Disruption of metal ion homeostasis in soils is associated with nitrogen deposition-induced species loss in an Inner Mongolia steppe

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Abstract

Enhanced deposition of atmospheric nitrogen (N) resulting from anthropogenic activities has negative impacts on plant diversity in grassland ecosystems globally. Several mechanisms have been proposed to explain the species loss. Ion toxicity due to N deposition-induced soil acidification has been suggested to be responsible for species loss in acidic grasslands, while few studies have evaluated the role of soil-mediated homeostasis of ions in species loss under elevated N deposition in alkaline grasslands. To determine whether soil-mediated processes are involved in changes in species composition by N deposition, the effects of 9 yr N addition on soil properties, aboveground biomass (AGB) and species composition were investigated in an Inner Mongolia steppe. Low to moderate N addition rate (2, 4, 8 g N m$^{-2}$ yr$^{-1}$) significantly enhanced AGB of grasses, while high N addition rate (> 16 g N m$^{-2}$ yr$^{-1}$) reduced AGB of forbs, leading to an overall increase in AGB of the community under low to moderate N addition rates. Forb richness was significantly reduced by N addition at rates greater than 8 g N m$^{-2}$ yr$^{-1}$, while no effect of N addition on grass richness was observed, resulting in decline in total species richness. N addition depleted base cations (Ca$^{2+}$, Mg$^{2+}$ and K$^{+}$) in soils, reduced soil pH and mobilized Mn$^{2+}$, Fe$^{3+}$ and Cu$^{2+}$ ions in soils. Soil inorganic-N concentration was negatively correlated with forb richness, explaining 27.2% variation of forb richness. The concentrations of base cations (Ca$^{2+}$ and Mg$^{2+}$) and metal ions (Mn$^{2+}$ and Cu$^{2+}$) showed positively and negatively linear correlation with forb richness, accounting for 25.9 and 41.4% variation of forb richness, respectively. These results reveal that disruption of metal ion homeostasis in soils by N addition, particularly enhanced release of soil Mn$^{2+}$ and Cu$^{2+}$ may be associated with reduction in forb richness in temperate steppe of Inner Mongolia.
1 Introduction

Nitrogen (N) is an essential nutrient for plant growth and development, and many terrestrial ecosystems are adapted to conditions of low N availability (Bobbink et al., 1998). Since the agricultural and industrial revolution, atmospheric deposition of biologically reactive N has increased drastically due to N fertilization and combustion of fossil fuels globally (Galloway et al., 2008; Canfield et al., 2010; Sutton and Bleeker, 2013), resulting in a large impact on ecosystem structure and function (De Schrijver et al., 2008). Elevated atmospheric N deposition generally has positive effects on ecosystem productivity (Smith et al., 1999; Galloway et al., 2008), while it imposes a great threat to biodiversity and stability of the terrestrial ecosystems (Stevens et al., 2004; Clark and Tilman, 2008; Bobbink et al., 2010; Jiang et al., 2010; Kim et al., 2011). Atmospheric N deposition has driven significant reductions in plant species richness in different grassland ecosystems (Bai et al., 2010; Clark and Tilman; 2008; Dupre et al., 2010; Van Den Berg et al., 2011), substantially altering the structure and functioning of grassland ecosystems (Cardinale et al., 2012; Yang et al., 2012a). Both large scale field survey and manipulated experiments to simulate N deposition have shown that N deposition can drive species loss in grasslands globally (Stevens et al., 2004; Suding et al., 2005). The decline in species richness by N deposition results in changes of community composition and reduction in ecosystem productivity consequently (Isbell et al., 2013).

Several hypotheses have been proposed to explain the N deposition-induced species loss in grassland ecosystems, such as NH$_4^+$ toxicity to plants (van den Berg et al., 2005; Stevens et al., 2006; Zhang et al., 2014), phosphorus limitation (Phoenix et al., 2003), mobilization of toxic metals in soils (Bowman et al., 2008; Stevens et al., 2009; Chen et al., 2013) and changes in soil microbial activity and biodiversity (Dean et al., 2014). Competitive exclusion driven by enhanced resource uptake by dominant species and preemption of light or space has been widely invoked as a key mechanism for species loss under elevated N regimes (Clark and Tilman, 2008; Hautier et al., 2009; Suding...
et al., 2005; Borer et al., 2014). For instance, it has been suggested that chronic N de-
position shifts grassland towards grass-dominated vegetation due to higher productivity
of grasses at elevated N concentrations, which is thought to outcompete herbaceous
species, forbs and shrubs (Heil and Diemont, 1983; Bobbink et al., 1998; Stevens
et al., 2006). However, N addition-induced reductions in plant biodiversity cannot sim-
ply be explained by competitive exclusion, because many species had already dis-
appeared before grasses became dominant (Houdijk et al., 1993) and fertilization also
reduces plant biodiversity of grassland even when light is not limiting (Dickson and Fos-
ter, 2011). Although extensive research has demonstrated that N deposition reduces
biodiversity, the primary mechanism underlying the N deposition-induced changes in
community composition remains largely unknown (Stevens et al., 2006; Phoenix et al.,
2003; Bowman et al., 2008; Clark and Tilman, 2008; Hautier et al., 2009; Suding et al.,
2005; Borer et al., 2014). In addition to competition hypothesis, belowground interac-
tions, such as response to N form (van den Berg et al., 2005) and acquisition of toxic
metals (Bowman et al., 2008; Horswill et al., 2008; Stevens et al., 2009), has also been
implicated in species loss induced by N deposition.

N deposition often concurs with soil acidification (Stevens et al., 2004; Bobbink et al.,
2010; Fang et al., 2012; Horswill et al., 2008; Yang et al., 2012b). Soil acidification sub-
sequently mobilizes some metal ions, thus rendering phytotoxicity to plants at high
concentrations (Kochian, 1995; Marschner, 1995). For instance, release of toxic alu-
minum (Al$^{3+}$) ions due to soil acidification has been suggested to be a driving force for
N deposition-induced species loss in grasslands (Carnol et al., 1997; Horswill et al.,
2008; Chen et al., 2013). Homeostasis of ions in soil depends on soil properties, for
instance, soil pH affects transformation of nitrogen forms (Dorland et al., 2004) and
the magnitude of availability of nutrients is closely dependent on soil pH (Marchner,
1995; Bowman et al., 2008). The involvement of soil acidification-mediated processes
in species loss under elevated N deposition has been extensively evaluated in acidic
grasslands (Stevens et al., 2006; Bowman et al., 2008; Horswill et al., 2008). Whether
this mechanism is also responsible for N deposition-induced changes in plant biodiver-
sity in other type’s grassland remains largely unknown.

Inner Mongolia grassland is an important part of widely distributed grasslands across
the Eurasian Steppe with typical calcareous soil distinguished by high pH and buffering
capacity due to abundant base cations (Chen et al., 2013). Although calcareous steppe
has higher species richness than acid grassland, it may be more sensitive to soil acidi-
fication (van der Putten et al., 2013). N deposition rate in China has increased dramat-
ically in recent decades (Liu et al., 2013; Jia et al., 2014), thus imposing great threats
to plant biodiversity in grassland ecosystems there. Moreover, a significant soil acidifi-
cation in grasslands across northern China over the past two decades has been reported
(Yang et al., 2012b). Soil acidification associated with N deposition in calcareous grass-
lands has been implicated in species loss (Roem and Berendse, 2000). In contrast to
acidic grasslands, few studies have investigated the role of soil acidification-induced
metal mobilization in species richness in calcareous grasslands under conditions of
elevated N deposition. To evaluate the role of soil-mediated chemical processes in N
deposition-induced changes in species composition, the chronic effects of N addition,
which simulates N deposition, on soil pH, nutrient availability and species composition
were investigated in a temperate steppe of Inner Mongolia. Our results demonstrate
that soil acidification-induced mobilization of Mn$^{2+}$ and Cu$^{2+}$, especially Mn$^{2+}$, played
an important role in loss of forb species by elevated N deposition.

2 Materials and methods

2.1 Study site

The field experiment was carried out in Duolun County (116°17′ E, 42°02′ N), Inner
Mongolia, China. The experiment site is located in a semiarid temperate steppe with
mean annual temperature of 2.1°C. Mean annual precipitation is 382.2 mm with ap-
proximately 60–80 % fallen from May to August. Soil in the site is classified as chest-
nut type according to China’s soil classification system (Hou, 1982) and calcic-orthic aridisol based on ISSS Working Group RB, 1998. Soil is composed of 62.75 ± 0.04 % sand, 20.30 ± 0.01 % silt and 16.95 ± 0.01 % clay. Mean soil bulk density and soil pH is 1.31 gcm$^{-3}$ and 6.84 respectively. The net N mineralization rates in this area were $-0.04$ to $0.52 \mu gN^{-1} g^{-1}$ during the growing seasons (Zhang et al., 2012). The ambient total N deposition in this region was about 1.6 gNm$^{-2}$ yr$^{-1}$ (Zhang et al., 2008). Vegetation in this area is dominated by perennial species, including *Stipa krylovii*, *Artemisia frigida*, *Potentilla acaulis*, *Potentilla tanacetifolia*, *Dianthus chinensis*, *Heteropappus altaicus*, *Cleistogenes squarrosa*, *Allium bidentatum*, *Leymus chinensis*, *Carex korshinskyi*, *Salsola collina*, *Melilotoides ruthenica*, *Agropyron cristatum*.

2.2 Experiment design

The experiment site was fenced to exclude livestock grazing in July 2003. A total of 64 plots (15 m x 10 m) were established and each of them was spaced by a 4-m-width buffer strip. Eight levels of N addition (0, 1, 2, 4, 8, 16, 32, 64 gNm$^{-2}$) were added as urea (N, 46 %) with eight replicates by evenly spreading with hand in July every year since 2003. In our study, samples were collected from 48 plots supplemented with six levels of N addition (0, 2, 4, 8, 16, 32 gNm$^{-2}$).

2.3 Determination of community biomass and composition, and soil sampling

Species composition and Aboveground biomass (AGB) classified artificially as forbs and grasses was conducted at the peak biomass time in the middle of August in 2012 using a randomly selected quadrat (1 m x 1 m) of each plot. Aboveground biomass was harvested by clipping every quadrat completely above the soil surface, and both of living and dead parts were separated. Biomass was measured separately after samples were oven-dried at 75°C for 48 h.

Soil samples were collected from each quadrat. Topsoil samples (0–10 cm below the litter layer) and subsoil samples (20–30 cm deep) were taken randomly using a 10 cm
diameter soil auger. Three-core soils were combined to one sample per quadrat. In this study, only soil samples from 0–10 cm layers were used. All soil samples were kept cool during transit and air dried in the laboratory. Soil samples were thoroughly mixed and sieved through a 2 mm mesh for laboratory analysis of soil pH and exchangeable ion concentrations.

2.4 Measurements of soil pH and electrical conductivity (EC)

For determination of soil pH, 6 grams of air-dried soil was shaken with 15 mL CO₂-free deionized water for a minute, and equilibrated for an hour to determine pH with a pH meter (HANNA, PH211, Italy). Water soluble salts in the soil solution are strong electrolytes to be electrical. The electric conduction can be expressed as electrical conductivity (EC). The content of salts in the solution is positively correlated with EC, and EC can be determined by a conduct meter to represent the content of ions in soil. For determination of soil EC, 10 grams of air-dried soil was shaken with 50 mL CO₂-free deionized water for three minutes, and filtered to get clear leachate for determination with the conduct meter (METTLER TOLEDO, FE30, Switzerland). EC was calculated with the following formula:

\[ L = C \cdot f_t \cdot K \]

\( L \) indicates electrical conductivity with 1 : 5 soil leachate at 25°C;
\( C \) indicates displayed electrical conductivity on the conduct meter;
\( f_t \) indicates correction coefficient of temperature;
\( K \) indicates electrode constant.

2.5 Determination of available soil P and inorganic N concentrations

Available P (Olsen-P) in soil was determinate by extracting 10 grams of air-dried soil with 50 mL 0.5 M NaHCO₃ (pH 8.5) for 30 min at 25°C, and analyzed after filtering by
molybdenum blue-ascorbic acid method (Olsen et al., 1954) with a UV-visible spectrophotometer (UV-2550, SHIMADZU Corporation, China).

Soil inorganic N (NH$_4^+$-N and NO$_3^-$-N) that was determined using fresh soil was analyzed calorimetrically using a continuous-flow analyzer (Seal XY-2, Australia) after extraction of 2 M KCl at the ratio of 1 : 5 (w/v) (Mulvaney, 1996; Wendt, 1999).

### 2.6 Determination of soil Fe$^{3+}$, Mn$^{2+}$, Cu$^{2+}$, Zn$^{2+}$ and Al$^{3+}$

The exchangeable Mn$^{2+}$, Fe$^{3+}$, Cu$^{2+}$, Zn$^{2+}$ in the soil were extracted with an extracting agent (pH 7.3) consisted of 5 mM diethylenetriamine pentaacetic acid (DTPA), 10 mM CaCl$_2$ and 0.1 M triethanolamine (TEA) in 1 : 2 ratio (w/v) for 2 h (Lindsay and Norvell, 1978). Exchangeable Al$^{3+}$ in the soil was extracted by 0.1 M BaCl$_2$ (pH 5.3) at the ratio 1 : 5 (w/v) for 30 min (Bowman et al., 2008). After filtering, samples were stored frozen prior to analysis by ICP-OES (Thermo Electron Corporation, USA).

### 2.7 Measurements of soil exchangeable Ca$^{2+}$, Mg$^{2+}$, K$^+$

Base cations (Ca$^{2+}$, Mg$^{2+}$, K$^+$) in the soil were extracted by 1 M NH$_4$OAc (pH 7.0) at a 1 : 10 ratio (w/v) for 30 min. The extraction solution was filtered to determine the concentration of Ca$^{2+}$, Mg$^{2+}$, K$^+$ by ICP-OES.

### 2.8 Statistical analysis

One-way ANOVA (Duncan’s test) was used to evaluate the difference between species richness and AGB under six levels of N addition. Linear regression was used to identify the significance of the correlation between soil exchangeable ions and pH, species richness and AGB, respectively (SPSS 17.0). Stepwise regression was used to identify potential predictors of variables of soil pH and N addition (SPSS 17.0).

Principal component analysis (PCA) was used to extract the principal component of variables of the exchangeable ions and to group them in terms of their high loading on principal axis (R. i386 3. 0. 3). Redundancy analysis (RDA) is used to explore to what
extent that species richness and AGB can be explained by ion concentrations in soils and which variables are responsible for N addition-induced species loss and changes in AGB (R. i386 3. 0. 3).

3 Results

3.1 N addition enhanced aboveground biomass and reduced species richness

At the community level, N addition at low rates stimulated plant growth and increased aboveground biomass (AGB) of the steppe, and AGB peaked at 4 g m$^{-2}$ yr$^{-1}$, and further increases in N addition rates led to decline in AGB, such that AGB values in plots added with 16 and 32 g N m$^{-2}$ yr$^{-1}$ were comparable to those in control plots (Fig. 1a). A similar pattern of N addition-induced increase in AGB of grasses was found (Fig. 1c). N addition at low rates (2–8 g N m$^{-2}$ yr$^{-1}$) had no effect on AGB of forbs, while it significantly reduced AGB of forbs at 16 g N m$^{-2}$ yr$^{-1}$ (Fig. 1c). Therefore, the increase in total AGB was driven entirely by the increase of grass biomass. In contrast to AGB, total species richness was significantly reduced at N addition rates of greater than 8 g N m$^{-2}$ yr$^{-1}$, and the magnitude of reduction was positively dependent on N addition rate (Fig. 1b). Moreover, grass richness was relatively insensitive to N addition, while a decline in forb richness was detected at 8 g N m$^{-2}$ yr$^{-1}$, and the decline was increased with increasing N addition rate (Fig. 1d). These results indicate that the reduction in total species richness by N addition is accounted for by the loss of forb species.

3.2 N addition acidified soil and increased soil electrical conductivity

In addition to productivity and species richness, N deposition in terrestrial ecosystems often acidifies soils (Stevens et al., 2004; Bobbink et al., 2010). Therefore, we investigated the effect of N addition on soil pH. Soil pH significantly reduced with increase of N addition rates, such that N addition rate at 16 and 32 g N m$^{-2}$ yr$^{-1}$ for 9 yr reduced soil pH from 6.82 to 6.29 and 5.37, respectively (Fig. 2). Electrical conductivity (EC) of soil
is an indicator to reflect exchangeable ion concentrations in soil (Friedman, 2005). N addition caused linear increase in soil EC ($r^2 = 0.36, P < 0.0001$) (Fig. S1 in the Supplement), indicating that N addition may lead to solubilization of some nutrients from soil minerals.

3.3 N addition altered availabilities of mineral nutrients

Soil inorganic N concentrations were significantly increased by N addition rate greater than 8 g N m$^{-2}$ yr$^{-1}$ (Fig. 3a). There was a significantly positive correlation ($P < 0.001$) between N addition rate and inorganic N (Table S1 in the Supplement). A significant increase in soil available P (Olsen-P) was detected at high doses of N addition (> 16 g N m$^{-2}$ yr$^{-1}$), whereas soil Olsen-P was not affected by low doses of N addition (Fig. 3a). Calcium (Ca$^{2+}$), magnesium (Mg$^{2+}$) and potassium (K$^+$) are main base cations in soils of calcareous grasslands. N addition across the rates used in the present study generally led to significant decline in these cation concentrations (Fig. 3b). A positively significant correlation existed between N addition rates and concentrations of Ca$^{2+}$ and Mg$^{2+}$ (Table A1). The positive correlation of Ca$^{2+}$ and Mg$^{2+}$ with soil pH indicates that soil acidification is likely to be a key cause for reduction in soil Ca$^{2+}$ and Mg$^{2+}$. In contrast to the base cations, N addition resulted in significant increases in availabilities of several metal ions, such that concentrations of iron (Fe$^{3+}$), manganese (Mn$^{2+}$), copper (Cu$^{2+}$) and aluminum (Al$^{3+}$) in soils were significantly enhanced by N addition (Fig. 3c). N addition-induced increases in soil Fe$^{3+}$ and Mn$^{2+}$ concentrations were most evident compared to other metal ions (Fig. 3c). Moreover, concentrations of Fe$^{3+}$, Mn$^{2+}$, Cu$^{2+}$ and Al$^{3+}$ were positive and negative response to N addition and soil pH, respectively (Table S1). These results suggest that N addition-induced soil acidification is a driver for mobilization of these metal cations.

Further stepwise regression analyses between these ion concentrations and soil pH indicated that Mn$^{2+}$ was the most closely related to soil pH, which individually explained 84.6 % of the variation of soil pH, while Cu$^{2+}$ and Al$^{3+}$ explained 1.6 and 1.2 % of the variation of soil pH, respectively. Similarly, the results of stepwise regression analysis
between concentrations of these ions and N addition rates showed that Mn\(^{2+}\) was mostly affected by N addition, with 88.6\% of variation reflected by the change in Mn\(^{2+}\), while inorganic-N, Fe\(^{3+}\), and Al\(^{3+}\), Cu\(^{2+}\) only showed 4.7, 1.7, 1.1\%, and 0.5\% of the variation, respectively.

3.4 Soil ion concentrations were correlated with AGB and species richness

To test whether the N addition-induced changes in soil nutrient and metal ions are involved in decline in species richness under N-added regimes, correlations among nutrient availabilities, aboveground biomass and species richness of forbs and grasses were explored. A negatively linear relationship between six soil nutrients (inorganic N, Olsen-P, Fe\(^{3+}\), Mn\(^{2+}\), Cu\(^{2+}\), Al\(^{3+}\)) and forb aboveground biomass and species richness was observed (Figs. 4 and 5). Soil Ca\(^{2+}\) (\(r^2 = 0.26, P = 0.0002\)) and Mg\(^{2+}\) (\(r^2 = 0.17, P = 0.0038\)) only exhibited positive correlation with forb species richness (Fig. 5). In contrast, both richness and aboveground biomass for grasses were not affected by the majority of nutrient ions, with their AGB showing no significant correlation with these ions and their species richness exhibiting negative correlation with Mn\(^{2+}\), Cu\(^{2+}\) and Al\(^{3+}\) concentrations (Figs. 4 and 5). Other soil metals such as K\(^{+}\) and Zn\(^{2+}\) exhibited no significant correlation with both AGB and species richness for grass and forb (Figs. 4 and 5). Therefore, these results rule out the possibility that changes in soil K\(^{+}\) and Zn\(^{2+}\) may contribute to decline in plant diversity in alkaline grasslands by N deposition.

3.5 Principle component and redundancy analysis

Principle Components Analysis (PCA) gave two axes of variation with eigenvalues greater than one, accounting for total 70.5\% of the variation in ten exchangeable ions variables (Fig. 6). The first principal component (PC1) based on strong positive loadings on axis 1 (Table 1) included Mn\(^{2+}\), Cu\(^{2+}\), Fe\(^{3+}\), Inorganic-N, Al\(^{3+}\), and Olsen-P, which explained 51.8\% of the variation (eigenvalues = 5.179). Linear regression showed that this group had significant correlation with soil pH (\(P < 0.001\)), indicat-
ing that PC1 is mainly pH-dependent (Table S1). Due to higher loadings on the axis 2, Ca\(^{2+}\), Mg\(^{2+}\) and K\(^+\) were clustered into the second principal component (PC2), accounting for 18.7% of the variation (eigenvalues = 1.874) (Fig. 6). Ca\(^{2+}\), Mg\(^{2+}\) and K\(^+\) were the fundamental constitutes of alkaline soils and play an important role in acid-buffering, thus the PC2 reflected the basic soil properties. Zn\(^{2+}\) had little contribution for variation in soil exchangeable ions.

Redundancy analysis (RDA) was conducted to further assess the extent to which significant species richness and aboveground biomass are affected by principal soil ions (Table 2). Based on PCA analysis, only soil ions in PC1 and PC2 were used for RDA. High doses of N addition reduced species richness and simultaneously enhanced P availability in soils (Figs. 1 and 3a), thus discounting the role of P deficiency in the species loss. Therefore we did not include P in the RDA analysis. We also excluded soil K\(^+\) in the RDA analysis because no significant correlation between soil K\(^+\) concentrations and AGB/species richness of grasses and forbs was detected (Figs. 4 and 5). Moreover, as a significant increase in soil Al\(^{3+}\) concentration was detected only in high rate of N addition (Fig. 3c), soil Al\(^{3+}\) was not included in the RDA analysis. Because forbs can only absorb Fe\(^{2+}\) (Marchner, 1995), an increase in Fe\(^{3+}\) in soil would have little effect on forbs, soil Fe\(^{3+}\) was also excluded in the RDA analysis. The remaining five variables were classified into three groups, i.e., the macro-element group (Inorganic-N), the heavy metal group (Mn\(^{2+}\) and Cu\(^{2+}\)) and the base cation group (Ca\(^{2+}\) and Mg\(^{2+}\)). Inorganic-N accounted for 27.2% of the variation in forb richness and 23.6% of the variation in forb aboveground biomass, while they can explain only 7.6 and 0.4% of the variation in grass species richness and biomass, respectively (Table 2). In contrast, soil Mn\(^{2+}\) and Cu\(^{2+}\) explained a higher proportion of the changes in both the species richness and aboveground biomass of forbs. For instance, 41.4 and 21.3% of the variation in forb species richness and aboveground biomass was explained by soil Mn\(^{2+}\) and Cu\(^{2+}\), respectively (Table 2). Variation in grass species richness and aboveground biomass was less explained by soil Mn\(^{2+}\) and Cu\(^{2+}\) than that in forbs, accounting for approximately 11.8 and 3.9% respectively. Similarly, soil Ca\(^{2+}\) and Mg\(^{2+}\) explained more
variation in forbs richness than in grass richness (Table 2). Both aboveground biomass of forbs and grasses were little affected by the base cations, explaining only 3.0 and 1.9 % of the variation (Table 2). These results suggest that metal ions, especially Mn$^{2+}$ and Cu$^{2+}$, are likely to be primary factors that drive loss of forb species by N addition in temperate alkaline grasslands.

4 Discussion

Nitrogen deposition can have multiple impacts on grassland ecosystems, including changes in productivity, reductions in species richness and soil acidification (Smith et al., 1999; Stevens et al., 2004; Galloway et al., 2008; Clark and Tilman, 2008; Bobbink et al., 2010). Elevated N deposition resulting from human activities in the past decades has negative impacts on growth and development of certain plant species, leading to reduction in plant diversity (Isbell et al., 2013; Maskell et al., 2010; Stevens et al., 2006). To evaluate the effects of N deposition on temperate grassland ecosystems, long-term N fertilization experiments were conducted in Inner Mongolia steppes by applying urea. The applied urea can be hydrolyzed to ammonia/ammonium, by the enzyme urease, and ammonium is further converted into nitrate by nitrification, leading to an increase in inorganic N in soils. Our results that the application of urea led to significant increases in soil nitrate concentrations (Fang et al., 2012) are consistent with this proposition. Therefore, our N addition experiments can simulate the natural N deposition. In the present study, we found that low and moderate rate of N addition, which simulate atmospherics N deposition, for consecutive 9 yr led to an enhanced total AGB (Fig. 1a). More specifically, we found that AGB of grasses and forbs displayed different responses to N addition, such that AGB of grasses and forbs was increased and relatively unchanged by moderate N addition, respectively (Fig. 1c). Species richness of grasses and forbs also differed in their responses to N addition. N addition significantly reduced forb species richness, while grass species richness was relatively unchanged in response to the N addition (Fig. 1d). These findings that forbs were more sensitive to
N deposition than grasses in terms of species richness are consistent with the results obtained in Europe grasslands (Stevens et al., 2006).

Species loss induced by N deposition on grasslands has been suggested to result from competition due to increase growth of grasses in response to N enrichment (Stevens et al., 2006). Although moderate rate of N addition, i.e. 2 and 4 g m\(^{-2}\) yr\(^{-1}\), stimulated grass growth, the total species richness and forb species richness under these N addition rates were relatively constant (Fig. 1). Moreover, at higher rates of N addition (16 and 32 g m\(^{-2}\) yr\(^{-1}\)), AGB of grasses was not enhanced, but species richness of forbs was dramatically reduced (Fig. 1). These results may suggest that loss of forbs is not simply be caused by competitive exclusion driven by increased growth of grass, rather these findings may highlight the involvement of other processes associated with N addition in inhibition of forb growth.

Enhanced N deposition may decrease plant diversity by disrupting nutrient homeostasis in soils, such as excess NH\(_4^+\) (van den Berg et al., 2005; Stevens et al., 2006; Zhang et al., 2014) and P limitation (Phoenix et al., 2003; Carroll et al., 2003). In our study, N addition led to significant increases in inorganic-N in soils (Fig. 3a). Forb species richness and aboveground biomass was negatively correlated to soil inorganic-N concentration, whereas grass did not response to increased inorganic-N (Figs. 4 and 5). Moreover, concentrations of inorganic-N in soil respectively accounted for 27.2 and 23.6 % of the variation in forb richness and aboveground biomass (Table 2). These results suggest that an increase in N availability due to N addition may directly contribute to N deposition-induced loss of forb species. Stevens et al. (2006) and Zhang et al. (2014) demonstrated that species richness is negatively correlated with soil NH\(_4^+\)-N concentrations in acidic grasslands and alkaline grasslands, respectively. Processes associated with N transformation in soils, including mineralization and nitrification, depend on soil pH, which determine homeostasis of NH\(_4^+\)-N and NO\(_3^-\)N (Dorland et al., 2004). Although it has been reported that species from acidic and alkaline soils usually prefer different forms of nitrogen (Falkengren-Grerup and Lakkenborg-Kristensen, 1994; van den Berg et al., 2005), both high NO\(_3^-\)N concentration and high NH\(_4^+\)-N con-
centration can suppress root elongation (Tian et al., 2005, 2009; Britto and Kronzucher, 2002). Therefore, it is necessary to further dynamically monitor changes in N forms in soils after N addition and to evaluate the different response of forbs and grasses to different N forms. In addition to increase of N availability, N deposition-induced P limitation has been implicated in the reduction of species richness (Phoenix et al., 2003). However, in the present study, N addition significantly increased Olsen-P concentration in soils (Fig. 3), suggesting that N addition is unlikely to result in P limitation, thus discounting the involvement of P deficiency in N-induced changes in species richness.

Soil acidification often concurs with N deposition due to the formation of hydrogen ions during ammonium nitrification (Guo et al., 2010; Yang et al., 2012b). Numerous studies across N deposition gradients (Maskell et al., 2010; Stevens et al., 2004) and field fertilization experiments (Bowman et al., 2008; Lan and Bai, 2012; Zhang et al., 2014) have demonstrated that N deposition leads to soil acidification. In the present study, we found a significant soil acidification by 9 yr N addition in the calcareous temperate grassland (Fig. 2a). Although relationship between species richness and soil pH is dependent upon vegetation types and climatic conditions (Chytrý et al., 2007), a positive correlation between soil pH and the overall species richness was found in this study (Fig. S1). Chytrý et al. (2007) also reported that the species richness-pH relationship tends to be positive in acidic to neutral soils. The positive correlation between soil pH and the overall species richness suggests that soil pH is an important factor in determination of species richness in Inner Mongolia steppe. Soil acidification would disturb nutrient homeostasis in soil, including depletion of base cations and mobilization of metal cations (Bowman et al. 2008; Horswill et al., 2008). Ca$^{2+}$, Mg$^{2+}$ and K$^+$ are dominant base cations in calcareous soils. Depletion of these base cations in soils by N addition (Fig. 3b) renders the soil less capable to buffer their soil pH. The insignificant correlation between K$^+$ concentrations and AGB and species richness of forbs rules out the possibility that changes in soil K$^+$ may contribute to decline in plant diversity (Figs. 4 and 5). The depletion of Ca$^{2+}$ and Mg$^{2+}$ explained 25.9 % of the vari-
ation of forb species richness (Table 2), implying that depletion of Ca\(^{2+}\) and Mg\(^{2+}\) may account for loss of forb species by N addition in our systems.

In addition to depletion of base cations, soil acidification can release some metal ions by increasing their solubility. N addition markedly enhanced concentrations of Mn\(^{2+}\), Fe\(^{3+}\) and Cu\(^{2+}\) in soils (Fig. 3c), and Mn\(^{2+}\), Fe\(^{3+}\) and Cu\(^{2+}\) concentrations were negatively correlated with forb species richness and aboveground biomass (Figs. 4 and 5), explaining more variation on forb species richness than inorganic-N and the base cations (Table 2). These results suggest that the release of metal cations is a main driving force for N addition-evoked loss of forb species. In contrast to Mn\(^{2+}\), Fe\(^{3+}\) and Cu\(^{2+}\), an increase in soil Al\(^{3+}\) concentration was not detected with N addition < 32 g m\(^{-2}\) yr\(^{-1}\) (Fig. 3c), while loss of forb species had already occurred at moderate N addition rate (8 g m\(^{-2}\) yr\(^{-1}\)) (Fig. 1d). Moreover, PCA analysis indicates that soil Mn\(^{2+}\), Fe\(^{3+}\) and Cu\(^{2+}\) concentrations had higher loadings on PC1 than Al\(^{3+}\) concentrations (Fig. 6 and Table 1). These results may imply that soil Al\(^{3+}\) is unlikely to be a key driving factor for species loss evoked by N deposition in our experimental systems. Several studies have demonstrated that Al toxicity is involved in N deposition-induced species loss in acidic grasslands with soil pH < 5 (Stevens et al., 2009). In our studies, soil pH was greater than 5 even under the highest N addition rate (Fig. 2). Given that Al phytoxicity normally occurs at soil pH < 5 (Tyler, 1996), the contribution of Al toxicity to species loss can be discounted in our studies. Similar to results reported by Bowman et al. (2008), we found that N addition led to a substantial increase in soil Fe\(^{3+}\) concentration. Because forb species can only take up Fe\(^{2+}\) after reduction of Fe\(^{3+}\) to Fe\(^{2+}\) by ferric chelate reductases in roots (Marchner, 1995), the N addition-induced increase in soil Fe\(^{3+}\) concentration can contribute little to the loss of forb species in the present study. Based on the results of stepwise regression analyses, compared with Cu\(^{2+}\), Mn\(^{2+}\) exhibited the closest correlation with soil pH and Mn\(^{2+}\) concentrations were most greatly affected by N addition (Fig. 3, Table S1). Therefore, mobilization of Mn\(^{2+}\) due to soil acidification induced by N addition is expected to be critical process responsible for forbs loss in the Inner Mongolia steppe under elevated N deposition.
5 Conclusions

We demonstrate that N addition reduced species richness, acidified soil and disturbed nutrient homeostasis in soil in an Inner Mongolia steppe. We further reveal that decline in species richness by N addition was mainly accounted for by loss of forb species as forbs were more sensitive to N addition than grasses. Our findings also show that an increase in inorganic-N concentration, depletion of base cations (Ca$^{2+}$ and Mg$^{2+}$) and mobilization of Mn$^{2+}$ and Cu$^{2+}$ in soils were involved in reduction in species richness in the temperate steppe of northern China. These findings highlight that soil-mediated nutrient depletion and mobilization are involved in decline in species richness, particularly in forb richness, in the alkaline, calcareous grasslands in northern China.

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Author contributions. W.-H. Zhang, Q.-Y. Tian, W.-M. Bai and L.-H. Li designed the experiments and Q.-Y. Tian, N.-N. Liu, W.-M. Bai and W.-H. Zhang conducted the experiments. Q.-Y. Tian and W.-H. Zhang prepared the manuscript with contributions from all co-authors.

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Table 1. Factor loadings of ten mineral nutrient variables on axes 1 and 2 of the principal components analysis.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Factor 1</th>
<th>Factor 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inorganic-N</td>
<td>0.878</td>
<td>-0.002</td>
</tr>
<tr>
<td>Olsen-P</td>
<td>0.775</td>
<td>0.082</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>-0.423</td>
<td>0.793</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>-0.279</td>
<td>0.841</td>
</tr>
<tr>
<td>K$^+$</td>
<td>0.014</td>
<td>0.765</td>
</tr>
<tr>
<td>Fe$^{3+}$</td>
<td>0.879</td>
<td>-0.204</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>0.932</td>
<td>-0.165</td>
</tr>
<tr>
<td>Cu$^{2+}$</td>
<td>0.916</td>
<td>-0.166</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>0.175</td>
<td>0.359</td>
</tr>
<tr>
<td>Al$^{3+}$</td>
<td>0.871</td>
<td>0.001</td>
</tr>
</tbody>
</table>
Table 2. Proportion explained by variables of three groups (the macro-element group, the heavy metal group and the base cation group) in forb and grass species richness and aboveground biomass by RDA analysis.

<table>
<thead>
<tr>
<th>Principal variables</th>
<th>Forb richness</th>
<th>Forb aboveground biomass</th>
<th>Grass richness</th>
<th>Grass aboveground biomass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inorganic-N</td>
<td>27.2 %</td>
<td>23.6 %</td>
<td>7.6 %</td>
<td>0.4 %</td>
</tr>
<tr>
<td>Mn$^{2+}$, Cu$^{2+}$</td>
<td>41.4 %</td>
<td>21.3 %</td>
<td>11.8 %</td>
<td>3.9 %</td>
</tr>
<tr>
<td>Ca$^{2+}$, Mg$^{2+}$</td>
<td>25.9 %</td>
<td>3.0 %</td>
<td>5.4 %</td>
<td>1.9 %</td>
</tr>
</tbody>
</table>
Figure 1. Effects of N addition on aboveground biomass and species richness of vegetation. Total aboveground biomass (AGB) (a), total species richness (b), grass and forb aboveground biomass (c) and species richness of grasses and forbs (d) in plots with different rates of N addition. Number of species and AGB were determined in quadrats (1 m × 1 m). *, **, and *** indicate significant difference with control plots with no N addition at $P < 0.05$, $P < 0.01$ and $P < 0.001$. Data are mean ± SE ($n = 8$).
Figure 2. Reduction of soil pH with N addition rate. Soil pH was measured after N addition for 9 yr. ANOVA analysis with Duncan’s test was used to determine the significance. *** indicates significant difference with control plots at $P < 0.001$. Data are mean ± SE ($n = 8$).
Figure 3. Effect of N addition on exchangeable ion concentrations in soils. Data are mean± SE (n = 8). *, **, and *** indicate significant difference between control (no N added) and N-added plots at P < 0.05, P < 0.01 and P < 0.001, respectively.
Figure 4. Correlation of ion concentrations in soils and aboveground biomass (AGB) of grasses and forbs. Linear regression was used to identify the significance of the correlation between soil ions and AGB. Inorganic-N ($r^2 = 0.24$, $P = 0.0005$), Olsen-P ($r^2 = 0.19$, $P = 0.0020$), Fe$^{3+}$ ($r^2 = 0.13$, $P = 0.0111$), Mn$^{2+}$ ($r^2 = 0.21$, $P = 0.0010$), Cu$^{2+}$ ($r^2 = 0.16$, $P = 0.0044$) and Al$^{3+}$ ($r^2 = 0.22$, $P = 0.0007$) showed linear correlation with AGB of forbs.
Figure 5. Correlation of ion concentrations in soil and species richness of grasses and forbs. Linear regression was used to identify the significance of the correlation between soil ions and species richness. Inorganic-N ($r^2 = 0.27$, $P = 0.0001$), Olsen-P ($r^2 = 0.11$, $P = 0.0190$), Ca$^{2+}$ ($r^2 = 0.26$, $P = 0.0002$), Mg$^{2+}$ ($r^2 = 0.17$, $P = 0.0038$), Fe$^{3+}$ ($r^2 = 0.32$, $P < 0.0001$), Mn$^{2+}$ ($r^2 = 0.41$, $P < 0.0001$), Cu$^{2+}$ ($r^2 = 0.27$, $P = 0.0002$) and Al$^{3+}$ ($r^2 = 0.30$, $P < 0.0001$) showed linear correlation with species richness of forbs.
Figure 6. Projection of ten elemental variables for principle component analysis factors one and two.