

ARTICLE

The changes in plant and soil C pools and their C:N stoichiometry control grassland N retention under elevated N inputs

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Abstract

Nitrogen (N) retention is a critical ecosystem function for maintaining soil fertility and mitigating pollution caused by anthropogenic N input. However, it has not yet been elucidated how responses of plant and soil regulate ecosystem N retention. Here, we combined a 14-year N addition experiment in a temperate steppe with a global meta-analysis in grasslands, to assess changes in carbon (C) pool size and stoichiometric C:N ratio of plant and soil components and evaluate the contribution of each component to grassland N retention under increasing N levels. We found that N addition increased N storage in the plant pool by stimulating biomass production and reducing tissue C:N at the community level. However, the non-random loss of forbs and legumes associated with a low C:N ratio partially offset the decline in community-level C:N ratio, thereby diminishing the positive net effect of N enrichment on plant N storage. The observed increase in soil N storage was predominantly determined by the decrease in C:N ratio of topsoil, while no changes were detected in the subsoil. On 14-year time scale, the upper limitation of N retention capacity in our study site was 167.02 g N/m². Global meta-analysis further indicated that a decade's N addition significantly increased the N storage in shoot, root and topsoil through enhancing the C pool and decreasing the C:N ratio, while did not affect those of subsoil. However, the positive correlation between the response of subsoil N storage and treatment duration further indicates that, though the accumulation of added N lagged behind that of topsoil, subsoil could play an important role in N retention on a longer time scale. Our study demonstrated that the enhanced plant productivity and altered physiological metabolism indicated by the decreased C:N ratio jointly determined grassland ecosystem N retention. The capacity of the grassland ecosystem to retain exogenous N input is limited, especially for a large amount of N input that occurs in a short period. However, in the context of chronically rising N deposition, the long-term N retention capacity of grasslands should largely depend on the response of subsoil, especially after topsoil N is saturated.

KEYWORDS

ecosystem N retention, N deposition, non-random species loss, soil N retention, subsoil, topsoil

INTRODUCTION

Anthropogenic creation of reactive nitrogen (N) has been increasing tenfold since the industrial revolution (IPCC, 2013), which dramatically enhanced global nitrogen (N) input to terrestrial ecosystems and resulted in environmental problems such as water eutrophication, soil acidification, and biodiversity loss (Bergström & Jansson, 2006; Bowman et al., 2008; Stevens et al., 2004). Nonetheless, recent studies have found that the N limitation of plant growth increases in major biomes (Craine et al., 2018; McLauchlan et al., 2013). Therefore, increasing understanding of the regulatory mechanisms involved in ecosystem N retention is crucial for the future management of N pollution risk and the achievement of a favorable ecosystem nutrient status.

Nitrogen within terrestrial ecosystems is primarily stored in living and dead organic matter. How much N an ecosystem can retain is primarily determined by the size of the plant or soil carbon (C) pools and their C:N stoichiometric ratio (Figure 1; Lovett & Goodale, 2011). Enhancement of N input could significantly increase community-level plant N storage via the stimulation of productivity and regulating the physiological metabolism of plants indicated by the reduction of C:N ratio (Elser et al., 2007; LeBauer & Treseder, 2008; Yang et al., 2011). According to the carbon–nutrient balance hypothesis, plants tend to use more absorbed N to synthesize N-rich proteins and second metabolites in more fertile ecosystems, which explains the faster N accumulation than C after N addition (Koricheva et al., 1998; Liu et al., 2016; Thamer et al., 2011). However, since the variation in the C:N ratio of plants is generally relatively narrow due to the homeostasis of biological stoichiometry, more significant changes in the C:N of plant biomass pools may be achieved through species reordering (Smith et al., 2009; Sterner & Elser, 2002). For example, plant biodiversity loss following N addition was widely reported (Harpole & Tilman, 2007; Hautier et al., 2009; Stevens et al., 2004). Previous studies found that species loss after N addition was non-random, as the loss of forbs and legumes was faster than grasses under increasing N input (Stevens et al., 2006; Tian et al., 2016). Since the forbs and legumes are more N-rich than grasses (Yuan & Chen, 2009), the higher C:N ratio of grasses could diminish the positive effect of enhanced plant biomass production on N storage under increasing N deposition (Figure 1a). In addition,

the declines in biodiversity can induce negative impacts on the plant biomass C pool and may partially offset increases in plant N storage driven by plant productivity (Isbell et al., 2013). However, our understanding of how changes in plant species composition can regulate the stoichiometry of the whole plant community or impact plant N retention remains inadequate (Figure 1a).

In the terrestrial ecosystems, the soil is the largest N pool, retaining 25 times more N than the plant pool (Chapin III et al., 2011). Increasing N deposition can enhance N storage in the soil pool via increasing the soil C storage and decreasing the C:N ratio (Figure 1b). Increasing plant-derived C input and inhibiting microbial decomposition following N addition were expected to stimulate soil C storage (Huang et al., 2020; Liu & Greaver, 2010; Wieder et al., 2015). However, the responses of soil C pool to N addition were not consistent, as both positive and neutral effects were reported (Crowther et al., 2019; Fornara & Tilman, 2012). Compared with the inconsistent response of soil C storage, the soil C:N ratio was widely reported to be reduced after N addition (Yang et al., 2011). Recent studies have highlighted the critical impacts of microbial metabolism on the formation and turnover of soil organic matters (Cotrufo et al., 2013; Liang et al., 2017). The emerging concept of the “microbial pump” emphasizes that microbial activities govern the efficiency of integrating exogenous organic matters into soil matrix (Liang et al., 2017). Therefore, it is to be expected that microbial stoichiometry should largely influence soil C:N through converting plant-derived C into metabolites and necromass. However, the impact of microorganisms on regulating soil N retention still has not been fully explored.

The amount of N retained in the soil pool is dependent on the response of the entire soil profile. As the most fertile and biologically active layer of the soil profile, topsoil conventionally responds strongly to N input (Lu et al., 2011). Added N can reach the subsoil through downward physical transfer, decomposer movement, and root-derived input (Chapin III et al., 2011). A large amount of subsoil presents great potential for storing N from exogenous input (Yang et al., 2007). However, changes in the subsoil N pool may lag behind the topsoil due to slow N downward transport (Lovett et al., 2018). A better understanding of the asynchrony of N accumulation between the topsoil and subsoil is very important for estimating the capacity of soil N retention, particularly

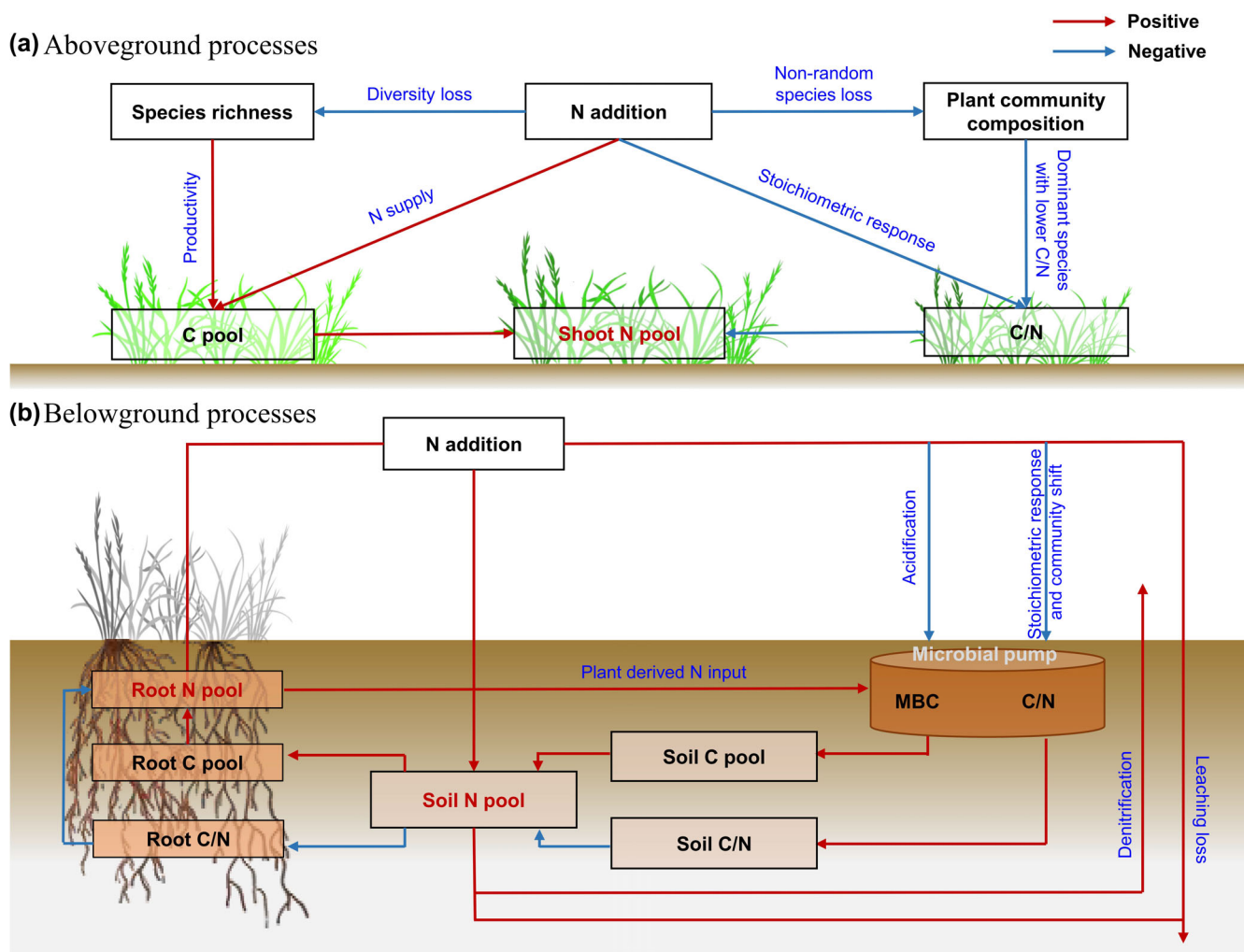


FIGURE 1 Schemes indicating how the responses of C pool and C:N ratio of aboveground (a) and belowground (b) components to N enrichment regulating grassland ecosystem N retention capacity. Red lines represent positive effects and blue lines represent negative effects

under chronically increasing N input. However, few experimental studies have evaluated the contributions of different soil layers to soil N retention capacity and the changes in top- and subsoil N storage over time.

This study incorporated data from a 14-year multi-level N addition experiment in a temperate steppe in Inner Mongolia. We investigated how changes in plant and soil C pools and their stoichiometric ratio (C:N) governed the capacity of grassland ecosystem N retention. To further assess whether our findings at site level can be extrapolated to other grassland ecosystems, we conducted a meta-analysis to explore how different ecosystem N pools respond to a decade-long N addition across grassland ecosystems. We aimed to test the following three hypotheses: (1) N enrichment will increase plant N storage by increasing plant biomass production and reducing the community-level C:N ratio. However, the decreased forb-to-grass ratio will diminish the positive net effect of N enrichment on the plant N pool by

partially offsetting the decline in community-level C:N ratio; (2) N enrichment will reduce microbial C:N stoichiometry. As microbial metabolites and necromass are the primary sources for SOM formation, the decrease in microbial C:N will drive the decrease in soil C:N; (3) grassland ecosystem N retention capacity is determined by N storage in topsoil rather than in subsoil soil as plant residual inputs and microbial actives are both concentrated in the topsoil.

MATERIALS AND METHODS

Field experiment design and measurements

The field experiment was established in Duolun County, Inner Mongolia, China (42°02' N, 116°17' E, 1,324 m above sea level) in 2003. The ecosystem consisted of a

typical temperate semiarid steppe with mean annual temperature (MAT) of 2.1°C and mean annual precipitation (MAP) of 382.2 mm. The local plant community is composed of forbs including *Artemisia frigida* Willd, *Potentilla bifurca* L., and *Potentilla acaulis* L.; grasses including *Stipa krylovii* Roshev., *Leymus chinensis* (Trin.) Tzvel., *Agropyron cristatum* (L.) Gaertn., *Cleistogenes squarrosa* (Trin.), and *Carex korshinskyi* Kom; and legumes like *Melissilus ruthenicus* (L.) Peschkova.

Sixty-four 10 × 15 m plots were arranged into eight rows and eight columns. Eight N addition rates (0, 1, 2, 4, 8, 16, 32, and 64 g N·m⁻²·year⁻¹) were randomly assigned to eight plots in each row, and urea was applied once a year in July. Since 2005, four rows were clipped every year. Field sampling was conducted in August 2016 in the four non-clipped plots under each N addition treatment. Aboveground plant samples were collected by species with a 1 × 1 m quadrat, and then oven-dried at 65°C for 48 h to estimate biomass. Three soil cores were randomly collected at three depths each (0–10 cm, 10–20 cm, and 20–40 cm) in each plot, and then mixed to obtain a composite sample for each soil depth. Roots were collected by passing soil cores through a 2-mm mesh. Following washing, the root samples were oven-dried at 65°C for 48 h to estimate root mass. Dried plant and soil samples were ground using a ball mill (Retsch MM400, Haan, Germany) to measure C and N concentration with an element analyzer (Vario EL III; Elementar Analysensysteme GmbH, Hanau, Germany).

The N storage was calculated as the N concentration and biomass production or bulk density respectively for plant and soil. The C storage was calculated with the same equation by replacing the N concentration with C concentration data. The community-level shoot C (shoot C_{tot}) or N (shoot N_{tot}) storage is the sum of C or N storage for all species occurring in the same plot, and the community level root C (root C_{tot}) or N (root N_{tot}) storage is the sum of root C or N storage in the three layers. Soil C and N storage were estimated at three soil layers as the product of bulk density and C or N concentration. The C-to-N ratio (C:N) of plant and soil is the ratio between C and N storage. The ecosystem N storage was calculated as the sum of N storage in the shoot, root, and the three soil layers.

Data analysis for the field experiment

Logarithmic fit was performed to assess how the C pool and C:N ratio of different plant and soil components changed following increases in N addition rate. One-way analysis of variance (ANOVA) was used to test the effects of N addition on the N pool for various plant and soil

components. Multiple comparison with Tukey's post hoc test was conducted whenever significant differences were detected. Values were considered statistically significant if $P < 0.05$.

The linear-plus-plateau model was used to find the tipping point where ecosystem N retention no longer statistically increased with the N addition rate. This model is defined with the following equations:

$$y = ax + b, \text{ if } x \leq x_0 \quad (1)$$

$$y = ax_0 + b, \text{ if } x > x_0 \quad (2)$$

where y is the ecosystem retained N amount (g N/m²), x is the N addition rate (g N·m⁻²·year⁻¹); a and b are the coefficients of the linear model, and x_0 is the tipping point. All coefficients were achieved using the nls function in the R package *stats*.

Multiple regression was conducted to assess the influence of plant and microbial C:N ratio on topsoil C:N. The independent variable was the topsoil C:N ratio, while the predictors were biomass C:N ratios of shoot, root, and microbes, respectively. All variables passed the collinearity test performed with R package *car*, with the variance inflation factors (VIFs) < 10 (García et al., 2014). The relative contributions of each predictor in the model were quantified using the Lindeman-Merenda-Gold (LMG) relative importance method with R package *relaimpo*. For the most relevant predictor, a partial regression was further performed to assess the relationships between the predictor and topsoil C:N ratio after removing the collinearity driven by N addition rates.

Meta-analysis

N addition studies in grassland ecosystem were searched using *Web of Science* (Thomson Reuters, New York, New York, USA). The PRISMA guidelines were followed in order to identify relevant literature (Appendix S1: Figures S1 and S2; Koricheva et al., 2013). The topsoil was defined as the soil layer at 0–10 cm and the subsoil as the soil layer at 10–30 cm. The mean, standard deviation/standard error, and sample size of plant biomass production, plant tissue chemistry (including C concentration, N concentration, and C:N ratio), plant C storage, plant N storage, soil chemistry (including C concentration, N concentration, and C:N ratio) were extracted from the publications when reported. Numeric data were obtained from the text, tables, figures, or appendices, and *engauge* software was employed when the data were graphically presented. A small proportion of studies reported plant C and/or N storage, which were directly

included in the data set. For the rest of the studies, plant C or N storage was calculated as the product of plant biomass production and plant C or N concentration. Soil C or N storage was calculated as the product of soil C or N concentration and bulk density, respectively. If not given in the article, the data of bulk density was extracted from the data set *Soilgrids* based on the coordinate information (Hengl et al., 2014). Soil C:N is the ratio between soil C and N storage. Finally, 19 and 46 papers were included to assess the effect of N addition on plant and soil pool, respectively (Appendix S1: Tables S1 and S2).

Information regarding experimental design (N addition rate and experimental duration) and study site (latitude, longitude, ecosystem type, MAT, and MAP) was also extracted. If multiple-year results were presented, only the data for the last year were retained for further analysis. Only data from N addition alone plus the control treatments were included in the database to avoid potential interactions with other factors such as warming and precipitation treatments.

Response ratio (RR) of the C storage, C:N ratio, and N storage of plant or soil to N addition was calculated with the following equation:

$$RR = X_e / X_c \quad (3)$$

where X_e is the data under N addition experimental treatments and X_c is the data under control treatments.

A preliminary analysis conducted a weighted meta-regression to assess whether N addition level and experimental duration affected RRs (Figures 7 and 8). Then the RR from studies with different N addition levels and experimental durations were normalized by using the following equation:

$$X_{e1} = [X_c + (X_e - X_c) / \log_N \times \text{mean}(\log_N)] \quad (4)$$

where $\log_N$ is the $\log_{10}$ -transformed N addition rate ($\text{g} \cdot \text{m}^{-2} \cdot \text{year}^{-1}$), and X_{e1} is the data of C storage, C:N ratio, and N storage under N addition following normalization to the mean value of $\log N$. The treatment duration was normalized to 10 year to calibrate the effect of treatment duration via the following equation:

$$X_{e2} = [X_c + (X_{e1} - X_c) / \log_D \times \log_{10} 10] \quad (5)$$

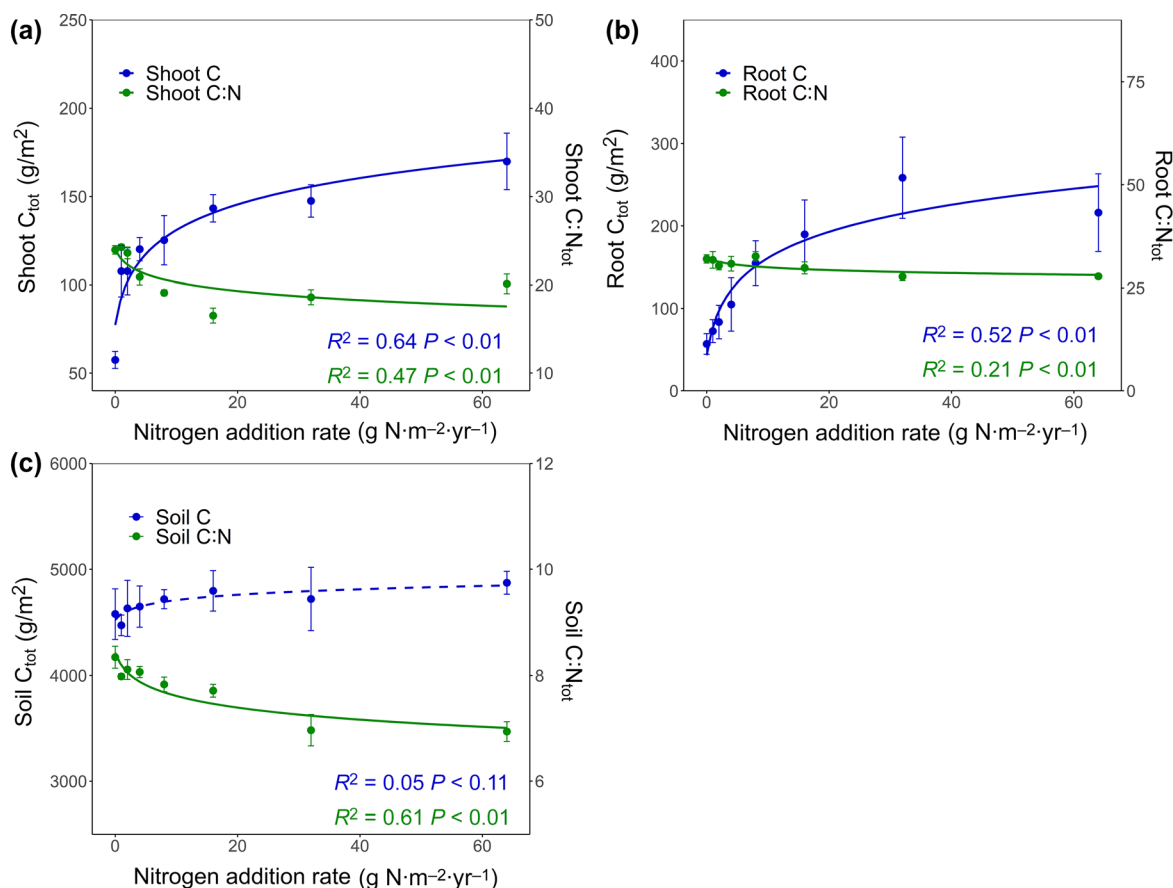


FIGURE 2 The effect of different N addition rates on C storage and the C:N ratio of different ecosystem pools, including (a) shoot, (b) root (0–40 cm), and (c) soil (0–40 cm)

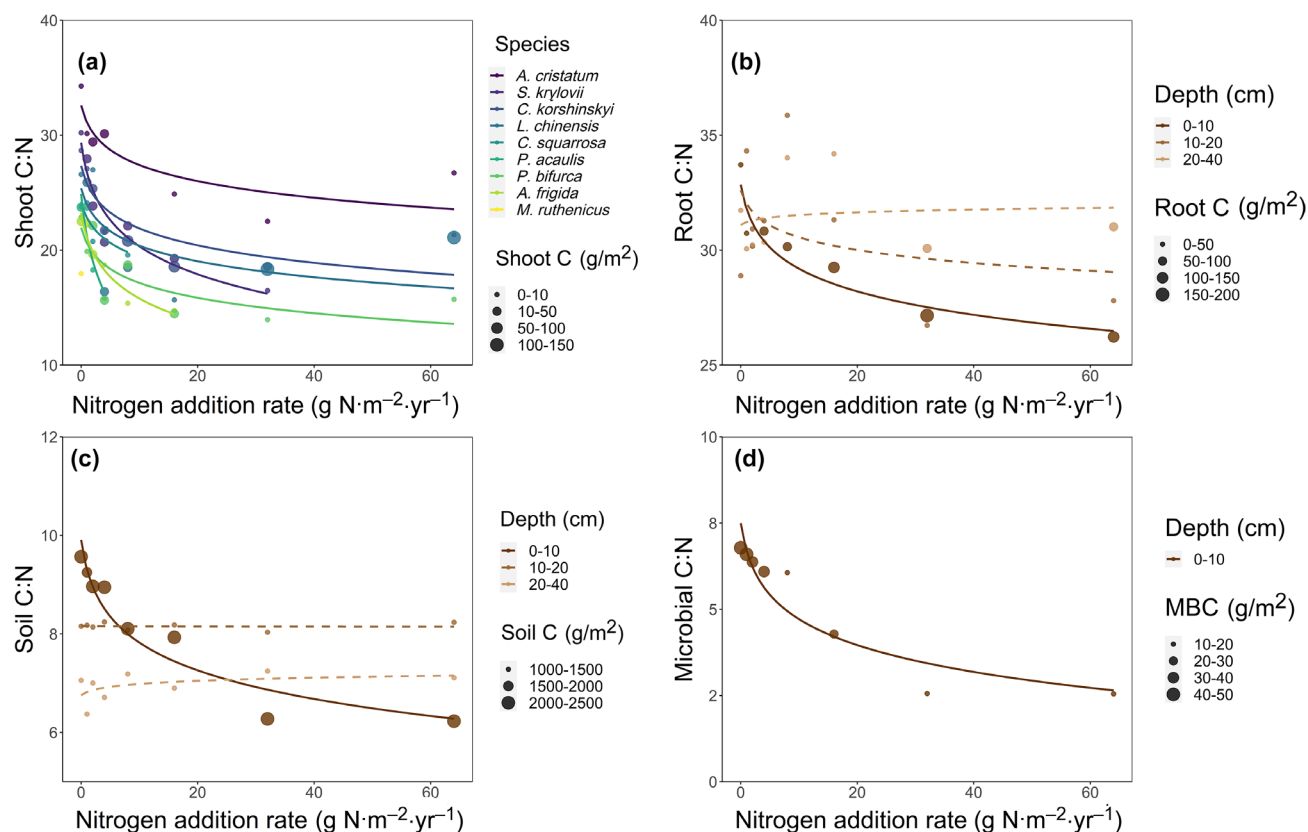


FIGURE 3 The effect of different N addition rates on (a) shoot C:N ratio of individual plant species, (b) root C:N ratio, (c) soil C:N ratio, and (d) microbial C:N ratio. The size of the points reflects the size of the C pool in each of the ecosystem components. Plant species in a are ranked in order from high (blue points) to low (yellow points) in accordance with their C:N rank, and missing points indicate that a species was lost under a corresponding treatment level

TABLE 1 The effects of different N addition rates on N storage in shoot, root, and soil components

N addition rate (g N·m ⁻² ·year ⁻¹)	Shoot N _{tot} (g N/m ²)	Root N _{tot} (g N/m ²)	Soil N _{tot} (g N/m ²)	Ecosystem N (g N/m ²)
0	2.39 ± 0.16 ^a	1.77 ± 0.38 ^a	547.60 ± 15.42 ^a	551.68 ± 15.65 ^a
1	4.44 ± 0.59 ^{ab}	2.27 ± 0.39 ^a	560.05 ± 9.62 ^a	566.63 ± 10.28 ^a
2	4.62 ± 0.70 ^{ab}	2.79 ± 0.71 ^a	569.71 ± 20.00 ^a	577.11 ± 20.74 ^a
4	5.76 ± 0.27 ^{bc}	3.33 ± 0.93 ^{ab}	576.30 ± 22.88 ^a	585.30 ± 22.71 ^a
8	6.55 ± 0.71 ^{bd}	4.74 ± 0.81 ^{ac}	602.58 ± 7.33 ^{ab}	613.43 ± 8.14 ^{ab}
16	8.80 ± 0.90 ^d	6.21 ± 1.56 ^{ac}	622.54 ± 27.77 ^{ac}	635.43 ± 26.04 ^{ac}
32	7.98 ± 0.63 ^{cd}	9.22 ± 1.52 ^c	677.24 ± 26.92 ^{bc}	694.37 ± 27.33 ^{bc}
64	8.47 ± 0.69 ^{cd}	7.88 ± 1.83 ^{bc}	702.89 ± 12.58 ^c	718.70 ± 11.14 ^c
P	<0.01	<0.01	<0.01	<0.01

Notes: N storage (mean ± SE) under each N addition rate are provided with four replicates. P values indicate the result of one-way ANOVA, and the letters next to values present the results of a multiple comparison based on Tukey' post-hoc test (different letters indicate significant differences at $P \leq 0.05$). Root N_{tot} and soil N_{tot} are the sum of root and soil N storage in all three soil layers (0–10 cm, 10–20 cm, and 20–40 cm), and ecosystem N is the sum of N storage for the shoot, root, and soil components.

$$\text{Normalized RR} = X_{e2}/X_c \quad (6)$$

where $\log_D$ is the $\log_{10}$ -transformed N duration (year), and X_{e2} is the soil N storage under N addition following

calibration by $\log_N$ and $\log_D$. The normalized RR was used to compare the response of soil N pool under N addition treatment among studies. Meta-analysis was conducted using the R package *metafor*.

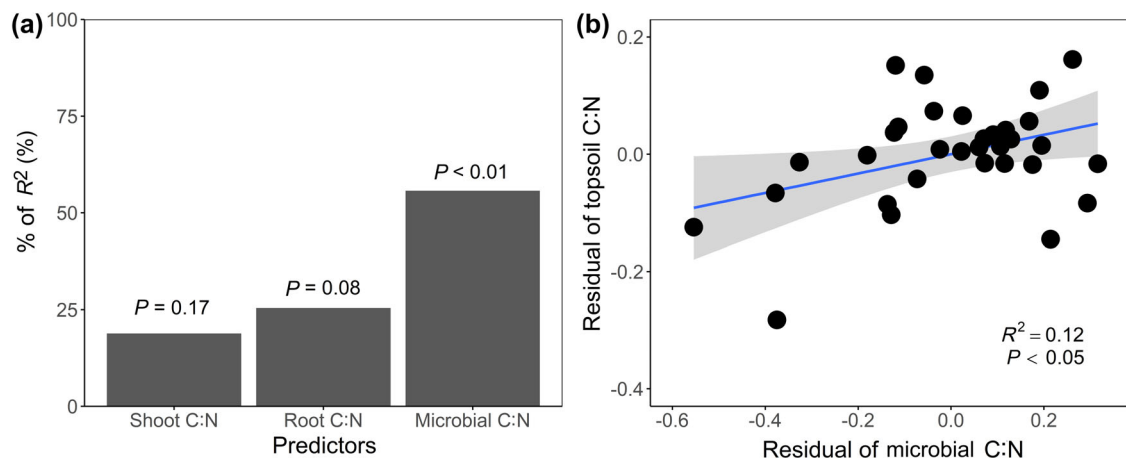


FIGURE 4 (a) The relative importance of shoot, root, and microbial C:N ratio on regulating the variation in topsoil C:N ratio after N addition and (b) the partial correlation between the most relevant predictor (microbial biomass C:N) and topsoil C:N ratio after controlling the influence of N addition rate. The letters and P values above bars indicate the significance of the parameters in the multiple regression

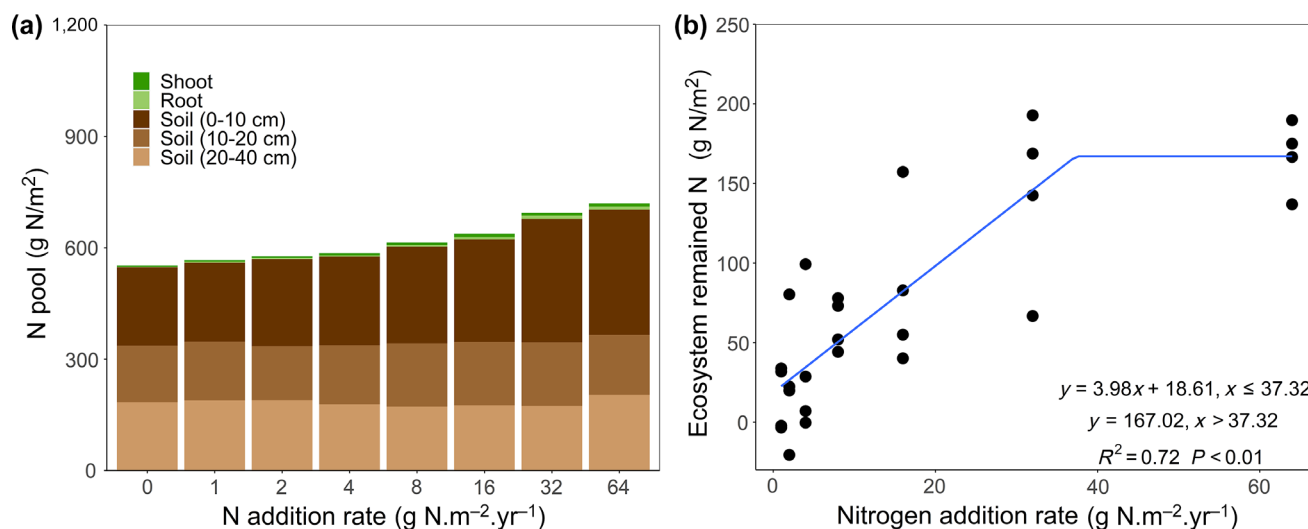


FIGURE 5 The effects of various N addition rates on (a) different ecosystem N pools and (b) ecosystem N retention amounts following the 14-year N addition treatment

All the statistical analyses were performed with R software (Version 4.0.3).

RESULTS

Response of plant N storage

Stimulated plant N storage was driven by the increase in both shoot and root biomass C pool and the decrease in their stoichiometric C:N ratios (Figure 2a, b). More specifically, N addition significantly reduced the C:N ratio of each plant species and altered the plant community composition (Figure 3a). The control plots were

dominated by forbs, and legumes only accounted for 1.6% of the total aboveground biomass (Appendix S1: Figure S3). In the presence of increasing N inputs, the dominant forb species with relatively low C:N ratio ultimately disappeared and were replaced by grass species with a relatively high C:N ratio (Appendix S1: Tables S3 and S4; Figure 3a; Appendix S1: Figure S3A). Therefore, the increase in shoot N storage was largely contributed by the increase in shoot biomass C (Table 1; Appendix S1: Figure S3B). The increase in root N storage was associated with the increased root biomass C pool in the three soil layers, and the reduced root C:N ratio in the topsoil (Appendix S1: Table S5; Table 1; Figure 3b).

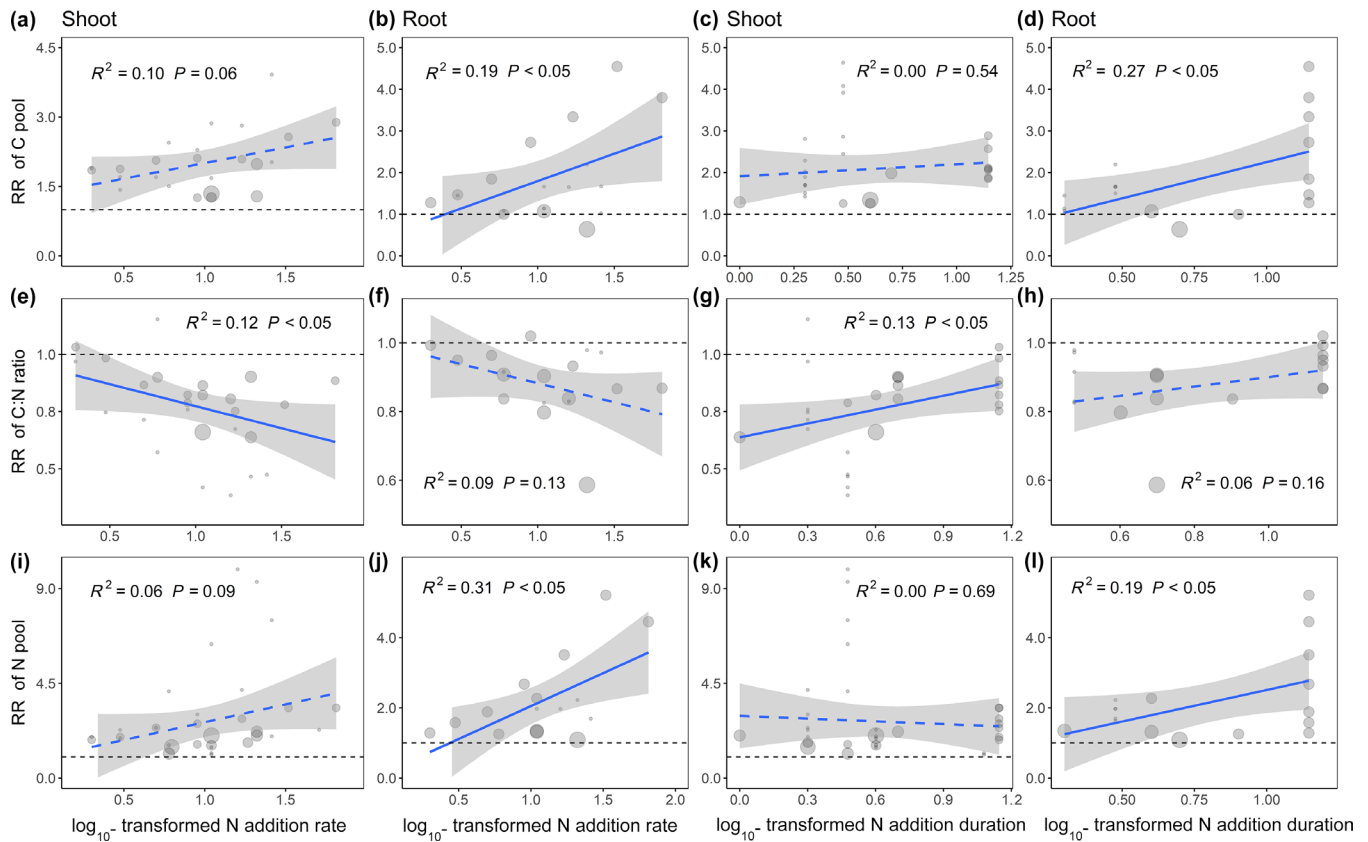


FIGURE 6 Bubble plots of the meta-regression results between response ratio (RR) of C pool, C:N ratio, N pool, and $\log_{10}$ -transformed N addition rate ($\text{g N}\cdot\text{m}^{-2}\cdot\text{year}^{-1}$) or treatment duration (year) for (a, c, e, g, i, k) shoots and (b, d, f, h, j, l) roots, respectively. The bubble size corresponds to study sample size. Larger bubbles indicate that the study results contributed a greater overall weight in the meta-regression

Response of soil N storage

The increase in soil N storage was driven by the decrease in stoichiometric C:N ratio as no significant response was detected in the soil C pool (Figure 2c). More specifically, N addition did not alter soil C storage in any of the three soil layers (0–10 cm, 10–20 cm, and 20–40 cm); however, it decreased the C:N ratio in the topsoil (0–10 cm) (Appendix S1: Table S6). Therefore, N addition increased N storage in the topsoil (0–10 cm) without altering the other two soil layers (10–20 cm and 20–40 cm) (Table 1; Figure 3c). N addition also reduced microbial biomass C:N in the topsoil (Figure 3d). The relative importance analysis showed that the changes in microbial biomass C:N ratio contributed 55.7% of the explained variation of topsoil C:N ratio, while shoot and root C:N ratio accounted for 18.9% and 25.5% of the explained variation, respectively (Figure 4a). The positive correlation revealed by the partial regression further suggested that the decline in topsoil C:N was mainly driven by the decrease in microbial C:N ratio after N addition (Figure 4b; Figure 3d).

Changes in ecosystem N storage and the upper limitation for ecosystem N retention

The increase in ecosystem N storage was predominantly contributed by topsoil retention of this element (Table 1; Figure 5a). Results of the linear-plus-plateau model indicate that, on a 14-year time scale, the upper limit of the grassland ecosystem N retention in this area was 167.0 g N/m^2 (Figure 5b).

Meta-analysis of grassland ecosystem N storage under N addition

The meta-regression indicated that the response ratios (RRs) of C pool and N pools of both shoot and root were positively correlated with experimental duration while presented no significant correlation with N addition rate (Figure 6a–d, i–l). The RR of shoot C:N ratio decreased with the N addition rate, and increased with experimental duration, while root C and N pools were not correlated with N addition rate or experimental duration (Figure 6e–h).

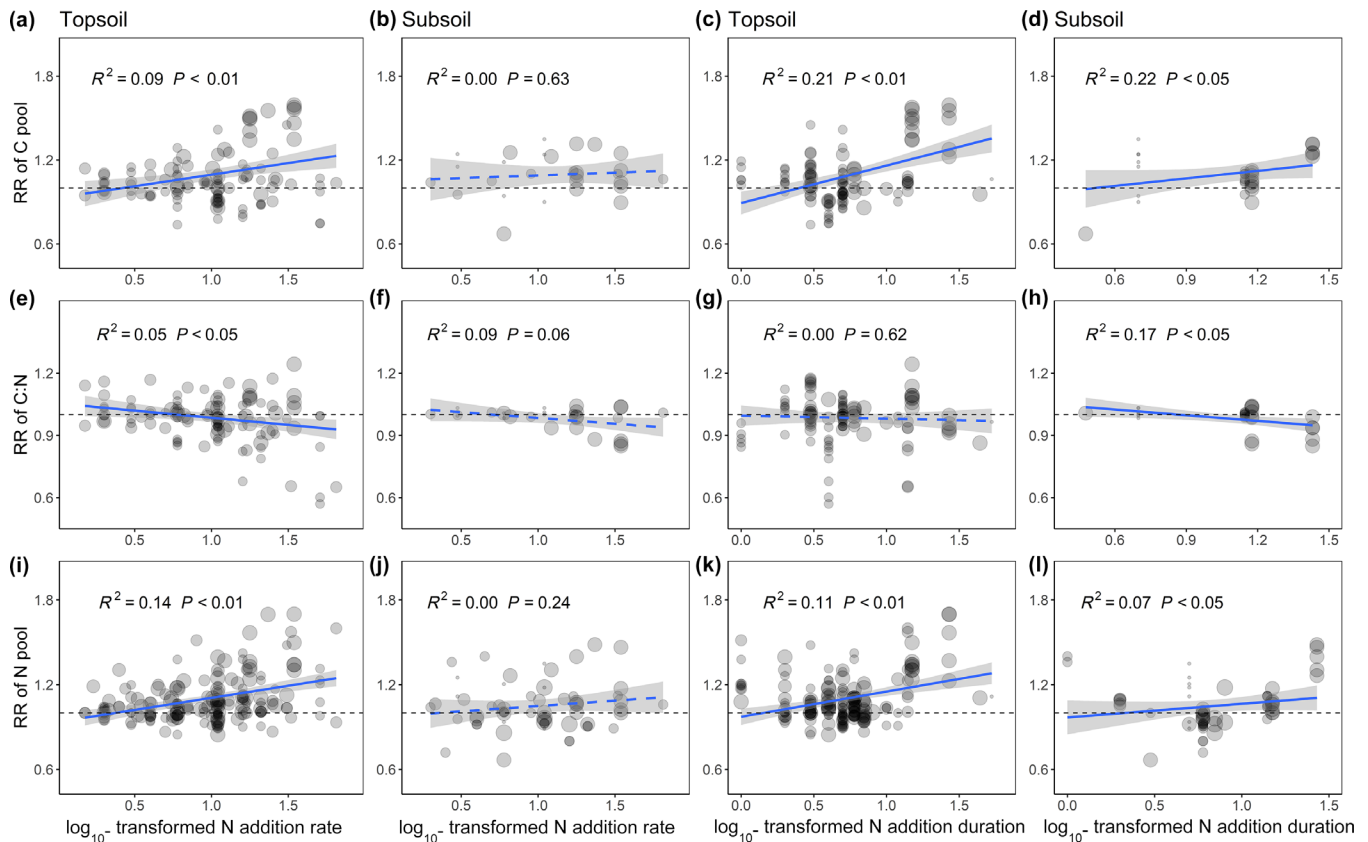


FIGURE 7 Bubble plots of the meta-regression results between response ratio (RR) of C pool, C:N ratio, N pool and $\log_{10}$ -transformed N addition rate ($\text{g N}\cdot\text{m}^{-2}\cdot\text{year}^{-1}$) or treatment duration (year) for (a, c, e, g, i, k) the topsoil and (b, d, f, h, j, l) subsoil, respectively. The bubble size corresponds to study sample size. Larger bubbles indicate that the study results contributed a greater overall weight in the meta-regression

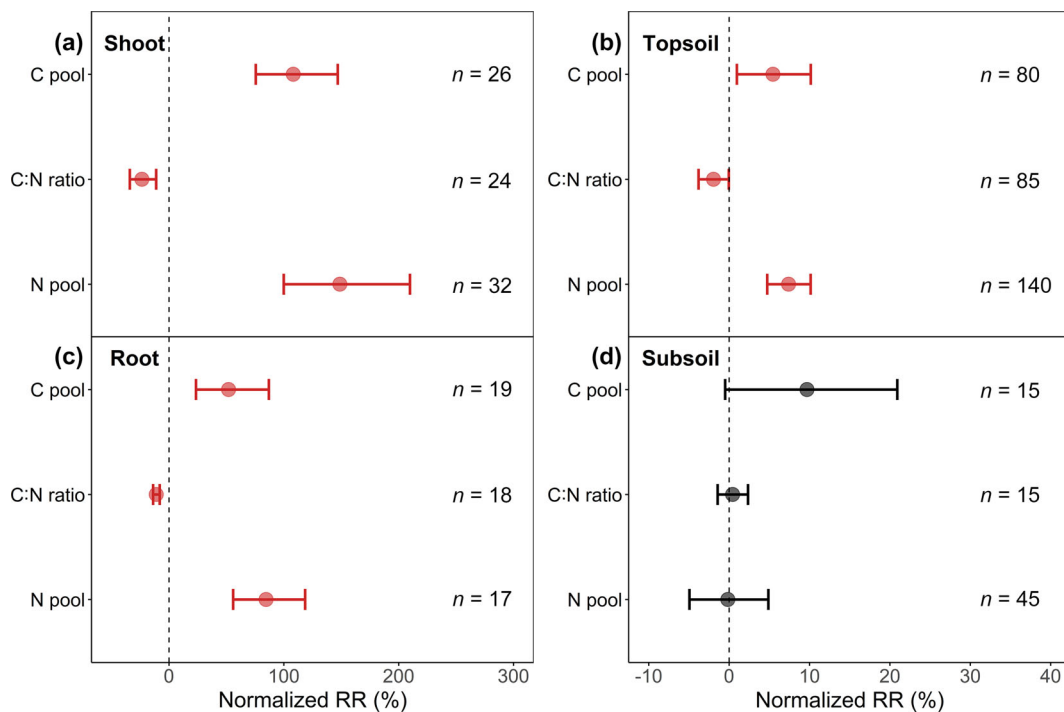


FIGURE 8 Normalized response ratio (%) of N pool to N addition in (a) shoot, (b) topsoil (0–10 cm), (c) root, and (d) subsoil (10–30 cm) in grassland ecosystem. The points with error bars indicate the mean of normalized response ratio with 95% confidence interval (CI). Red points represent the significant effect of N addition, and black points represent that no significant effect was detected. The number of observations is given next to each error bar

The RRs of topsoil C and N pool both increased with N addition rate and experimental duration, while subsoil only positively responded to experimental duration (Figure 7a–d, i–l). The RR of topsoil C:N ratio was negatively correlated with N addition rate, while the RR of subsoil C:N ratio was negatively correlated with experimental duration (Figure 7e–h).

To account for the impacts caused by the difference in N addition level and experimental duration, all observations were normalized by $\log_{10}$ -transformed N addition level and experimental duration. The meta-analysis showed that N addition enhanced the normalized C pools of shoot, root, and topsoil by 73.3%, 41.8%, and 5.3%, respectively (Figure 8a, b). N addition reduced C:N ratios of shoot, root and topsoil by 26.8%, 11.7%, and 2.0%, respectively. The changes in C pool and C:N ratio under N addition together increased the N pools of shoot, root and topsoil by 91.2%, 61.3% and 7.1%, respectively (Figure 8a, b). N addition did not alter C, N pool, and C:N ratio of subsoil (Figure 8b).

DISCUSSION

In this study, we explored how plant and soil C pool size and their C:N stoichiometric ratio regulated grassland ecosystem N retention capacity under elevated N input. We demonstrated that the increase in plant biomass C pool, decrease in plant tissue C:N ratio, and non-random loss of forb species together drove the changes in shoot and root N retention. Our field experiment and global meta-analysis further found that grassland ecosystem N retention was mainly controlled by the response of topsoil C storage and its C:N ratio. These findings indicated that the non-random loss of forbs and legumes and the lag-behind response of subsoil N pool could jointly constrain the capacity of grassland ecosystem to retain exogenous N input, especially for a large amount of N input that occurs in the short term.

Non-random species loss constrained shoot N retention capacity

Our field experiment and meta-analysis both found that, although anthropogenic N input significantly enhanced shoot N storage through stimulating aboveground productivity, the decrease in plant tissue C:N also played an important role (Figures 2a and 8a; Appendix S1: Figure S4). The C:N ratio of plant tissue determines how much N can be stored in the plant biomass pool and regulates the transformation of N from the plant litter to the soil organic matter. The stimulation effect of N addition

on plant productivity has an upper limit, which is governed by factors such as the short supply of other nutrients, soil acidification, and biodiversity loss (Chen et al., 2020; Isbell et al., 2013; Kimmel et al., 2020). Thus, considering that plant biomass production shows a saturation response to N addition rate, the decrease in plant C:N ratio was important for N retention. Our results have showed the synergistic effect of the increase in plant biomass and the decrease in stoichiometry on the N retention of plant community (Figure 2a, b), indicating the enhanced plant growth and changes in physiological metabolism jointly contributed to the N retention of plant community.

At our experimental site, N addition led to non-random loss of dominant species with relatively lower C:N ratio (Figure 3a), which reduced the potential of plant biomass pool to store N. Species with higher C:N ratio exhibited higher N-use efficiency and became more dominant under increasing N input. This phenomenon was also observed in other N addition experiments, suggesting that plants with a high C:N ratio could have an advantage via intensified interspecific competition following N addition (Craine et al., 2001; Liu et al., 2017; Sonnier et al., 2012). More specifically, most of the lost species were forbs with lower C:N ratio (Figure 3a; Appendix S1: Figure S3A). Our result further confirmed that the increase in shoot N pool was mainly contributed by the rapid increase in grass biomass production (Appendix S1: Figure S3B). In addition to the non-random loss of forbs, N addition often resulted in the loss of legumes (Bassin et al., 2007). Legumes play a virtual role in supporting high biodiversity and promoting ecosystem N supply (Pirhofer-Walzl et al., 2012; Spehn et al., 2002; Wu et al., 2017). However, legumes in our experimental site accounted for less than 1.6% of the total biomass (Appendix S1: Table S3; Figure 3a). Therefore, N addition induced legume loss had a small impact on shoot N retention at our site.

Our field experiment revealed that although the shoot C:N of individual plant species decreased logarithmically with the increase in N addition rate (Appendix S1: Table S4; Figure 3a), the non-random loss of species with a lower C:N ratio could constrain plant N retention at the ecosystem scale. Because plant community reordering lags behind the response of plant physiological metabolism under environmental changes (Smith et al., 2009), we expected that as the experimental duration increases, the negative effect of non-random species loss on community-level C:N ratio will become stronger. Indeed, our meta-regression found that the negative impact of N addition on C:N gradually diminished as indicated by the positive correlation between RR of shoot C:N ratio and experimental duration (Figure 6g).

The stoichiometric response and shift in functional groups regulated root N retention

Our field experiment and meta-analysis both indicated that N addition enhanced root C storage in all three soil layers, but the most pronounced increase was observed in the topsoil (Figure 5a). In terms of root C:N ratio, N addition reduced it in topsoil, but not in the other two soil layers (Appendix S1: Table S5; Figure 3b). The higher root C storage in topsoil may be due to the fact that N addition reduced C allocation to deep roots (Chapin et al. 2002), and increased the abundance of shallow-rooted species (Yang et al., 2011). Previous research has found that N addition reduced the root biomass of forbs while it enhanced that of grasses (Bai et al., 2015). Compared with forbs with taproot structure, the fibrous roots of grasses are mainly distributed in the surface soil (Li et al., 2020). Therefore, the decrease in forb/grass ratio under elevated N input could alter the vertical distribution of root biomass at the community level, resulting in higher root biomass in the topsoil. Studies have found that roots could proliferate in nutrient-rich soil zones to enhance resource uptake (Hodge, 2004). Since N addition only increased N storage in the topsoil (Figure 5a), the spatially synchronous response of root C allocation and C:N ratio along the soil profile further confirms that plant roots could adjust their morphology and stoichiometry to maximize the use of the added N in the soils.

Soil stoichiometry is more sensitive to N addition than to C storage

Soil N pool accounted for 97.5%–99.2% of the ecosystem N storage at our study site. Therefore, how the soil N pool responded to N addition determined ecosystem N retention capacity. The increase in soil N storage was induced by reducing the soil C:N ratio rather than an increase in soil C storage after the 14-year N addition (Figure 2c). Although the meta-analysis revealed a positive effect of N addition on topsoil C storage (Figure 8b), no significant increase in soil C pool was observed at any depth in our field experiment (Appendix S1: Table S6; Figure 3c). Our previous study at this site found that N addition decreased the turnover rate of the litter layer (Yang et al., 2019), which may reduce the transformation of plant litter C to soil C through decomposition. We also found that N addition decreased litter quality at the community level (Yang et al., 2019). As microbial C use efficiency of the recalcitrant components was reduced after N addition, less C in plant litter could be incorporated into the soil C pool through microbial-driven integration

(Cotrufo et al., 2013; Liu et al., 2018). Thus, we did not observe significant changes in topsoil C storage at our site.

Although N addition could reduce the rate of new C formation through inhibiting microbial decomposition, the soil C pool is still expected to be enhanced in the long term as indicated by the positive correlation between the RR of the topsoil C pool and the experimental duration (Figure 7c). Our results further revealed that the increase in soil N pool was driven by the decrease in soil C:N ratio after N addition (Appendix S1: Table S6, Figure 2c). The vast majority of soil N exists in the soil organic matter (Appendix S1: Figure S5), either in the form of partly decomposed plant organic matter or in soil microbial biomass and residues (Liang et al., 2017). It is thus reasonable to expect that soil C:N ratio should be largely controlled by the stoichiometry of plants and microorganisms. Indeed, our relative importance analysis indicated that the decrease in soil C:N ratio was mainly associated with the decrease in microbial C:N ratio (Figure 4). Similar to the response of plant tissue C:N, the decrease in microbial C:N can also be attributed to a shift in microbial community composition. Consistent with many other studies (Zhang et al., 2018), N addition decreased the fungi-to-bacteria ratio at this site (Liu et al., 2018). Since bacteria generally have lower C:N ratio than that of fungi (Strickland & Rousk, 2010), the decrease in the fungi-to-bacteria ratio partially contributed to the decrease in the microbial C:N ratio.

Divergent responses of topsoil and subsoil N storage to decade-long N addition

The field experiment revealed that the 14-year N addition only increased N storage in the topsoil, not in the subsoil. The meta-analysis also revealed a similar pattern that a decade N addition only significantly increased N storage in the topsoil, while not altering N storage of subsoil in grassland ecosystem (Figure 8b). As root derived litter and exudates are the main source of soil organic matters, the increased grass abundance after N addition promoted topsoil organic N storage by increasing the root biomass (Figure 3b; Bai et al., 2015). In addition, the dense and fibrous grass roots could also efficiently intercept and retain the added N in the topsoil, which further promote the higher topsoil N storage after N addition.

Compared to the topsoil, subsoil often possesses lower biological activity and less frequent nutrient exchanges with the environment (Bahram et al., 2018). However, the results of meta-regression revealed a positive correlation between subsoil RR and treatment duration both in the topsoil and subsoil (Figure 7k,l), suggesting that

subsoil could eventually play an important role in ecosystem N retention in the long-term, particularly when N stored in the topsoil became saturated (Lovett et al., 2018). The differences between the responses of top- and subsoil N pools to N input over time indicated that soil could retain more N if it received chronic low-levels of N input. However, since most of the subsoil observations were from N addition experiments lasting less than 20 years, the robustness of this finding requires further testing with long-term N addition experiments.

In conclusion, our field experiment found that N addition increased ecosystem N storage by enhancing plant productivity indicated by the increased plant biomass C pool and altering physiological metabolism indicated by the reduced the stoichiometric C:N ratio of plant tissues and topsoil. The non-random loss of forbs and legumes after N addition diminished the ability of the plant community to retain the exogenous N. The results from the field experiment and meta-analysis also emphasized that ecosystem N retention was primarily determined by the response of the topsoil (0–10 cm). The contribution of subsoil (10–30 cm) to N retention was limited in the short-term, but it could play an important role in N retention under long-term chronic N input, especially when the topsoil N storage is saturated. Long-term experiments are required in order to refine these estimates and improve our understanding of the above- and belowground processes that regulate ecosystem N retention.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

AUTHOR CONTRIBUTIONS

Lingli Liu and Sen Yang designed the experiments. Sen Yang, Lingli Liu, and Weixing Liu analyzed the data and wrote the manuscript. Lulu Guo, Chengzhang Wang, Meifeng Deng, and Ziyang Peng helped to conduct the field and lab work.

DATA AVAILABILITY STATEMENT

Data and code (Yang, 2021) are available in Figshare: <https://doi.org/10.6084/m9.figshare.15131307>

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