

Changes in Species Abundances with Short-Term and Long-Term Nitrogen Addition are Mediated by Stoichiometric Homeostasis

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Research Article

Keywords: ecological stoichiometry, global change, ecosystem structure, plant functional traits, species dominance

Posted Date: February 24th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-250958/v1>

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Version of Record: A version of this preprint was published at Plant and Soil on September 7th, 2021. See the published version at <https://doi.org/10.1007/s11104-021-05141-2>.

Abstract

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

Methods Here we address these two knowledge gaps with three representative species in a long-term N addition experiment and a sand culture experiment.

Results The abundance of *Leymus chinensis* decreased with short-term N addition, and increased with long-term N addition, while *Chenopodium glaucum* showed opposite pattern. *Cleistogenes squarrosa* was only favored by 1-year N addition, and depressed by two and more years of N addition. The H values of *L. chinensis* and *C. glaucum* decreased significantly with long-term N addition however did not change for *C. squarrosa*.

Conclusion 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. We anticipate our research to be a starting point for explaining ecosystems function and process in response to global change from the perspective of species adaptability mediated by H .

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). Changes in plant species composition are mainly 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). 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 and increasing abundance of competitive species (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 & Elser 2002), is a trait that describes the degree in which a species maintains relatively constant elemental concentrations or ratios within its tissues despite fluctuations in the environment (Elser et al. 2010). A high H value is indicative of a species that is homeostatic (i.e., maintains low tissue nutrient concentrations irrespective of environmental availability), whereas a species with a low H value has nutrient concentrations in its tissues that track 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 abundances over time when compared to plant species with low H values (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 abundances were 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 abundances 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. A previous study showed that species H depended 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.

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 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 dominance? We hypothesized that the dominant grass, *L. chinensis*, with high H would decrease with short-term N addition due to its 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 tested these predictions with 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.

Materials And Methods

Study site

Our experiments were conducted in a temperate steppe near the Inner Mongolia Grassland Ecosystem Research Station (IMGERS, 43° 38' N, 116° 42' E, 1250 m), China. Mean annual precipitation in the study area is 350 mm, with 60 - 80% falling during the growing season (May to August). Mean annual temperature is 0.3 °C, with mean monthly temperature ranging from -21.6 °C (January) to 19.0 °C (July) (Yu et al. 2010, 2011).

Experimental design

Data used in our study came from two sources: 1) species abundances from a long-term field N addition experiment and 2) calculations of species *H* from a sand culture experiment. Three species were studied: *L. chinensis* (a perennial rhizomatous C₃ grass), *C. squarrosa* (a perennial C₄ bunchgrass) and *C. glaucum* (an annual C₃ forb), which represent the dominant, subdominant, and the minor species, respectively.

The field long-term N addition experiment was established in 2006 in a *L. chinensis* grassland that was fenced to prevent grazing by large animals. The N addition experiment had two treatments with six replicates each: control (no N added) and N addition as 1.6 mol N m⁻² yr⁻¹ (added as urea). Further details of this experimental design can be found Yu *et al.* (2010).

The sand culture experiment was conducted after 7 years of N addition in 2012. The three focal species were transplanted from control and N addition plots of the field experiment (after 7 years N addition) to explore the effect of long-term N addition on species foliar *H*. 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). 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 content to negligible amounts. For each plant species, there were six N treatments (2, 4, 8, 16, 24, 32 mmol N L⁻¹, added as NH₄NO₃ solution). Each treatment had three replicates. Further details of this experimental design can be found in Yu *et al.* (2011).

Plant Sampling and calculations

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. 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.

Fifty mature and healthy leaves of the three species in the sand culture experiment were selected, ground and mixed evenly for foliar N analyses. N concentrations (% of dry mass) of the leaves were analyzed using the micro-Kjeldahl method. The stoichiometric homeostasis of each plant species was estimated by calculating the homeostatic regulation coefficient (H) according to the following equation (Sterner & Elser 2002):

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

where y is the plant foliar N concentration (% dry mass), x is the soil inorganic N equal to the N concentration of the solution in the sand culture experiment, and c is a constant. To estimate species H , we used regression analysis to fit the homeostatic model equation $\log(y) = \log(c) + \log(x)/H$ to the data.

One-way ANOVA was used to compare whether change in abundance was different from zero, as well as whether the sign of the change differed among years. The difference in species H between the control and N addition treatments was compared by single factor analysis of covariance (ANCOVA). All statistical analyses were performed using IBM SPSS Statistics 25. All plotting were performed using OriginPro version 9.1.

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. In the first three years (2006-2008), the abundance of *L. chinensis* significantly decreased by 19.34% on average (Fig. 2 (a)). However, after not changing from 2009 to 2011, the abundance of this C₃ grass increased by more than 35% in the last two years of N addition (Fig. 2 (a)). Thus, there was a significant reversal in the abundance response of *L. chinensis* between short-term (2006-2008) and long-term (2009-2013) N addition (Fig. 2 (a)). For *C. squarrosa*, the abundance of this C₄ grass significantly increased by 6.74% after one year of N addition but decreased by 0.1% - 4.1% in the following seven years (Fig. 2 (b)). As for *C. glaucum*, abundance of this minor forb tended to increase over time, and was significant in 2007 and 2010, but decreased by 5.38% in the eighth year (Fig. 2 (c)). However, there was a significant difference in the change in abundance of *C. glaucum* between short-term (2006-2010) and long-term (2013) N addition (Fig. 2 (c)).

Foliar N concentrations increased with increasing N addition rates in the sand culture experiment. This pattern was well captured by the homeostasis model: $\log(y) = \log(c) + \log(x)/H$ (Fig. 3, $p < 0.0001$). 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. 3 (a) (c), $P < 0.05$). However, foliar H of *C. squarrosa* did not change significantly with long-term N addition (Fig. 3 (b), $P > 0.05$).

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 after short-term N addition 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. Many related studies assumed constant responses of plant species to short-term vs. long-term N addition (Song et al. 2019; Zarzycki and Kopeć 2020), which ignored the change of species strategies to long-term N addition and may lead to an inaccurate conclusion about the effect of N addition on species abundances. 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 even 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 after long-term N addition, supporting the view that nutrient supply could influence species stoichiometric homeostasis (Güsewell 2004). However, our previous study found that plant H did not change with 2-year N addition (Yu et al. 2011), suggesting that foliar H may 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). These results also suggest that H hypothesis might still work in an ecosystem with long-term eutrophication if we consider the possibility that H may change over time with 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 the increase of N availability.

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 with short-term N addition as predicted by Hypothesis I (Fig. 1) and 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 its high H . However, after long-term N addition, H decreased significantly for *L. chinensis*, which suggests its 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 I, Fig. 1). However, it shifted to a relatively high H species as its H did not change while H of other species decreased with long-term N addition. 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). However, the H value of *C. glaucum* decreased with long-term N addition but 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 its H 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.

In summary, our study found a contrasting response 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 may 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.

Declarations

Funding. The research was supported by the National Key R&D Program of China (2017YFA0604802), and the National Natural Science Foundation of China (31971533, 31270476).

No Conflicts of interest/Competing interests.

Availability of data and material. 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

Authors' contributions. Qiang Yu and Nianpeng He conceived the ideas and designed methodology; Min Long and Chong Xu collected the data; Tian Yang analysed the data; Tian Yang and Qiang Yu led the

writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Acknowledgments

The research was supported by the National Key R&D Program of China (2017YFA0604802), and the National Natural Science Foundation of China (31971533, 31270476). The authors are grateful to the Inner Mongolia Grassland Ecosystem Research Station for supporting the fieldwork and providing long-term meteorological records.

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Figures

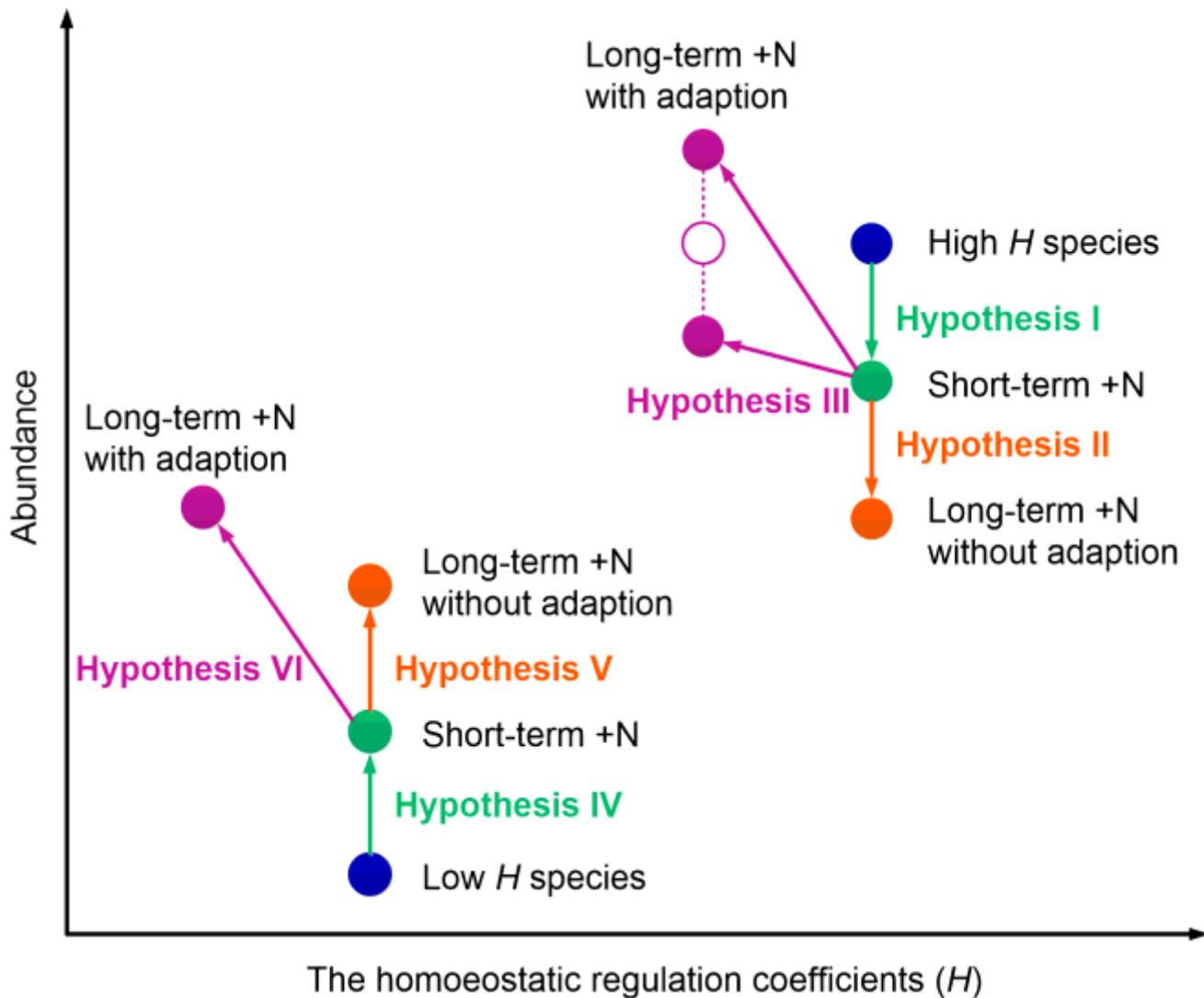


Figure 1

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 .

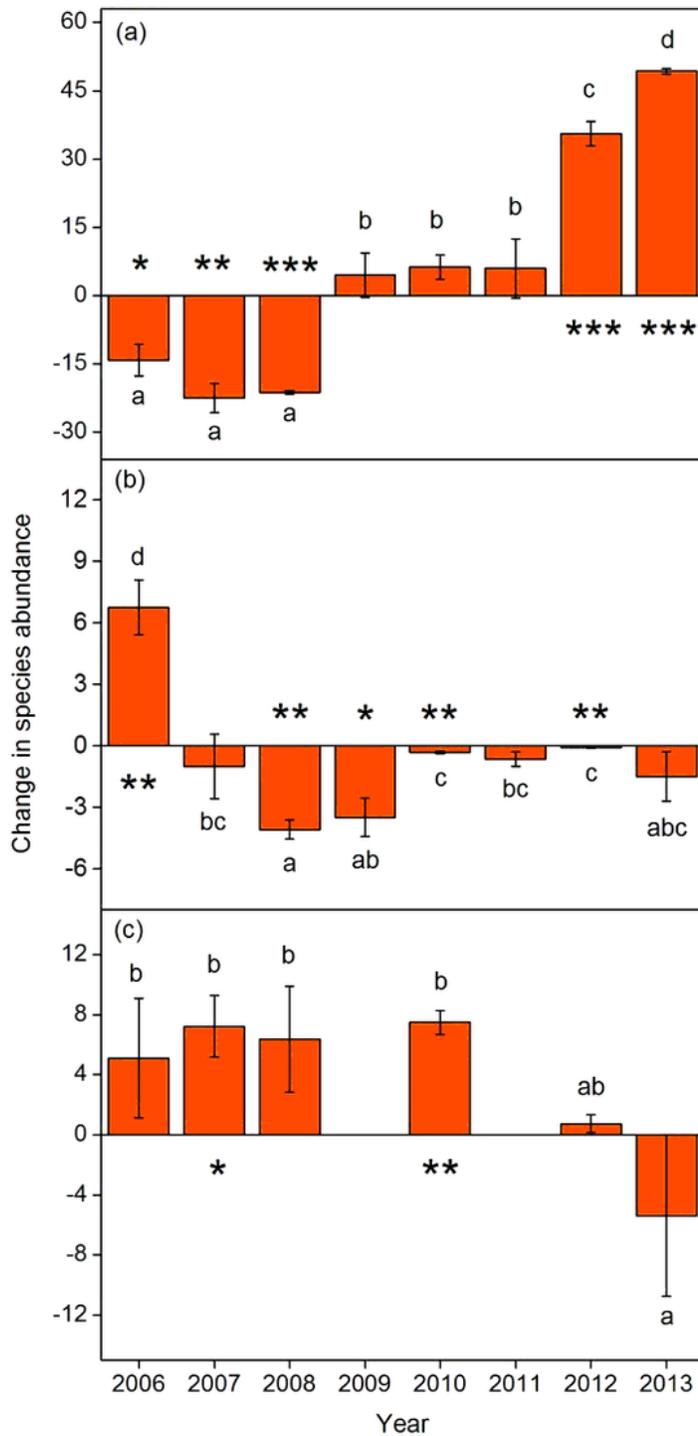


Figure 2

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 between the change in species abundance and zero. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The error bars indicate the standard error. Note *C. glaucum* was not present in 2009 and 2011.

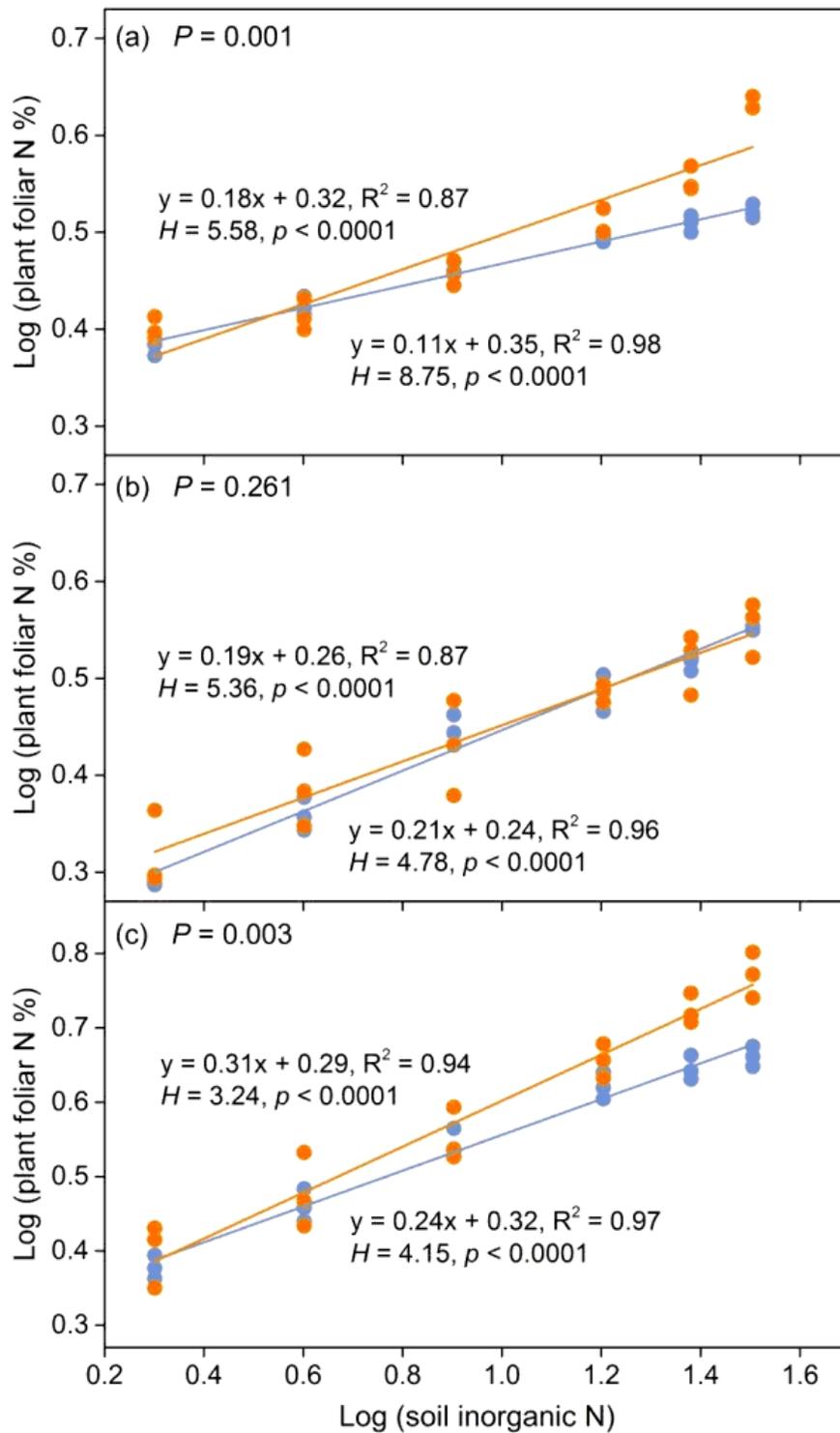


Figure 3

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 content (y) and soil inorganic N content (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).