

# Impacts of nitrogen addition on plant biodiversity in mountain grasslands depend on dose, application duration and climate: a systematic review

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## Abstract

Although the influence of nitrogen (N) addition on grassland plant communities has been widely studied, it is still unclear whether observed patterns and underlying mechanisms are constant across biomes. In this systematic review, we use meta-analysis and metaregression to investigate the influence of N addition (here referring mostly to fertilization) upon the biodiversity of temperate mountain grasslands (including montane, subalpine and alpine zones). Forty-two studies met our criteria of inclusion, resulting in 134 measures of effect size. The main general responses of mountain grasslands to N addition were increases in phytomass and reductions in plant species richness, as observed in lowland grasslands. More specifically, the analysis reveals that negative effects on species richness were exacerbated by dose ( $\text{ha}^{-1} \text{ year}^{-1}$ ) and duration of N application (years) in an additive manner. Thus, sustained application of low to moderate levels of N over time had effects similar to short-term application of high N doses. The climatic context also played an important role: the overall effects of N addition on plant species richness and diversity (Shannon index) were less pronounced in mountain grasslands experiencing cool rather than warm summers. Furthermore, the relative negative effect of N addition on species richness was more pronounced in managed communities and was strongly negatively related to N-induced increases in phytomass, that is the greater the phytomass response to N addition, the greater the decline in richness. Altogether, this review not only establishes that plant biodiversity of mountain grasslands is negatively affected by N addition, but also demonstrates that several local management and abiotic factors interact with N addition to drive plant community changes. This synthesis yields essential information for a more sustainable management of mountain grasslands, emphasizing the importance of preserving and restoring grasslands with both low agricultural N application and limited exposure to N atmospheric deposition.

**Keywords:** conservation, cumulative effects, fertilization, fertilisation, global change, nitrification, nutrient, vegetation

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## Introduction

Reactive nitrogen (N) addition to terrestrial ecosystems through agricultural fertilization or atmospheric deposition has increased substantially in recent decades and is today considered to be one of the most widespread drivers of global change (Galloway *et al.*, 2008). This dramatic increase concerns both the rate of N application or deposition, and its spatial extent (Galloway *et al.*, 2004; Erisman *et al.*, 2008). Although N addition to terrestrial ecosystems has recently levelled off in

some areas of the globe, it is predicted to increase further on a global scale (Dentener *et al.*, 2006; Erisman *et al.*, 2008). Among terrestrial ecosystems, grasslands, especially in the lowlands, have received considerable research attention. Several empirical studies and reviews have demonstrated the general response of grassland plant communities to N addition, notably in terms of decreases in species richness and resulting increases in phytomass productivity (e.g. Bobbink *et al.*, 2010; Maskell *et al.*, 2010; De Schrijver *et al.*, 2011; Borer *et al.*, 2014). These results have raised several conservation concerns and policy responses, such as the critical load policy concept (a policy tool for the control of air pollution, see Payne *et al.*, 2013 and Roth *et al.*, 2013). Some studies, however, have shown that responses can

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differ among plant communities as well as along large environmental gradients, with considerable variation in the magnitude of the responses (Clark *et al.*, 2007).

The composition of the original plant community, including the relative proportion of functional groups (i.e. grasses, forbs, legumes and sedges), can influence the direction and magnitude of the changes to N addition (e.g. Tilman *et al.*, 2001; Bassin *et al.*, 2007; Marquard *et al.*, 2009; Bai *et al.*, 2010; Onipchenko *et al.*, 2012). Grasses are generally favoured by N addition, while legumes are not, and forb responses tend to be species specific (Theodore & Bowman, 1997; Leto *et al.*, 2008; Niu *et al.*, 2008; Duprè *et al.*, 2010).

Regional environmental conditions such as climate and local soil characteristics also influence the response of the original plant community to N addition. Climate may influence responses to N addition by controlling important aspects of energy supply that contribute to plant productivity and diversity maintenance (Hawkins *et al.*, 2003; Cross *et al.*, 2015), or by influencing secondary stress impacts such as frost damage in cold climates (e.g. Clark *et al.*, 2007), and heat and water constraints in hot climates (Rustad *et al.*, 2001; Ren *et al.*, 2010). It has been shown that relative productivity responses to N addition increase with latitude (Lebauer & Treseder, 2008). At very local scales, responses can further differ due to different original soil nutrient limitation and/or soil moisture (Theodore & Bowman, 1997; but see Seastedt & Vaccaro, 2001). Relevant to these findings, resource ratio theory (Tilman, 1982) predicts that responses to N addition may be contingent on the supply of other limiting resources such as phosphorus or potassium (Ren *et al.*, 2010; Harpole *et al.*, 2011). Soil pH may also alter responses to N supply by influencing soil microbial activity and rates of N and carbon cycling (Kemmitt *et al.*, 2006; Duprè *et al.*, 2010).

In addition to environmental conditions, plant community responses may vary according to the form (oxidized vs. reduced) and type (ammonium nitrate, urea, etc.) of N addition (Gaudnik *et al.*, 2011). Nitrogen fertilizer origin (mineral or organic) also appears to play a role, but we lack quantitative evidence about its effects (but see Kirkham *et al.*, 2014; Pacurar *et al.*, 2012). Finally, biomass removal via grazing and mowing, especially within seminatural agricultural grasslands, also influences grassland community diversity and composition (e.g. Marriott *et al.*, 2009; Humbert *et al.*, 2012) and responses to N addition (e.g. Kamppmann *et al.*, 2008; Lanta *et al.*, 2009; Pavlù *et al.*, 2011; Borer *et al.*, 2014).

Overall, research to date tends to show that local biotic and abiotic conditions play an important role in moderating plant response to N addition. As the vast majority of studies were carried out in lowland

grasslands, conclusions drawn from experiments at low altitude are not readily transferable to other types of ecosystems, notably to mountain grasslands (Sebastia, 2007). Biome-specific systematic syntheses are therefore required before we can generalize (Pullin, 2012). This systematic review thus focuses on temperate mountain ecosystems including montane, subalpine and alpine grasslands. It aims at assessing the available evidence regarding the effects of N addition upon biodiversity and productivity of temperate zone mountain grasslands. In particular, it investigates how abiotic factors that potentially interact with N addition drive the variable plant community responses that are commonly observed in nature. The term N addition refers here to N from anthropogenic origin, either in the form of agricultural fertilization (sometimes in combination with other nutrients) or atmospheric deposition. The present review not only provides a basis for sound predictions about community changes but also informs land managers and policymakers about the conservation threats potentially affecting mountain grasslands and remedies for biodiversity more friendly management (Maurer *et al.*, 2006; Maskell *et al.*, 2010; Bobbink & Hettelingh, 2011).

## Materials and methods

We followed the review methodology of the Collaboration for Environmental Evidence partnership (Pullin & Stewart, 2006) and published an *a priori* protocol that was peer-reviewed (Dwyer *et al.*, 2010 provided in Appendix S1).

### Search strategy

The following Web databases were searched for documents: ISI Web of Science, Science Direct, JSTOR, Google (100 first hits) and Google Scholar (100 first hits). A high-sensitivity and low-specificity approach was used to ensure that all important relevant information was found (Dwyer *et al.*, 2010 in Appendix S1; Pullin & Stewart, 2006). The databases searches were carried out between September 2012 and January 2013 (see Appendix S2 for exact term lists and dates). Any apparently relevant citations or links were followed one step away from the original hit. In addition, national and international experts on the subject were asked for any related literature and unpublished data.

### Study inclusion criteria

All references retrieved from the Web search (2285) were scanned at the title, abstract and full-text filter levels by a first reviewer. From the 2285 initial references, 20% were randomly selected and rescanned by a second reviewer to check for inclusion consistency. The following inclusion criteria were used:

- Relevant subjects: natural or seminatural grasslands in temperate mountain zones. Grasslands were defined as generally treeless, dominated by graminoid and forb species (>50% graminoid and herbaceous cover prior to interventions), which excluded heath or other shrub-dominated lands. Mountain grasslands were defined as those occurring on mountain ranges within temperate regions that experience winter snow cover. Temperate regions were defined as those: (i) within temperate latitudes and (ii) classified in the Köppen–Geiger climate classification system as: Cfb, Cfc, Cwb, Cwc, Dfb, Dfc, Dfd, Dwb, Dwc, Dwd and Et (Kottek *et al.*, 2006). The use of these eleven categories excluded Mediterranean, subtropical and arid climates that occur within the temperate latitudes.
- Types of intervention: addition of nitrogen (alone or in combination with other nutrients).
- Types of comparator: nonfertilized control plots (experimental studies) or suitable reference areas that have not been fertilized (observational studies). Control plots had to be managed in the same way as treatment plots with the exception of fertilizer addition.
- Types of outcome: species richness or Shannon index of diversity ( $H'$ ) of at least one taxonomic group. Changes in biomass production or absolute abundance of functional groups were also recorded if provided.

Manipulative micro- and mesocosm experiments were not included as they cannot be considered 'seminatural' grasslands. While the definition of what is or what is not a mountain grassland is difficult to state, the majority of the authors defined their study sites as mountain, montane, subalpine or alpine grasslands. Importantly, study sites had to be located on the slopes or on the top of a recognized mountain range. Elevation *per se* was not a criterion, as high plateaus, such as the Xilin River Basin, Inner Mongolia, were not considered mountain sites (e.g. Bai *et al.*, 2010; study site at 1250 m). Included taxonomic groups were restricted to aboveground communities, which excluded soil microbial, faunal and fungi communities as well as seed banks. Inclusion consistency was checked with kappa statistics, and agreement between the reviewers was satisfactory ( $k = 0.81$ ) (Pullin & Stewart, 2006).

#### Data extraction

Many studies reported more than one treatment (different amounts or types of fertilizer added), and some studies reported the results of the same experiment replicated in different habitats [e.g. Theodose & Bowman (1997) duplicated their experiment in dry and wet meadows]. In these cases, all comparisons were recorded as independent data points, and this is why there are more data points (units of analysis) than studies (Pullin & Knight, 2003; Humbert *et al.*, 2012). The majority of studies (40 of 42) that respected inclusion criteria were on vascular plants, with only two studies on either bryophytes (Bergamini & Pauli, 2001) or Coleoptera (Majzlan & Gajdoš, 2007). We therefore decided to limit this review to vascular plants.

The following information was extracted for each relevant treatment from the selected studies: (i) species richness and/

or  $H'$ ; (ii) total vascular plant biomass and biomass per functional group (i.e. grass, sedge, legume or forb); (iii) number of replicates per treatment; (iv) study duration in years; (v) Köppen–Geiger climate (hereafter 'K-G climate'); (vi) country where the study was carried out; (vii) precipitation per year in mm; (viii) mean monthly temperature; (ix) altitude; (x) latitude; (xi) soil pH before the experiment started; (xii) nitrogen fertilizer origin, classified as mineral or organic; (xiii) dose of N [ $\text{kg}\cdot\text{ha}^{-1}\cdot\text{year}^{-1}$ ] applied; (xiv) dose [same units] of phosphorus (P) and potassium (K) if applied with N; (xv) fertilization frequency per year; and finally (xvi) management type of the plots during the experiment, classified as unmanaged, grazed, mown, or grazed and mown.

For studies with multiple replicates per treatment, standard deviations (SD) were retrieved from published standard errors (SE) or variances. If no estimate of variance was provided, we requested it from the original authors. If original authors could not provide estimate of variance, or sample size was equal to one (i.e. no variance), the corresponding study was included only in the unweighted analyses (see Statistical analysis section below). K-G climates were rarely provided in the publications, but could be inferred from study site averaged monthly precipitations and temperatures. These values were either found in the original publication, provided by the authors, or found on web pages linked to the corresponding publication (e.g. from the Niwot Ridge Long-Term Ecological Research Site at <http://niwot.colorado.edu/index.html>). In some cases, we relied on the data from the nearest available weather station (e.g. for Swiss studies at: <http://www.meteosuisse.admin.ch/>) or highest resolution (30 arc-seconds latitude, i.e. ca 1 km) WorldClim global climate data ([www.worldclim.org](http://www.worldclim.org)). Values for soil pH could not always be extracted from the publication or obtained by the authors and therefore some values were missing.

Additional potential sources of heterogeneity were also extracted such as exact fertilizer form (e.g. ammonium nitrate or urea), type of geological substrate, habitat type (e.g. dry, wet or mesophilous), plot size of vegetation relevés, original plant community, and former management. However, these factors could not be sourced for all studies and were later disregarded from analyses due to insufficient data.

#### Statistical analysis

Meta-analyses (MAs) were conducted on three response variables: (i) species richness; (ii)  $H'$ ; and (iii) biomass. Studies lasted up to 65 years, and if multiple time-points were available along the time series, only the data for the last year (longest time period) were considered.

The response ratio statistic was used to estimate effect sizes. The response ratio (lr) for a given comparison is the difference between the mean ln-transformed treatment value and the mean ln-transformed control value (Hedges *et al.*, 1999):

$$\text{lr} = \ln(\bar{X}_F) - \ln(\bar{X}_C),$$

where  $\bar{X}_F$  and  $\bar{X}_C$  are the means of the fertilized and control (unfertilized) groups. The lr is symmetric around 0, and negative values indicate a negative effect of N addition on the

response variable. The  $l_r$  and its variance (see Hedges *et al.*, 1999; eq. 1) were calculated using the function *escalc* of the R package *metafor* (Viechtbauer, 2010).

Random- and mixed-effects meta-analytical models were used (Gurevitch *et al.*, 2001). Under random- and mixed-effects models, the true effect size, that is the effect size as if there were no sampling error, can vary from study to study, but is assumed to do so under a normal distribution (Perera, 2009; Viechtbauer, 2010). Here, the  $Q$  test and  $I^2$  statistic were used to assess heterogeneity among studies. The  $Q$  test is the test of significance, and the  $I^2$  statistic estimates how much of the total variability in the mean effect size (composed of heterogeneity and sampling error) can be attributed to heterogeneity among the true effect sizes (Borenstein *et al.*, 2009; Perera, 2009).

Following Johnson & Omland (2004), a set of candidate models was generated including moderator(s) related to potential biotic and abiotic processes than can be biologically or agronomically interpreted. Candidate models comprised all models including one of the following moderators: study duration in years; K-G climate; mean summer temperature (i.e. mean monthly averages of May–August); number of month(s) with mean temperature  $\geq +10^\circ\text{C}$ ; fertilizer origin (organic vs. mineral); fertilizer type (i.e. N, NP, NK or NPK); fertilization doses of N, P, and K; management type (nominal variable with four classes: unmanaged, grazed, mown, or grazed and mown); and management occurrence (binary variable with two classes: managed or unmanaged, with managed including grazed, mown, and grazed and mown). Candidate models also consisted of all models that included N dose applied plus one of the above-mentioned moderators, and the following more complex model: N dose + P dose + K dose. Influences of mean yearly precipitation, altitude and latitude were not tested independently as they are all encompassed in the K-G climate variable. The set of candidate models were ranked based on AIC values (Akaike information criterion) and on the level of significance of the estimates (Johnson & Omland, 2004; Borenstein *et al.*, 2009). Influences of soil pH and productivity ratio (phytomass production in fertilized plots/phytomass production in control plots) were also investigated, but could not be included in the model selection process because of missing values for several studies. Publication bias was assessed using funnel plots, by applying a regression test for funnel plot asymmetry (Borenstein *et al.*, 2009; Viechtbauer, 2010). Normal quantile–quantile (Q–Q) plots were inspected to assess normality of the residuals. Only the results where corresponding Q–Q and funnel plots were satisfactory are presented here.

In addition to the weighted MAs that utilized variances among replicates, unweighted meta-analyses were applied to a larger data set that included effect sizes without associated variances. Unweighted MAs were limited only to null models (i.e. without moderators) to assess overall effects of N addition. Bootstrapping was used to calculate 95% confidence interval (CI) of the estimated effect size; if CIs overlapped zero, the effect size was considered to be nonsignificant. All statistics were performed using R version 3.1.2 (R Core Team, 2014).

## Results

A total of 2285 references were retrieved from the Web. Only 43 articles matched inclusion criteria, that is dealt with the influence of N addition on grassland biodiversity (Appendix S3). Among them, eight

**Table 1** List of studies included in the meta-analysis, with study area and number of extracted data point(s). Appendix S4 provides a more comprehensive overview of variables considered for each data point

Source (study reference)	Country	Number of data points
Bassin <i>et al.</i> (2007)	Switzerland	4
Bassin <i>et al.</i> (2012)	Switzerland	4
Baumberger <i>et al.</i> (1996)	Switzerland	2
Bergamini & Pauli (2001)	Switzerland	2
Bonanomi <i>et al.</i> (2009)	Italy	3
Bowman <i>et al.</i> (2006)	USA, Colorado	3
Bowman <i>et al.</i> (2012)	USA, Colorado	3
Brinkmann & Reif (2006)	Romania	12
Britanak <i>et al.</i> (2007)	Slovakia	2
Chytrý <i>et al.</i> (2009)	Germany	4
Delpech (1984)	France	1
Elisseou <i>et al.</i> (1995)	Greece	9
Fahnestock & Detling (1999)	USA, Wyoming	2
Gross <i>et al.</i> (2009)	France	2
Jeangros & Troxler (2008)	Switzerland	2
Kassioumi (2003)	Greece	4
Kohler <i>et al.</i> (2004)	Switzerland	4
Kohler <i>et al.</i> (2005)	Switzerland	4
Krajčovič <i>et al.</i> (1990)	Slovakia	3
Kralovec <i>et al.</i> (2009)	Czech Republic	4
Lanta <i>et al.</i> (2009)	Czech Republic	3
Leto <i>et al.</i> (2008)	Croatia	2
Li <i>et al.</i> (2010)	China, Tibet	1
Majzlan & Gajdoš (2007)	Slovakia	3
Mamolos <i>et al.</i> (2005)	Greece	4
Mudrak & Leps (2010)	Czech Republic	1
Niu <i>et al.</i> (2012)	China, Tibet	2
Olofsson & Shams (2007)	Sweden	2
Onipchenko <i>et al.</i> (2012)	Russia	4
Pauli <i>et al.</i> (2002)	Switzerland	2
Pavlù <i>et al.</i> (2011)	Germany	4
Pavlù <i>et al.</i> (2012)	Czech Republic	2
Ren <i>et al.</i> (2010)	China, Tibet	4
Rixen <i>et al.</i> (2008)	Switzerland	1
Seastedt & Vaccaro (2001)	USA, Colorado	6
Sebastia (2007)	Spain, Pyrenees	2
Song <i>et al.</i> (2012)	China, Tibet	3
Suding <i>et al.</i> (2008)	USA, Colorado	1
Tenz <i>et al.</i> (2010)	Switzerland	1
Theodore & Bowman (1997)	USA, Colorado	4
Veresoglou <i>et al.</i> (2011)	Greece	4
Wang <i>et al.</i> (2010)	China, Tibet	4

articles were excluded due to paper content duplication. This was apparent when two distinct references stemming from the same experiment presented the same data to address different questions or presented results over different time periods. Seven additional articles were found in the bibliography sections of the retained papers or obtained after contacting experts, which resulted in a total of 42 suitable studies. In some studies, more than one treatment or more than one habitat type were investigated, resulting in a total of 134 treatment-control comparisons (i.e. data points, Table 1).

From these 134 data points, 98 reported results on plant species richness, 60 on Shannon index ( $H'$ ) for plants (in some cases, the original reference did not report results on  $H'$ , but the corresponding author provided the raw data for its calculation), and 103 on changes in phytomass production. In 43 cases (12 for plant species richness, 20 for  $H'$  and 11 for phytomass), the study did not report SD, or replication per treatment was one. Consequently, these data could only be included in the unweighted MA. All studies were experimental, except one which was observational (Jeangros & Troxler, 2008) but of sufficient quality to be included (quality category II-2 of Pullin & Knight, 2003). Most observational studies were excluded because they did not report the quantity of nitrogen applied (e.g. Spiegelberger *et al.*, 2006). A list of all studies considered and of all treatment-control comparisons is provided in Appendix S4. Appendix S5 lists the articles excluded after full-text filtering, mentioning reasons for exclusion.

We present the results of the different MAs as follows: (i) all null models (models without moderators) for plant species richness,  $H'$  and biomass production; (ii) the best-supported models for species richness and  $H'$ ; and (iii) several single-moderator models that did not emerge as 'best' models, but included moderators of high agronomical or biological relevance.

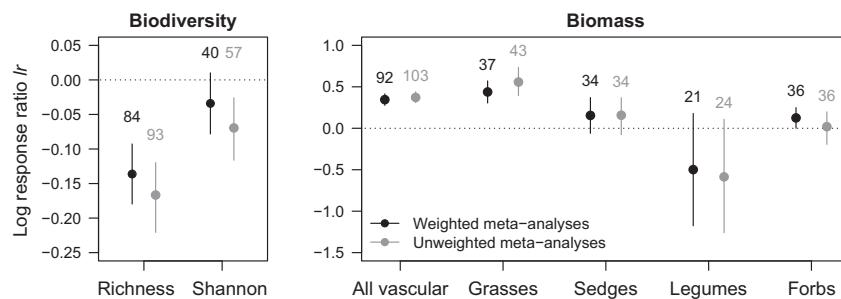
### Null models

Species richness was reduced by N addition in weighted and unweighted MAs (Fig. 1). Regarding  $H'$  (Shannon index) weighted MA indicated no effect of N addition, while the unweighted MA indicated a significant decrease of  $H'$  in response to N addition. Overall, vascular plant and grass biomass were higher in fertilized compared to unfertilized plots. Sedge and legume biomass did not change following N addition, while forb biomass exhibited a significant increase with weighted MA and no change with unweighted MA (Fig. 1; see Appendix S6 for detailed model outputs).

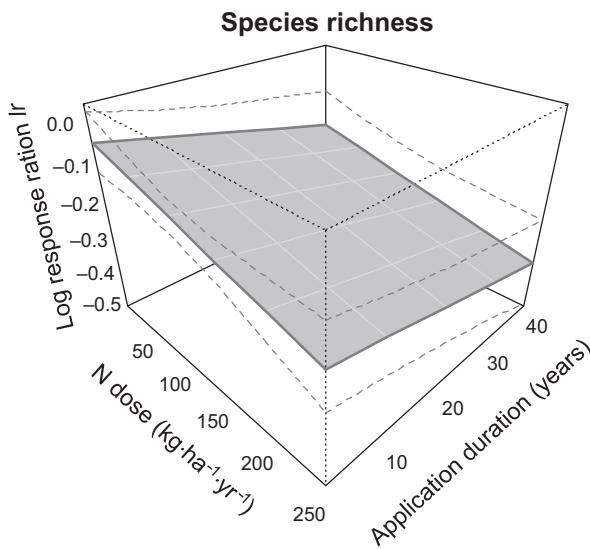
### Best-supported models

The model with best-support explaining changes in species richness was the bivariate model including N dose and study duration as moderators (Fig. 2 and Appendix S7). Both moderators had a significant negative effect on plant species richness effect size (N dose:  $lr = -0.0007$ ,  $z = 0.0003$ ,  $P = 0.026$ ; study duration:  $lr = -0.0041$ ,  $z = 0.0014$ ,  $P = 0.003$ ). Heterogeneity among studies was significant ( $Q = 605.28$ ,  $df = 83$ ,  $P < 0.001$ ), indicating that other moderators likely influence responses to N addition.

The best model explaining  $H'$  was the single-moderator model including the number of months with mean temperature  $\geq +10^\circ\text{C}$  (Fig. 3b and Appendix S7), which indicated reduced  $H'$  in warmer regions. However, the resulting funnel plot showed significant asymmetry due to an outlying data point ('Site.T ii', from Kassio-umi, 2003 unpublished PhD Thesis). Excluding this data point improved model diagnostics, but did not alter conclusions (number of months with mean temperature  $\geq +10^\circ\text{C}$ :  $lr = -0.044$ ,  $z = 0.008$ ,  $P < 0.001$ ) or selection of the 'best' model. As for the species richness models, heterogeneity among studies was significant, indicating that other moderators likely influence effect sizes.



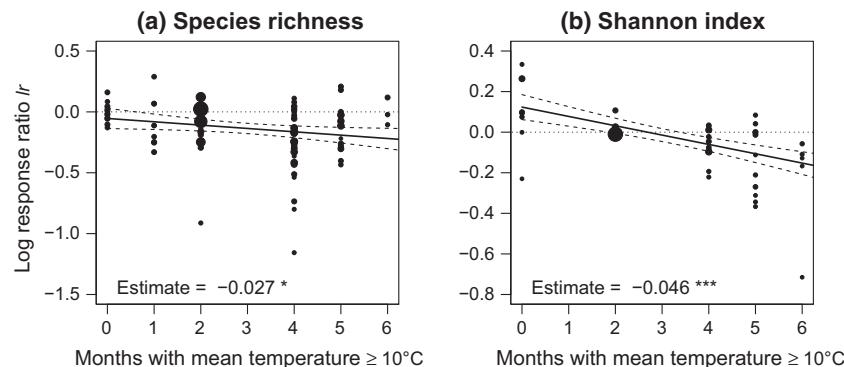
**Fig. 1** Influence of N addition on plant species richness, diversity (Shannon index) and biomass production. Effect sizes are response ratios ( $lr$ ), with negative values meaning a negative effect of N addition. Points represent means and error bars  $\pm 95\%$  CI. Sample size (numbers of data points) is given above each bar.



**Fig. 2** 3D perspective plot of species richness response ratio (lr) vs. the amount of nitrogen applied per year and application (study) duration. The grey plane represents the mean response and dashed lines are corresponding 95% CI.

#### Other single-moderator models

The negative effect of N addition on species richness was more pronounced in warmer than in cooler regions (Fig. 3a) and where phytomass responses to N addition were largest (higher productivity ratios; Fig. 4a). In contrast, there was no significant relationship between  $H'$  effect size and the productivity ratio (Fig. 4b). The negative effect of N addition on species richness was significantly more pronounced where experimental plots were managed (lr =  $-0.1070$ ,  $z = -2.5717$ ,  $P = 0.0101$ ; Fig. 5). Species richness effect sizes were also moderated by fertilizer type (i.e. N, NP, NK or NPK): there was a stronger negative effect on species richness when NPK was applied compared to N alone (Appendix S8). Initial soil pH did not influence the effect of N addition on species richness (Appendix S8).



**Fig. 3** Species richness and Shannon index response ratios (lr) vs. the number of months with mean temperature  $\geq 10^\circ\text{C}$ . Significance levels for slope estimates are given for each regression line (\* $P < 0.05$ ; \*\*\* $P < 0.001$ ). Symbol size depicts study weight.

Unsatisfactory funnel plots prevented further metaregression on  $H'$ .

#### Discussion

Overall, this systematic review indicates that N addition generally reduces plant species richness and diversity and increases biomass in temperate mountain grassland systems, but these responses are also strongly influenced by N dose, application duration and management practice. Our results also suggest that plant community responses to N addition are modulated by mean summer monthly temperatures.

Both weighted and unweighted MAs showed that N addition typically induced a decrease in plant species richness. This is in agreement with the general negative pattern found in broad-scale studies of lowland grassland fertilization (e.g. Bobbink & Hettelingh, 2011; De Schrijver *et al.*, 2011; Van Den Berg *et al.*, 2011). Shannon index ( $H'$ ) also decreased with N addition but to a lesser extent, and the decrease was significant only when using unweighted MA. Vascular plant biomass increased with N addition, and this pattern was mostly driven by an increase in grass biomass. Biomass of sedges and legumes did not change with N addition while forb biomass exhibited a small significant increase, but only when weighted MA was applied. Mean effect sizes for legume biomass were clearly negative, but the variances of both weighted and unweighted MAs were large with the 95% CI overlapping 0 (i.e. no effect). It suggests that adding N has the tendency to decrease legume biomass, although other factors, such as the addition of P with N, can have interactive effects (e.g. Willems *et al.*, 1993; Ren *et al.*, 2010).

#### Best-supported models

The model with best support for explaining changes in species richness following N addition was the bivariate

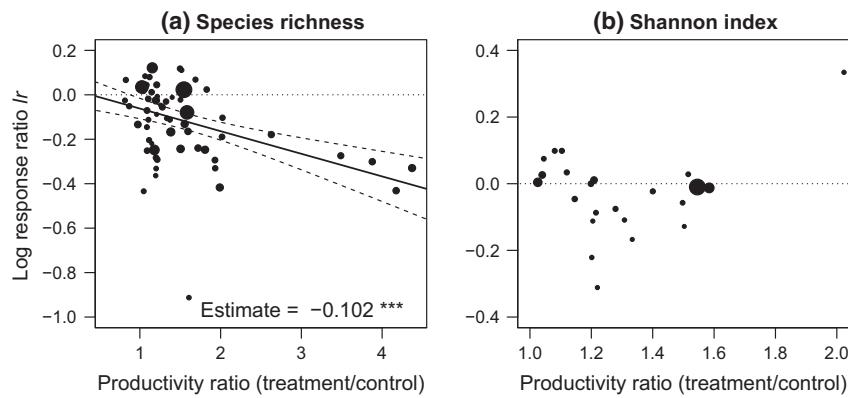


Fig. 4 Species richness and Shannon index ( $H'$ ) response ratios ( $lr$ ) vs. phytomass productivity ratio (treatment/control). Significance level for slope estimate is given (\*\* $P < 0.001$ ). Symbol size depicts study weight.

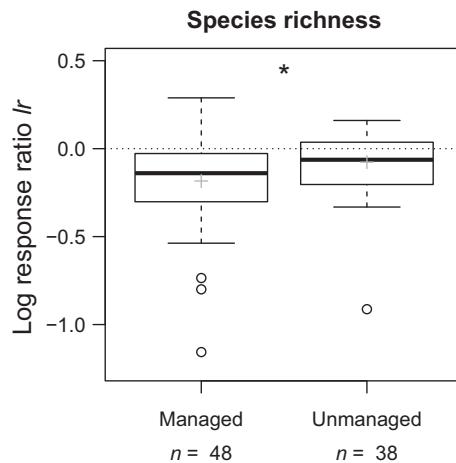


Fig. 5 Boxplot presentation of species richness response ratio ( $lr$ ) with respect to management occurrence. Managed control and fertilized plots were grazed, mown, or grazed and mown. Bold transversal bars represent medians; grey crosses means; box boundaries the first and last quartiles; whiskers the interquartile distance multiplied by 1.5; and open circles the outliers. The asterisk indicates a significant difference between managed and unmanaged plots at  $P < 0.05$ .

model including N dose and study duration (years of N application, which indicates cumulative N; see Fig. 2). Both moderators had pronounced additive negative effects on species richness, revealing that low N doses applied for long periods lead to similar richness declines as high N doses applied for short periods. This additive effect has been recurrently presumed in the literature about grasslands. To the best of our knowledge, it is here demonstrated for the first time (but see Clark & Tilman, 2008). Because the effect sizes are on a log scale, significant negative linear relationships with N dose and study duration indicate that the steepest richness declines occur at low N doses and in the first few

years of N application, respectively. These results are consistent with common grassland models that predict the steepest species declines occurring as N supply initially increases (Kleijn *et al.*, 2009), and with findings from longer-term fertilization and N atmospheric deposition studies (Clark *et al.*, 2007; Clark & Tilman, 2008; Duprè *et al.*, 2010; De Schrijver *et al.*, 2011).

For both species richness and  $H'$ , the negative effect of N addition was weaker in cooler regions (regions with fewer months with  $\geq +10^\circ\text{C}$  mean monthly ambient temperature) than in warmer regions. This is consistent with some findings from alpine and Arctic tundra regions (Bowman *et al.*, 2006; Dittomaso & Aarssen, 1989; but see Seastedt & Vaccaro, 2001), but contrasts with findings from multiple sites across North America where the greatest species losses were observed in colder regions (Clark *et al.*, 2007). It is likely that colder growing season temperatures limit the extent to which plant species can respond to increased N supply (Cross *et al.*, 2015). Slower growth rates in cold adapted species have been widely reported and attributed to a trade-off between freezing tolerance and growth rate (Savage & Cavender-Bares, 2013). It has also been shown in cold ecosystems that warming alone can enhance plant productivity (Rustad *et al.*, 2001), mostly because warming increases nitrogen and phosphorus uptake capacity by plants (Jonasson *et al.*, 1999).

#### Influences of management, productivity ratio and initial soil pH

Further analyses of data showed that responses varied according to management occurrence (presence or absence of management). The relative negative effect of N addition on species richness was significantly more pronounced in managed communities (grazed, mown, or grazed and mown). This result reflects our choice of

control-treatment comparisons – we always compared the effects of N addition between experimental communities that experienced the same management. Control plots in managed communities generally had higher richness than control plots in unmanaged communities, and this richness was maintained through time in longitudinal studies (e.g. Krajčovič *et al.*, 1990; Kohler *et al.*, 2004; Bonanomi *et al.*, 2009). As such, these managed communities had more species to 'lose' after N addition. In unmanaged communities, it is likely that successional processes had already reduced diversity in control plots (Pavlů *et al.*, 2012; Gaisler *et al.*, 2013), and so further losses due to N addition were not as pronounced in relative terms. In the subset of studies that applied combinations of fertilization and management treatments to the same community (Kohler *et al.*, 2004, 2005; Bonanomi *et al.*, 2009; Lanta *et al.*, 2009), fertilized-managed plots displayed higher species richness than fertilized-unmanaged plots in all cases, indicating that mowing and grazing actually maintains a higher level of species richness following fertilization compared with no phytomass removal. This corroborates the findings of Borer *et al.* (2014) that grazing can rescue richness losses in fertilized plots by allowing more light to reach ground level and by preventing competitive exclusion via intense light competition.

Species richness, but not  $H'$ , was strongly negatively related to N-induced increases in phytomass, that is the greater the phytomass response to N addition, the greater the decline in species richness (Fig. 4). Clark *et al.* (2007) found a similar pattern, suggesting that changes in productivity play a key ecological role regarding species richness responses to N addition.

It is known that fertilization often decreases soil pH and that subsequent soil acidification has negative effects on plant communities (e.g. Duprè *et al.*, 2010; Bobbink & Hettelingh, 2011; Van Den Berg *et al.*, 2011; Liu *et al.*, 2012); however, the influence of initial soil pH on plant community responses to N addition is still unclear (De Schrijver *et al.*, 2011; Veresoglou *et al.*, 2011). Although Clark *et al.* (2007) found greatest species losses following N addition in plant communities with lower soil cation exchange capacity, that is in soils most prone to acidification, we did not find evidence of such a link with soil pH.

#### Mechanistic link to species loss

There are several ecological mechanisms that can drive grassland plant community changes following N addition. First, it has been demonstrated that fertilization can negatively impact species richness by reducing the number of available limiting resources, which diminishes trade-off opportunities that allow species coexistence

(Levine & Hillerislambers, 2009; Harpole *et al.*, 2011). This increases the biomass of exploitative species which deter smaller species with low growth rates through intensified light competition (Hautier *et al.*, 2009). Accordingly, species losses would be expected to be smaller in sites with smaller relative biomass responses to N, because small changes in foliage quantity would induce only small changes in overall light availability for the whole plant community (Ren *et al.*, 2010; Borer *et al.*, 2014). This mechanism is supported by the data at hand. In line with the concept of limiting resources reduction, stronger negative effects on plant species richness were found when P and K were jointly added to N, compared to N alone, suggesting nutrient colimitation [Appendix S8, see also Ren *et al.* (2010) and Harpole *et al.* (2011)]. Fertilization can also increase belowground root competition, causing additional competitive exclusion among species (Dickson & Foster, 2011; Rajaniemi, 2002; but see Hautier *et al.*, 2009).

#### Limitations and research gaps

Other factors that have been shown to influence plant community responses to N addition include the following: the form of N input (i.e. ammonium, nitrate; see Song *et al.*, 2012), the scale (i.e. size of the experimental plot; see Gross *et al.*, 2009 and Spiegelberger *et al.*, 2006), habitat (e.g. dry or wet; see Theodose & Bowman, 1997) and grassland type (e.g. Wang *et al.*, 2010). Unfortunately, the effects of these factors could not be investigated in this MA due to incomplete data. We have also to recognize that changes in management from pre-experimental conditions may also generate confounding effects (Kralovec *et al.*, 2009); such changes could not be investigated as information about pre-experimental conditions was rarely provided.

The main research gap identified by this systematic review is certainly the lack of studies on invertebrate responses to grassland N addition. All studies that met inclusion criteria were on vascular plants, except two: one on bryophytes (Bergamini & Pauli, 2001) and one on Coleoptera (Majzlan & Gajdoš, 2007). While there are few observational studies on invertebrate responses to fertilization (e.g. Grandchamp *et al.*, 2005; Boschi & Baur, 2008), these did not meet our inclusion criteria. Given that insect herbivory has been demonstrated to influence both nutrient cycling and plant production (Blumer & Diemer, 1996; Belovsky & Slade, 2000), the dearth of information about the role of herbivory in grassland responses to abiotic change is a serious issue (Scherber *et al.*, 2010; Borer *et al.*, 2014). We need more comprehensive experimental research on the influence of nutrient addition on both plant and invertebrate communities, and interactions in between, to better

appraise the functional ecology of grasslands (Scherber *et al.*, 2010; Littlewood *et al.*, 2012; Andrey *et al.*, 2014).

## Conclusions

Plant species losses and biomass increases following N addition appear to be a universal pattern across grassland systems. Here, we further establish that effects on species richness are negatively and additively influenced by the dose of N applied and duration of application. This finding has important conservation implications; it implies that sustained addition of relatively small N doses will ultimately reduce plant diversity in the long term. Consequently, it is important to protect grasslands not only where N application is limited if not null, but also which are not affected by N atmospheric deposition (Payne *et al.*, 2013). In addition, refined estimates of exposure thresholds below which no harmful effects can be detected (e.g. in the critical load concept, Bobbink & Hettelingh, 2011) have to be defined from the dual point of view of quantity and time, this given their additive effects (see also Clark & Tilman, 2008).

We also found that the effects of N addition on species richness and diversity are less pronounced in cool summer mountain areas than in warm summer mountain areas (see also Ditommaso & Aarssen, 1989). These two areas closely match the segregation between subalpine and alpine grasslands. We even observed a positive effect of N addition on  $H'$  in the coldest summer mountain areas, that is where  $<4$  months have a mean ambient temperature  $\geq +10^\circ\text{C}$ . In the face of global warming, this finding indicates that the magnitude of the effects of N addition upon mountain plant communities might increase as summer temperatures increase (Rustad *et al.*, 2001). Given that climate warming is more pronounced in mountain ranges and in boreal regions (Nogues-Bravo *et al.*, 2007; Engler *et al.*, 2011), it is in subalpine and boreo-alpine grassland ecosystems that we might expect major changes in vegetation.

Of course, it remains to be seen how subtle changes in the species richness and diversity and productivity of these mountain systems may alter their functioning and resilience to further environmental change. Anthropogenic N enrichment is likely to become more widespread in the future, touching remote mountain regions that have so far remained unaffected. Research is urgently needed to predict its impacts on ecosystems and their services so as to take appropriate conservation action (Bobbink & Hettelingh, 2011; Manning, 2012).

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## Supporting Information

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

**Appendix S1.** Systematic review protocol published in 2010.

**Appendix S2.** Search terms used and dates when literature searches were conducted.

**Appendix S3.** Flow diagram reporting the number of references identified, excluded and added during the literature screening process.

**Appendix S4.** Lists of all data points included in the review with details on extracted variables.

**Appendix S5.** List of all studies excluded after abstract or full text filtering and reasons for exclusion.

**Appendix S6.** Influence of N addition on plant species richness, Shannon index and biomass production. The statistical outputs of all null models are presented (related to Fig. 2).

**Appendix S7.** Model selection processes and detailed statistical outputs of the most parsimonious models about the influence of N addition on plant species richness and Shannon index.

**Appendix S8.** Model outputs on the influence of fertiliser type or soil pH on species richness.