

# A trait-based analysis of the role of phosphorus vs. nitrogen enrichment in plant species loss across North-west European grasslands

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## Summary

1. Both nitrogen (N) and phosphorus (P) enrichment have been identified to drive plant species losses from nutrient-poor semi-natural grasslands. The relative contribution of N vs. P to species loss remains unclear, however.

2. We investigated how soil N and P availability affect the occurrence of 61 grassland species across North-western Europe. We selected 132 study sites, located in Great Britain, Belgium and France, along a soil fertility gradient based on variability in atmospheric N deposition and on nutrient input from adjacent agricultural land. To gain insight into the underlying ecological mechanisms of species loss, we examined the role of a suite of plant traits that may mediate a species' response to increased N or P availability.

3. Mixed logistic regression showed that the occurrence of 24 plant species (39.3%) was affected by soil nutrient availability. Of these species, 18 were negatively affected by increased P (29.5%) and five by increased N (8.2%). Regionally declining plant species were absent from both P-rich and N-rich grasslands. Higher susceptibility to elevated P was associated with stress tolerance, low maximum canopy height and symbiosis with arbuscular mycorrhizae.

4. *Synthesis and applications.* Although we also identified negative effects on plant diversity through N enrichment, our results strongly suggest that P enrichment is a more important driver of species loss from semi-natural grasslands. Species in symbiosis with mycorrhizae and with low canopy height are especially at risk. Because detrimental effects of P enrichment are very difficult to mitigate due to the persistence of P in the soil, nature management should give absolute priority to preventing P input in grasslands through fertilization, agricultural run-off or inundation with P-polluted surface water. To restore species-rich grasslands on P-enriched soils, top soil removal appears crucial and more research regarding alternative removal strategies is essential.

**Key-words:** eutrophication, mycorrhizae, N deposition, nutrient enrichment, plant functional traits, semi-natural grasslands

## Introduction

Through the burning of fossil fuels and large-scale application of agricultural fertilizers, natural and semi-natural ecosystems are continuously being enriched with nitrogen (N) and phosphorus (P) (Newman 1995; Carpenter *et al.* 1998; Galloway *et al.* 2008). The specific effects on plant species are often complex and include direct toxicity, increased susceptibility to

herbivores and altered patterns of competition (Bobbink *et al.* 2010). Numerous field surveys and fertilization experiments have provided evidence that this enrichment drives significant losses of biodiversity (e.g. Janssens, Peeters & Tallowin 1998; Stevens *et al.* 2004; Wassen *et al.* 2005; Clark & Tilman 2008; Gilbert, Gowing & Wallace 2009; Maskell *et al.* 2010). This is especially true for plant species typically found in nutrient-poor semi-natural grasslands, which have dramatically declined in Western Europe, resulting in local or even regional extinction (Roem & Berendse 2000; Van Landuyt *et al.* 2008). This species loss is likely to be driven by trait-based constraints

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on the ability to respond to changes in the competitive environment following nutrient enrichment (Aerts 1999; Suding *et al.* 2005; Harpole & Tilman 2007). The limited supply of nutrients in nutrient-poor grasslands can be expected to have driven selection for traits involved in acquiring sufficient amounts of nutrients to sustain growth (Robinson *et al.* 1999; Vance, Uhde Stone & Allan 2002; Craine *et al.* 2003). Enhanced nutrient supply, however, may cause larger above-ground biomass production, leading to a gradual decrease in light penetration because of increasing interception of light by subsequent layers of leaves (Chapin, Matson & Mooney 2002; Craine 2009). Those plant species typically found in nutrient-poor environments generally lack traits to cope with this increased shading, and they are susceptible to reduced seedling recruitment and even competitive exclusion following nutrient enrichment (Hautier, Niklaus & Hector 2009).

Although several studies have provided very valuable insights into the negative effects of increased input of N (Stevens *et al.* 2004; Clark & Tilman 2008; Bobbink *et al.* 2010; Dupré *et al.* 2010) and P (Janssens, Peeters & Tallowin 1998; Wassen *et al.* 2005; Gilbert, Gowing & Wallace 2009), it remains unclear whether either N or P enrichment is the most important driver of species loss from semi-natural grasslands. The main reason is that very few studies have directly addressed the relative impact of N vs. P enrichment by measuring both N and P availability (Gordon, Wynn & Woodin 2001; Güsewell 2004); especially at larger scales (but see Stevens *et al.* 2010a). Furthermore, most studies have focused on the response of plant community composition and total species richness to N or P enrichment, rather than on the response of individual species. The latter approach, however, is expected to provide more detailed information on specific species loss (Dorrough & Scroggie 2008; Gross *et al.* 2009). Finally, whereas the importance of functional traits in our understanding of plant responses to environmental change has been widely acknowledged (Lavorel & Garnier 2002; McGill *et al.* 2006; Suding *et al.* 2008), few studies have established a relationship between specific plant traits and the response to N or P enrichment (e.g. Diekmann & Falkengren Grerup 2002; Suding *et al.* 2005; Dorrough & Scroggie 2008). Yet, identifying the plant traits mediating the response to enhanced nutrient supply may provide additional insights into the ecological mechanisms behind enrichment related species losses.

Incomplete insights into the detrimental effects of N vs. P enrichment may have important implications for environmental and agricultural policy as a bias towards reducing one single nutrient may prove to be inadequate. Therefore, the overall aim of this study is to quantify the relative importance of N vs. P enrichment for the occurrence of semi-natural grassland species and to identify associated ecological mechanisms through studying specific plant traits that may mediate species' response to nutrient enrichment. We investigated the occurrence of 61 plant species in relation to soil N and P availability in 132 semi-natural grasslands in North-western Europe across a gradient of N and P availability. More specifically, we addressed the following questions: (i) Is species occurrence in semi-natural grasslands mainly constrained by N or by P

availability, or are both nutrients equally (un)important? (ii) Is the occurrence of regionally declining plant species more constrained by N or by P enrichment than the occurrence of non-declining species? (iii) Do plant traits associated with effective nutrient acquisition and capturing of light under increased shading mediate the susceptibility of species to N or P enrichment?

## Materials and methods

### FIELD METHODOLOGY

In June and July 2009 and 2010, we surveyed 132 semi-natural grasslands belonging to the *Violin caninae* alliance (Schwickerath 1944). We covered 40 grasslands in the United Kingdom (spread across north-east England and southern Scotland), 40 in France (spread across the southern departments of the central region) and 52 across the whole of Belgium. The grasslands were located in 15 geologically distinct regions (four in the UK, three in France and eight in Belgium). All grasslands were located in nature reserves, National parks or Sites of Special Scientific Interest and had received continuous extensive management by cutting or cutting and grazing for at least 15 years.

The grasslands were selected to cover a range of nutrient enrichment. For this, we first determined a gradient of atmospheric N deposition, ranging from 13 kg N ha<sup>-1</sup> year<sup>-1</sup> in southern Scotland (near the critical threshold of 10–15 kg N ha<sup>-1</sup> year<sup>-1</sup> for these grasslands; Bobbink *et al.* 2010) up to 50 kg N ha<sup>-1</sup> year<sup>-1</sup> in the north of Belgium. Estimates of N deposition, comprising wet and dry deposition of oxidized and reduced nitrogen for the years 2000, 2004 and 2008, for 50 × 50 km<sup>2</sup> grid cells were downloaded from the UNECE/EMEP air quality data base and averaged to provide an estimate of long term N input (<http://www.emep.int/>). Secondly, along this gradient, we chose within each EMEP grid cell 'pristine' grasslands as well as grasslands with adjacent arable land, therefore presumably subject to varying degree of runoff and seepage of nutrients.

We established a quadrant of 2 × 2 m per grassland, positioned to closely represent the overall species composition of the site. We recorded the presence of all vascular plant species in each quadrant. Then, ten topsoil samples (0–10 cm below the litter layer) were randomly taken with an auger (2 cm diameter) and mixed to give one bulk soil sample.

### LABORATORY METHODOLOGY

All analyses were conducted on fresh soil samples (stored in a refrigerator at 5 °C for maximum 1 month prior to analysis). Soil pH was determined using a pH probe in a 1:25 soil/deionized water mixture. As a measure of soil inorganic N availability, we extracted NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> by shaking 10-mg dry weight equivalent of soil in 100 mL of 1 M KCl solution for one hour (N<sub>KCl</sub>; Robertson *et al.* 1999). Extracts were subsequently filtered through Whatman No. 42 filter paper and analysed colorimetrically by a segmented flow auto analyser (Skalar, Breda, the Netherlands). As a measure of soil inorganic P availability, we determined Olson P values (P<sub>ols</sub>) by shaking 2-g dry weight equivalent of soil for 30 min with 0.5 M NaHCO<sub>3</sub> at pH 8.5 and subsequent colorimetric analysis of the extracts using molybdenum blue (Lajtha *et al.* 1999). As chemical nutrient extractions from soil may not necessarily reflect true nutrient availability as experienced by plants, we measured N and P concentration in the vegetation biomass to control whether N<sub>KCl</sub> and P<sub>ols</sub> represent plant N and P uptake in this study

(Güsewell & Koerselman 2002). For this, we clipped two randomly chosen plots of 0.25 m<sup>2</sup> per quadrant and pooled the harvested biomass into a bulk vegetation sample. N concentration of the biomass sample was measured by combustion and subsequent gas detection (Flash 1112; Interscience, Breda, the Netherlands), and P concentration was determined after nitric acid destruction and subsequent ICP MS analysis. Finally, we also calculated the molar ratio of the N and P concentration of the vegetation samples. This ratio provides a relative measure of nutrient availability based on plant uptake over a longer period of time and therefore also contributes to assess whether  $N_{KCl}$  and  $P_{ols}$  represent plant N and P availability in this study (Güsewell 2004).

#### COLLECTION OF PLANT TRAIT DATA

Information regarding specific plant traits of the investigated species was collected from three online available data bases (Fitter & Peat 1994; Klotz, Kühn & Durka 2002; Kleyer 1995). The traits were selected on the basis of their expected effect on nutrient uptake (support or no support of N fixing symbionts and arbuscular mycorrhizae; Vance 2001) and light acquisition under intensified shading (maximum canopy height, specific leaf area and maximum seedling growth rate; Lambers, Chapin & Pons 2006; Craine 2009). We limited the selection to relevant traits available for at least 50% of the surveyed species. Some traits of possible interest were therefore omitted (e.g. root hair diameter and rooting depth).

Next, we retrieved data on each species' tolerance to environmental stress or disturbance *sensu* the C S R typology (Grime, Hodgson & Hunt 1988). Species showing an intermediate profile were allocated to the group 'CSR' whereas 'competitive' species or 'competitive ruderal' species were allocated to the group 'C' and 'stress tolerant' and 'stress tolerant ruderal' species were allocated to the group 'S'.

Finally, we retrieved the change index of each species in the United Kingdom and in Flanders (northern part of Belgium). Positive values indicate that the species increased in occurrence between survey periods (1939–1971 and 1972–2004 for Flanders; 1970–1986 and 1987–1999 for UK), and negative values indicate a decline. For detailed information, we refer to Preston, Pearman & Dines (2002) and Van Landuyt *et al.* (2008). As species' change indices for both regions were highly correlated ( $r = 0.65$ ,  $P < 0.0001$ ), we also calculated their mean value for further analysis. We could not retrieve change indices for France and Wallonia (southern part of Belgium). The index from Flanders is further denoted as Belgian change index. An overview of collected plant trait data is provided in Table S1 (Supporting Information).

#### STATISTICAL ANALYSIS

First,  $N_{KCl}$  and  $P_{ols}$  were log transformed to obtain homoscedasticity. Then, we built a mixed logistic regression model to examine the influence of soil N and P availability on the occurrence of each species, using the GLIMMIX procedure in SAS (Hosmer & Lemeshow 1989; Littell *et al.* 2006). Because very rare or very abundant species are not suitable for logistic modelling, our analyses were restricted to species occurring in between 10% and 90% of all plots (61 species). Nomenclature follows Stace (2010). Region ( $n = 15$ ) was incorporated as a random effect in the models to account for the nonindependence of quadrants in the same region. Soil pH may also have an effect on species occurrence and effects of nutrient enrichment may covary with soil pH (Diekmann & Falkengren Grerup 2002; Stevens *et al.* 2010b). To clearly distinguish between the effects of N and P availability and the effects of pH, we assigned each quadrant to one of eight pH

classes, ranging from pH 3.5–4 (class 0) to pH 7.7–8 (class 8) and incorporated pH class as a second random effect in the model. The resulting logistic regression coefficients,  $\beta-N$  and  $\beta-P$ , can be considered as an indicator of the strength of the response of individual species to increased N or P availability (Dupré & Ehrlén 2002).

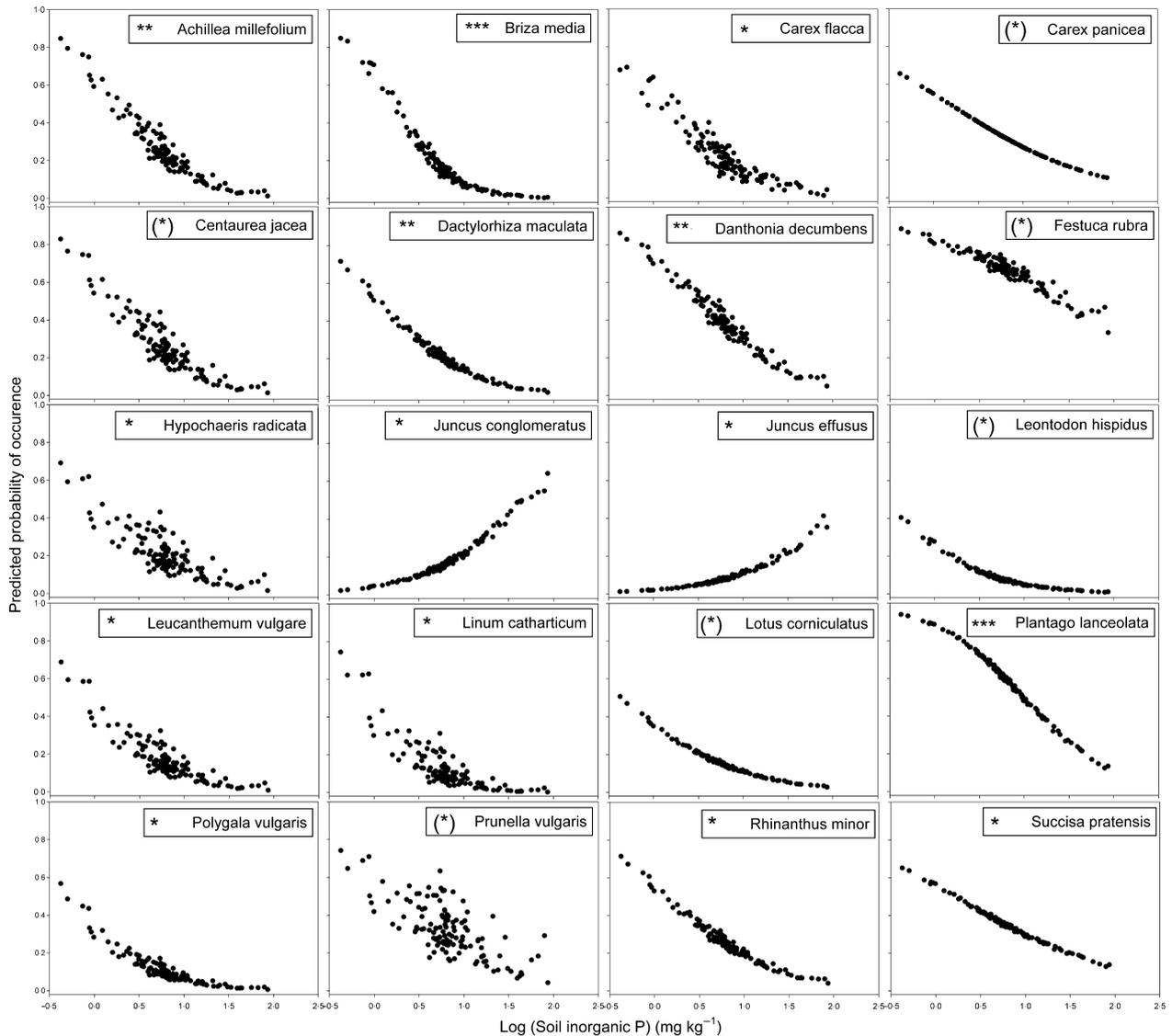
Next, the relationship between these regression coefficients on the one side and the change index and the selected plant traits on the other was quantified. We calculated Pearson correlations for relating continuous variables to the logistic regression coefficients and one-way ANOVAS for relating nominal traits to the logistic regression coefficients. For some traits, the ANOVA assumption of homoscedasticity was violated and we then used the nonparametric Mann–Whitney *U*-test. We also checked for intercorrelations between the traits using Pearson correlations for continuous traits and Pearson chi-square statistics for nominal traits. Maximum canopy height was first log transformed to meet the requirements of the statistical methods used. The latter analyses were performed using SPSS v17.0 (SPSS Inc., Chicago, IL, USA).

## Results

Soil nutrients varied considerably across grasslands, constituting a gradient of both N and P availability. Soil inorganic N ( $NH_4^+ + NO_3^-$ ,  $N_{KCl}$ ) ranged from 2.3 to 49.8 mg N per kg soil (average 15.1 mg N per kg soil, SE: 0.85) and soil inorganic P ( $P_{ols}$ ) from 0.4 to 86.1 mg P per kg soil (average 10.7 mg P per kg soil, SE: 1.25). There was no relationship between soil N and P ( $r = 0.13$ ,  $P = 0.14$ ). Soil N was significantly correlated with N concentration of plant biomass ( $r = 0.21$ ,  $P = 0.019$ ), and soil P correlated significantly with plant P concentration ( $r = 0.33$ ,  $P < 0.0001$ ) (Fig S1, Supporting Information). Soil N and P also correlated significantly with the vegetation N/P ratio ( $r = 0.18$ ,  $P = 0.039$  and  $r = -0.34$ ,  $P < 0.0001$  respectively). This demonstrates that the used soil chemical extractions ( $N_{KCl}$  and  $P_{ols}$ ) sufficiently represent nutrient availability in this study.

The GLIMMIX analysis with soil N and P as fixed effects and region and pH class as random effects yielded significant regression coefficients of N or P for 16 of 61 plant species (26.2%). P was the most important variable showing a significant effect on the occurrence of 14 species (22.9%). N had a significant effect on only two species (3.2%). Furthermore, six additional species had marginally significant regression models for P and four species for N ( $0.05 \leq P < 0.09$ ). Including these marginally significant relations, the occurrence of 24 plant species (39.3%) was affected by soil nutrient availability. Of these species, 18 were negatively affected by P (29.5%) and five by N (8.2%). Only two species showed a higher incidence with increased P and one with increased N (Figs 1 and 2). More detailed results of the GLIMMIX procedure results are provided in Table S2 (Supporting Information).

There was a significant positive relationship between the  $\beta-P$  coefficients on the one side and the Belgian ( $r = 0.32$ ,  $P = 0.017$ ), the British ( $r = 0.41$ ,  $P = 0.002$ ) and the mean change index ( $r = 0.40$ ,  $P = 0.002$ ) on the other, indicating that species experiencing a negative effect of increased P are more likely to have a declining distribution (Fig. 3). We also found a significant positive relationship between the  $\beta-N$



**Fig. 1.** Probability of occurrence of a species provided by mixed logistic regression modelling as a function of the log transformed soil inorganic P availability in grasslands ( $P_{ois}$ ). \*\*\*,  $P < 0.001$ ; \*\*,  $0.001 < P < 0.01$ ; \*,  $0.01 < P < 0.05$ ; (\*),  $0.05 < P < 0.09$ .

coefficients on the one side and the Flemish change index ( $r = 0.37$ ,  $P = 0.006$ ) and mean change index ( $r = 0.29$ ,  $P = 0.025$ ) on the other. This was not the case for British change index ( $r = 0.19$ ,  $P = 0.16$ ), indicating that increased N availability is only related to species decline in Belgium.

CSR profiles were significantly related to species'  $\beta$ -P coefficients but not to  $\beta$ -N coefficients ( $F = 8.69$ ,  $P < 0.001$  and  $F = 0.87$ ,  $P = 0.43$  respectively). Stress-tolerant species showed a higher preference for P poor, and competitive species showed a higher preference for P richer grasslands whilst species with an intermediate profile showed intermediate  $\beta$ -P coefficients (Fig. 4).

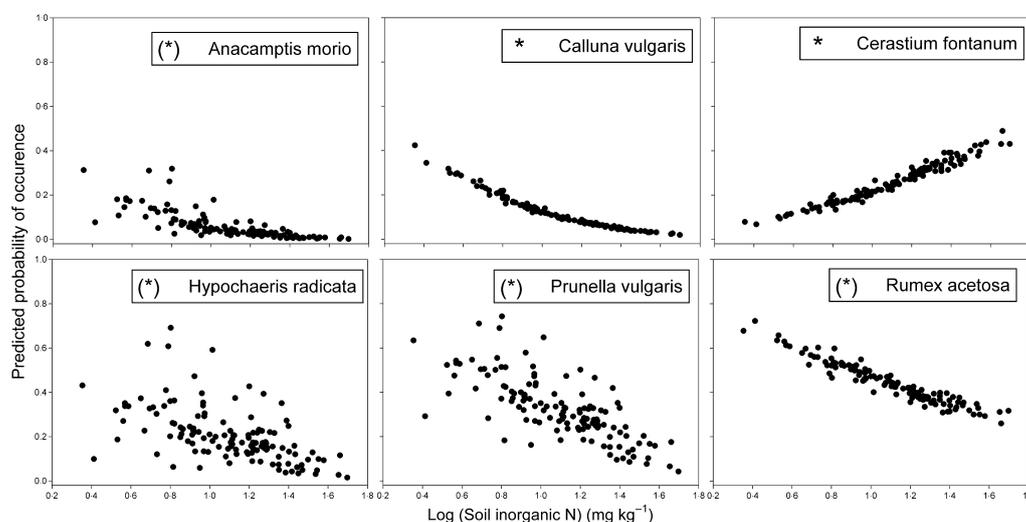
There were no intercorrelations between the different plant traits. The differences between  $\beta$ -P coefficients of the species were related to maximum canopy height ( $r = 0.30$ ,  $P = 0.031$ ) and support of mycorrhizae ( $F = 6.60$ ,  $P = 0.013$ ) and differences in  $\beta$ -N coefficients could be

explained by support of N fixing symbionts ( $Z = -1.98$ ,  $P = 0.047$ ) (Figs 5 and 6).

## Discussion

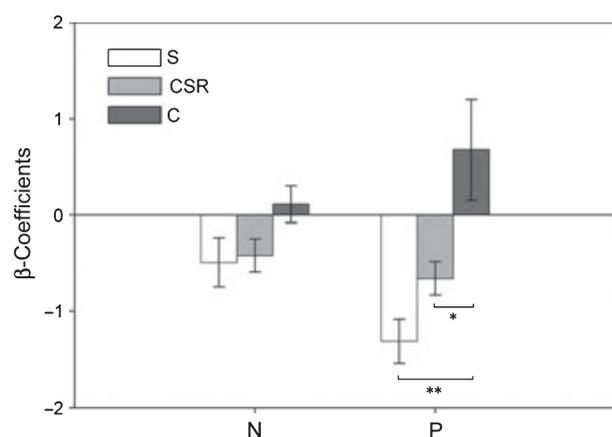
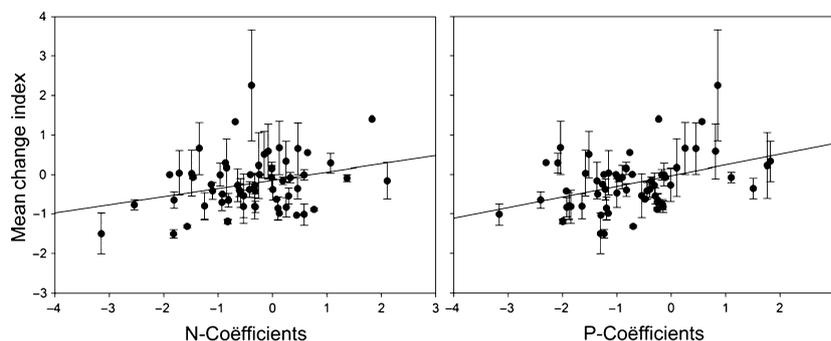
### EFFECTS OF N AND P ON THE OCCURRENCE OF PLANT SPECIES

Nutrient availability is an important factor structuring plant species composition by significantly affecting the occurrence of up to 40% of the plant species present, in at least 10% of all surveyed grasslands. Not surprisingly, plant species negatively affected by increased N or P were typically species from nutrient-poor habitats such as *Calluna vulgaris*, *Briza media* and *Dactylorhiza maculata*. The inclining response of *Cerastium fontanum*, *Juncus effusus* and *Juncus conglomeratus* is also supported by observations that these species frequently respond



**Fig. 2.** Probability of occurrence of a species provided by mixed logistic regression modelling as a function of the log transformed soil inorganic N availability in grasslands ( $N_{KCl}$ ). \*\*\*,  $P < 0.001$ ; \*\*,  $0.001 < P < 0.01$ ; \*,  $0.01 < P < 0.05$ ; (\*),  $0.05 < P < 0.09$ .

**Fig. 3.** The mean of the Belgian and British change index against the  $\beta$ -N and  $\beta$ -P coefficients of the investigated grasslands species. Bars denote standard error. Pearson correlations revealed significant relationships between the calculated mean change index and the  $\beta$ -N coefficients ( $r = 0.29$ ,  $P = 0.025$ ) and  $\beta$ -P coefficients ( $r = 0.40$ ,  $P = 0.002$ ).



**Fig. 4.**  $\beta$  coefficients of the GLIMMIX analysis with soil N availability (N) and soil P availability (P) for stress-tolerant (S), intermediate (CSR) and competitive (C) species *sensu* Grime, Hodgson & Hunt (1988). ANOVA showed significant differences between S, CSR and C species for P coefficients ( $F = 8.69$ ,  $P < 0.001$ ), but not for the N coefficients ( $F = 0.87$ ,  $P = 0.43$ ). Brackets indicate significant differences between groups (\*,  $P < 0.05$  and \*\*,  $P < 0.01$ ; Tukey's *post hoc* comparisons).

positively to higher soil nutrient availability (Preston, Pearman & Dines 2002; Smolders *et al.* 2008). Strikingly, only these three species (5%) responded positively whilst 38% showed a

declining response (Figs 1 and 2). This lack of replacement by nutrient-tolerant species indicates a high potential loss of species richness following nutrient enrichment and supports the common hypothesis that enrichment can drive significant losses of biodiversity. Interestingly, however, our results show that almost 30% of the investigated species are likely to be lost following P enrichment, whereas only 8% of the species are at risk following N enrichment (Figs 1 and 2). These results clearly indicate that P enrichment is a more important contributor to the loss of plant diversity in European grassland than N enrichment, as has been suggested by Wassen *et al.* (2005).

There may be several, not mutually exclusive, explanations for this apparently larger effect of increased P availability on plant species loss in grasslands. First, grasslands may be more resilient to increased N input than to P input, leaving enhanced P availability predominantly affecting plant species composition. N can volatilize through denitrification, especially in intermittently wet and dry soils, and excess N may also be lost through leaching of  $NO_3^-$  (Gough & Marrs 1990). P, on the other hand, is very immobile and remains particularly tightly bound in soils containing high clay fractions, calcium and sesquioxides (Addiscott & Thomas 2000; Hinsinger 2001). Indeed, it has been shown that former agricultural P amendments have caused chronic enhanced P supply for several

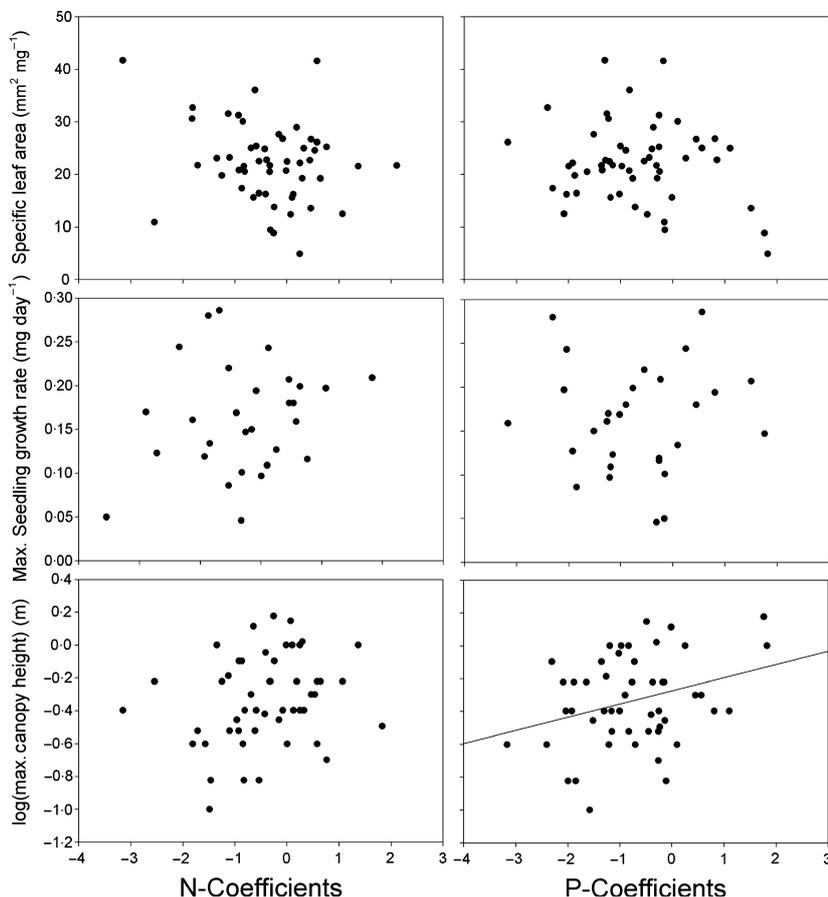


Fig. 5. Specific leaf area, maximum seedling growth rate and maximum canopy height (log transformed) against the  $\beta$ -N and  $\beta$ -P coefficients of the investigated grasslands species. Pearson correlations only revealed a significant relationship between maximum canopy height on the one hand and the  $\beta$ -P coefficients ( $n = 51$ ;  $r = 0.30$ ,  $P = 0.031$ ) on the other.

decades, seriously hindering species-rich grassland restoration projects (Fagan *et al.* 2008), whilst grassland diversity appears to be able to recover after cessation of N addition (Clark & Tilman 2008). Secondly, grasslands may have shifted from N limitation in the past, to P limitation at present through chronic atmospheric N deposition (Güsewell 2004). These novel P-limited grasslands can be expected to be susceptible to further detrimental changes in species composition through increased P availability. Therefore, part of the detrimental effects of P enrichment may be preceded by effects of enhanced N, possibly indicating that increased N and P may work synergistically in their effect on species loss (Allgeier, Rosemond & Layman 2011). Finally, Maskell *et al.* (2010) suggested that the negative effects of enhanced N on species richness are related to the secondary effect of soil acidification by input of acidic nitrogenous compounds. Indeed, Stevens *et al.* (2010b) found that effects of acidification, linked with increased N deposition, could better explain shifts in species richness than effects of N eutrophication through increased competition. As we corrected for the effect of pH on the occurrence of species in this study, it is unlikely that our results reflect effects of increased N availability through acidification. We may therefore have underestimated some deleterious effects of N enrichment through exclusion of acidifying effects.

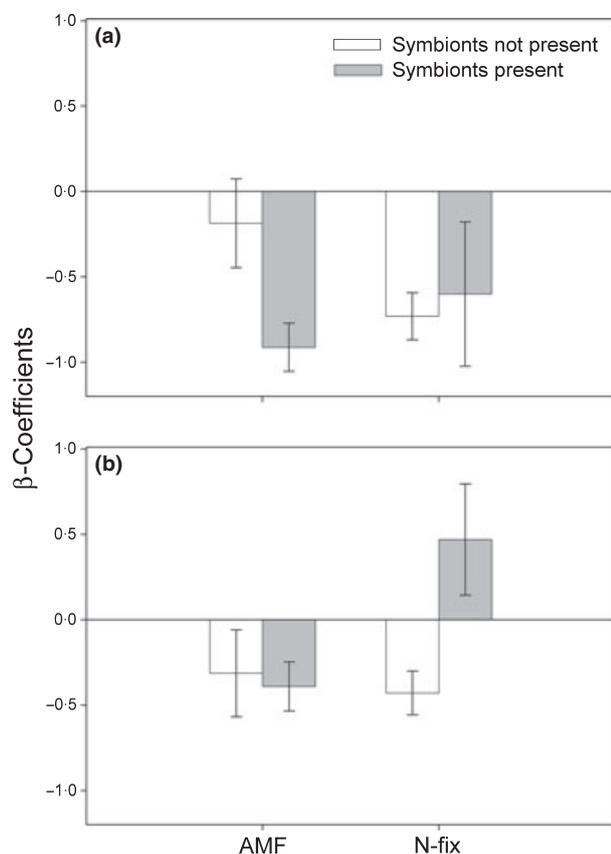
We also found that species with an increasingly negative response to N or P were more likely to have a smaller change index (Fig. 3). Although a negative change index may result

from a variety of factors (Preston, Pearman & Dines 2002), these results confirm that nutrient enrichment may play an important role in species' decline. As the Belgian change index was related to both N and P sensitivity, whereas the UK change index was only related to P sensitivity, these findings also support earlier results indicating a more dominant role of P enrichment.

#### THE MEDIATING ROLE OF PLANT TRAITS

P-sensitive species were typically smaller statured species (Fig. 5). This suggests that species loss is caused by P-induced eutrophication through increased biomass production and the subsequent intensified shading in the vegetation understory. The resulting competition for light may cause competitive exclusion of small species, unable to acquire sufficient light to sustain growth and reproduction (Hautier, Niklaus & Hector 2009). The observation that competitive species (*sensu* Grime, Hodgson & Hunt 1988) preferred P-rich grasslands, as opposed to stress-tolerant species which prefer P-poor grasslands (Fig. 4), supports this conclusion. Interestingly, Stevens *et al.* (2010b), who studied similar grassland communities, failed to produce evidence for N-induced eutrophication and therefore suggested that other nutrients or limiting resources may be more important. Our results identify increased P as a cause for eutrophication related species loss.

Species loss following P enrichment may also be provoked by altered cost/benefit relationships of supporting symbiotic



**Fig. 6.**  $\beta$  coefficients of the GLIMMIX analysis with soil P availability (a) and soil N availability (b) for support of arbuscular mycorrhizal symbionts (AMF) or N fixing symbionts (N fix). Bars denote standard error. ANOVA showed significant differences between P coefficients of non-mycorrhizal species and mycorrhizal species ( $F = 6.60$ ,  $P = 0.013$ ). Mann–Whitney  $U$ -tests showed significant differences for N coefficients between species with and without N fixing symbionts ( $Z = -1.98$ ,  $P = 0.047$ ).

arbuscular mycorrhizal fungi because mycorrhizal species appeared to be more susceptible to increased soil P than non-mycorrhizal species (Fig. 6). As arbuscular mycorrhizal fungi can increase P uptake by plants in exchange of carbon assimilates, growth and fitness of mycotrophic species are linked to mycorrhiza-mediated P acquisition, especially in P-poor environments (Read 1991; Vance, Uhde Stone & Allan 2002). The carbon drain towards the fungal partner on obligate mycorrhizal species may possibly explain the observed P susceptibility of mycorrhizal species. This potentially causes a competitive disadvantage under P enrichment, especially for competition for light as less carbon can be invested in above-ground biomass for light acquisition (Johnson 1993; Johnson *et al.* 2008). It has also been demonstrated that beneficial effects of this symbiosis on host physiology differs depending on the identity of the fungal partner (Van der Heijden, Boller & Iain 1998; Hart, Reader & Klironomos 2003; Vogelsang, Reynolds & Bever 2006). As arbuscular mycorrhizal species richness is known to decrease following P enrichment (Helgason *et al.* 1998; Murray, Frank & Gehring 2010), the observed susceptibility of certain mycotrophic species in this study may thus also

be explained through the loss of specific suitable fungal partners (Bever *et al.* 2010). Interestingly, the only species significantly negatively affected by increased N availability, *C. vulgaris* (Fig. 2), is also the only species in this study that supports ericoid mycorrhizae. As ericoid mycorrhizae are known to increase N uptake in exchange for carbon assimilates (Aerts 2002), the susceptibility to increased N of species that support ericoid mycorrhizae may be similar to the susceptibility of arbuscular mycorrhizal species to increased P (Bever *et al.* 2010).

Support of N fixing symbionts was the only plant trait related to N sensitivity, showing that species that support N fixing symbionts showed a higher incidence in more N-rich grasslands (Fig. 6). It is not unlikely that this result reflects increased N availability induced by N fixation of these symbiotic species, rather than a specific preference of N fixing species for N-rich grasslands.

## Conclusion

Although we may have underestimated some secondary effects of N enrichment through controlling for pH differences among sites, our results clearly demonstrate the detrimental effects of increased P availability for biodiversity in grasslands. Species loss appears to occur through eutrophication but interestingly, we also identified mycorrhizal dependence as an important risk factor to increased P availability. Continued P enrichment may thus provoke a systematic and widespread species loss, as nearly 80% of all terrestrial plants are associated with arbuscular mycorrhizae (Smith & Read 1997). Further research is required to investigate the interaction between (species specific) fungal partners and plant performance, to assess the impact of nutrient enrichment on mycotrophic species.

This study has indicated that nature management strategies and agricultural policies biased towards N enrichment alone are inadequate for effective conservation of nutrient-poor semi-natural grasslands. First, a stronger focus on preventing P enrichment is necessary considering the difficulty to remove the tightly bound P from the soil, which contrasts with the much more mobile N. Therefore, we strongly advocate prevention of P enrichment of species-rich semi-natural grasslands through fertilization, run-off or inundation with P-polluted surface water. For the ecological restoration of former species-rich grasslands amended with P, removal of the P-rich top soil is crucial. To determine the required depth of soil removal, our data provide important data on the level of P sensitivity of specific target species. Finally, we stress the need to develop alternative P-reducing measures, as top soil removal is often too expensive or radical to be included in environmental management schemes.

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## Supporting Information

Additional supporting information may be found in the online version of this article.

**Fig. S1.** Relationship between soil nutrient availability and nutrient concentration of the vegetation biomass.

**Table S1.** List of the investigated plant species with their functional traits.

**Table S2.** Detailed results of the GLIMMIX analyses for the occurrence of 61 species in function of soil N and P.

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