



Changes in species abundances with short-term and long-term nitrogen addition are mediated by stoichiometric homeostasis

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Abstract

Background Increasing nitrogen (N) deposition has altered plant communities globally, however the changes in species abundances with short-term vs. long-term N enrichment remains unclear. Stoichiometric homeostasis, quantified by the homeostatic regulation coefficient (H) is a key trait predictive of plant species dominance and species responses to short-term global changes. It is unknown whether H changes with N enrichment over time, thereby affecting species responses to long-term N addition.

Methods Here we investigated three representative plant species how species dominance changed to

short-term and long-term N addition with a field N addition experiment (2006–2013) in an Inner Mongolia grassland. Changes in species H with long-term N addition were analyzed using a sand culture experiment, and the correlation between species H and species abundances were explored to address the above research gaps. **Results** The abundance of *Leymus chinensis* decreased with short-term N addition, and increased with long-term N addition, while *Chenopodium glaucum* exhibited the opposite pattern. *Cleistogenes squarrosa* was only favored by 1-year N addition, and depressed by two or more years of N addition. The H values of *L. chinensis* and *C. glaucum* decreased significantly with long-term N addition, but did not

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change for *C. squarrosa*. The H values were significantly related with the abundance both in Control and long-term N addition treatments.

Conclusion Species abundance had opposite responses to short-term vs. long-term N addition. The decrease of H suggested the nutrients use strategy became more progressive, which mediated the responses of species abundances to short- and long-term N addition.

Keywords Ecological stoichiometry · Global change · Ecosystem structure · Plant functional traits · Species dominance · Grassland ecosystems

Introduction

Increasing fertilizer application and fossil fuel combustion has drastically accelerated atmospheric nitrogen (N) deposition (Zhu et al. 2016; Yu et al. 2019), and in turn has altered plant species composition of various ecosystems globally in the last several decades (Harpole et al. 2016; Sundert et al. 2021). Changes in plant species composition are primarily controlled by the different responses of species to increasing N availability (Bai et al. 2010; Zheng et al. 2019). In the early stage of N addition, invasive species, forbs and annual species often increase in abundance when N availability is relatively low (Rao and Allen 2010; You et al. 2017; Zheng et al. 2019; Vetter et al. 2020). However, the accumulation of N over time often results in a decline in plant species richness and altered species dominance through loss of more vulnerable species such as annual forbs and increasing abundance of competitive species such as rhizomatous grasses or perennial bunchgrasses (Suding et al. 2005; Koerner et al. 2016; Burson et al. 2018). Thus, there is evidence that the patterns of species responses can vary with the accumulation of N over time (Bobbink et al. 2010), but the factors determining this temporal variation in response are relatively unexplored.

There are several potential mechanisms underlying community composition change with N addition, including light competition (Hautier et al. 2009), nutrient competitive exclusion (Burson et al. 2018), soil acidification (Chen et al. 2017), manganese poisoning (Tian et al. 2016), and litter accumulation (Fang et al. 2012). It is clear that these

mechanisms are not generalizable across ecosystems or with respect to N addition amount or duration (Dickson and Foster 2011; Liu et al. 2018). For example, competition for light caused plant diversity loss after N addition in a Switzerland grassland (Hautier et al. 2009), but not in a US Kansas grassland where light was not limiting (Dickson and Foster 2011). However, what is often not taken into account is that species traits related to nutrient strategies may change with long-term N addition (Li et al. 2017; Zheng et al. 2019), which might induce shifts in the relative importance of different mechanisms.

Stoichiometric homeostasis, as quantified by the homeostatic regulation coefficient (H , Sterner and Elser 2002), is a trait that describes the degree in which a species maintains relatively constant elemental concentrations or ratios within tissues despite fluctuations in the environment (Elser et al. 2010). A high H -value indicates a species is homeostatic (i.e., maintains tissue nutrient concentrations irrespective of environmental availability), whereas a low H -value indicates the nutrient concentrations of a species varied a lot with environmental availability. The H hypothesis proposes that vascular plant species with high H dominate ecosystems (i.e., highly abundant) and also have higher stability of abundance over time when compared to plant species with low H value (Yu et al. 2010, 2015). Thus, the H hypothesis suggests that H is a powerful indicator of species dominance and responses of species to environmental changes (Yu et al. 2015). Indeed, both in an Inner Mongolia grassland and Kansas grassland, species abundance was positively related to species' H (Yu et al. 2010, 2015). In both studies, H was also predictive of the short-term response of species to N addition, but H failed to predict species abundance after long-term N addition (Yu et al. 2015). This suggests that the H hypothesis may not extend to N-rich environments or potential changes in H with long-term N addition. Previous studies showed that species H could not be affected by short-term N addition (Yu et al. 2010, 2011), however H values were dependent on overall nutrient supply (Güsewell 2004), suggesting that H may change with the accumulation of long-term N addition. To our knowledge, there have been no studies that explore the effect of long-term N addition on a species H and how changes of H are related to changes in species abundance.

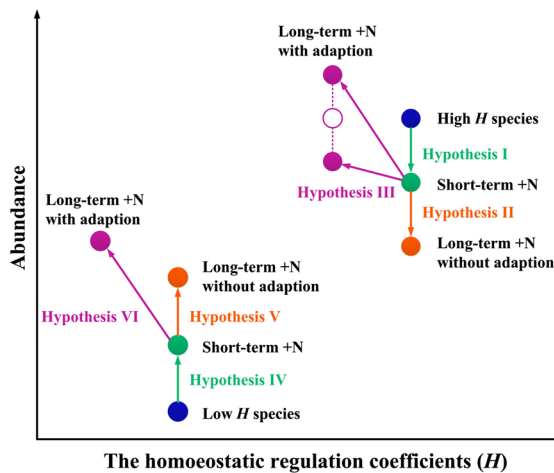


Fig. 1 A conceptual representation of the predicted changes in species abundances with short-term vs. long-term nitrogen (N) addition as mediated by stoichiometric homeostasis (H). The blue dots represent the initial abundance of high- H species and low H -species: high H species dominant the community and low H species are less abundant. Hypothesis I (the green arrow and dot): the abundance of high H species decreases with short-term N addition. Hypothesis II (the orange arrow and dot): abundance of high- H species will continue to decrease with long-term N addition if their H does not change. Hypothesis III (the purple arrow and dot): the decrease in abundance will be weakened or even reversed if high- H species decrease H to respond to the enrichment of N. Hypotheses IV, V and VI: the abundance of low H species will increase with short-term N addition and long-term N addition despite the change of H

Here, we studied the effects of both short- and long-term N addition on the abundances of three representative plant species (*Leymus chinensis*, *Cleistogenes squarrosa* and *Chenopodium glaucum*) in an Inner Mongolia grassland and on foliar H over time. We focused on three questions: (1) Will the effects of N addition on species abundance change with the accumulation of N addition over time (short-term vs. long-term N addition)? (2) Does foliar H of the three species change with the accumulation of N addition over time? And (3) Are changes of species foliar H related to the changes of the species abundance? We hypothesized that the dominant grass, *L. chinensis*, with high H would decrease with short-term N addition due to conservative nutrient utilization strategy (hypothesis I, Fig. 1). Such high- H (homeostatic) species are expected to continue to decrease in abundance with long-term N addition if H does not change over time (hypothesis II, Fig. 1). However, the decrease in abundance of high- H species would

be weakened or even reversed over time if foliar H decreased over time with N enrichment (hypothesis III, Fig. 1). In contrast, less homeostatic (low- H) species are expected to increase after N addition over the short- or long-term, even if there is a change in H because of the ability to take advantage of increased resource availability more rapidly than high- H species (hypotheses IV, V, VI, Fig. 1). We conducted a long-term N addition study to assess changes in species abundances with N enrichment over time, and we used a sand culture experiment to assess changes in H with long-term N addition (Fig. 2). We then investigated whether the changes of species homeostasis over time could affect species responses to short- and long-term N addition (Fig. 2).

Materials and methods

Study site

Our experiments were conducted in a temperate steppe near the Inner Mongolia Grassland Ecosystem Research Station (43° 264 N, 116° 046 E, 1100 m), which is located in the Xilin River Basin, Inner Mongolia. Long-term (1982–2019) mean annual precipitation in the study area was 333 mm, with 60–80% falling during the growing season (May to August), and long-term mean annual temperature was 1.1 °C, with mean monthly temperature ranging from –26.5 °C (January) to 22.8 °C (July). The soil is classified as Calcic-Orthic Aridisol according to the U.S. Soil Classification System (USCS).

Experimental design

Data used in our study came from two sources: 1) species abundances from a field N addition experiment and 2) calculations of species H from a sand culture experiment. Three species were studied: *L. chinensis* (a perennial rhizomatous C_3 grass), *C. squarrosa* (a perennial C_4 bunchgrass) and *C. glaucum* (an annual C_3 forb), which represent the dominant, subdominant, and the minor species, respectively.

The field N addition experiment was established in 2006 in a *L. chinensis* grassland that was fenced to prevent grazing by large animals. The field experiment was designed originally with seven

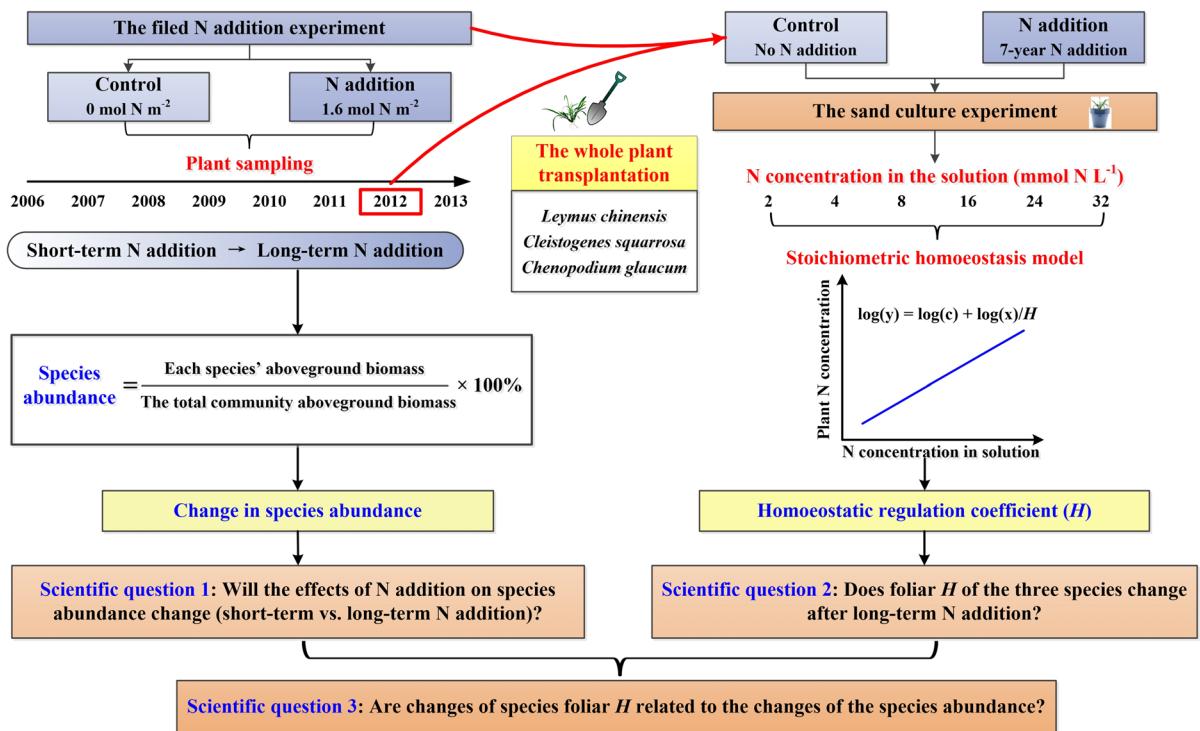


Fig. 2 The layout of experimental design

treatments including No nutrients, 0, 0.4, 0.8, 1.6, 2.8, 4.0 mol N m⁻² (added as urea, 1 mol N m⁻² equals 14 g N m⁻²) with six replicates for each treatment. Each plot, except for the No nutrients, also received 0.05 mol P m⁻² (as KH₂PO₄). The fertilizer was thoroughly mixed with sand and then applied to the plot (6×7 m) surfaces in May 2006–2013. In this study, we focused on two treatments: Control (0 mol N m⁻²) and N addition (1.6 mol N m⁻², i.e., 22.4 g N m⁻²) (Fig. 2). We chose the 1.6 mol of N addition, as it is a rate that significantly affected community composition and does not produce excessive accumulation of N (Tian et al. 2016; Zhang et al. 2014). For plant sampling, all living vascular plants rooted within a 1 m² quadrat located in each Control and N addition plot were clipped at ground level at the end of July from 2006 to 2013 (Fig. 2). Biomass was sorted to species, oven-dried at 60 °C and weighed to obtain species biomass data. Note the quadrats were randomly located each year, and previous locations of quadrats were not resampled. Species abundance was determined using the relative biomass, i.e. each species' aboveground biomass as a fraction of the total

community aboveground biomass. Change in species abundance was estimated as the difference between species abundance in the N addition treatment minus species abundance in the Control treatment (Fig. 2).

The sand culture experiment was conducted in 2012 (Fig. 2). The three focal species were transplanted on May 1st from Control and N addition plots (1.6 mol N m⁻²) of the field experiment to explore the effect of long-term N addition (after 7 years N addition) on species foliar H (Fig. 2). Plants of similar height, size and root amount were selected to minimize individual differences. The roots were well washed with water to remove all of the soil and the entire plant was planted in plastic pots (30 cm diameter, 35 cm height, 4 drainage holes). Sand between 0.2 and 2.0 mm was screened with mesh and washed five times using tap water prior to filling the pots to reduce soil nutrient concentration to negligible amounts. All the plants were cultured in pots with nutrient solutions to calculate H. For each plant species, there were six N levels (2, 4, 8, 16, 24, 32 mmol N L⁻¹, added as NH₄NO₃ solution) and three replicates (2

treatments \times 3 species \times 6 levels \times 3 replicates, 108 pots totally) (Fig. 2). Each pot received the same amount of macro- and micronutrients except for N. The macro- and microelement composition of the solution was based on Hoagland and Arnon (1950) and Jensen and Collins (1985). Each pot received 250 mL nutrient solutions every day to maintain a relatively constant concentration. To avoid ionic toxicity, the pots were washed with 500 mL water twice every 10 days immediately followed by 250 mL solutions. All pots were covered when it rained. If rainwater entered pots, additional 250 mL solutions were added. There were 10–30 individuals in each pot depending on the plant size. The density was controlled to ensure that the plant individuals did not shade each other. The aboveground and belowground parts of three species were harvested at the end of August in 2012.

N concentrations (% of dry mass) of the leaves in the sand culture experiment were analyzed using the micro-Kjeldahl method (Sparks 1996). Fifty mature, healthy and fully expanded leaves of the three species in the sand culture experiment were oven-dried at 60 °C and ground with 0.1-mm mesh. The plant samples were digested in a sample tube with a portion of sulfuric acid and Kjeldahl tablet. After digestion of the sample, the ammonia was distilled into a boric acid solution by steam distillation and titrated with sulfuric acid to calculate N concentration of the leaves in the samples.

The stoichiometric homeostasis of each plant species was estimated by calculating the homeostatic regulation coefficient (H) according to the following equation (Sterner and Elser 2002):

$$\log(y) = \log(c) + \log(x)/H,$$

where y is the plant foliar N concentration (% dry mass), x is the N concentration of the nutrient solutions in the sand culture experiment, and c is a constant.

The heterogeneity of variance test was conducted prior to all statistical analyses, and the original data met the assumption of variance normality. We analyzed the effects of N addition on response variable - species abundance using Two-way ANOVA with block as a random factor, N addition as fixed factor. One-way ANOVA was conducted to assess the significant differences of the change in species

abundance from 2006 to 2013. To estimate species H , we used regression analysis to fit the homeostatic model equation $\log(y) = \log(c) + \log(x)/H$ to the data. The difference in species H between the Control and N addition was compared by single factor analysis of covariance (ANCOVA). The Pearson correlation between species H and species abundance was evaluated using correlation analysis. All statistical analyses were performed using IBM SPSS Statistics 25, IBM Company, US. All plotting were performed using OriginPro version 9.1, OriginLab, US.

Results

The abundances of the three species changed significantly with N addition, and the direction of changes reversed with the accumulation of N over time (Fig. 3). For *L. chinensis*, in the first three years (2006–2008) of N addition, the abundance significantly decreased by 19.34% on average (Fig. 3(a), $p < 0.05$). There were no significant effects in 2009–2011 (Fig. 3(a), $p > 0.05$). However, the abundance of this C₃ grass increased by more than 35% in the last two years of N addition (Fig. 3(a), $p < 0.05$). There was a significant difference (reversal from negative to positive) in the changes of species abundance of *L. chinensis* between the first three years (2006–2008) and the last five years (2009–2013) of N addition (Fig. 3(a), $P < 0.05$). Short-term N addition inhibited the growth of *L. chinensis*, while long-term N addition enhanced species abundance (Fig. 3(a)).

For *C. squarrosa*, the abundance of this C₄ grass significantly increased by 6.74% after one year of N addition in 2006 (Fig. 3(b), $p < 0.05$). However, N addition led to a significant reduction in the abundance of *C. squarrosa* in 2008, 2009, 2010 and 2012 (Fig. 3(b), $p < 0.05$). In addition, N addition also reduced the abundance of *C. squarrosa* in 2007, 2011 and 2013, but not significantly (Fig. 3(b), $p > 0.05$). The change of species abundance in 2006 was significantly different with that in 2007–2013 (Fig. 3(b), $P < 0.05$), which was positive in the first year and negative in the other years.

As for *C. glaucum*, abundance of this minor forb increased from 2006 to 2012, but only significantly in 2007 and 2010 (Fig. 3(c), $p < 0.05$). *C. glaucum* was absent in all the plots in 2009 and 2011.

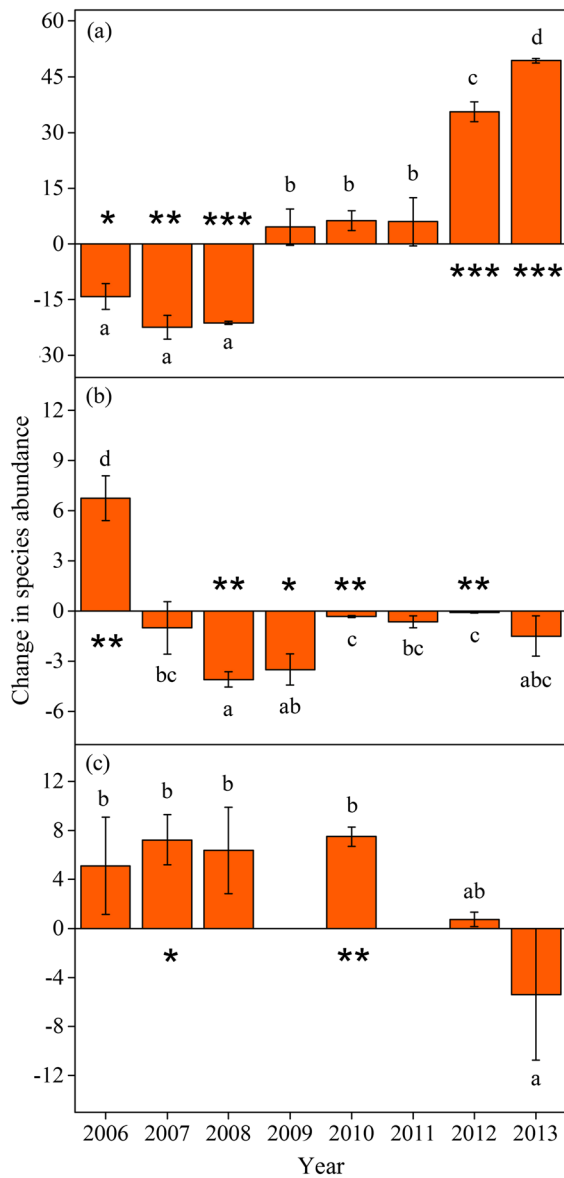


Fig. 3 Change in species abundance with long-term nitrogen addition of three species: (a) *Leymus chinensis*; (b) *Cleistogenes squarrosa*; (c) *Chenopodium glaucum*. The different letters indicate significant differences among different years ($P < 0.05$). The asterisks indicate significant differences of species abundance between Control and N addition (all block effects: $p > 0.05$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The error bars indicate the standard error. Note *C. glaucum* was not present in 2009 and 2011

However, in the eighth year of N addition, the abundance of *C. glaucum* was reduced by N addition through not significantly (Fig. 3(c), $p > 0.05$). The change in species abundance of *C. glaucum* in the

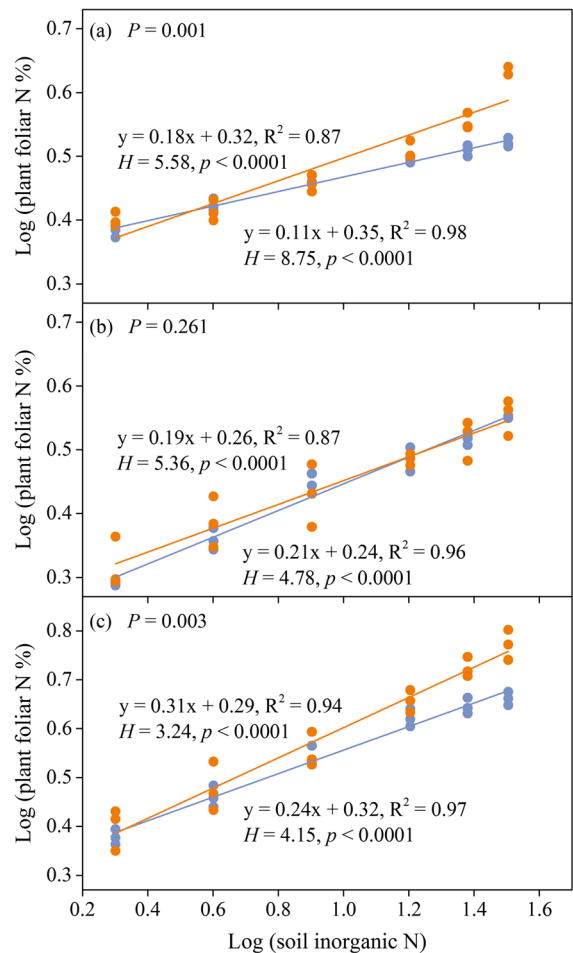
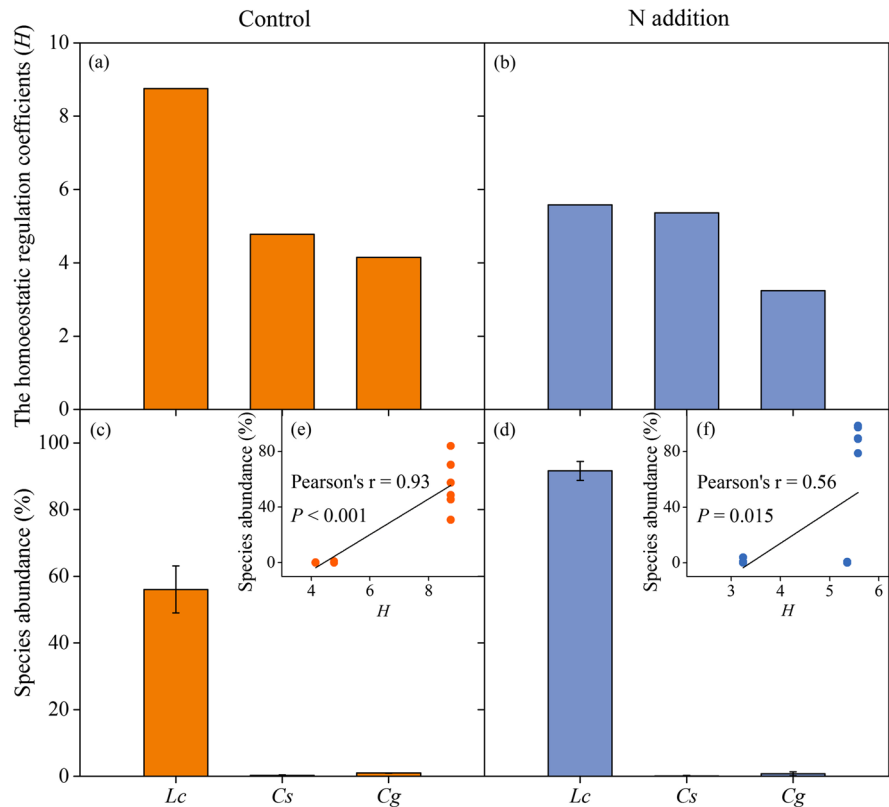


Fig. 4 The effect of long-term nitrogen (N) addition on the homeostatic regulation coefficients (H , see text for details) of three species: (a) *Leymus chinensis*; (b) *Cleistogenes squarrosa*; (c) *Chenopodium glaucum*. H values and associated R^2 of the three species in the sand culture experiment were calculated by using $\log(y) = \log(c) + \log(x)/H$ to describe the relationship between plant foliar N concentration (y) and soil inorganic N concentration (x). Lower-case p -values indicate the significance for linear fitting; upper case P -values indicate the significance differences between the slopes for the control (blue dots and line) and long-term N addition treatments (orange dots and line)

eighth year (2013) of N addition was the opposite to that of the first five years (2006–2010) (Fig. 3(c), $P < 0.05$).

In the sand culture experiment, the relationships between plant N concentrations and solution N concentrations could be rigorously described by the stoichiometric homeostasis model (Sterner and Elser 2002): $\log(y) = \log(c) + \log(x) / H$, (Fig. 4,

Fig. 5 The correlation between the homeostatic regulation coefficients (H) and species abundances in Control and N addition plots in 2012. Control (orange): (a), (c), (e); N addition (blue): (b), (d), (f). The homeostatic regulation coefficients (H): (a), (b); species abundances: (c), (d); the correlation between species H and species abundance: (e), (f). *Lc*: *Leymus chinensis*; *Cs*: *Cleistogenes squarrosa*; *Cg*: *Chenopodium glaucum*. The error bars indicate the standard error



all $p < 0.0001$). And the response patterns were similar for the three species: foliar N concentrations increased with increasing N addition rates in the solutions. On average, the homeostasis model could explain $93 \pm 5\%$ (mean \pm SD) of variation in plant tissue N. The H values in the Control treatment for *L. chinensis*, *C. squarrosa* and *C. glaucum* were 8.75, 4.78 and 4.15, respectively. After 7 years of N addition, foliar H of *L. chinensis* and *C. glaucum* decreased significantly (Fig. 4(a) (c), $P < 0.05$). However, foliar H of *C. squarrosa* did not change significantly with long-term N addition (Fig. 4(b), $P > 0.05$).

The ranks of H values for the three species were *L. chinensis* $>$ *C. squarrosa* $>$ *C. glaucum* both in Control and N addition plots (Fig. 5). The rank of species abundance in the Control was the same with that of H , while the abundance of *C. glaucum* was higher than *C. squarrosa* in N addition plots. The correlation analysis showed that the species H and species abundance were consistently related significantly and positively both in the Control ($P < 0.001$) and N addition treatments (Fig. 5, $P = 0.015$).

Discussion

In this study, the changes of species abundances with N addition exhibited two main patterns: 1) the dominant species (*L. chinensis*) decreased in abundance, while the subdominant species (*C. squarrosa*) and the minor species (*C. glaucum*) increased in abundance with short-term N addition; 2) the direction of changes in abundance of the three species reversed with long-term N addition, though the timing was different for the three species. It is very common that different species respond differently with N addition because nutrient use strategies differ among species (Chen et al. 2017; Burson et al. 2018). However, it is not well studied how species abundances change with the duration of N addition. For example, one study in a typical steppe showed that the annual species increased, while grasses and forbs decreased with N addition in the mature community, whereas perennial rhizomatous grasses increased while other species decreased in the degraded community (Bai et al. 2010). Another 14-year N addition experiment showed the abundance of grasses increased with

short-term N addition, while decreased with long-term N addition (Zheng et al. 2019). These results suggest the patterns of species responses might shift with the duration of N addition. However, most previous studies only focused on the average effects ignoring the different responses of plant species to short-term vs. long-term N addition (Song et al. 2019; Zarzycki and Kopeć 2020). Our study provides evidence that the directionality of responses of species abundances (i.e., positive vs. negative change or vice versa) reversed with short-term vs. long-term N addition, suggesting the acclimation or adaptation of species to eutrophication.

We estimated foliar H of three species with long-term N addition and found that H of *L. chinensis* and *C. glaucum* was significantly reduced, supporting the view that nutrient supply could influence species stoichiometric homeostasis (Güsewell 2004; Martínez et al. 2021). However, our previous study found that plant H did not change with 2-year N addition (Yu et al. 2011), suggesting that foliar H might only change with extended exposure to elevated N in the soil. The change of species H that we observe may also explain why the H calculated from control or short-term N addition failed to predict species abundances with long-term N addition (Yu et al. 2015). Species H and species abundance were positively related both in Control and long-term N addition, suggesting that the H hypothesis still works in an ecosystem with long-term eutrophication. Thus, the response of species H to long-term N addition should be considered in explaining the change in response pattern of species abundance with the duration of N addition. However, H of *C. squarrosa* did not change after long-term N addition, which suggests not all species may be able to actively adjust nutrient absorption strategies with an increase of N availability.

In this study, the switch in directionality of responses in species abundances to short- and long-term N addition could be well explained by the change in H of *L. chinensis* and *C. squarrosa*. *L. chinensis*, a perennial, rhizomatous C_3 grass with high H , is dominant in N-limited grasslands. With short-term N addition, the abundance of this high- H species was depressed greatly as predicted by Hypothesis I (Fig. 1), which is consistent with previous studies (Bai et al. 2010; Yu et al. 2010). This decrease in abundance is likely due the conservative

nutrient use strategy of *L. chinensis*, as indicated by the high H value. However, after long-term N addition, H decreased significantly for *L. chinensis*, which suggests the resource use strategy changed from conservative to less conservative. As predicted by Hypothesis III (Fig. 1), this shift in H resulted in an increase of *L. chinensis* abundance after long-term N addition. In contrast, *C. squarrosa*, a C_4 bunchgrass with low H , was favored by short-term N addition (Hypothesis IV, Fig. 1). However, it became a relatively high H species with long-term N addition as H of other species decreased though H of *C. squarrosa* did not change. This may explain why the abundance of *C. squarrosa* decreased with long-term N addition as expected by Hypothesis II (Fig. 1). Finally, *C. glaucum*, the annual forb with low H , was favored by short-term N addition (Hypothesis IV, Fig. 1). The H value of *C. glaucum* decreased with long-term N addition, however the abundance of *C. glaucum* also decreased, which does not support Hypothesis VI (Fig. 1). Previous studies showed that manganese poisoning with N addition depressed forbs greatly (Tian et al. 2016), which may explain why the growth of *C. glaucum* was inhibited even though the H value decreased with long-term N addition. However, we have no direct evidence that manganese poisoning or other possible mechanisms inhibited the growth of *C. glaucum* (Fang et al. 2012; Burson et al. 2018), therefore further research is needed to identify the underlying mechanisms (Smith et al. 2019; Anderson et al. 2021).

In summary, our study found contradictory responses of plant species abundances to short-term vs. long-term N addition. To our knowledge, we found for the first time that the H of two species decreased with long-term N addition, suggesting the acclimation or potential adaption of some species to nutrient enrichment. More importantly, we found evidence that the reversal in the directionality of responses in species abundances to short-term and long-term N addition were mediated by stoichiometric homeostasis (H), indicating H should be a key trait regulating plant species responses to N eutrophication over time. Moreover, our study suggests that adaptation of nutrient regulation in plant tissues via changes in stoichiometric homeostasis may be a mechanism underlying of plant community response to eutrophication and other global changes over the long-term. We anticipate our study to inspire further research to

explore how changes in stoichiometric homeostasis may mediate species responses to N enrichment or global change.

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Authors' contributions Qiang Yu conceived the ideas and designed methodology; Min Long, Chong Xu and Honghui Wu collected the data; Tian Yang analysed the data; Tian Yang and Qiang Yu led the writing of the manuscript. Qiang Yu and Honghui Wu provided the financial supports. Nianpeng He, Qian Gu, Yadong Yang, Melinda D. Smith, Anke Jentsch, Leena Vilonen and Jinling Zhao made substantial contributions to the interpretation of our data and the revision of the manuscript.

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Data availability Data used in this study are publicly achieved on Figshare and are available at: <https://doi.org/10.6084/m9.figshare.13502355.v1> (Yang, Tian 2020).

Code availability Not applicable.

Declarations

Conflicts of interest/Competing interests None.

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