

Rapid plant species loss at high rates and at low frequency of N addition in temperate steppe

YUNHAI ZHANG^{1,2,3}, XIAOTAO LÜ², FOREST ISBELL⁴, CARLY STEVENS⁵, XU HAN^{1,3}, NIANPENG HE⁶, GUANGMING ZHANG¹, QIANG YU^{2,7}, JIANHUI HUANG¹ and XINGGUO HAN^{1,2}

¹State Key Laboratory of Vegetation and Environmental Change, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China, ²State Key Laboratory of Forest and Soil Ecology, Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang 110016, China, ³University of Chinese Academy of Sciences, Yuquan Road, Beijing 100049, China, ⁴Department of Plant Biology, University of Georgia, Athens, GA, USA, ⁵Lancaster Environment Centre, Lancaster University, Lancaster LA1 4YQ, UK, ⁶Synthesis Research Center of Chinese Ecosystem Research Network, Institute of Geographic Sciences and Natural Resources Research, Chinese Academy of Sciences, Beijing 100101, China, ⁷Department of Biology, Graduate Degree Program in Ecology, Colorado State University, Fort Collins, CO 80523, USA

Abstract

Humans are both intentionally (fertilization) and unintentionally (atmospheric nutrient deposition) adding nutrients worldwide. Increasing availability of biologically reactive nitrogen (N) is one of the major drivers of plant species loss. It remains unclear, however, whether plant diversity will be equally reduced by inputs of reactive N coming from either small and frequent N deposition events or large and infrequent N fertilization events. By independently manipulating the rate and frequency of reactive N inputs, our study teases apart these potentially contrasting effects. Plant species richness decreased more quickly at high rates and at low frequency of N addition, which suggests that previous fertilization studies have likely over-estimated the effects of N deposition on plant species loss. N-induced species loss resulted from both acidification and ammonium toxicity. Further study of small and frequent N additions will be necessary to project future rates of plant species loss under increasing aerial N deposition.

Keywords: acidification, ammonium toxicity, biodiversity, Inner Mongolia, N addition frequency, peak effect, pulse effect, temperate grassland

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Introduction

With the rapidly increasing demand for food and energy, human activities have dramatically increased the mobility and deposition of reactive N (Galloway *et al.*, 2008; Canfield *et al.*, 2010; Erisman *et al.*, 2013). Atmospheric N deposition, with average rate of $2 \text{ g m}^{-2} \text{ yr}^{-1}$ at the global scale, has increased 5- to 20-fold over preindustrial conditions (Galloway *et al.*, 2008). In recent years, total N deposition has increased to $15 \text{ g m}^{-2} \text{ yr}^{-1}$ in some areas of the North China Plain (He *et al.*, 2010). Furthermore, global ecosystems will continue to receive increasing deposition of reactive N over the next century (Galloway *et al.*, 2008; Sutton & Bleeker, 2013).

Despite the positive effects of N fertilization/deposition on primary production (Smith *et al.*, 1999; Bai *et al.*, 2010), its threats to biodiversity in diverse ecosystems have received increasing attention (Stevens *et al.*, 2004a;

Clark & Tilman, 2008; Bai *et al.*, 2010; Bobbink *et al.*, 2010). High plant species diversity is important for sustainable supply of ecosystem services (Isbell *et al.*, 2011). To simulate increasing atmospheric N deposition and explore its effects on ecosystem functioning, N compounds are usually added to ecosystems during the growing season up to a few times per year in controlled experiments (Wright & Rasmussen, 1998; Aber & Magill, 2004; Lü *et al.*, 2013). However, atmospheric N deposition occurs much more frequently than the frequency of additions made in many experiments. Despite decreased agricultural activities, atmospheric N deposition remains high over winter due to increased coal combustion. It is speculated that such studies might have overestimated the effects of N deposition, due to the excessively large and infrequent pulses of nutrient that was experimentally added to ecosystems (Smith *et al.*, 2009), but the frequency of N addition has rarely, if ever, been tested in previous empirical studies. Furthermore, N is often added as urea or ammonium nitrate (NH_4NO_3) in either a wet or dry form only whereas atmospheric N is deposited in

Correspondence: Xiaotao Lü, tel. +86 24 83970301, fax +86 24 83970300, e-mail: lxiaotao@iae.ac.cn; Xingguo Han, e-mail: xghan@ibcas.ac.cn

both dry and wet form. Soil acidification (Bobbink *et al.*, 2010), ammonium toxicity (Nihlgård, 1985), light competition (Hautier *et al.*, 2009), and litter accumulation (Clark & Tilman, 2010) have been proposed as the potential mechanisms accounting for species loss in response to N enrichment. New experiments with a wide-range of rates and frequencies of N addition are needed to better understand how N deposition influences communities and ecosystems.

Temperate steppe is a floristically diverse and productive grassland type of both ecological and economic importance in Eurasia (Kang *et al.*, 2007). There is increasing evidence that the temperate steppe is sensitive to global change (Xia *et al.*, 2009; Niu *et al.*, 2011), particularly to N addition (Bai *et al.*, 2010; Lü *et al.*, 2013; Zhang *et al.*, 2014). It remains unclear whether Eurasian steppe will continue to maintain its biodiversity and provide important ecological services under the projected increasing N deposition scenarios (Kang *et al.*, 2007; Liu *et al.*, 2011). Given that biodiversity is fundamentally important for ecosystem functioning and services (Isbell *et al.*, 2011; Tilman *et al.*, 2012), it is imperative to understand the extent to which the biodiversity of temperate steppe will be diminished by N deposition. For example, N enrichment can initially promote productivity, but later drive declines in plant diversity that erode productivity over time (Isbell *et al.*, 2013).

The objectives of our study were to determine the response of grassland plant diversity to independent manipulations of both the frequency and rate of N addition. Since 2008, we carried out an N deposition experiment in the temperate steppe with two N addition frequencies, twice per year and monthly, and with nine rates of N addition, ranging from 0 to 50 g N m⁻² yr⁻¹, with the aim of examining the effects of N deposition on plant species diversity, as well as the possible underlying mechanisms. Consistent with previous predictions (Smith *et al.*, 2009), we hypothesized that lower N addition frequency might induce greater plant species loss in this semiarid grassland (i.e. greater species loss with two than with 12 N additions per year). If so, then the negative impacts of N deposition on plant diversity have likely been over-estimated by previous nutrient enrichment experiments, which have often added a larger quantity of nutrients in fewer discrete pulses than occurs under N deposition.

Materials and methods

Study site and experimental design

Our experiment site, fenced since 1999 to exclude large animal grazing, is located at a temperate steppe (116°14'E, 43°13'N) in

Inner Mongolia Autonomous Region, China. Long-term (1983–2012) mean annual temperature is 0.9 °C, with mean monthly temperatures ranging from -21.4 °C in January to 19.7 °C in July. Mean annual precipitation is 355.3 mm with approximately 60–80% fallen from May to August. The soil is classified as Haplic Calcisols and Calcic-Orthic Aridisols by the FAO and the US soil classification system respectively. The plant community was dominated by *Leymus chinensis* and *Stipa grandis* which together accounted for >60% of the total peak aboveground biomass. There were no legumes in our experimental site. This site had received no fertilizer prior to this experiment. The ambient total N deposition in the field was <1.5 g N m⁻² yr⁻¹ for recent two decades (Jia *et al.*, 2014).

This N deposition experiment began in September 2008, following a randomized complete block design. There were nine rates of N addition (0, 1, 2, 3, 5, 10, 15, 20, and 50 g N m⁻² yr⁻¹), and two frequencies of N addition (2 or 12 N additions yr⁻¹). Moreover, we setup one control treatment (neither N nor water addition) to detect the influence of the water and sand added without N by comparing responses of species number between the control and 0 N addition plots. Hence, there were 19 experimental treatments in total, each with 10 replicates. Each plot was 8 m × 8 m in size. All 19 treatments were randomized to plots, which were separated by 1 m walkways, within each of ten replicate blocks. Each block was 45 m × 70 m, and there were 2-m walkways between blocks. Purified NH₄NO₃ (>99%) addition started on 1 September 2008 and continued on the first day of each month for the high frequency (monthly) N addition treatment, and started on 1 November 2008 and continued on the first day of June and November for the low frequency (2 N additions yr⁻¹) N addition treatment. When the monthly N is added in August per year, it is with total annual equal N loadings between 2 and 12 N additions yr⁻¹. During the growing season from May to October, the fertilizer was weighed, mixed with purified water (9.0 l total for all treatments receiving water: either 9.0 l once in June or 1.5 l monthly from May to October), and sprinkled evenly using a sprayer to each plot. It was estimated that less than 1 mm of water was added to plots annually, except the control plots, which had no water added. In winter (from November to next April), NH₄NO₃ was mixed with clean sand (0.5 kg total for all treatments receiving sand: either 0.5 kg once in November or 0.08 kg monthly from November to April) and broadcast by hand. Sand sieved through less than 1 mm in size, hydrochloric acid dipped, washed in purified water, and then heated at 120 °C for 24 h in an oven.

Field sampling and measurements

Number of plant species (richness) of community was sampled each year for 2008–2013 between 10 and 15 August using a single 0.5 m × 2 m quadrat, which was randomly placed in each plot without spatial overlap of quadrats among years and at least 50 cm inside the border of each plot to avoid edge effects. The assessment was made with one quadrat per plot per year. We classified all plant species into three plant functional groups, grasses, perennial forbs, and annuals and

biennials (see taxonomic details in Table 1). The frequency of each species from each sampling plot was calculated each year across 2009–2013. Species frequency was summed for the community and each of plant functional group analysis at every N addition rate of each frequency of N addition. Concurrent with aboveground plant sampling between 10 and 15 August in 2012 and 2013, three soil cores (0–10 cm depth and 50 cm apart) were collected using a 3 cm diameter soil auger adjacent to each aboveground plant sample plot. The soil samples were thoroughly mixed and sieved through a 2 mm mesh to obtain one composite sample for laboratory analysis of soil ammonium ($\text{NH}_4^+\text{-N}$; mg kg^{-1}) and nitrate ($\text{NO}_3^-\text{-N}$; mg kg^{-1}) concentration and soil water content (%), and subsamples were air-dried for analysis of soil pH. For soil $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ measurements, 10 g fresh soil sample was extracted with 50 ml KCl (2.0 M) solution and then analyzed using a flow injection auto analyzer (FLAstar 5000 Analyzer; Foss Tecator, Hillerød, Denmark). The soil $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ concentration is expressed on a dry weight basis. The soil pH was measured in water suspension (soil : water = 1 : 2.5) by a pH meter (FE20–FiveEasy). Soil temperature ($^{\circ}\text{C}$) was automatically recorded within each plot at a 1.5 h interval by iButton digital temperature loggers (DS1922L). These devices were buried to a depth of 10 cm in the soil in April 2012. We used the average temperature of growing seasons (from May to 9 August) for further analysis in both 2012 and 2013.

Statistical analyses

We calculated the Shannon–Wiener diversity (H) for the equation as

$$H = - \sum_{i=1}^N \left(\frac{b_i}{\sum_{i=1}^N b_i} \times 100 \right) \times \ln \left(\frac{b_i}{\sum_{i=1}^N b_i} \times 100 \right)$$

where N is plot species richness and b_i is the aboveground biomass of species i .

Repeated-measures analysis of variance (ANOVA) was employed to detect for the significance of plant species richness in the community, Shannon–Weiner diversity, and species richness in each of three plant functional group (grasses, perennial forbs, and annuals and biennials), and soil properties (soil water content, temperature, pH, $\text{NH}_4^+\text{-N}$, and $\text{NO}_3^-\text{-N}$), using N addition frequency, N addition rate, year, and their interactions as fixed-factors, and using the value in 2008 (pretreatment investigation) as a covariate for richness analysis. Two-way ANOVA was used to test the effects of the frequency and rate of N addition, and their interaction on species frequency of community, each plant functional group and each species across 2009–2013. The proportional loss (or decrease) of species, soil water content, soil temperature and soil pH relative to controls was calculated as one minus the ratio of the plot average value (species number, soil water content, soil temperature and soil pH, respectively) divided by

Table 1 Major species of different functional groups within the quadrats (0.5 m × 2 m) in control across 2009–2013

Functional groups	Species scientific name	Family	Genus	Relative aboveground biomass in community (%)
Grasses	<i>Stipa grandis</i> P. Smirn.	Gramineae	<i>Stipa</i>	35.60
	<i>Leymus chinensis</i> (Trin.) Tzvel.	Gramineae	<i>Leymus</i>	25.22
	<i>Achnatherum sibiricum</i> (L.) Keng	Gramineae	<i>Achnatherum</i>	16.49
	<i>Agropyron cristatum</i> Roshev.	Gramineae	<i>Agropyron</i>	11.64
	<i>Cleistogenes squarrosa</i> (Trin.) Keng.	Gramineae	<i>Cleistogenes</i>	3.29
	<i>Koeleria cristata</i> (L.) Pers.	Gramineae	<i>Koeleria</i>	1.01
	<i>Poa subfastigiata</i> Trin.	Gramineae	<i>Poa</i>	0.65
	<i>Festuca dahurica</i> (St.-Yves) V.Krecz. et Bobr.	Gramineae	<i>Festuca</i>	0.12
Perennial forbs	<i>Carex korshinskyi</i> Kom.	Cyperaceae	<i>Carex</i>	4.55
	<i>Allium tenuissimum</i> L.	Liliaceae	<i>Allium</i>	0.40
	<i>Iris tenuifolia</i> Pall.	Iridaceae	<i>Iris</i>	0.10
	<i>Allium bidentatum</i> Fisch. Ex Prokh.	Liliaceae	<i>Allium</i>	0.06
	<i>Allium ramosum</i> L.	Liliaceae	<i>Allium</i>	0.06
	<i>Thalictrum petaloideum</i> L.	Ranunculaceae	<i>Thalictrum</i>	0.04
Annuals and biennials	<i>Potentilla bifurca</i> L.	Rosaceae	<i>Potentilla</i>	0.03
	<i>Chenopodium glaucum</i> L.	Chenopodiaceae	<i>Chenopodium</i>	0.25
	<i>Axyria amaranthoides</i> L.	Chenopodiaceae	<i>Axyris</i>	0.13
	<i>Salsola collina</i> Pall.	Chenopodiaceae	<i>Salsola</i>	0.12
	<i>Artemisia scoparia</i> Waldst et Kit.	Compositae	<i>Artemisia</i>	0.05
	<i>Saussurea japonica</i> (Thunb.) DC.	Compositae	<i>Saussurea</i>	0.05
	<i>Dontostemon micranthus</i> C.A.Mey.	Cruciferae	<i>Dontostemon</i>	0.04
<i>Chenopodium aristatum</i> L.	Chenopodiaceae	<i>Chenopodium</i>	0.01	

the average number of species in the control treatment for both 2012 and 2013 respectively. The proportional increase of soil $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ relative to controls was calculated as the ratio of the plot average value divided by the average value in the control treatment minus one in both 2012 and 2013 respectively. Multiple linear regression was performed to identify the strength of all five environmental factors (i.e. soil properties) that accounting for the variation of species loss, and forward stepwise multiple regression analysis also used to find the best-fitted model. Pearson correlation analysis was used to test the linear correlations among five soil factors. We chose a significance level of 0.05. All the statistical analyses were performed with SPSS software package (SPSS 17.0 for windows, SPSS Inc., Chicago, IL, USA).

Results

Effects on species richness

At community level, plant species richness decreased with the increase of N addition rates (Table 2), especially during the later years of our study (Figs 1a, 2b). Plant species richness did not depend on the rate of N addition in the first year of study (2009) at low frequency of N addition, and at high frequency of N addition in both 2009 and 2010 (Fig. 1a). By the other years, species richness strongly declined as the rate of N addition increased for both N addition frequencies (Figs 1a, 2b). Species frequency of the community was declined with the increasing rate of N addition (Table 3). Uniquely, we also found both plant species richness (Table 2; Fig. 1a; $P = 0.007$) and species frequency (Table 3; $P = 0.016$) decreased less under high frequency of N addition across 2009–2013. The effect of N addition frequency on plant species richness was slightly, although nonsignificantly, stronger during later years (Fig. 2a,b) and at high rates of N addition

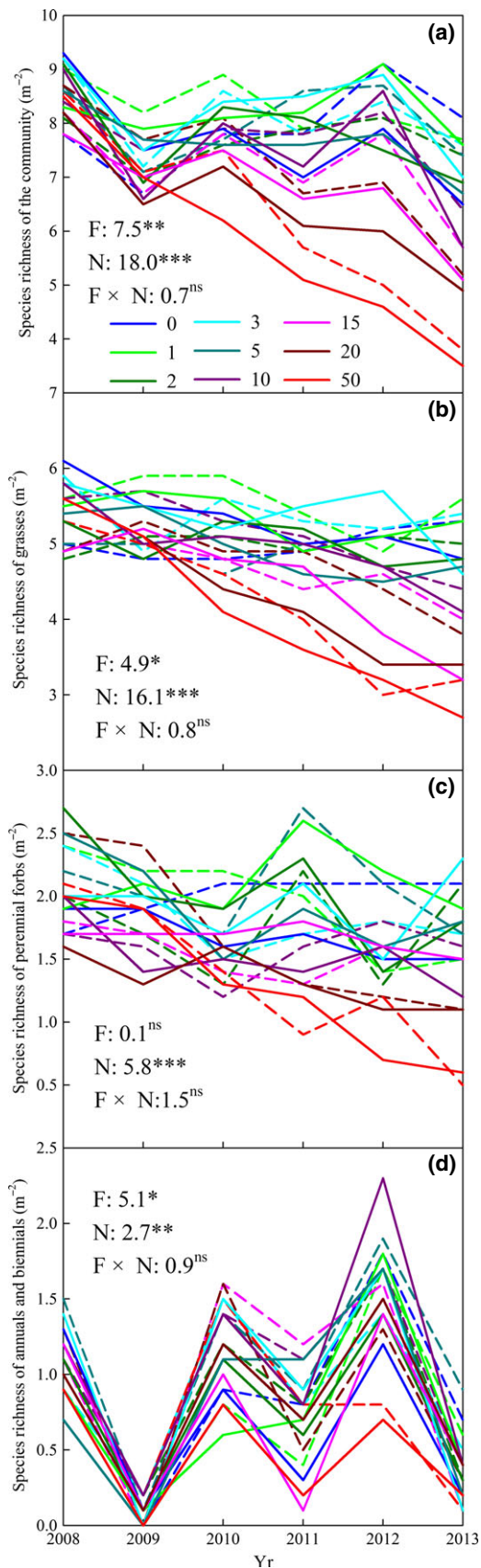
(Fig. 2c). Significant differences were detected in intercepts of regressions of both N rates vs. species richness (Fig. 2c) and N rates vs. species frequencies (Fig. 2d) between 2 and 12 N additions yr^{-1} . Shannon–Wiener diversity decreased with the increase of N addition rates and showed significant interannual variation across 2009–2013 (Table 2). No significant effects of N addition frequency were detected on Shannon–Wiener diversity. The temporal rate of decrease in Shannon–Wiener diversity was marginally lower at higher frequency of N addition ($P = 0.077$; Table 2).

At plant functional group level, increasing the frequency of N addition resulted in a significant reduction in the rate of species loss of grasses, and of annuals and biennials, but had no impacts on perennial forbs (Table 2; Fig. 1b–d). For grasses ($P = 0.059$) and annuals and biennials ($P = 0.035$), the decrease rate in the species frequency was lower at higher frequency of N addition (Table 3). Increasing the rate of N addition significantly decreased both species richness and species frequency of all three functional groups (Tables 2, 3; Fig. 1b,c,d), especially during the later years for grasses and perennial forbs (Table 2; Fig. 1b,c). Species richness of both perennial forbs and annuals and biennials significantly varied across years (Table 2; Fig. 1c,d).

For specific species, increasing the rate of N addition decreased the frequency of *S. grandis*, *C. squarrosa*, *K. cristata*, *P. subfastigiata*, *C. korshinskyi*, *A. tenuissimum*, *C. aristatum*, and *A. scoparia* (All $P < 0.05$). Frequency of *A. cristatum* and *A. amaranthoides* differed between levels of N addition ($P < 0.05$), but did not consistently increase or decrease with increases in the rate of N addition. Frequency of *Ch. glaucum* nonlinearly increased with increases in the rate of N addition ($P = 0.035$). Adding N twice per year instead of monthly decreased the frequency of *C. squarrosa*

Table 2 Results of repeated-measures ANOVAs testing the effects of the frequency and rate of N addition on the number of plant species (richness) in the community, Shannon–Weiner diversity index (H), and species richness in each of three plant functional groups (grasses, perennial forbs, and annuals and biennials). The initial value in 2008 was included as a covariate (R_0) across 2009–2013. Degrees of freedom (df), F -test values, and P -values are given

	df	Community		H		Grasses		Perennial forbs		Annuals and biennials	
		F	P	F	P	F	P	F	P	F	P
R_0	1,161	19.1	<0.001	5.9	0.016	5.9	0.016	23.1	<0.001	20.1	<0.001
N frequency (F)	1,161	7.5	0.007	2.0	0.156	4.9	0.029	0.1	0.744	5.1	0.025
N rate (N)	8,161	18.0	<0.001	10.3	<0.001	16.1	<0.001	5.8	<0.001	2.7	0.008
$F \times N$	8,161	0.7	0.661	0.4	0.917	0.8	0.616	1.5	0.170	0.9	0.534
Year (Y)	4,644	1.3	0.263	3.1	0.018	0.4	0.762	3.2	0.013	32.2	<0.001
$Y \times F$	4,644	0.7	0.617	2.2	0.077	1.9	0.117	0.6	0.656	0.8	0.486
$Y \times N$	32,644	3.0	<0.001	3.2	<0.001	3.0	<0.001	2.1	<0.001	1.2	0.200
$Y \times F \times N$	32,644	0.7	0.846	1.3	0.110	1.0	0.523	0.9	0.580	0.9	0.627



($P = 0.003$), *K. cristata* ($P = 0.063$), *P. subfastigiata* ($P = 0.081$), *D. micranthus* ($P = 0.036$) and *A. amaranthoides* ($P = 0.103$) across 2009–2013.

Effects on soil conditions

Increasing the rate of N addition significantly decreased soil water content, soil temperature, and soil pH, and significantly increased soil NH_4^+-N and $NO_3^- - N$ (Table S1; Fig. 3, Figure S1). In contrast, increasing the frequency of N addition significantly increased soil NH_4^+-N , but had no significant effect on other soil variables (Table S1; Fig. 3). Results of Pearson correlation showed that soil water content, temperature, and pH showed significantly positive relationships with each other, but negatively correlated with soil NH_4^+-N and $NO_3^- - N$ (Table S2).

Relationships between species richness and soil conditions

In 2012 and 2013, proportional species richness loss was significantly positively correlated with proportional soil water content decrease (Fig. 4a), proportional soil temperature decrease (Fig. 4b), proportional soil pH decrease (Fig. 4c), proportional soil NH_4^+-N increase (Fig. 4d), and proportional soil $NO_3^- - N$ increase (Fig. 4e) after N addition. Multiple regression analyses revealed that all soil factors together explained 86.8% of the variation in species richness. Stepwise regression analyses showed that changes of soil pH explained 79.5% and changes of soil ammonium explained additional 5.7% of the variation in proportional plant species richness loss after N addition.

Discussion

Effects of the rate and the frequency of N addition on species richness

Plant species loss was highly dependent on N addition/deposition rates, which is in line with previous

Fig. 1 Effects of N additions on the average number of plant species (richness). Responses of species richness of community (a) and three plant functional groups (b, grasses, c, perennial forbs, and d, annuals and biennials) to the N addition frequencies (solid = 2 N additions yr^{-1} , dash = 12 N additions yr^{-1}) and N addition rates (different colors; $g N m^{-2} yr^{-1}$). $N = 10$. The values in 2008 show pretreatment richness levels. Average plant species richness at both the community level and the plant functional group level significantly declined with increasing rates of N addition at both N addition frequencies across 2009–2013. F, N addition frequency and N, N addition rates. ^{ns}, *, **, and ***, statistically significant at $P > 0.05$, $P < 0.05$, 0.01, and 0.001 respectively.

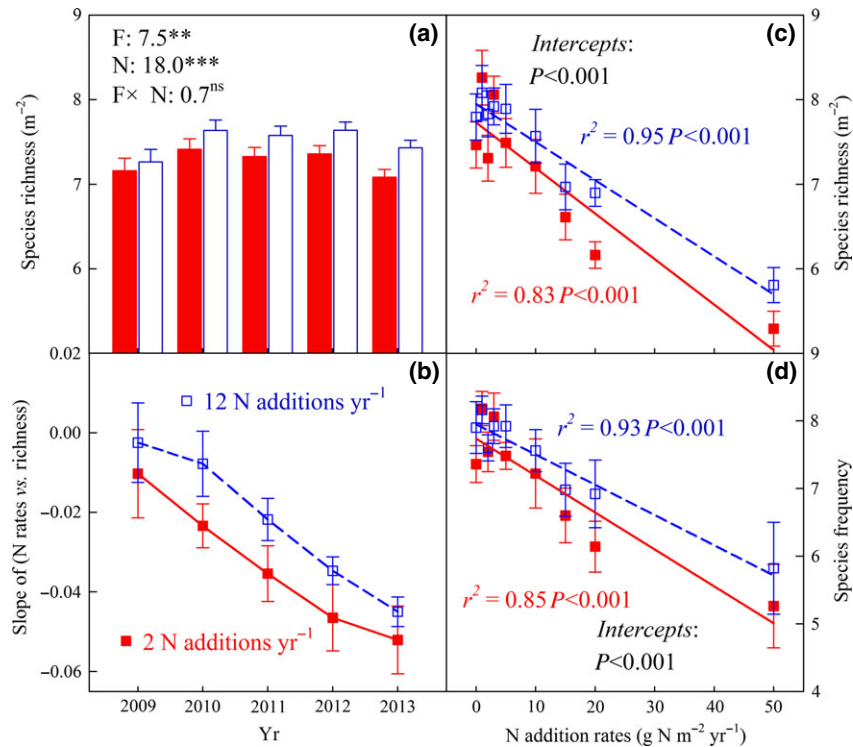


Fig. 2 Effects of N addition frequencies on species richness across 2009–2013. Plant species richness (a) and slopes of (N rates vs. species richness) (b) from 2009 to 2013. Species richness (c) and species frequency (d) responded to N addition rates. Filled symbols (■; solid line) correspond to 2 N additions yr^{-1} and open symbols (□; dash line) correspond to 12 N additions yr^{-1} , respectively. Error bars indicate 1 SEM. *F*, N addition frequency and *N*, N addition rates. ^{ns}, **, and ***: statistically significant at $P > 0.05$, $P < 0.01$, and 0.001 respectively.

Table 3 Results of two-way ANOVAs testing the effects of the frequency and rate of N addition on species frequency in the community, grasses, perennial forbs, and annuals and biennials across 2009–2013. Degrees of freedom (*df*), *F*-test values, and *P*-values are given

	<i>df</i>	Community		Grasses		Perennial forbs		Annuals	and
		<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	biennials	<i>P</i>
<i>F</i>	1,162	5.9	0.016	3.6	0.059	0.2	0.629	4.5	0.035
<i>N</i>	8,162	17.4	<0.001	16.7	<0.001	5.3	<0.001	2.5	0.014
<i>F</i> × <i>N</i>	8,162	0.6	0.801	0.8	0.605	0.9	0.542	1.0	0.431

studies (Stevens *et al.*, 2004a; Bai *et al.*, 2010). Grassland ecosystems with a history of low atmospheric N inputs, are very sensitive to N enrichment. The impacts of N addition rates on species composition and richness are relatively well studied in grasslands worldwide. For example, Stevens *et al.* (2004a) found that plant species richness decreased linearly with increasing N deposition rates in acid grasslands across Great Britain. Isbell *et al.* (2013) found that the effects of N addition rates on species richness became increasingly negative over time

in an experiment lasting over two decades in Minnesota, United States. Bai *et al.* (2010) found a significant negative relationship between species richness and N addition rates in mature and degraded grasslands of northern China. In a synthesis paper, Bobbink *et al.* (2010) also reported a significant negative relationship between species richness and N addition rates in semi-natural grasslands in six countries across Europe. Overall, N addition rates have negative impacts on plant species diversity over time.

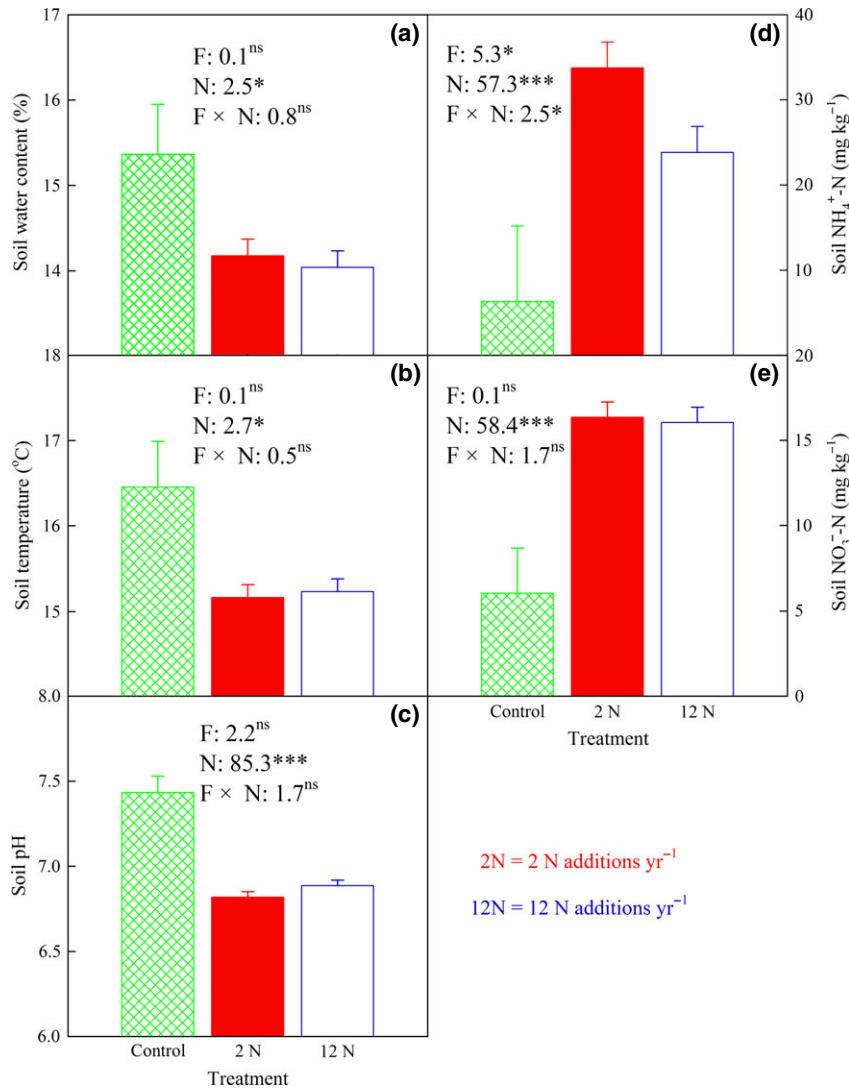


Fig. 3 Effects of N addition on the top 10 cm soil factors across 2012–2013. The top 10 cm soil water content (a), soil temperature (b), soil pH (c), soil ammonium concentration (NH₄⁺-N; d), and soil nitrate concentration (NO₃⁻-N; e) in response to the two N addition frequencies (2 or 12 N additions yr⁻¹) and the control. Error bars indicate 1 SEM. *F*, N addition frequency and *N*, N addition rate. ^{ns}, *, and ***: statistically significant at $P > 0.05$, $P < 0.05$ and 0.001 respectively.

Uniquely, our results show that plant species loss can depend not only on the rate of N addition, but also on the frequency of N addition. Consistent with our hypothesis, we found greater species loss with less frequent N addition. Species richness gradually diverged between the two N addition frequencies across the study period (Fig. 2). For a particular N addition rate, large and infrequent N pulses led to greater and more rapid species loss. The difference in the response of species richness might be explained by accumulating ammonium toxicity (Nihlgård, 1985; van den Berg *et al.*, 2005) due to less ammonia loss from soils as gas under low frequency of N addition (Zhang *et al.*, 2014). Moreover, despite decreased agricultural activities in winter, rates of atmospheric N

deposition in many temperate ecosystems remain high over winter due to increased coal combustion. It has been reported that only a small fraction of the added N that accumulates in the winter snow pack may be available to plants in the subsequent spring (Joseph & Henry, 2009). Thus, most of winter added N may be lost from the root zone via leaching, runoff or denitrification under the water-saturated soil conditions during spring snow melt in our system. Consequently, less available N would accumulate in root zone in high frequency of N addition in spring. The accumulated ammonium in top soils may have toxicity effects on plants under low frequency of N addition. Other factors (i.e. soil cations exchange capacity, elements availability) might also contribute to the

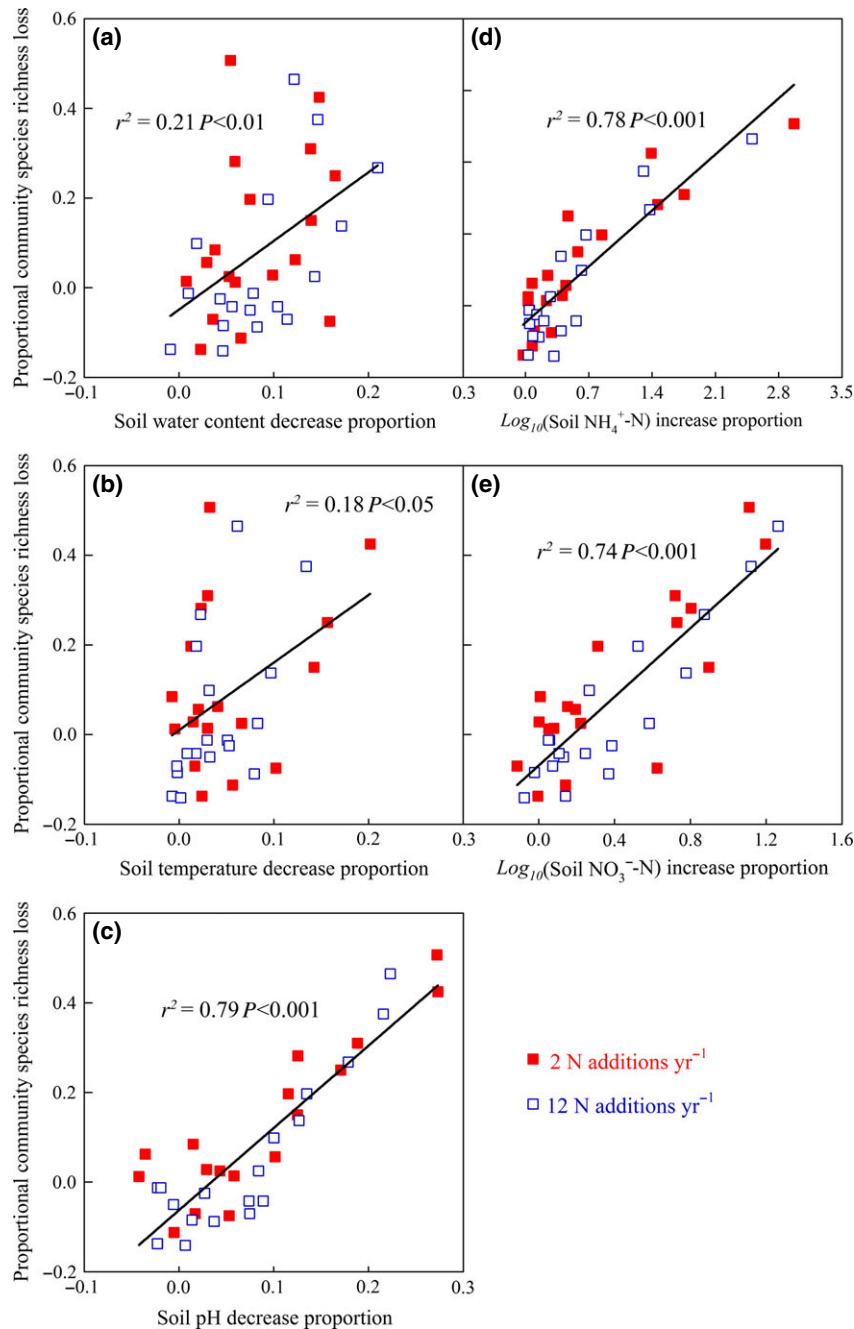


Fig. 4 Potential mechanisms for explaining the declines in plant species richness due to N addition. Proportional decrease of soil water content (a), soil temperature (b), and soil pH (c), proportional increase of soil $\text{Log}_{10}(\text{NH}_4^+\text{-N})$ (d) and soil $\text{Log}_{10}(\text{NO}_3^-\text{-N})$ (e) with proportional species richness loss across 2012–2013 respectively. Each symbol indicates mean of ten replications.

difference in species richness (Clark *et al.*, 2007; Zhang *et al.*, 2013). Together, these factors likely explain how lower frequency of N addition leads to greater species loss.

Our results suggest that previous studies using low frequency of N fertilization might thus overestimate the extent to which, and the rate at which, N deposition drives plant species loss. Atmospheric biologically N

deposition is expected to further increase in the coming decades due to increasingly ubiquitous anthropogenic impacts (Galloway *et al.*, 2008). With slow plant species loss at high frequency of N addition, our findings call for a re-evaluation of the rate of species loss in response to future increasing aerial N deposition in developing regions (i.e. in Latin America, East Asia, South Asia, North Africa, and the Near East). It also calls for an

increase in studies using ambient gradients of N deposition (Stevens *et al.*, 2004a).

Mechanisms for N-induced species loss

The result that the same total N loading reduced species richness more when applied in less frequent and higher doses, suggests a peak effect of N addition on plant diversity (Smith *et al.*, 2009). We found that species losses were linked to increases in soil available ammonium and soil pH, and to a lesser extent to soil available nitrate (Fig. 4). Together, these results suggest that large and infrequent pulses of N decrease species diversity by increasing soil available N and by decreasing soil pH. Combined both acidification and ammonium toxicity accounted for 85.2% of the variation in species richness, which might be the primary mechanisms for species loss (Fig. 4). Previous studies proposed that recruitment limitation is important in underpinning the N enrichment-induced loss of plant species (Stevens *et al.*, 2004b; Bobbink *et al.*, 2010; Clark & Tilman, 2010). Plant species loss from the forest understory (Nihlgård, 1985) and grasslands (Stevens *et al.*, 2010) might generally be a consequence of anthropogenic ammonium input and associated soil acidification. For example, de Graaf *et al.* (1998) found that soil ammonium concentrations, $\geq 0.5 \text{ mM l}^{-1}$, were detrimental for germination, growth, and survival of plants; and accelerated local species losses in European species-rich heathlands and acidic grasslands. van den Berg *et al.* (2005) found that ammonium toxicity and soil acidification had strong detrimental and interacting effects on acid-sensitive plant species loss in greenhouse experiments. Moreover, soil water content and temperature decreased with increasing N addition rates, which also had negative effects on species establishment (Haugland & Froud-Williams, 1999). Furthermore, previous studies also showed that species' ammonium-tolerance can be acid-tolerance (Yan *et al.*, 1992; van den Berg *et al.*, 2005). For instance, a perennial rhizome C₃ grass, *L. chinensis*, was favored by N enrichment, and other species became somewhat rarer (Bai *et al.*, 2010). Overall, suppressing seed germination and inhibiting seedling establishment and plant growth by acidification and ammonium toxicity and restriction by decreased soil water content and temperature, may increase the mortality of acid and ammonium sensitive plants, and decrease species diversity in temperate grasslands in the future with increasing N deposition. In all, results from this study showed that the negative effects of N addition in a singular pulse were related to increases in the toxic effect of soil ammonium and soil acidification.

The changes of stoichiometric ratios in soil following N addition likely play an important role in modulating

plant growth and interspecific competition (Güsewell & Bollens, 2003). Increases in reactive N inputs have substantially altered the magnitude and pattern of both N and phosphorus (P), altering the N : P ratios of organisms (Peñuelas *et al.*, 2012). In a meta-analysis, Marklein & Houlton (2012) found that N addition enhanced soil P availability over most terrestrial ecosystems, which could be a greater threat to biodiversity than soil N enrichment in some terrestrial ecosystems (Ceulemans *et al.*, 2013). Soil acidification following N enrichment may accelerate the P release from rock weathering (Vitousek *et al.*, 2010). In a nearby ecosystem with our study, Lü *et al.* (2013) found that plant available P in soil increased following N addition and that both plant and soil N:P ratios were quite sensitive to N addition.

In conclusions, atmospheric biologically N deposition is expected to further increase in the coming decades due to increasingly ubiquitous anthropogenic impacts. Our results suggest that there is significantly greater plant species loss under large and infrequent N fertilization events than under small and frequent N deposition events, as the toxicity of soil ammonium tends to succeed in soils when there is a high loading. N-induced species loss is due to the peak effect instead of the cumulative effect of N addition. Thus, results from previous N addition experiments, which have almost exclusively considered the effects of large and infrequent N addition, might over-estimate plant species losses in response to N deposition. More experiments that independently manipulate the rate and frequency of N addition will be needed to examine the generality of our results in other ecosystems. Long-term and continuing experiments are needed too. Our study showed that species richness declined via excluded acid and ammonium sensitive plants under N enrichment in this grassland. Management practices that reduce soil acidification and ammonium toxicity might help to slow plant species loss under N enrichment.

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Supporting Information

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

Figure S1. Changes in the soil factors across 2012–2013.

Table S1. Repeated-measure ANOVA for soil factors.

Table S2. Pearson correlation among soil factors.