0.05). Plant variables include above-ground biomass (AB), below-ground biomass (BB), biomass of perennial rhizome species (PR), biomass of perennial bunchgrasses (PB) and BB : AB ratio (BB/AB). Soil microbial variables include bacteria (Ba), fungi (Fu), arbuscular mycorrhiza fungi (AMF) and fungi : bacteria ratio (F/B).

The pathways of soil microbial communities together explained 75% of the total variance of microbial respiration in that decreases in microbial respiration in response to N enrichment were closely associated with decreases in total FAs and microbial components (i.e., Ba FAs, Fu FAs and AMF FAs) and increase in F/B (Fig. 4). Linear regression further confirmed that total FAs, Ba FAs, VAM FAs, and F/B explained 46–68% of the variation in microbial respiration (Fig. 6). The total FAs, Ba FAs, and VAM FAs showed positive linear relationships with microbial respiration while a negative linear relationship with microbial respiration. In addition, the acid addition experiment showed that the total soil respiration and soil microbial respiration increased with soil pH while root respiration declined with soil pH for both experiments (Fig. 3g–i).

Discussion

BIOTIC RESPONSES TO LONG-TERM N ENRICHMENT

Our results demonstrate that, in systems subjected to long-term N enrichment, increases in plant biomass and shifts in plant community structure result from both the pathways of N availability and soil acidification. Our observation of dramatic increases in above- and below-ground biomass under N enrichment and associated with the soil N availability pathway is consistent with previous meta-analyses at the global-scale, which indicated that plant biomass in grasslands increases with increasing N supply because plant growth is generally limited by N availability (Xia & Wan 2008; Liu & Greaver 2010). With respect to plant community structure, we found a strong compensatory shift in biomass between the two dominant plant functional groups (i.e., PRs and PBs) across both the N

Fig. 5. Relationships between root respiration and below-ground biomass (a) and biomass of perennial rhizome species (PR) (b), plus responses of specific root respiration (c) and root N content (d) of the five dominant species [belonging to PR or perennial bunchgrasses (PB)] to N enrichment. Error bars represent means of five replicate plots. Regression was estimated using a linear model. Statistics ($r$ and $P$ values) for linear regression are also indicated (***, $P < 0.001$). PB species include Stipa grandis (Sg), Achnatherum sibiricum (As), and Agropyron cristatum (Ac). PR species include Leymus chinensis (Lc) and Carex korshinskii (Ck).

Fig. 6. Relationships between soil microbial respiration and total fatty acids (FAs) (a), Ba FAs (b), VAM FAs (c) and F : B ratio (d). Regression was estimated using a linear model. Statistics ($r^2$ and $P$ values) for linear regression are also indicated (***, $P < 0.001$).
enrichment and acid addition levels. This strong compensatory shift in biomass may be explained by the higher H\(^{+}\), Al\(^{3+}\) and NH\(_{4}^{+}\) tolerances (Van Den Berg et al. 2005; Chen et al. 2013) and N use efficiency (Bai et al. 2010) of PRs than PBs.

Our study also showed that the soil acidification pathway rather than the N availability pathway explains most of the effects of long-term N enrichment on soil microorganisms. We found that N enrichment negatively affected total, bacterial, fungal biomass and AMF biomass. These results are consistent with previous studies, which indicated that total biomass (Treseder 2008), bacterial biomass, fungal biomass (Treseder 2008) and AMF biomass were reduced by N enrichment. Several mechanisms have been proposed to explain how soil acidification suppresses microbial biomass under N enrichment. First, the increases in soil H\(^{+}\) caused by N enrichment greatly affect the microbial community composition, as evidenced in our acid addition experiment in which we found that a high level of acid addition also determined the total, AMF and bacterial FAs. Second, loss of base mineral cations and especially of Ca\(^{2+}\) and Mg\(^{2+}\) has been linked to increased susceptibility of microorganisms to various stresses and with a decline in soil microbial biomass. Third, acidification could also change the physiological capabilities of microorganisms and decrease extracellular enzyme activity (Waldrop & Zak 2006). Although N enrichment may increase litter production and thereby increase the supply of organic C to microorganisms, this positive effect on microorganisms was more than offset by the negative effects of soil acidification.

**ALTERATION OF SOIL RESPIRATION IN RESPONSE TO N ENRICHMENT**

Our results also indicated that differential responses of soil microbial and root respiration to N enrichment may largely stem from different responses to plant and soil microbial communities. N enrichment caused a decline in total soil respiration because the negative impact of N enrichment on microbial respiration was greater than its positive impact on root respiration. The decline in microbial respiration coincided with substantial declines in total microbial biomass and changes in the soil microbial community structure. Previous reports have proposed that the N-induced reduction of C input into soil explains the negative effects of N enrichment on microbial respiration (Janssens et al. 2010). According to this explanation, a large increase in N availability reduces the need for plants to invest C in N-absorbing fine roots and mycorrhizal fungi, and induces a shift in C allocation towards above-ground tissue production (Litton, Raich & Ryan 2007). This reduction in below-ground carbon allocation has been shown to directly reduce rhizosphere respiration and thus microbial respiration (Phillips & Fahey 2007; Sayer, Powers & Tanner 2007). However, our data indicate that N enrichment increases above-ground litter production, root biomass production and litter quality because of a shift in plant community composition. These observed patterns show that the quantity and quality (indicated by C : N ratio) of plant-derived C allocated to soil increases with increasing N enrichment rate, although N enrichment reduced the proportion of gross primary production allocated to soils, i.e., the BB : AB ratio decreased. Since soil microorganisms are often C-limited (Treseder 2008; Janssens et al. 2010), one might reason that the increase in plant-derived C input to soils should lead to higher microbial respiration. However, our results of significantly lower microbial respiration indicate that other factors rather than C allocation dominated N-addition effects in our experiment.

Consideration of both our N enrichment experiment and acid addition experiment indicates that the reduction in microbial respiration caused by long-term N enrichment is driven more by soil acidification than N availability. This decline in microbial respiration was associated with declines in the biomass of most components of the microbial community. That acidification could greatly affect soil microorganisms in the studied grassland is consistent with the higher leaching of soil base cations of the sandy soil. Other studies have also demonstrated that soil acidification can have detrimental effects on microbial community structure or function, and thus on the decomposition of plant litter and soil organic matter (SOM) (Fog 1988; Janssens et al. 2010). Microorganisms that are less tolerant to acidic soil are likely to be out-competed by those microorganisms that are tolerant (Rousk, Brookes & Bäath 2009). Results from both of our experiments showed greater changes in bacterial FAs than fungi FAs (see Fig. S5), which suggests that a decline in bacteria rather than the slight decline in fungi played a larger role in determining the response of microbial respiration to N enrichment. In addition, soil acidification and accompanying shifts in the composition of the microbial community could reduce the activity or expression of key extracellular microbial enzymes and hence reduce the decomposition of SOM (Waldrop & Zak 2006). Taken together, our results indicate that the N enrichment-induced decline in microbial respiration was mainly mediated by the soil acidification pathway and the associated shifts in the soil microbial community.

In contrast with microbial respiration, root respiration increased in response to N enrichment, and this increase was associated with an increase in BB, root N content and specific root respiration of the dominant plant species. Root respiration (including root, mycorrhizal and rhizosphere respiration) is a major component of total soil CO\(_{2}\) efflux (Kuzyakov 2006) and is closely related to below-ground productivity and root N content (Burton & Pregitzer 2002; Chen et al. 2010). Our results show that the BB and root N content of the five dominant plant species increased with N enrichment; these changes are consistent with a previous meta-analysis (Xia & Wan 2008). Root respiration of plant community reflects the specific root respiration rates (respiration per unit dry mass) of the
plant species in that stand (Burton et al. 2012) and the specific root respiration of the five dominant plant species at our study site also increased with N enrichment due to their increase in root N content. In addition, the shift in the plant community towards PRs may also have contributed to the increase in root respiration because PRs have a higher specific root respiration than PBs. This difference in specific root respiration between species in the two plant function groups may have contributed to the higher root N content of PRs than PBs. The N enrichment-induced increase in BB and shift in the plant community towards PRs were also found in the acid addition experiment (see Fig. S5). This further confirms that the increase in root respiration following N enrichment may occur mainly via the soil acidification pathway. It is important to note that the measurement of soil respiration was conducted in the growing seasons, in which the root respiration may be maximal relative to the whole year. Therefore, data from non-growing seasons (root respiration may be lower at that moment) warrants further research in the arid and semi-arid grasslands. Overall, the positive effect of N enrichment on root respiration was substantially smaller than the negative effect on microbial respiration in our experiments, leading to a significant reduction in total soil respiration.

UNCERTAINTIES REGARDING THE MICROBIAL AND ROOT RESPIRATION

It is important to note that our estimation of the microbial and root respiration includes some uncertainties. First, the trenching method separating total CO2 efflux into microbial and root respiration components has inherent drawbacks in that the residual roots undoubtedly affect CO2 efflux by changes in soil environmental conditions and microbial community (Kuzuyakov 2006; Subke, Inglima & Cotrufo 2006). Although our investigation showed that 2-year trenching treatment did not alter the soil moisture, the total microbial biomass (i.e., bacterial biomass, fungal biomass and F/B) were lower in the trenched subplots (see Fig. S1). Therefore correcting for the effects of residual roots and caused by trenching warrants further research the semi-arid grasslands. Second, the trenched subplots surrounded by polyethylene board inserted into 50 cm depth still allows root ingrowth underneath barriers (>50 cm depth) into trenched subplots, although these live roots at 50–100 cm depth contribute only 8% of total live roots (0–100 cm depth) in this semi-arid grassland. In this situation, the trenching method probably contributed to an overestimation of microbial respiration and an underestimation of root respiration (Sayer & Tanner 2010). The first two drawbacks, in part, were the reasons why our estimation of the contribution of microbial respiration to total soil respiration (72–79%) was slightly higher than the mean contribution (68%) from temperate grassland ecosystems summarized by Subke, Inglima & Cotrufo (2006). Therefore, future studies may need to trench deeper to ensure no root movement into the trenched plots, particularly in highly sandy, loose soils. Third, the estimation of soil respiration in this study was based on the period of growing season in semi-arid grassland ecosystem, while we are not sure whether the conclusion drawn during the growing season is general for the non-growing season. The seasonal pattern of total CO2 efflux and its components has been well-documented in grasslands (Raich & Schlesinger 1992; Xu & Baldocchi 2004). We therefore suggest that, with respect to the responses of total CO2 efflux and its two components to N enrichment, the results of this study might be more meaningful for growing season than non-growing season in the semi-arid grasslands.

N-INDUCED SOIL ACIDIFICATION: CAUSES AND CONSEQUENCES IN SEMI-ARID GRASSLANDS

While application of mineral N fertilizer is the main cause of acidification in agricultural soils (Guo et al. 2010; Liu et al. 2013), N and S deposition may be primary causes of soil acidification in natural systems (Likens, Driscoll & Buso 1996; Bowman et al. 2008). The impact of long-term N-induced soil acidification on semi-arid grassland in Eurasia has attracted limited attention, probably for the following reasons: (i) low rainfall slows cation depletion from soil exchangeable pools and slows cation leaching (Stewart, Capo & Chadwick 2001) and (ii) relatively high soil pH and carbonates may represent a buffer against soil acidification (Yang et al. 2012). N-induced soil acidification in semi-arid grasslands of Eurasia, however, has been increasing because the rapid expansion of industrial, vehicular and agricultural activity in semi-arid regions has greatly increased energy consumption and NOX emissions (Zhao et al. 2009). In addition, soils in semi-arid grasslands are usually sandy and generally have low buffering capacity against soil acidification (Grayston et al. 2001). The rapid decrease in soil pH in response to N enrichment reported here suggests that sandy soils in semi-arid grasslands have a very limited capacity to buffer against N deposition and acidification. Higher soil base cation losses in response to N-induced acidification in our experiment also suggest that the semi-arid grasslands, especially the agricultural grasslands, may be very vulnerable to base cation losses under future N deposition scenarios.

IMPLICATIONS FOR TERRESTRIAL ECOSYSTEM C BALANCE

Continuous N deposition remains a major concern in the rapidly developing countries of East Asia (Liu et al. 2013). Our results indicate that N enrichment reduces soil respiration primarily because soil acidification greatly reduces microbial respiration, i.e., the effects of soil acidification are greater than those of soil N availability. We therefore suggest that N-induced soil acidification should be included in models that predict C cycling in terrestrial ecosystems under future scenarios of N deposition. It is
important to point out that the N addition rates and acidity inputs used in our experimental gradient extend well beyond possible inputs from atmospheric deposition in this semi-arid grassland (Zhao et al. 2009; Liu et al. 2011) or in North American and European grasslands (0.5–3.5 g N m⁻² year⁻¹) (Bobbink et al. 2010), while they are well into the range associated with intensive agriculture inputs (Guo et al. 2010). We therefore suggest that, regarding the N-induced changes in soil respiration and C cycling, the results of this study from the low levels of N enrichment might be more meaningful for natural and semi-natural grasslands, while the high levels of N enrichment might be more meaningful for the intensively-managed grasslands.

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Data accessibility

Data are available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.rk987 (Chen et al. 2015).

References


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Supporting Information

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

**Fig. S1.** Responses of soil moisture and soil microbial community in trenched (T) and un-trenched (UT) subplots to N enrichment.

**Fig. S2.** The schematic of the specific root respiration measuring system designed to deliver controlled concentrations of CO₂ to roots with simultaneous specific root respiration measurement.

**Table S3.** Results of a principal components analysis (PCA) of four groups (soil N availability, soil base cations, plants, and soil microorganisms).

**Fig. S4.** Bivariate correlations among N levels, pH, PC1 of four groups, and the two components of soil respiration included in the SEM model.

**Fig. S5.** Responses of soil cations, plants and microbes to acid addition level.