Balance matters

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Balance matters

N:P stoichiometry and plant diversity in grassland ecosystems

Een kwestie van balans
N:P stoichiometrie en plantendiversiteit in graslandecosystemen
(met een samenvatting in het Nederlands)

Proefschrift

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geboren op 13 juli 1976 te Fujinomiya, Japan

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Contents

1 Introduction to the thesis

Eutrophication affects grassland ecosystems

Ecosystems have changed dramatically by human interferences in the last decades (Millennium ecosystem assessment 2005). Disturbance in nitrogen (N) and phosphorus (P) cycles is one of the most important impacts on the Earth's environment (Vitousek *et al.* 1997, Rockstrom *et al.* 2009; also see Figure 1.1).

N and P are the primary elements for living organisms that frequently limit the production of ecosystems (Elser *et al.* 2007). The anthropogenic influence on nutrient cycles, therefore, will inevitably have consequences for the functioning of ecosystems. In Europe, increasing atmospheric N deposition, mainly caused by intensive agriculture, is identified as a crucial factor deteriorating biodiversity of species-rich grassland ecosystems (Bobbink *et al.* 1998, Stevens *et al.* 2004). The current levels of atmospheric N deposition in many ecosystems remain above the levels where a substantial species loss is expected to occur (Stevens *et al.* 2004, Clark & Tilman 2008). Availability of P is also increasing due to human activities but via different pathways than N. Besides the agricultural inflow, P availability in ecosystems may also increase via internal eutrophication triggered by human activities, e.g. enhanced P mobilization by groundwater extraction (Wassen *et al.* 1996), increased wetting-drying dynamics (Turner & Haygarth 2001), or sulphate pollution (Lamers *et al.* 1998). The negative impacts of P eutrophication on biodiversity, in particular on the richness of endangered plant species, are of increasing concern (Olde Venterink *et al.* 2003, Wassen *et al.* 2005). Nevertheless, there has been little attention to the effects of P availability on grassland ecosystems compared to those of N availability.

A well recognized impact of eutrophication on grasslands is the enhanced primary productivity of plant communities. The increased above-ground productivity intensifies competition for light, allowing fast growing or taller species to preempt the directionally supplied resource and thus outcompeting other species (Hautier *et al.* 2009). Competitive exclusion leads to dominance of a few, superior species, that results in the loss of biodiversity (Grime 1973).

Eutrophication may also induce extra negative impacts on grassland communities by changing the balance among major nutrients, such as N and P. Liebig's Law of the Minimum states that growth is determined not by the total resource available, but by the supply of the scarcest, limiting resource. It indicates that organisms need to maintain certain proportions of each element to grow efficiently. Although plants have various homeostatic mechanisms and adaptive traits to regulate the nutrient balance in their biomass (Lambers *et al.* 1998), altered nutrient availability in their environment may lead to unbalanced nutrient concentrations in biomass, with possible negative impacts on plant performance (Sterner & Elser 2002). In natural habitats, more than 10-fold differences in plant N:P ratio are observed among plant communities from different locations (Güsewell & Koerselman 2002). This large variation in nutrient balance in plants highlights the need to separately consider the effects of stoichiometry (i.e. the *relative* balance of multiple elements in availability and requirement), besides those of the productivity alone, when we assess and predict the impacts of changing nutrient availability in ecosystems (Cardinale *et al.* 2009). For aquatic ecosystems, it is widely recognized that a stoichiometric balance between elements matters for growth, species competition, and trophic interactions of organisms (Elser *et al.* 2000, Sterner & Elser 2002, Moe *et al.* 2005). For terrestrial ecosystems, there is a need for a better understanding of how the balance between nutrients matters for plant functioning and vegetation composition (e.g. Güsewell 2004, Agren 2004).

N:P stoichiometry in grassland ecosystems

N and P are necessary nutrients for plant growth, and they have mutually dependent but physiologically contrasting roles. N is the major component of proteins, while P forms a large part of rRNA which is used to generate the proteins. The different functions of N and P lead to different requirements of plants for N and P in relation to their functional characteristics, such as plant growth or plant reproduction strategies. This underlies the observed patterns that biomass N:P ratio is negatively correlated with growth rate (Agren 2004, Niklas *et al.* 2005, Agren 2008); (but also see Matzek & Vitousek 2009) and that N:P ratio is higher in structural organs than in metabolically active organs (Kerkhoff *et al.* 2006). The different roles of N and P pose the question of how the relative availability of N and P in the environment influences plant species which have different requirements for N and P and/or different characteristics in response to N:P stoichiometry (e.g. adaptive traits and plastic responses), and how these interspecific variations consequently influence species interactions and assemblages. Below I outline the current knowledge of the effects of N:P stoichiometry on plants on different levels: i.e. plant traits and growth, competition, community, and ecosystems.

Plant traits adaptive to N and P limitation

On an evolutionary time scale, plants have developed to have various traits with which they adapted to better cope with nutrient deficiency in the environment (Vance *et al.* 2003, Lambers *et al.* 2008). Species inherent traits that are regarded as adaptations to nutrient limitations include those which enhance uptake of the limiting nutrients. Examples of such traits are mycorrhizal associations, N-fixing symbiosis, formation of cluster roots, and various kinds of root exudates (e.g., organic acid, phosphatase enzyme, chitinase enzyme). Plant traits for P uptake are especially diversified (Vance *et al.* 2003, Raghothama & Karthikeyan 2005, Lambers *et al.* 2006), because a large proportion of P (ca. 30-65%; Harrison 1987) is immobile in the soil and the immobile P exists in diverse forms (Turner *et al.* 2003). Plants, therefore, need to biochemically release P from these immobile P sources, i.e. inorganic and organic P compounds, to make it bio-available (Vance *et al.* 2003, Lambers *et al.* 2006). Interspecific variation in the ability to use different forms of soil phosphorus is large, which could be a mechanism to explain coexistence of plant species (Lambers *et al.* 2008, Turner 2008).

Effective resorption of nutrients from senescing leaves is another important trait to cope with nutrient poor environments (Aerts & Chapin 2000). Although N resorption and P resorption are positively correlated with each other, plants generally resorb P more efficiently than N (Killingbeck 1996, Aerts & Chapin 2000, Güsewell 2005b), indicating that efficient resorption is beneficial especially in P-limited environments.

Plastic responses of plant traits and growth to N:P stoichiometry

Plant individuals respond plastically to changing availability of N and P. I define plastic responses as flexible reactions of organisms induced by environmental input, which are seen as phenotypic variations rather than genetic variation. Plastic responses of plants include a change in concentrations of N and P in their biomass in response to fluctuations in the relative availability of N and P (Güsewell 2005c). Plants biomass has generally a larger variation in P than in N concentration (Güsewell & Koerselman 2002) and the plasticity largely differs among species (Garnier 1998). N and P concentrations in plant biomass may have similar but also somewhat contrasting effects on plant growth, resulting in different responses of plants to N-limitation (i.e. low N:P ratio in the environment) and to P-limitation (i.e. high N:P ratio in the environment).

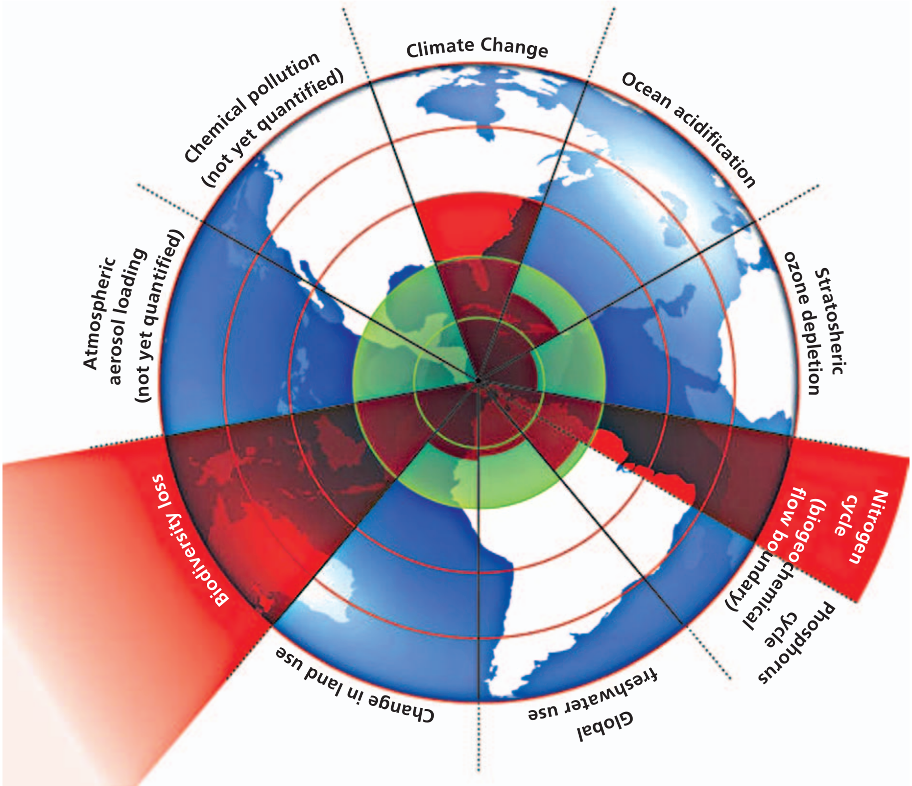

Figure 1.1 The nine Earth systems and associated planetary boundaries which, if crossed, could generate unacceptable environmental change for humanity. The inner green shading represents the proposed safe operating space for the systems. The red wedges represent an estimate of the current position for each variable. Three of the Earth-system processes (i.e. climate change, rate of biodiversity loss, and interference with the nitrogen cycle) are suggested to have already transgressed their boundaries. Source: Rockstrom et al (2009). Reprinted by permission from Macmillan Publishers Ltd: Nature (Rockstrom *et al.* 2009), copyright 2009.

For example, decreases in growth rate and biomass allocation to shoots are more sensitive to N than to P limitation, while increases in senescence of leaves and roots are more sensitive to P than to N limitation (Shaver & Melillo 1984, de Groot *et al.* 2003, El-Kahloun *et al.* 2003, Güsewell 2005a, Güsewell 2005c).

Some of the adaptive traits are also plastically regulated by N or P concentrations in plant biomass or the concentrations in the abiotic environment. For example, phosphatase, the enzyme which hydrolyzes P from organically bound P, is stimulated by N addition and prohibited by P addition (Johnson *et al.* 1999, Olander & Vitousek 2000). Resorption efficiency of N and P from senescing leaves may be promoted by deficiency of the element, although the relationship is generally weak and not constantly observed (Aerts 1996) and largely depends on species (van Heerwaarden *et al.* 2003, Güsewell 2005a). These plastic responses help plants to maintain a proper balance of nutrients in their biomass, enabling them to survive under fluctuating nutrient conditions.

N:P stoichiometric effects on plant competition, community, and ecosystems

The associations between the type of nutrient limitations and plant community properties (e.g. species diversity, species composition) are of particular interest for the management and conservation of grasslands (Verhoeven *et al.* 1996, Aerts *et al.* 2003, Olde Venterink *et al.* 2003). The interspecific differences in response to N:P stoichiometry, as mentioned in the previous paragraphs, are expected to have consequences for all these community properties. The mechanisms of stoichiometric effects on the community level are less understood than that on an individual plant level, but theoretical studies and field observations have indicated several ways in how the relative availability of N and P may influence grassland communities.

Theoretical models provide concepts of how N:P stoichiometry may influence species interactions in plant communities. As the niche theory suggests (Chase & Leibold 2003), a species with advantageous characteristics under a certain environmental condition wins the competition. In this context, if a species has better adaptive traits or more efficient plastic responses to a particular type of nutrient limitation, for example a high phosphatase activity under P limited conditions, then they are likely to become a dominant species. Furthermore, the resource ratio theory (Tilman 1982) predicts that a species will be likely to persist better if its requirement ratio of multiple resources is closer to the supply ratio of the resources, than a species with a more strongly deviated ratio. Thus, species which have inherited a high requirement of P and a low requirement of N would have a relatively high survival probability in N-limited conditions.

Field observations support such relations between N:P stoichiometry and the competitive strength of species. They include more graminoids and/or less forbs in P-limited grasslands or after N fertilization (Bobbink *et al.* 1998, Beltman *et al.* 2007), different effects on species composition after N- and P-fertilizations (Limpens *et al.* 2003, van der Hoek *et al.* 2004), and more threatened species in P-limited grasslands (Olde Venterink *et al.* 2003, Wassen *et al.* 2005). It is, however, still unclear which characteristics of species are responsible for the observed patterns of species-specific responses to a particular type of nutrient limitation (Perez-Corona *et al.* 1996, Güsewell *et al.* 2003). Nevertheless, the shift of species along a gradient of N- to P-limited conditions implies the risk that eutrophication of a single element would change species composition depending on their functional traits, and thus have a directional effect on the community structure.

As a consequence, N:P stoichiometry may have strong effects on species diversity in grassland ecosystems. The resource balance hypothesis (Braakhekke & Hooftman 1999) proposes that species diversity is highest at an intermediate resource ratio, because different species may be limited by different resources and will therefore be able to co-exist. Several field studies show that species diversity is indeed highest at intermediate N:P ratios (Olde Venterink *et al.* 2003, Güsewell *et al.* 2005, Wassen *et al.* 2005), although the relations are only weakly supported by statistical analyses.

Nutrient retention of N and P in ecosystems may also be influenced by N:P stoichiometry. A long-term field study has indicated that bio-availability of P increases after decades of N eutrophication (Malmer & Wallen 2005). A modeling study addressing the role of the availability of multiple nutrients has emphasized the possibility that, due to coupled dynamics of N and P in soil and in plants, an increased N input can increase P retention in the ecosystem (Perring *et al.* 2008). This raises the question in what way N eutrophication may have additional effects on the availability of other nutrients, which eventually may increase productivity, and thus affect species composition and diversity.

To comprehensively understand such N:P stoichiometric effects on grassland communities and ecosystems, more empirical, experimental, and theoretical examination is needed, especially about the link between the responses of plants on the individual species level and those on the community level. This will help us to understand how anthropogenic influences on the relative availability of N and P affect functioning of grassland ecosystems.

Aims and outline of this thesis:

To properly assess the impacts of eutrophication on grassland ecosystems, it is necessary to understand how the stoichiometric balance between N and P affects the functioning of plants at the individual plant species level as well as at the plant community level. As conceptualized in Figure 1.2, I examine the effects of eutrophication on plant communities in terms of availability of N and P, as well as the balance between them. Particular attention is paid to species variations in the effects, possibly caused by interspecific differences in their functional traits, and its consequence on species competition, species composition, and biodiversity. Focusing on functional traits enables us to better understand the general ecological patterns (McGill *et al.* 2006) and to provide useful predictions for ecological restoration (Pywell *et al.* 2003).

The specific aims of my thesis are:

- To clarify N:P stoichiometric effects on the growth of individual plants, besides the effect of overall nutrient availability
- To identify species traits which influence N:P stoichiometric effects on plant individual growth on one hand, and species composition of plant communities along a gradient of N- to P-limited grasslands on the other hand
- To predict N:P stoichiometric effects on community- and ecosystem-level properties, such as plant species competition, plant species composition, plant diversity, and ecosystem nutrient retention
- To provide information for ecosystem conservation about possible additional effects of eutrophication on grassland ecosystems via its N:P stoichiometric effects

To tackle these aims, I carry out the following studies:

- Greenhouse experiments in order to understand responses of individual plants to N:P stoichiometry under controlled nutrient supply on a short term (one to two years)
- A meta-analysis of field vegetation data in order to identify patterns of plant species distributions along a gradient from N- to P-limited plant communities, and to link the patterns to species functional strategies
- A modeling study in order to predict the consequence of N:P stoichiometric effects on individual plant species for plant community and ecosystem properties.

In chapter 2, the interspecific difference in P uptake ability under varying levels of N supply is examined in two 1-year greenhouse experiments with eight grassland species, including those typical from N- and P-limited grasslands. Here I test how different plant species take up P from not-readily available P sources (bound-P) under varied N levels, and which uptake traits contribute to the interspecific difference in P uptake. Furthermore, I examine whether the plant uptake traits are more strongly affected by N:P stoichiometry than by the availability of single elements. Finally, I discuss whether species that are typical for P-limited grasslands have better uptake traits, and thus grow better than those that are typical for N-limited grasslands.

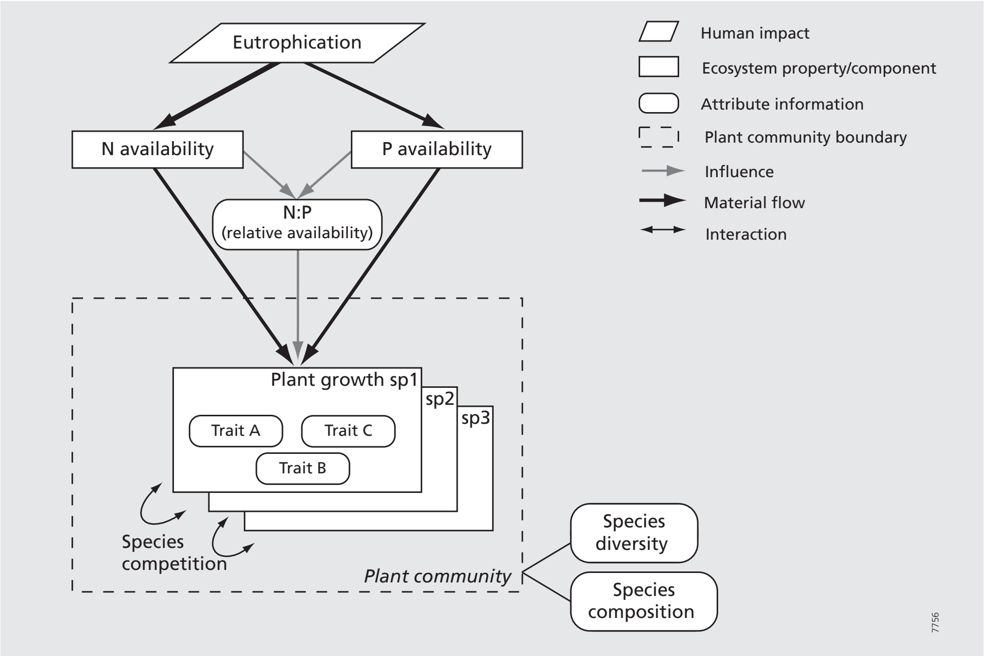

Figure 1.2 Schematic presentation of the focus of this thesis in examining the effects of eutrophication on plant communities. Anthropogenic eutrophication increases N and P availability in the ecosystem. Increased N availability is especially widespread and significant. The availability of N and P, as well as the stoichiometric balance between N and P, influence plant growth. The strength of these influences differs among species because of the interspecific variation in their functional traits. This may lead to changes in the competitive hierarchy among species under different nutrient conditions, eventually leading to changed species composition and biodiversity.

In chapter 3, with the same eight species, I study how different N:P supply ratios influence plant individual growth in a 2-year greenhouse experiment. Here nutrient economy of the plants (i.e. inflow and outflow of N and P in below- and above-ground biomass) are examined for each year in order to clarify the underlying physiological mechanisms which cause the stoichiometric effects of N and P on plant individual growth. Special attention is paid to interspecific differences among eight species in responses to N:P supply ratio.

In chapter 4, the plant community structure along a gradient from N- to P-limited conditions are analyzed by using a large dataset of Eurasian herbaceous ecosystems. The association between the species distribution patterns and functional strategies of the plants (e.g. growth and reproduction strategies) are examined in order to test if interspecific differences in the functional traits play a role in the assembly of the plant community structure.

In chapter 5, the knowledge from the greenhouse experiments on the physiological responses of plants is used to study how differences in balance between N and P availability may affect species competition. For this I present a species competition model in which plants plastically adjust the nutrient concentrations in their biomass, and in which plants have an adaptive trait for P uptake, i.e. phosphatase activity. I examine how different magnitudes of phosphatase activity affect species competition under increased N input levels with varying input levels of P. Furthermore, I examine whether ecosystem P retention changes with increasing N input levels.

Finally, in chapter 6, I integrate the findings from all the chapters and give a mechanistic explanation on how the balance between N and P availability may influence grassland ecosystems on the individual plant level as well as on the plant community level. Furthermore, I discuss the possible influence of eutrophication via the associated N:P stoichiometric effects on plant community properties to provide implications for grassland conservation.

2 Increased N affects P uptake of eight grassland species: the role of root surface phosphatase activity

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Abstract

Increased N deposition may change species composition in grassland communities by shifting them to P limitation. Interspecific differences in P uptake traits might be a crucial yet poorly understood factor in determining the N effects. To test the effects of increased N supply (relative to P), we conducted two greenhouse fertilization experiments with eight species from two functional groups (grasses, herbs), including those common in P and N limited grasslands. We investigated plant growth and P uptake from two P sources, orthophosphate and not-readily available P (bound-P), under different N supply levels. Furthermore, to test if the N effects on P uptake was due to N availability alone or altered N:P ratio, we examined several uptake traits (root-surface phosphatase activity, specific root length (SRL), root mass ratio (RMR)) under varying N:P supply ratios. Only a few species (*M.caerulea*, *A.capillaris*, *S.pratensis*) could take up a similar amount of P from bound-P to that from orthophosphate. These species had neither higher SRL, RMR, phosphatase activity per unit root ($\text{Pase}_{\text{root}}$), nor higher total phosphatase activity (Pase_{tot}: $\text{Pase}_{\text{root}}$ times root mass), but higher relative phosphatase activity (Pase_{rel}: Pase_{tot} divided by biomass) than other species. The species common from P-limited grasslands had high Pase_{rel}. P uptake from bound-P was positively correlated with Pase_{tot} for grasses. High N supply stimulated phosphatase activity but decreased RMR and SRL, resulting in no increase in P uptake from bound-P. $\text{Pase}_{\text{root}}$ was influenced by N:P supply ratio, rather than by only N or P level, whereas SRL and RMR was not dominantly influenced by N:P ratio. We conclude that increased N stimulates phosphatase activity via N:P stoichiometry effects, which potentially increases plant P uptake in a species-specific way. N deposition, therefore, may alter plant community structure not only by enhancing productivity, but also by favouring species with traits that enable them to persist better under P limited conditions.

Introduction

Increased nitrogen deposition is a major threat for plant species richness in terrestrial ecosystems (Bobbink *et al.* 1998, Stevens *et al.* 2004). A well known effect of increased N availability is that enhanced productivity causes fast growing plant species to benefit more than others (Diekmann & Falkengren-Grerup 2002, Suding *et al.* 2005), resulting in outcompeting of slow growing species. Increased N availability may also change the relative availability of various nutrients such as nitrogen (N), phosphorus (P) and potassium (K). Ecosystems previously limited by N can switch to limitation by P and/or K after receiving high N (Mohren *et al.* 1986, van der Woude *et al.* 1994). P is a crucial element, which limits productivity of aquatic and terrestrial ecosystems equally frequently as N (Elser *et al.* 2007). Enhanced N supply may even suppress biomass production, because extreme nutrient balance, i.e. very high N:P ratio, could impose impaired responses (e.g. greater turnover of leaves and roots in response to N addition or to P deficiency) by plants (Fisher *et al.* 2002, El-Kahloun *et al.* 2003, Bai *et al.* 2008). Clearly, the balance of elements available for plant uptake, rather than the availability of single elements, is important as has been empirically demonstrated for aquatic ecosystems (Sterner & Elser 2002) and for terrestrial plant communities (Güsewell 2004).

Several nutrient uptake traits of plants play an important role in regulating tissue nutrient concentrations to avoid elemental imbalance (Treseder & Vitousek 2001, Lambers *et al.* 2008). Because only a small fraction of P in the soil is readily available for plants, P uptake traits are important, especially under P limited conditions (Vance *et al.* 2003). For example, root surface phosphatase, an enzyme which is excreted by plant roots, fungi or bacteria and which can hydrolyze ester-phosphate bonds in soil organic P, may contribute to as much as 65% of the annual P uptake of grasses (Kroehler & Linkins 1988). A series of studies have shown that phosphatase activity is suppressed by P addition but promoted by N addition (Johnson *et al.* 1999, Olander & Vitousek 2000, Phoenix *et al.* 2003). Mycorrhizal infection, which helps P acquisition of plants, also decreases with P availability and, though less evident, increases with N availability (Furlan & Berniercardou 1989). Although not specific for P uptake but for nutrient uptake in general, root morphology and allocation may also contribute to avoid elemental imbalance. Nutrient limitation is known to stimulate root length, formation of cluster roots, and root mass ratio (Lambers *et al.* 2006).

A number of field studies indicated that interspecific differences in responses to P or N limitations may affect plant communities, e.g. in terms of species composition (Roem & Berendse 2000, Olde Venterink *et al.* 2003), high richness of threatened species in P limited grasslands (Wassen *et al.* 2005), and the divergent impacts of N and P fertilization on species composition (Limpens *et al.* 2003, van der Hoek *et al.* 2004). Interspecific differences in response to P limitation are likely caused by differences in traits related to P uptake (Verhoeven *et al.* 1996). Mycorrhizal association, for example, tend to be more intensive for dicots than monocots (Read *et al.* 1976, Harley & Harley 1987), whereas root surface phosphatase activity is higher for monocots than for dicots (Phoenix *et al.* 2003) and differs among plant species (Rejmankova & Macek 2008). Although interspecific differences in these traits are recognized, there has been little understanding about which traits are the most likely to influence species specific performance along N:P gradients in the field (Perez-Corona *et al.* 1996, Güsewell *et al.* 2003).

Our study aims to explore the interspecific differences in response to increased N supply relative to P of eight grassland plant species from two functional groups (grasses and herbs). First, we investigated plant growth and P uptake from not readily available P sources (hereafter referred to as bound-P), which is the major form of P in terrestrial ecosystems, under different N levels. Here the contributions of several uptake traits (phosphatase activity, root length, root mass) to the P uptake were examined. Second, in order to test if the effects of increased N supply on P uptake was due to availability of N only or altered N:P balance, we examined the effects of varying N:P supply ratios on the uptake traits.

We hypothesized that 1) growth and P uptake from bound-P differ among functional groups and among species due to differences in the uptake traits. We also hypothesized that 2) increased N supply would result in increased plant P uptake from bound-P but also increased P loss by stimulating turnover of leaves and roots. Furthermore, we expect that 3) phosphatase activity would be enhanced not by availability of only N or only P but by increased N:P ratio. Finally, we hypothesized that 4) uptake traits of species typically from P limited grasslands would be stronger or more sensitive to P limitation than those from N limited grasslands.

Materials and Methods

We carried out two growth experiments with eight grassland species. The first experiment (hereafter referred to as Bound-P Experiment) aimed at examining plant growth and P uptake from bound-P by using two types of P sources (readily available orthophosphate and non-readily available bound-P) under two levels of N. In the second experiment (hereafter referred to as

Stoichiometry Experiment) we studied the effects of N:P stoichiometry on three uptake traits (root surface phosphatase activity, specific root length and root mass ratio) independent from the effects of only N or P supply, by using readily available P in three N:P ratios and two supply levels.

Greenhouse experiments

Plant species and growth conditions

We used eight species which naturally occur at a range from wet to moist grasslands in The Netherlands. Species were selected based on field data from 381 European grassland sites (Ertsen 1998, Wassen *et al.* 2005, Hölzel unpublished) and included species which typically occur in P and N limited grasslands. The selected species were five grasses (*Alopecurus pratensis* L., *Agrostis capillaris* L., *Festuca rubra* L., *Festuca ovina* L., and *Molinia caerulea* (L.) Moench) and three herbs (*Lychnis flos-cuculi* (L.) Greuter & Burdet, *Lycopus europaeus* L., and *Succisa pratensis* Moench). *A. pratensis* and *L. flos-cuculi* occur more frequently in N limited grasslands, whereas *M. caerulea* and *S. pratensis* occur more frequently in P limited grasslands ($p<0.05$, chi-square test). See Appendix I Table 2.A1 for field distribution data of the eight species.

Plants were cultivated from seeds in a germination chamber from May 2007. In June 2007, all seedlings were transplanted to plastic pots (c. 0.6L) filled with *c.* 500g quartz sand and placed in an open greenhouse with a transparent roof and walls of coarse mesh. Chemical composition of the quartz sand was analyzed to check if it contained none to negligible amounts of nutrients, which was the case.

Nutrient treatments

We supplied different amounts of N and P for each treatment, while all other nutrients (K, Ca, S, Mg, Fe, B, Mn, Zn, Mo, Cu) were supplied in constant, non-limiting amounts (see Appendix II Table 2.A2 & 2.A3).

In the Bound-P Experiment, we combined two forms of P (orthophosphate and bound-P) and two nitrogen supply levels (high and low) in a full factorial design. The resulting four treatments were replicated five times. The total amount of P provided per pot per year was 1.29 mg, and was equal for all treatments. P was provided as KH_2PO_4 for the orthophosphate treatments. In bound-P treatments, we supplied a mixture of organic P compound (inositol hexaphosphate ($C_6H_{18}O_{24}P_6$), 20%) and inorganic P compounds (calcium phosphate ($Ca_{10}(PO_4)_6(OH)_2$), 50%), and aluminum phosphate ($AlPO_4$), 30%) (see footnote 4 of Appendix II Table 2.A2 for the choice of the compounds). Calcium phosphate and aluminum phosphate were supplied into pots in the beginning of the experiment and lightly mixed with the sand. Inositol phosphate suspension was supplied weekly with a micropipette. N was provided as KNO_3 and $Ca(NO_3)_2$ or KNO_3 only (see footnote 2 of Appendix II Table 2.A2),.

In the Stoichiometry Experiment we combined two levels of nutrients (high and low) at three ratios of N:P (1.7, 15 and 135) in a full factorial design. See Appendix II Table 2.A2 for the N and P supply levels of each treatment. The resulting six treatments were replicated five times. N was supplied as KNO_3 and $Ca(NO_3)_2$ or KNO_3 only (see footnote 2 of Appendix II Table 2.A2), and P was supplied as KH_2PO_4. Following previous studies (Güsewell 2005a), N and P supply were set in such a way that the geometric means of N and P supply became equal for all the treatments within each supply level. The geometric means of N and P supply per plant per year were 15 mg for high and 5 mg for low supply.

The nutrient solution was supplied weekly over a period of 15 weeks, starting from the end of June 2007. Demineralised water was provided into trays placed under each pot every 1 – 3 days. Once every 3 weeks each pot was leached with demineralised water three times, in order to prevent accumulation of nutrients and toxic compounds. The alignment of the pots was randomized every 3 weeks.

Measurements

We have measured the following plant properties for both the Bound-P Experiment and the Stoichiometry Experiment. For plant individuals that had very small root biomass, the measurements for either phosphatase activity or root length was omitted, yet in the constraint that at least three samples were taken for every treatment for each trait. See Tables and Figure captions for the specifications of the sample numbers.

Biomass and nutrient concentration

Before the fertilization period, for each species, five pots were randomly selected and harvested to determine initial biomass and nutrient content. After 15 weeks of fertilization, above and below ground biomass were harvested from the remaining pots. Dead leaves were collected throughout the experiment. Biomass was sorted into dead and alive parts, dried at 70°C for 48 h., weighed and ground. After a digestion procedure (Kjeldahl; 1h at 200°C and 2h at 340°C in a mixture of sulphuric acid, salicylic acid, copper sulphate, sodium sulphate and selenium), N and P concentrations in plants were determined colorimetrically on a continuous flow analyzer (Skalar SA-40). N and P contents were calculated by multiplying concentration by biomass. Biomass production was calculated by subtracting the initial biomass (at week 0) from the final living biomass (at week 15). Similarly, P uptake was estimated by subtracting the initial P content from the final P content. Death rate was calculated as the percentage of dead biomass over total biomass. P productivity, i.e. the rate of biomass production per unit P acquired (Berendse & Aerts 1987), was approximated by dividing total dry biomass by total P content. This equals to the reciprocal of P concentration. P loss was calculated as a percentage of P content in dead biomass over total P content. Resorption rate of P from senescing leaves was calculated as the percentage of the difference of P concentration in dead and living aboveground biomass over that in living aboveground biomass. Root mass ratio (RMR) was calculated as the proportion of belowground biomass to total biomass.

Root length

Approximately 100 mg of root was subsampled for each pot to estimate root length. Each subsample was dyed with 0.0005 % methylene blue solution and a digital photo was taken. Digital images were analyzed to estimate root length using NIH image software (Kimura & Yamasaki 2003), after which specific root length (SRL; cm g^{-1} root FW) was calculated. Total root length was calculated by multiplying SRL by total root fresh weight. *L. flos-cuculi* was excluded from this analysis, because their dense root hairs hampered length estimation with the software.

Phosphatase bioassay

Approximately 100 mg of root was taken from each pot in order to measure root surface phosphatase activity, using a *p*-nitrophenyl phosphate (*p*NPP) assay method (see Johnson *et al.* 1999). Within one hour after harvesting, the root subsample was assayed in artificial substrate *p*NPP at pH 7.4, which is similar to conditions in the pots (average pH 7.5, SE 0.5), for two hours.

Every 30 minutes, the concentrations of *p*-nitrophenol (*p*NP), a released compound from *p*NPP by phosphatase, were measured using a spectrophotometer (Hach colorimeter DS/890) at 410 nm. Phosphatase activity (Pase$_{root}$; μg *p*NP h^{-1} g^{-1} root FW) was estimated on the fresh root weight basis.

Since the method for expressing phosphatase activity might influence comparisons among species (Rejmankova & Macek 2008), we used two additional units to evaluate phosphatase activity. Total phosphatase activity (Pase$_{tot}$; μg *p*NP h^{-1}), the amount of phosphatase activity from the whole root system, was calculated by multiplying Pase$_{root}$ by total root fresh weight excluding rhizomes. Furthermore, as an indicator of how sufficiently a plant produces phosphatase independently from the effect of plant size, we calculated relative phosphatase activity (relative to biomass), Pase$_{rel}$, by dividing Pase$_{tot}$ by total biomass (μg *p*NP h^{-1} g^{-1} biomass DW).

Mycorrhizal infection

Approximately 100 mg of root was subsampled for three individuals of all species from each treatment. Roots were boiled with 10% KOH and stained with ink (Parker black) and 5% acetic acid (Vierheilig *et al.* 1998). Mycorrhizal infection was counted using the gridline intersect method (Giovannetti & Mosse 1980). Counting was restricted up to 100 intersections, as this proved to provide proper accuracy (Giovannetti & Mosse 1980). Mycorrhizal infection was expressed as the percentage of intersections with infected roots over all intersections.

Since the infection route of mycorrhizal fungi was neither controlled nor stimulated in our experiment, we limited our analysis to use the mycorrhizal infection rate as an explanatory variable for plant P uptake, but not as a response variable to test treatment effects.

Statistical analysis

Bound-P Experiment

Effects of P source, N supply level, functional group, species as nested within functional groups, and their interactions (all fixed factors) on various plant properties were analyzed with 3-way nested ANOVA. When functional group and any of its interactions had no significant effect, ANOVA was repeated with factors excluding functional group (source of P, N supply level, species and their interactions: all fixed factors) as 3-way ANOVA. The following data were used as the plant properties: square-root-transformed P uptake, biomass production, P loss, SRL, and mycorrhizal infection; log-transformed P productivity, Pase$_{root}$, Pase$_{tot}$, and Pase$_{rel}$; P resorption rate, death rate, and RMR. Consequently, differences in these variables between two P sources and two levels of N were compared for each species by a t-test when data was normally distributed, else by a Mann Whitney U-test. Additionally, interspecific differences in these variables were compared by multiple comparisons (Games Howell test (Sokal & Rohlf 1995)). Finally, the relationships between P uptake and four uptake traits (root mass, Pase$_{tot}$, total root length, and mycorrhizal infection) for bound-P treatments were tested with Pearson's correlation analysis for each functional group. Because the former three traits are correlated, partial correlation analysis was also conducted with these traits. *L. flos-cuculi* was excluded from the analysis concerning total root length due to absence of their SRL data

Stoichiometry Experiment

Effects of supply level, N:P supply ratio, functional groups, species as nested within functional groups, and their interactions (all fixed factors) on three uptake traits (Pase$_{root}$, RMR, and SRL) were analyzed with 3 way nested ANOVA. The factors which showed a significant effect were compared with Games Howell test. Pase$_{root}$ and SRL were log-transformed to obtain normal distributions of residuals.

Results

Bound-P Experiment: growth and P uptake from bound-P under different N levels

Effects of P source on plant growth properties

P uptake was less when plants were grown with bound-P compared to orthophosphate (Table 2.1, Figure 2.1a), yet in a species specific manner (see Sp x P interaction effect in Table 2.1). Five species (*A. pratensis*, *F. ovina*, *F. rubra*, *L. europaeus*, and *L. flos-cuculi*) had significantly reduced P uptake when grown with bound-P (Figure 2.1a, $p<0.05$ with t-test).

Biomass production, a proxy of overall plant performance, also decreased when plants were grown with bound-P (Table 2.1), but the decrease was not as large as when P uptake was compared (Figure 2.1b). The interaction between P source and species was nearly significant ($p=0.059$) and only two species (*F. rubra* and *L. europaeus*) significantly reduced biomass production when grown with bound-P (Figure 2.1b, $p<0.05$ with U-test).

P productivity was influenced by species, P source, and their interaction (Table 2.1). Five species (*A. pratensis*, *F. ovina*, *F. rubra*, *L. europaeus*, and *L. flos-cuculi*) had significantly higher P productivity when grown with bound-P ($p<0.05$ with U-test).

P loss was also significantly affected by P source and species (Table 2.1). Plants lost more P when grown with bound-P (significant for *A. pratensis*, *F. rubra*, *L. europaeus*, *L. flos-cuculi*, and *S. pratensis*). The increase of P loss was attributed to two factors: higher death rate and lower P resorption rate when grown with bound-P (Table 2.1).

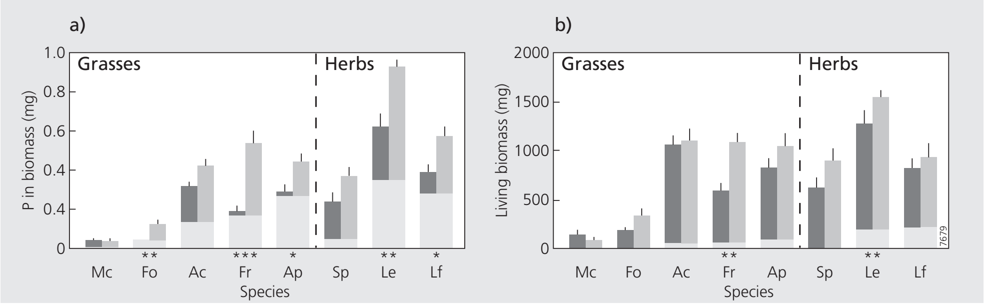

Figure 2.1 P content in biomass (a) and living biomass (b) after 15 weeks of fertilization with bound-P or orthophosphate treatments. White bars represent initial P content and initial biomass before the start of the fertilization ($N=5$); dark grey bars represent P uptake and biomass production with bound-P ($N=10$); light grey bars represent P uptake and biomass production with orthophosphate ($N=10$). Standard errors of total P and total living biomass at the end of the fertilization are shown. Low N and high N treatments were combined. Asterisks indicate significant differences in P uptake and biomass production between bound-P and orthophosphate treatments (***, $p<0.001$; **, $p<0.01$; *, $p<0.05$). Species abbreviations are; Mc: *M. caerulea*, Fo: *F. ovina*, Ac: *A. capillaris*, Fr: *F. rubra*, Ap: *A. pratensis,* Sp: *S. pratensis*, Le: *L. europaeus,* Lf: *L. flos-cuculi*.

Table 2.1 Effects of species, N supply level, source of P, and their interactions on several plant properties: P uptake, biomass production, P productivity, P loss, death rate, P resorption, phosphatase activity (Pase$_{root}$), specific root length (SRL), and root mass ratio (RMR). Results of a 3-way ANOVA (F values and significant levels) and treatment means are shown. Effect of functional group, examined in a 3-way nested ANOVA where species is nested within functional group, is also shown. None of the interactions with functional group were significant. Number of samples within a treatment is five unless specified. Significance levels are; ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$.

Factors		**P uptake**	**Biomass production**	**P productivity**	**P loss**	**Death rate**	**P resorption**	**Pase$_{root}$**	**SRL**	**RMR**
ANOVA results										
	df									
Species	7[*1]	20.8^{***}	46.6^{***}	47.1^{***}	7.6^{***}	7.4^{***}	4.3^{***}	111.3^{***}	105.7^{***}	69.5^{***}
N level	1	0.2	11.8^{**}	31.3^{***}	0.0	19.2^{***}	7.8^{**}	53.8^{***}	14.3^{***}	57.4^{***}
P source	1	68.7^{***}	13.6^{***}	189.1^{***}	40.0^{***}	12.1^{**}	16.8^{***}	1.1	1.1	0.6
Sp x N	7[*1]	1.9	1.9	15.1^{***}	1.8	4.8^{***}	1.9	2.2^{*}	4.9^{***}	2.3^{*}
Sp x P	7[*1]	4.1^{***}	2.0	7.6^{***}	1.5	2.6^{***}	1.7	0.9	1.8	2.0
N x P	2	0.5	0.3	17.0^{***}	0.5	1.3^{*}	0.5	0.0	2.9	7.0^{**}
Sp x N x P	7[*1]	0.8	1.1	2.4^{*}	1.8	1.4	1.4	0.7	1.1	2.0
Functional group	1	5.8	1.0	10.0^{*}	2.2	0.0	0.1	11.4^{*}	8.2^{*}	0.2
Treatment means ± SE										
	N	**(mg)**	**(mg)**	**(g mg^{-1})**	**(%)**	**(%)**	**(%)**	**(µg *p*-NP h^{-1} g^{-1} root FW)**	**(cm mg^{-1} root FW)**	**(Fraction)**
Low N	80[*2]	0.19±0.02	623±47	2.9±0.1	6.2±0.6	9.7±0.7	43.7±6.4	60.8±5.2	3.5±0.3	0.54±0.01
High N	80[*3]	0.17±0.02	770±55	3.3±0.1	6.0±0.5	13.7±0.9	60.7±2.8	82.9±6.8	3.1±0.2	0.46±0.02
PO$_4$	80[*2]	0.26±0.02	789±55	2.6±0.1	4.4±0.4	10.1±0.7	64.7±2.6	72.4±6.3	3.3±0.2	0.49±0.02
Bound-P	80[*3]	0.10±0.02	603±46	3.7±0.2	7.8±0.6	13.3±0.9	39.7±6.3	71.4±6.1	3.4±0.2	0.50±0.02
Grasses	100[*4]	0.12±0.02	595±45	3.6±0.1	5.5±0.5	11.9±0.8	53.6±5.4	98.0±5.6	4.1±0.2	0.47±0.02
Herbs	60[*5]	0.29±0.03	865±56	2.4±0.1	7.0±0.5	11.4±0.9	49.9±2.9	31.5±2.1	1.5±0.1	0.51±0.01

[*1] d.f. = 6 for SRL, because SRL of *L. flos-cuculi* was not measured (see Methods). [*2] *N=64* for SRL, *N=76* for Pase$_{root}$, [*3] *N=64* for SRL, *N=77* for Pase$_{root}$, [*4] *N=92* for SRL, *N=93* for Pase$_{root}$, [*5] *N=37* for SRL, *N=60* for Pase$_{root}$

Species difference in uptake traits

The magnitudes of several uptake traits and its sensitivity to P source was compared among functional groups and among species in order to speculate why three species (*A. capillaris, M. caerulea,* and *S. pratensis*) did not reduce P uptake from bound-P. Variation in $Pase_{root}$ was large between functional groups (higher for grasses than for herbs) as well as among species (Table 2.1), although neither of the variations depended on P source (interaction effects in Table 2.1). The rank order of species in $Pase_{root}$ was: *A. capillaris* > *M. caerulea* > *F. ovina* > *F. rubra* > *A. pratensis* > *L. europaeus* > *S. pratensis* > *L. flos-cuculi*, where the difference between the lowest and highest species was approximately 10-fold. $Pase_{tot}$ was not significantly different between functional groups ($F_{1,139}= 2.0$, $p=0.21$), but varied among species ($F_{6,139}=41.8$, $p<0.001$). *A. capillaris* had the highest $Pase_{tot}$, followed by *F. rubra*. $Pase_{rel}$ was also significantly higher for grasses than for herbs ($F_{1,139}= 11.2$, $p<0.05$). Interspecific variation in $Pase_{rel}$ was significant for both grasses and herbs: two grasses (*M. caerulea* and *A. capillaris*) and one herb (*S. pratensis*) had significantly higher $Pase_{rel}$ than other species in their functional group (Figure 2.2; $p<0.01$).

Variation in SRL was large between functional groups (higher for grasses than for herbs), as well as among species (Table 2.1), although neither of the variations depended on P source (interaction effects in Table 2.1). *A. pratensis* and *F. ovina* had higher SRL than other species ($p<0.01$).

Variation in RMR was large among species, but not significant between functional groups (Table 2.1). Neither of the variations depended on P source (interaction effects in Table 2.1). RMR was higher for *M. caerulea*, *S. pratensis*, and *A. pratensis* than for the other species ($p<0.05$).

Variation in mycorrhizal infection was large among species ($F_{6,82}= 16.2$, $p<0.001$) but not significant between functional groups ($F_{1,82}= 0.11$, $p=0.95$). Interactive effects of P source on the variations are not discussed here because of the limitation in mycorrhiza measurements (see Methods).

Uptake traits related to P uptake from bound-P

The contributions of several uptake traits to the plant P uptake from bound-P were examined by correlation analyses. P uptake from bound-P was positively correlated with root mass for both functional groups, and positively correlated with $Pase_{tot}$ and with total root length for grasses but not for herbs (Table 2.2). Mycorrhizal infection was not correlated with P uptake from bound-P. Partial correlation analysis showed that the correlation between $Pase_{tot}$ and P uptake was positive and significant for grasses even when root mass and total root length were kept constant (Table 2.2). In contrast, the correlation between root mass and P uptake was positive and significant for herbs when the effects of $Pase_{tot}$ and total root length were kept constant (Table 2.2).

Effects of increased N

With the exception of *F. ovina*, N supply level did not have a significant effect on P uptake when plants were grown with bound-P ($p>0.05$ with t-test). This contrasted with the results of phosphatase activity that increased with increased N supply in any units, i.e. $Pase_{root}$ (Table 2.1), $Pase_{tot}$ ($F_{1,121}= 26.9$, $p<0.001$) and $Pase_{rel}$ ($F_{1,121}= 4.7$, $p<0.05$). Decrease in RMR and SRL with increased N supply (Table 2.1) could have mitigated the effect of promoted phosphatase activity. Despite unchanged P uptake, biomass production increased significantly with increased N supply (Table 2.1). This corresponds with higher P productivity with increased N supply (Table 2.1; significant for *A. capillaris, A. pratensis*, *F. rubra*, *L. europaeus*, and *L. flos-cuculi*, $p<0.05$ with t-test).

Table 2.2 Pearson's correlation coefficients of root mass, total phosphatase activity (Pase_{tot}), total root length and mycorrhizal infection with P uptake, as well as partial correlation coefficients of the former three traits with P uptake. Only bound-P treatments were considered for these analyses. Coefficients are shown for five grasses and three herbs, except that *L. flos-cuculi* was excluded from the analysis concerning total root length due to absence of their SRL data. Values in parenthesis are number of samples examined. Significance levels are; ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$; *ns*, not significant.

Variables correlated with P uptake	Variables kept constant	(Partial) correlation coefficient			
		Grasses		Herbs	
Root mass	-	0.457^{**}	(50)	0.715^{***}	(30)
Total phosphatase activity	-	0.632^{***}	(47)[*1]	*ns*	(30)
Total root length	-	0.403^{**}	(45)[*1]	*ns*	(19)[*1]
Mycorrhizal infection	-	*ns*	(30)	*ns*	(18)
Root mass	Total phosphatase activity Total root length	*ns*	(43)[*2]	0.594^{*}	(19)[*2]
Total phosphatase activity	Root mass Total root length	0.509^{**}	(43)[*2]	*ns*	(19)[*2]
Total root length	Root mass Total phosphatase activity	*ns*	(43)[*2]	*ns*	(19)[*2]

[*1] Sampling for either root length or phosphatase activity was omitted for a few plant individuals due to their very small root size.

[*2] Partial correlation analysis was conducted only for the plant individuals which had complete data set of root mass, total phosphatase activity, and total root length.

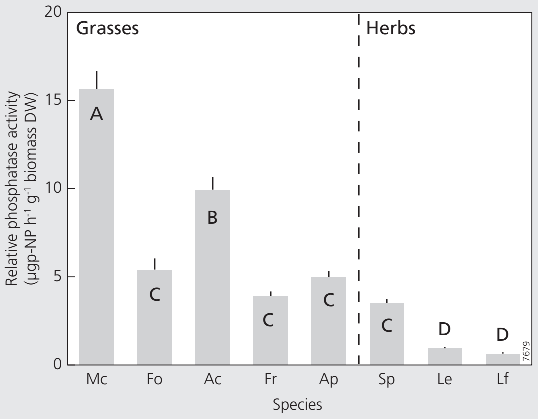

Figure 2.2 Relative phosphatase activity (Pase_{rel}), expressed as total phosphatase activity (Pase_{tot}) divided by total biomass, for each species (*N=20*, except for *M. caerulea*: *N=13*). Values are means + 1 standard error. Letters represent results of multiple comparison test. Species abbreviations are; Mc: *M. caerulea*, Fo: *F. ovina*, Ac: *A. capillaris*, Fr: *F. rubra*, Ap: *A. pratensis,* Sp: *S. pratensis*, Le: *L. europaeus,* Lf: *L. flos-cuculi*.

There was no significant effect of N level on P loss (Table 2.1). Although high N level resulted in higher death rates, it also promoted P resorption, resulting in no significant effect on P loss (Table 2.1) except for *F. rubra* ($p<0.05$ with t-test).

Stoichiometry Experiment: effects of N:P ratio on uptake traits

$Pase_{root}$ was highly influenced by N:P supply ratio ($F_{2,213}= 273.1$, $p<0.001$) and to a lesser extent by supply level ($F_{1,213}= 6.4$, $p<0.05$) (Figure 2.3a). $Pase_{root}$ was significantly lower at N:P of 1.7 compared to that at the two higher N:P ratios (15 and 135) ($p<0.001$). The N:P ratio effects on $Pase_{root}$ was not significantly different between two functional groups ($F_{2,213}= 1.2$, $p=0.30$) nor between two nutrient levels ($F_{2,213}=1.8$, $p=0.17$).

SRL was not influenced by N:P supply ratio ($F_{2,179}=2.6$, $p=0.07$), but decreased with increased supply level ($F_{1,179}= 5.7$, $p<0.05$) (Figure 2.3b). The N:P ratio effects on SRL was not significantly different between the two functional groups ($F_{2,179}= 0.7$, $p=0.48$) nor between two nutrient levels ($F_{2,179}=2.3$, $p=0.10$).

RMR decreased with increased N:P supply ratio ($F_{2,222}=29.6$, $p<0.001$) and decreased with supply level ($F_{1,222}=44.6$, $p<0.001$) (Figure 2.3c). The N:P ratio effects on RMR was not significantly different between the two functional groups ($F_{2,222}= 0.1$, $p=0.90$) nor between two nutrient levels ($F_{2,222}=0.4$, $p=0.67$). The relation between RMR and N:P supply ratio seems to be a pseudo relation caused merely by N effects, because RMR decreased with increased N supply, but did not change with P supply (Appendix III Figure 2.A1).

Discussion

Plant growth and P uptake from bound-P

We showed that the ability of plants to take up P from bound-P was species specific, and the species variation was, at least partly, explained by their phosphatase activity. Therefore, our first hypothesis was not rejected. Although there was no difference between grasses and herbs in P uptake from bound-P, the importance of several traits for their P uptake seemed contrasting. For grasses, P uptake from bound-P was related to $Pase_{tot}$ even when the other potentially confounding P uptake traits (i.e. root mass and root length) were controlled. In contrast, for herbs, P uptake from bound-P was not directly related to $Pase_{tot}$. This coincides with our observation that $Pase_{root}$ was considerably lower for herbs than grasses (also see Phoenix *et al.* 2003). Moreover, P uptake of herbs was higher than that of grasses when individuals with the same amount of $Pase_{tot}$ were compared, implying that mechanisms other than phosphatase might be more important for herbs. Since neither SRL, RMR, nor mycorrhizal infection were higher for herbs compared to grasses in our experiment, other unexplored factors such as phosphorus carrier enzymes in roots (Treseder & Vitousek 2001) or excretion of other root exudates (Lambers *et al.* 2006) might have contributed to the better P uptake of herbs. Note that the mycorrhizal infection rates were low in our experiments (average for herbs 14.7 %; grasses 14.0 %) due to the restricted infection route. Higher mycorrhizal infection rates of herbs in natural habitats (Weishampel & Bedford 2006) would promote their P uptake even more.

The importance of phosphatase in P uptake from bound-P was further supported by the trend that species which did not differ in P uptake from both P sources had neither high RMR nor high SRL, but high $Pase_{rel}$. This was a rather surprising result, because organic P, which is the only P form that phosphatase hydrolyzes, was only 20 % of the total bound-P supplied in our

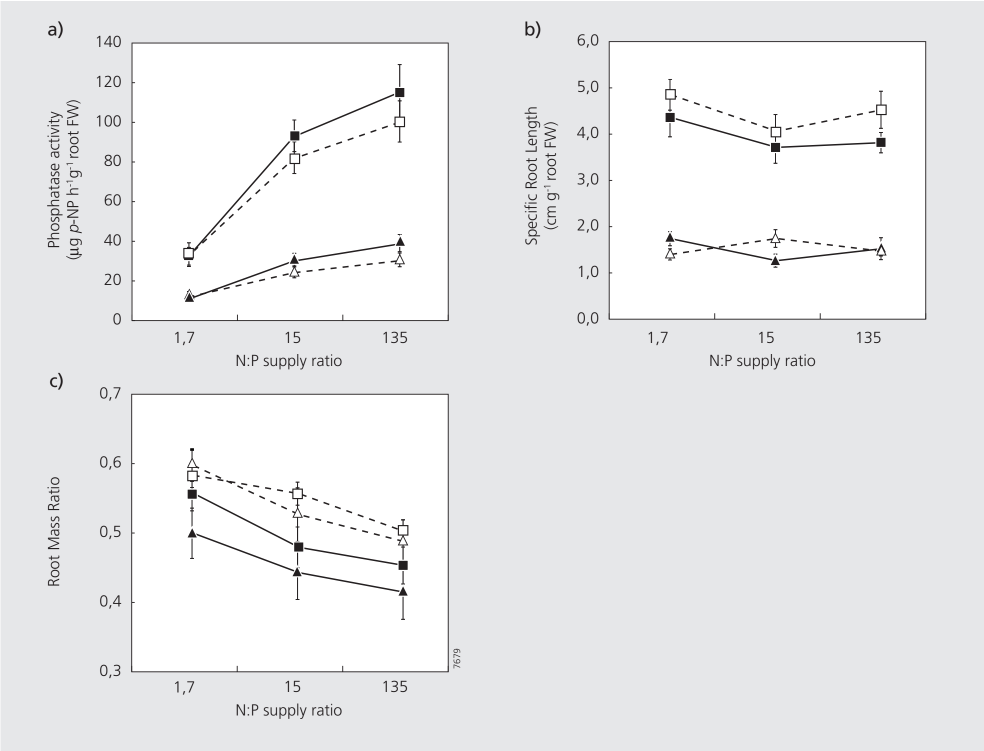

Figure 2.3 a) Phosphatase activity (Pase$_{root}$), b) specific root length (SRL), and c) root mass ratio (RMR) after 15 weeks of fertilization with different nutrient supply ratios for grasses (squares) and herbs (triangles). *L. flos-cuculi* is not included in the SRL graph. Closed and open symbols represent high and low nutrient supply levels, respectively. Average values and standard errors are shown. *N*= *25* for grasses and *N*=*15* (Pase$_{root}$ and RMR) or *N*=*10* (SRL) for herbs, with the following exceptions: for Pase$_{root}$ of grasses, *N*=*23* at all N:P ratios of low level and at N:P 135 of high level, *N*=*24* at N:P 1.7 of high level; for SRL of herbs, *N*=*8* at N:P 135 of high level.

experiment. Considering the possible P adsorption to the sand (see Appendix IV), presumably as inorganic calcium phosphate, a large part of the difference between bound-P and orthophosphate treatments may lay on organic P. This explains why phosphatase was related to P uptake from bound-P in our experiment regardless of the limited proportion of organic P. It should also be noted that phosphatase activity measured by *p*NPP assay (i.e. phosphomonoesterases) and hydrolysis of phytates have only indirectly a causal relationship (Tibbett 2002) although they are positively related (Tarafdar & Claassen 1988, Helal 1990). In natural soils where the proportion of organic P would be much higher (Perez-Corona *et al.* 1996) and in which organic P is present in various forms (Lambers *et al.* 2006), the importance of phosphatase for P uptake is expected to be even greater.

Both *M. caerulea* and *S. pratensis*, which occur typically in P limited grasslands, had the highest Pase$_{rel}$ among the grasses and herbs, respectively. These species did not have the highest, though moderately high, Pase$_{root}$ among species. However, they had a higher RMR than other species. Apparently, species variation in phosphatase activity does not coincide with their field distribution patterns when its rate per unit root (Pase$_{root}$) is concerned, but does coincide with its relative amount to the whole plant biomass (Pase$_{rel}$). This indicates that phosphatase activity should be evaluated on the whole plant level rather than the activity rate per unit root to properly assess its importance.

Effects of increased Nitrogen

Contrary to our second hypothesis, higher N supply increased neither P loss nor P uptake from bound-P. Although phosphatase activity was enhanced by increased N supply both on the whole root level and per unit root, it did not result in higher P uptake. This contradicts with our finding that P uptake from bound-P was related to phosphatase activity (see previous paragraph). Decreased RMR and SRL under high N supply may have hampered the positive effects of increased phosphatase activity. Furthermore, not all excreted phosphatase might have contributed to cleaving organic P because of the relatively small amount of P supplied in the Bound-P Experiment.

In contrast to the unchanged P uptake, biomass production was enhanced in high N treatments by increasing P productivity. This is because biomass production is more strongly regulated by the availability of N rather than P (de Groot *et al.* 2003). Interestingly, three species which did not increase P productivity with increased N (*M. caerulea*, *S. pratensis*, and *F. ovina*) tend to occur in P limited grasslands. Because high death rate is related to low P concentration (this study (results not shown), Fisher *et al.* 2002, El-Kahloun *et al.* 2003), these three species avoided increased senescence by not producing more biomass from extra N supply. Although the increased death rate did not result in higher P loss in our experiment due to an improved P resorption rate in P poor conditions (Richardson *et al.* 2008), maintaining too low P concentration might cause other negative impacts on a longer time scale, such as impaired reproductive activity (Brouwer *et al.* 2001). In this regard, a less plastic response to changing nutrient conditions, which is more common for species from nutrient poor habitats (Garnier 1998), might be advantageous under enhanced N deposition. This may account for the trend that P poor conditions commonly promote abundance of low productive, non-typical nitrophilous species (Güsewell 2004) or threatened species (Wassen *et al.* 2005).

N:P ratios regulate phosphatase activity

Our study showed that phosphatase activity is strongly influenced by N:P stoichiometry, which is in line with our third hypothesis. Pase$_{root}$ increased with N:P supply ratio, and the effect of N:P supply ratio was stronger than the effects of supply level of N and P. Although several studies have already shown that individual nutrients affect Pase$_{root}$, i.e. an inhibitory effect of P addition and a stimulating effect of N addition (Johnson *et al.* 1999, Olander & Vitousek 2000, Phoenix *et al.* 2003), we here explicitly show that the N:P stoichiometry is the most important regulatory factor by testing effects of each N:P supply ratio by two combinations of N and P supply. The mechanism responsible for the N:P stoichiometry effects on phosphatase activity could be a higher flexibility of plants for P concentration than for N concentrations in their biomass (Güsewell & Koerselman 2002). Due to the dominant role of N for biomass production, low P concentrations in biomass occur only when P supply is *relatively* low to N supply (where plants grow by increasing productivity per unit P). Thus, not merely low P supply but high N:P supply ratio results in P starvation in the plant, which induces synthesis and secretion of phosphatase (Duff *et al.* 1994). In contrast to Pase$_{root}$, SRL and RMR were not dominantly influenced by N:P stoichiometry. RMR was more strongly regulated by N than by P (Andrews *et al.* 1999, this study), while SRL was hardly influenced by N:P supply ratio. The minor influence of N:P stoichiometry effects on SRL and RMR seems plausible, because these traits are involved not only for P uptake but nutrient uptake in general. Extrapolating these trait responses in the Stoichiometry Experiment to the observed plant responses to high N supply in the Bound-P Experiment, we argue that enhanced phosphatase activity under high N supply was not due to increased N supply but increased N:P ratio, whereas decreased RMR was mainly due to solo effect of N. The contrasting responses of uptake traits to (relative) availability of N and P imply that the effects of N enrichment of a certain level on grassland ecosystems may differ depending on the relative P availability of the site.

In conclusion, our study showed that increased N supply stimulates phosphatase activity, most likely via N:P stoichiometric effects, although the increased phosphatase activity did not result in higher plant P uptake. This is probably due to decreased RMR and SRL, as well as limited P supply in our experiment. Nevertheless, the important role of phosphatase activity in plant P uptake from bound-P was indicated. Furthermore, we found that the species common in P limited grasslands were associated with effective phosphatase activity (i.e. high Pase_{rel}), which is in line with our fourth hypothesis. This provides an example of how a species trait possibly alters the competitive balance of species along a gradient of nutrient availability (Grime 2001). The trait-driven distribution pattern has been intensively tested for low to high nutrient gradients (e.g. Campbell & Grime 1992, Fynn *et al.* 2005), but it is still poorly understood for stoichiometric gradients of N and P. Our results implied that N deposition may affect plant community composition not only by enhancing site productivity, but also by favouring species that have adaptive traits to P limited conditions, which may cause changes in the species composition.

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Appendix I. Field distribution pattern of eight species in N and P limited grasslands

Table 2.A1 381 field sites from Dutch, Belgium, German and Polish grasslands were classified into either N, co, or P limited sites based on biomass N:P ratio of plant community (<13.5, 13.5 to 16, and >16, respectively). For the eight species used in the experiment, number of sites of occurrence in each type of nutrient limitation was shown. Species were considered to have affinity to N or P limited sites when they had significantly more frequent occurrence in N or P limited sites tested by chi-square test. Positive or negative associations to the limitation type were shown as "+" or "-", respectively. Significant levels of the chi-square test were shown as; ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$; *n.s.*, not significant.

	Species	Number of sites of occurrence (N=381)			Affinity to N limited sites	Affinity to P limited sites
		N limited	Co limited	P limited		
Grass	*Molinia caerulea*	10	16	72	$-^{***}$	$+^{***}$
	Festuca ovina	4	1	8	$-^{*}$	*n.s.*
	Agrostis capillaris	23	6	14	*n.s.*	*n.s.*
	Festuca rubra	84	12	38	*n.s.*	*n.s.*
	Alopecurus pratensis	34	0	0	$+^{***}$	$-^{*}$
Herb	*Succisa pratensis*	4	4	36	$-^{***}$	$+^{***}$
	Lycopus europaeus	25	3	18	*n.s.*	*n.s.*
	Lychnis flos-cuculi	66	12	5	$+^{**}$	$-^{**}$

Appendix II. Composition of nutrient solution

Table 2.A2 Amount of N and P in nutrient solution

Treatment		N (mg pot^{-1})		P (mg pot^{-1})	
Bound-P Experiment					
<P Source>	<N level>				
Orthophosphate	Low	19.4	*1	1.3	*3
	High	174.3	*2	1.3	*3
Bound-P	Low	19.4	*1	1.3	*4
	High	174.3	*2	1.3	*4
Stoichiometry Experiment					
<Supply level>	<N:P ratio>				
Low	1.7	6.5	*1	3.9	*3
	15	19.4	*1	1.3	*3
	135	58.1	*1	0.4	*3
High	1.7	19.4	*1	11.6	*3
	15	58.1	*1	3.9	*3
	135	174.3	*2	1.3	*3

*1 Supplied as KNO_3.

*2 Supplied as KNO_3 and $Ca(NO_3)_2$ in order to provide the same amount of potassium for all treatments. The amount of calcium associated with the $Ca(NO_3)_2$ was subtracted from $CaCl_2$, so that the same amount of calcium was supplied for all treatments.

*3 Supplied as KH_2PO_4.

*4 Supplied as a mixture of organic and inorganic P compounds. For the organic P compound we used inositol hexaphosphate ($C_6H_{18}O_{24}P_6$), since phytates typically comprise the largest fraction of organic P in natural soils (Dalal 1977). Because organic P typically comprise 30-65 % of total P (Harrison 1987) and phytates comprise ca. 50% of total organic P (Dalal 1977) in natural soils, we supplied 20% of our bound-P supply as inositol hexaphosphate. For the inorganic P compound we chose an acid-labile and an alkaline-labile compounds: calcium phosphate ($Ca_{10}(PO_4)_6(OH)_2$) and aluminum phosphate ($AlPO_4$), respectively. To resemble natural soils (Scheffer & Schachtschabel 1970), we supplied more calcium phosphate than aluminum phosphate (50% and 30% of our bound-P supply, respectively).

Table 2.A3 Amount of other elements in nutrient solution

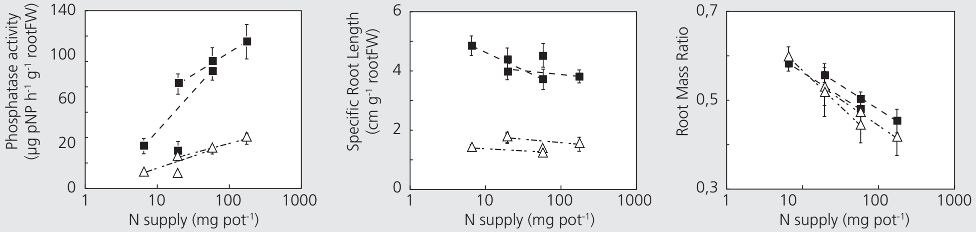
Element	mg pot^{-1}	Compound
Mg	25.64	$MgSO_4$ $7H_2O$
S	37.11	$MgSO_4$ $7H_2O$, $FeSO_4$ $7H_2O$, $CuSO_4$ $5H_2O$, $MnSO_4$ $4H_2O$, $ZnSO_4$ $7H_2$
Fe	5.13	$FeSO_4$ $7H_2O$
Cu	0.01	$CuSO_4$ $5H_2O$
B	0.24	H_3BO_3
Mn	0.55	$MnSO_4$ $4H_2O$
Mo	0.01	Na_2MoO_4 $2H_2O$
Zn	0.03	$ZnSO_4$ $7H_2O$
Ca	79.76	$CaCl_2$*1, $Ca(NO_3)_2$ $4H_2O$*2
K	332.39	KCl*1, KNO_3, KH_2PO_4

*1 Not for the treatments of high supply level x N:P ratio135 and orthophosphate x high N level.

*2 Supplied only for the treatments of high supply level x N:P ratio135 and orthophosphate x high N level.

Appendix III. Effects of N and P supply on plant uptake traits

a) Effects of N supply



b) Effects of P supply

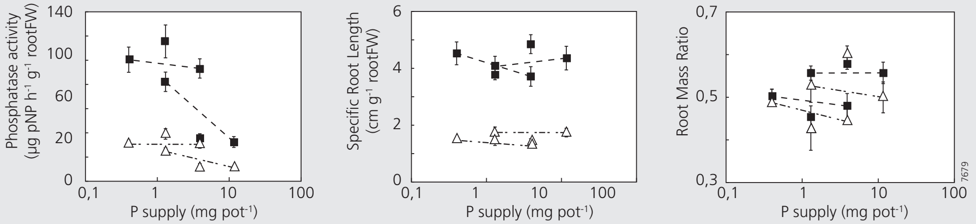


Figure 2.A1 Effects of N supply (a) and P supply (b) on phosphatase activity (Pase$_{root}$), specific root length (SRL), and root mass ratio (RMR) are shown for grasses and herbs. Closed squares represent grasses, while open triangles represent herbs. Treatments are connected with dotted lines when supply of the other nutrient was the same. Average values and standard errors are shown.

Appendix IV. Adsorption capacity of quartz sand

The amount of P uptake was low even with orthophosphate treatments; the highest P uptake was approximately 50 % of the total P supply. Therefore it was suspected that some of the supplied P was adsorbed to the quartz sand. To test the magnitude of the adsorption, we performed an additional lab experiment to check adsorption capacity of the quartz sand for orthophosphate. We monitored the phosphorus adsorption capacity of 50g of quartz sand in 100ml of KH_2PO_4 solution for one week. Although adsorption was ignorable when P concentration of the solution was high (50mg P l^{-1}), considerable amount of adsorption (c.a. 45% and 30%) was observed after one week when P concentration was low (0.5mg P l^{-1}) and intermediate (5mg P l^{-1}), respectively. The concentration of P in the solution used in our Bound-P Experiment was 2.6mg P l^{-1}.

Although the observed high percentages of P adsorption in the extra lab experiment were considerable, the effects of adsorption on the results of our fertilization experiments may be questioned, since plants may take up P quickly after fertilization before P will be adsorbed. Güsewell (2005c) showed that *Carex* species took up around 80 % of P from solution in seven hours when N:P supply ratio was larger than 5. In addition, percentage of N uptake relative to supplied N was also low in our experiment, ranging from 3% to 66%. This indicates that transportation of solution to roots in general, rather than adsorption of P to sand, was a major problem for plants in taking up nutrients.

3 Time-dependent, species-specific effects of N:P stoichiometry on grassland plant growth

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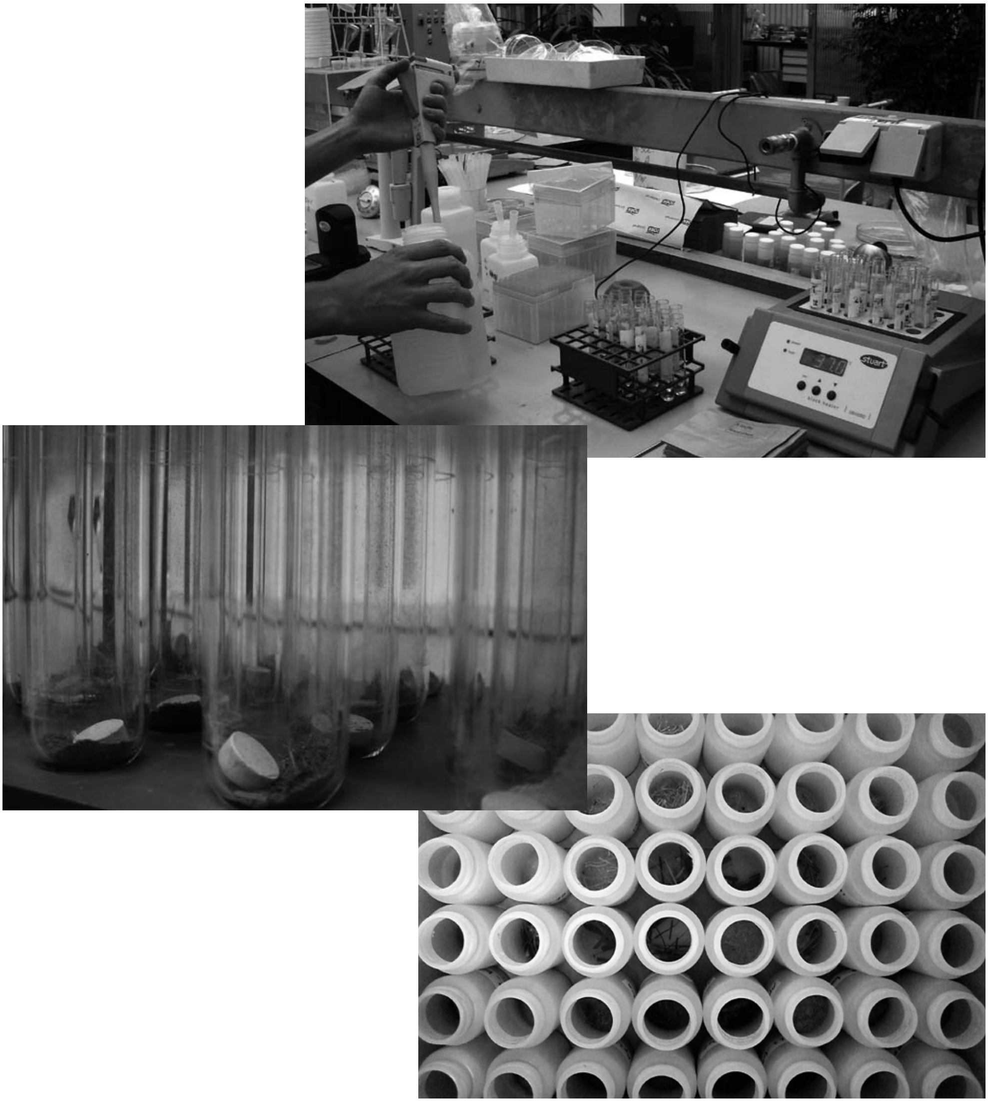

Abstract

N and P have different eutrophication effects on grassland communities, yet the underlying mechanisms are poorly understood. To examine plant growth in response to the varying (relative) supply of N and P, we conducted a two-year greenhouse experiment. Five grasses and three herbs were grown with three N:P supply ratios at two overall nutrient supply levels. During the first year the plant growth was relatively low at both high and low N:P supply ratios, whereas during the second year the growth was especially low at a high N:P supply ratio. This second-year low growth was attributed to the high root death rate, which was influenced by a high N:P supply ratio rather than by the nutrient supply level. Species responded differently, especially in P uptake and loss at a high N:P supply ratio. Each species seemed to have a different strategy for P limitation, e.g. an efficient P uptake or a high P resorption rate. Species typical of P-limited grasslands had neither better P uptake nor better P retention at a high N:P supply ratio. This study quantitatively demonstrates an increased plant root death triggered by strong P limitation. This finding indicates a possible extra effect of N eutrophication on ecosystem functioning via changed N:P stoichiometry.

Keywords
Eutrophication; plant growth and death; long-term experiment; interspecific differences; N:P ratio; nutrient loss

Introduction

Increased nutrient availability is one of the major causes of degradation among grassland plant communities in Europe. Nitrogen (N) and phosphorus (P) are the two main nutrients responsible for biodiversity decline in grassland ecosystems (Bobbink *et al.* 1998, Stevens *et al.* 2004, Wassen *et al.* 2005). Plant growth can respond differently to N compared to P enrichment. N fertilization immediately increases the biomass of a plant community, whereas the effects of P fertilization appear slowly (e.g. Güsewell *et al.* 2002) but last longer (El-Kahloun *et al.* 2003, van der Hoek *et al.* 2004). On top of that, some species-specific effects have been reported. Legumes, for example, decrease their abundance after N fertilization (Kirkham *et al.* 1996, Beltman *et al.* 2007). The contrasting effects of N and P fertilization indicate that plants are sensitive to the ratio of nutrients (besides their sensitivity to the overall supply level) but also that these sensitivities may vary among species (Güsewell 2004). It is known that plants maintain a certain stoichiometric balance of elements, such as a N:P, to function properly (Sterner & Elser 2002). Changing the N:P ratio, for instance by adding one element, may induce different responses in different species, which may then affect competition among those species and alter the structure of the community. Several field observations demonstrate the importance of a balance between N and P for the functioning of plant communities. Three observations illustrate this: the productivity level at the peak of a hump-shaped curve is lower for P-limited than for N-limited wetlands (Olde Venterink *et al.* 2003); the species composition shifts from N- to P-limited grasslands (Verhoeven & Schmitz 1991, Olde Venterink *et al.* 2003, Güsewell 2004); and endangered species persist better at P- rather than N-limited sites (Wassen *et al.* 2005).

In search of an explanation for these field observations, a number of greenhouse experiments have been carried out to investigate the morphological and physiological responses of grassland plants to varying balances of N and P (Shaver & Melillo 1984, Ryser *et al.* 1997, Güsewell 2005a, Macek & Rejmankova 2007). Short-term experiments show that adding N, leading to high N:P supply ratios, increases plant biomass, whereas adding P, leading to low N:P supply ratios, does not promote plant growth (Güsewell *et al.* 2003, Güsewell 2005c). Longer-term experiments show contrasting results: after a period of two years, high N:P ratios reduce nutrient retention (i.e., the amount of nutrients retained in biomass), whereas low N:P ratios do not (Güsewell *et al.* 2003, Güsewell 2005a). The poor retention at high N:P ratios is assumed to be caused by nutrient loss from roots (Güsewell 2005a), although this relation has not been quantitatively examined. Increased root turnover or root death was also observed in other studies under conditions of P limitation (e.g. El-Kahloun *et al.* 2003) or upon increased N availability (e.g. Fransen & de Kroon 2001). However, it remains a matter of speculation whether the root death is caused by the balance between N and P availability rather than by the availability of single elements. Nor is it clear what the underlying mechanisms are. Additionally, interspecific difference in response to N:P ratio has been also suggested by previous greenhouse experiments with a large number of species. Species that react strongly to a change in N supply do not necessarily also react strongly to a change in P supply (Keddy *et al.* 2001, Güsewell *et al.* 2003). This implies that for different species it could be N- or P-limited conditions that are favorable. These interspecific differences in the response to the availability of nutrients also call for research into an explanatory mechanism. Such investigations might fill the gap in understanding how the anthropogenic nutrient enrichment of ecosystems, which is often asymmetric between N and P (Lamers *et al.* 2002, Olde Venterink *et al.* 2002), may affect communities of plant species.

Hence, the aim of the present study is to examine the effects of the relative availability of N and P on plant growth. Concretely, we investigate whether a relatively high N to P availability reduces the nutrient retention of plants in a species-specific way. In order to examine both immediate effects and longer term effects, we conducted a two-year greenhouse fertilization experiment with grasses and herbs, including those typical of P- and N-limited grasslands. We varied both the overall N and P supply and the N:P supply ratio, enabling us to distinguish the effects due to the supply level from those due to the supply ratio. Based on the prior work, we hypothesized that (1) a high N:P supply ratio reduces plant growth mainly due to high root death and nutrient loss in the second year; and (2) that the high death rate and nutrient loss is influenced by N:P supply ratios rather than by overall nutrient supply levels. Moreover, we hypothesized that (3) different species respond differently to a high N:P ratio because of the interspecific difference in root death in the second year. Furthermore, we expected that (4) species that typically occur in P-limited grasslands would have less nutrient loss at a high N:P ratio and thus be better able to cope with P limitation than other species.

Materials and methods

Plant species and growth conditions

We used five grass and three herb species, all of which occur naturally in the Netherlands on grasslands ranging from wet to moist. These species were selected on the basis of field data from 381 European grassland sites (Ertsen 1998, Wassen *et al.* 2005; Hölzel unpublished) in order to include species typically found at P- and N-limited sites in the field. The selected species are *Alopecurus pratensis* L., *Lychnis flos-cuculi* (L.) Greuter & Burdet, *Agrostis capillaris* L., *Festuca rubra* L., *Lycopus europaeus* L., *Festuca ovina* L., *Molinia caerulea* (L.) Moench, and *Succisa pratensis* Moench. The first two and last two species in this list have a significantly more frequent distribution in N- and P-limited grasslands, respectively (chi-square test, $p<0.05$; see Chapter 2).

Plants were cultivated from seed in a germination chamber starting in May 2007. In June 2007 all seedlings were transplanted to plastic pots filled with *c.* 500g quartz sand and placed in an open greenhouse with a transparent roof and walls of coarse mesh. Plants were fertilized with nutrient solution once a week for two growing seasons. The environmental conditions within the greenhouse – such as temperature, humidity, and light intensity – were similar to the ambient conditions. The fertilization periods lasted 15 weeks in 2007 (starting at the end of June) and 14 weeks in 2008 (starting at the end of March). The chemical composition of the quartz sand was analyzed to ensure that it contained no nutrients or only negligible amounts, which was the case. Every one to three days, demineralized water was poured into trays placed under each pot. Once every three weeks, the pots were leached with demineralized water three times in order to prevent the accumulation of nutrients and toxic compounds. The alignment of the pots was randomized once every three weeks.

Nutrient treatments

We combined two nutrient levels, high and low, and three N:P supply ratios, low (1.7), intermediate (15), and high (135), in a full-factorial design. The resulting six treatments were replicated eleven times. N was provided as KNO_3 and $Ca(NO_3)_2$, while P was provided as KH_2PO_4. In line with previous studies (Güsewell 2005a, Güsewell 2005c), the supply of N and P was determined such that their geometric means came out equal for all treatments at a distinct supply level (5 mg and 15 mg per plant per year for high and low supply, respectively; Table 3.1). All other essential elements were supplied at sufficient levels (per pot per year: 25.6 mg Mg, 37.1 mg S, 5.1 mg Fe, 0.01 mg Cu, 0.24 mg B, 0.55 mg Mn, 0.01 mg Mo, 0.03 mg Zn, 79.8 mg Ca, 332 mg K). The amount of N and P in the high-level treatment at an N:P ratio of 15 is similar to the annual influx of those nutrients occurring in productive wetlands in the Netherlands (Koerselman *et al.* 1990a, Wassen & Olde Venterink 2006).

Table 3.1 Annual supply of Nitrogen and Phosphorus for six treatments

Treatment		**Nutrient supply (mg pot^{-1} year^{-1})**	
Nutrient supply level	**N:P supply ratio**	**N**	**P**
Low	Low (1.7)	6.5[a]	3.9
	Intermediate (15)	19.4[a]	1.3
	High (135)	58.1[a]	0.4
High	Low (1.7)	19.4[a]	11.6
	Intermediate (15)	58.1[a]	3.9
	High (135)	174.3[b]	1.3

[a] Supplied as KNO_3

[b] Supplied as KNO_3 and $Ca(NO_3)_2$. For this treatment, $CaCl_2$ was reduced so that total amount of supplied Ca is the same for all the treatments.

Measurements

Before the fertilization period in 2007, five pots of each species were randomly selected and harvested to determine the initial biomass and nutrient content. Dead leaves were collected throughout the experiment. After the 2007 fertilization period, the above- and below-ground biomass was harvested from five pots per treatment. At the same time, the above-ground biomass of the remaining six pots was clipped approximately 1 cm above the sand. After the fertilization period in 2008, the above- and below-ground biomass of these six pots was harvested. The harvested biomass was sorted into dead and alive parts, dried at 70°C for 48 h, weighed, and ground up. Dead roots were separated from living ones based on visual inspection of their color and testing elasticity with a tweezers. After digestion using the Kjeldahl procedure (1h at 200°C and 2h at 340°C in a mixture of sulfuric acid, salicylic acid, copper sulfate, sodium sulfate, and selenium) (Bremner & Mulvaney 1982), N and P concentrations were determined colorimetrically on a continuous flow analyzer (Skalar SA-40). Total contents of N and P were calculated for each part of the biomass (above-alive, above-dead, below-alive, below-dead) by multiplying their concentration by the biomass. For each part, when fewer than three samples within a treatment weighed more than 50 mg, all samples of the treatment were combined for digestion.

Growth in 2007 (mg per 15 weeks of fertilization) was calculated by subtracting average initial biomass from biomass (including dead and alive) at the end of the 2007 fertilization period. Growth in 2008 (mg per 14 weeks of fertilization plus the preceding winter) was calculated by subtracting the average living below-ground biomass in 2007, for each treatment, from the biomass at the end of the fertilization period in 2008. When growth in 2008 was negative, we considered the negative part as the below-ground dead biomass in 2008. Death rate was calculated as the percentage of dead biomass relative to total biomass. Root mass ratio (RMR) was calculated as the percentage of living below-ground biomass relative to the total amount of living biomass. P uptake in 2007 was estimated by subtracting the average initial P content from the total amount of P content in 2007. P uptake in 2008 was estimated by subtracting the average P content of living below-ground biomass in 2007 for each treatment from the total P content in 2008. When P uptake in 2008 was negative, we considered the negative part as loss from below-ground dead biomass in 2008. P loss rate was calculated as the percentage of P content in dead biomass relative to the total P content. Resorption rate of P from senescing leaves was calculated for 2007 as the percentage difference in the P concentration between dead and living above-ground biomass, relative to that in living above-ground biomass. Plants that did not regenerate after the winter were excluded from the calculations for 2008. Plants that did not have any living biomass left in 2008 were excluded from the calculations of RMR.

Statistical analysis

The effects of the nutrient supply level, N:P supply ratios, species, and their interactions (all fixed factors) on various plant responses (growth, death rate, above-ground death rate, below-ground death rate, P uptake, P loss, N uptake, N loss in 2007 and 2008, and leaf resorption rate of P and N in 2007) were tested with a three-way ANOVA. Note that some of these plant response variables are autocorrelated; there is a correlation among growth, P uptake, and N uptake as well as among death rate, above-ground death rate, below-ground death rate, P loss, and N loss. All variables except those expressed as percentages were log transformed or square root transformed to obtain normal distributions of the residuals. For the leaf resorption rate, three-way interaction was omitted, because we aggregated replicas of some treatments for N and P determination and therefore used average values of each treatment for each species in the ANOVA. *L. flos-cuculi*

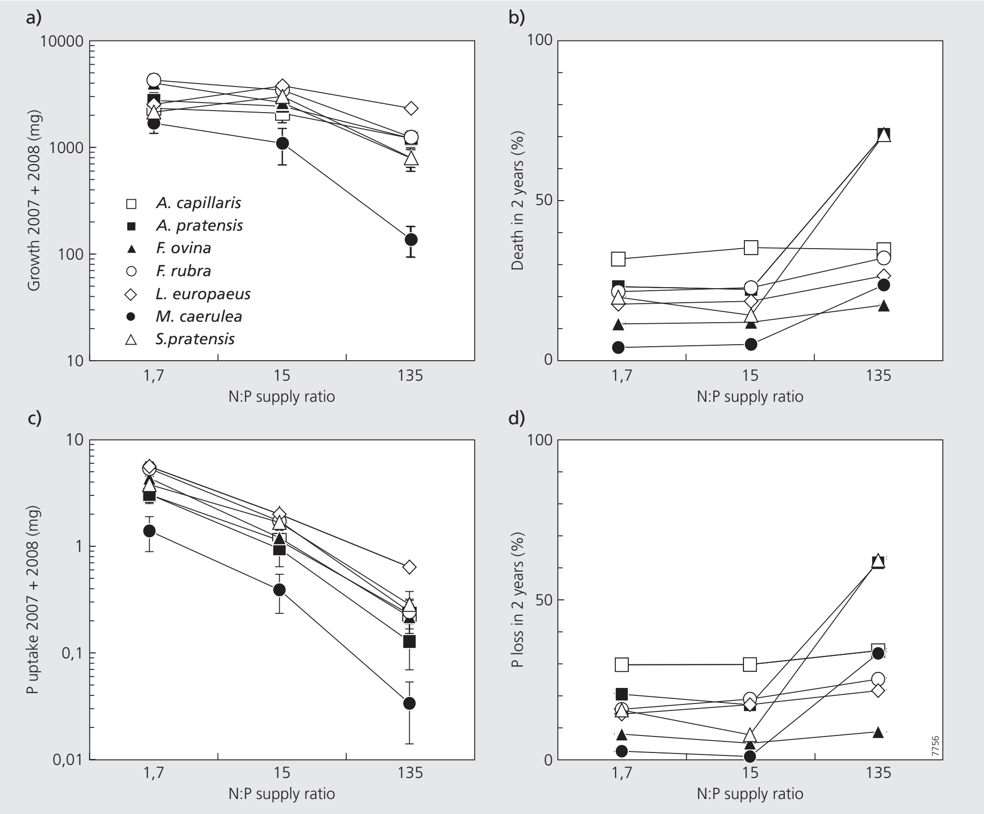

Figure 3.1 Species difference in a) growth, b) death rate, c) P uptake, and d) P loss over two years (15 weeks of fertilization in 2007, 14 weeks of fertilization in 2008, plus the winter in between) in response to different N:P supply ratios. See Materials and Methods for the definition of each of the variables. Means of high and low nutrient supply levels combined are shown. For growth and P uptake, the sums of SE for 2007 and SE for 2008 are shown

was excluded from ANOVA tests for 2008, as its regeneration rate in 2008 was very low (6%). The factors that showed a significant effect in ANOVA were compared by a multiple comparison test, the Games-Howell test (Sokal & Rohlf 1995). We also tested the difference in growth and death rate between N:P supply ratios within each nutrient supply level (Games-Howell test) as well as between nutrient supply levels within each N:P supply ratio (Mann-Whitney *U* test). Additionally, differences in death rates between 2007 and 2008 were analyzed by the *U* test.

Relationships between death rate and biomass concentrations (N, P, and N:P) were tested by Spearman's correlation analysis. To check whether the treatment and species effects had influenced the relationships, we repeated the correlation analyses with corrected variables (i.e., after dividing their values by the average values of the treatment for each species).

⇨ **Table** 3.2 Effects of species (Sp), nutrient supply levels (L), and N:P supply ratios (NP) and their interactions on eight plant responses in 2007 and 2008. F values and P values (***, $p<0.001$; **, $p<0.01$; *, $p<0.05$) determined by 3-way ANOVA, as well as treatment means (over all species) ± SE for nutrient supply levels and N:P supply ratios, are shown

		Growth (mg)	Death (%)	Death above ground (%)	Death below ground (%)	P uptake (mg)	P loss (%)	N uptake (mg)	N loss (%)
2007									
ANOVA results									
	df								
Species	7	88.0***	21.0***	28.6***	28.8***	79.8***	18.1***	97.1***	26.5***
Level	1	131.4***	49.1***	88.5***	0.2	608.5***	68.9***	440.0***	77.6***
N:P ratio	2	12.2***	88.9***	81.2***	0.6	730.2***	46.0***	25.3***	75.3***
Sp x L	7	4.0***	6.4***	6.4***	1.4	3.6**	6.3***	3.3**	5.5***
Sp x NP	14	3.4***	4.5***	5.9***	1.1	7.0***	4.7***	10.7***	4.6***
L x NP	2	4.2*	1.1	2.5	1.4	34.5***	3.4*	6.6**	1.5
SpxLxNP	14	0.6	2.3**	2.4**	1.3	1.8*	2.6***	1.1	3.2***
Error	192								
Treatment means ± SE									
Low & N:P=	1.7	676±53	6.6±0.9	15.1±2.0	0.8±0.2	0.96±0.07	3.3±0.6	4.3±0.2	5.6±0.9
	15	779±77	8.6±1.0	17.5±2.1	1.7±0.5	0.27±0.04	3.8±0.6	7.4±0.7	4.1±0.5
	135	685±73	15.0±0.9	29.5±2.0	1.4±0.3	0.07±0.02	7.1±0.9	7.9±0.9	8.9±0.8
High & N:P=	1.7	1190±81	4.6±1.0	8.0±1.5	0.9±0.2	2.86±0.23	2.3±0.5	12.4±0.7	2.7±0.5
	15	1427±126	5.8±1.1	9.5±1.7	0.9±0.2	1.07±0.09	2.3±0.7	22.4±1.7	3.2±0.7
	135	976±93	11.6±1.1	20.9±2.2	0.9±0.2	0.26±0.03	3.8±0.5	15.9±1.4	7.1±1.0
2008									
ANOVA results									
	df								
Species	6	21.2***	45.9***	31.2***	58.4***	16.5***	62.1***	27.1***	82.4***
Level	1	107.2***	2.2	0.0	0.1	83.2***	0.0	71.4***	0.1
N:P ratio	2	133.4***	51.8***	19.8***	49.2***	180.4***	67.2***	82.6***	61.5***
Sp x L	6	4.0**	3.3**	1.5	5.0***	4.0**	2.8*	2.3*	3.1**
Sp x NP	12	5.7***	3.3***	0.8	4.7***	5.6***	8.4***	7.2***	6.1***
L x NP	2	36.4***	6.7**	4.1*	4.9**	19.4***	12.4***	21.3***	7.5**
SpxLxNP	12	2.4**	6.4***	1.5	6.5***	2.6**	6.0***	3.1**	6.7***
Error[1]	173								
Treatment means ± SE									
Low & N:P=	1.7	1437±151	17.5±2.6	24.6±2.9	15.1±2.9	1.16±0.11	14.8±2.6	6.6±0.5	21.1±2.7
	15	474±76	27.6±3.8	34.4±3.5	24.8±4.2	0.15±0.02	28.1±3.8	4.7±0.6	28.0±3.7
	135	189±41	49.7±6.2	49.9±5.6	49.5±6.5	0.05±0.01	51.5±5.9	2.5±0.7	49.6±6.2
High & N:P=	1.7	2483±262	29.5±5.3	34.4±5.3	26.1±5.4	2.52±0.26	28.4±5.6	12.3±1.0	30.6±5.4
	15	2798±203	23.3±4.2	25.7±5.0	21.5±4.3	1.09±0.08	21.4±4.7	22.8±1.5	23.3±4.6
	135	378±787	52.1±5.6	49.2±5.8	52.2±6.0	0.12±0.02	53.7±6.1	5.8±1.3	52.9±5.6

Results

Effects of N and P supply on plant growth

The average plant growth across all species was significantly influenced by the nutrient supply level and N:P supply ratio in both years (Table 3.2). In 2007, growth was higher at the high supply level for all N:P ratios ($p<0.05$). Per supply level, the intermediate N:P supply ratio resulted in the highest growth. In 2008, average growth was higher at the high supply level ($p<0.001$, except for N:P supply ratio 135). At the high supply level, the low and intermediate N:P supply ratio resulted in high growth; at the low supply level, in contrast, the low N:P supply ratio yielded the highest growth. The ANOVA confirms that N:P ratio affected plant growth differently per supply level in 2008 ($p<0.001$). Still, for both supply levels, growth was significantly lower at the N:P supply ratio 135 than at the other ratios ($p<0.01$).

Looking at the responses per plant species, the results revealed a large interspecific variation in growth in both years (Table 3.2). Note that, for species-specific effects, we give the results for the whole 2007-2008 period (Figure 3.1). We do so because the year effects were similar for most species, with some minor deviation for a particular year (see Appendix I Figure 3.A1, 3.A2). Although there was a significant interaction effect between species and N:P supply ratio in both years (Table 3.2), all species had the lowest total growth at N:P supply ratio 135 over two years (Figure 3.1a).

Effects of N and P supply on plant death rate

In 2007 the average death rate of the whole biomass (below- and above-ground biomass together) across all plant species increased with an increasing N:P supply ratio and a decreasing nutrient supply level (Table 3.2). Death of below-ground biomass was small, amounting to less than a few percent (Table 3.2). In 2008 the average death rate of the whole biomass was much higher than in 2007 for all six treatments ($p<0.001$). In 2008, the death rate rose with an increasing N:P supply ratio independent of the nutrient supply level (Table 3.2), and the death rate of the below-ground biomass was much higher than in 2007 (Table 3.2). The death rate of the whole biomass in 2008 was particularly high at an N:P supply ratio of 135, where the death rate did not differ significantly between low and high nutrient levels ($p=0.35$). Although interaction effects between nutrient supply level and N:P supply ratio were significant for the year 2008, the trend remained that for both supply levels the death rate rose as the N:P ratio increased. (There was one exception: at the high nutrient level, the death rate was slightly higher for the intermediate than for the high N:P ratio.)

Correlation analysis showed that the death rate of the whole biomass was negatively related to both concentrations of N and P in plant biomass, but more strongly to the P ($r=-0.42$, $p<0.001$) than to the N ($r=-0.17$, $p<0.001$) concentration. These negative correlations were also found when the treatment and species effects were corrected ($r=-0.49$ and $p<0.001$ for P concentration; $r=-0.57$ and $p<0.001$ for N concentration). In addition, the plant death rate was positively correlated with the biomass N:P ratio ($r=0.30$, $p<0.001$).

There was a high interspecific variation in the death rates, especially in 2008 (Table 3.2; see also Appendix I Figure 3.A1b, 3.A2b). In both years, species responded differently to N:P supply ratios (Table 3.2). The total death rate over two years at an N:P ratio of 135 was the lowest for *F. ovina*, followed by *M. caerulea* and *L. europaeus* (Figure 3.2b).

Effects of N and P supply on nutrient uptake

We observed somewhat different patterns in the P and N uptake of plants in response to N:P supply ratio than those in growth, despite that the uptakes were autocorrelated with growth. The P uptake decreased with a rising N:P supply ratio in both years, while the N uptake differed from one year to the next. The N uptake was lowest at the low N:P ratio in 2007 but lowest at the high N:P ratio in 2008 (Table 3.2). The proportion of N and P taken up from the supplied N and P in two years decreased with an increasing N:P supply ratio. The N uptake was 92 ± 9 % for N:P supply ratio 1.7, 41 ± 5% for N:P 15, and 8 ± 2% for N:P 135. The P uptake was 31 ± 4% for N:P 1.7, 25 ± 4 for N:P 15, and 15 ± 4% for N:P 135. This means that the plants that were grown at the high N:P supply ratio not only received less P but also took up lesser percentages of the P supplied. With an N:P supply ratio of 135, the P uptake was especially small in 2008 (18.5% of total P uptake). At this N:P supply ratio, the initial content of P from the pre-fertilization period consisted of a large proportion (38.8%) of the total P content.

Total P uptake over two years largely differed among species at the N:P supply ratio of 135 (Figure 3.1c). *L. europaeus* took up 82.9% of the supplied P whereas *M. caerulea* took up only 4.1%.

Effects of N and P supply on nutrient retention

Similar to the plant death rates (and partly due to the autocorrelation), the losses of N and P increased with an increasing N:P supply ratio in both years, while they were not significantly influenced by the nutrient supply level in 2008 (Table 3.2). The P loss over two years became larger as the N:P ratio increased (Figure 3.2a, see dotted lines): the loss amounted to 18.1% for N:P

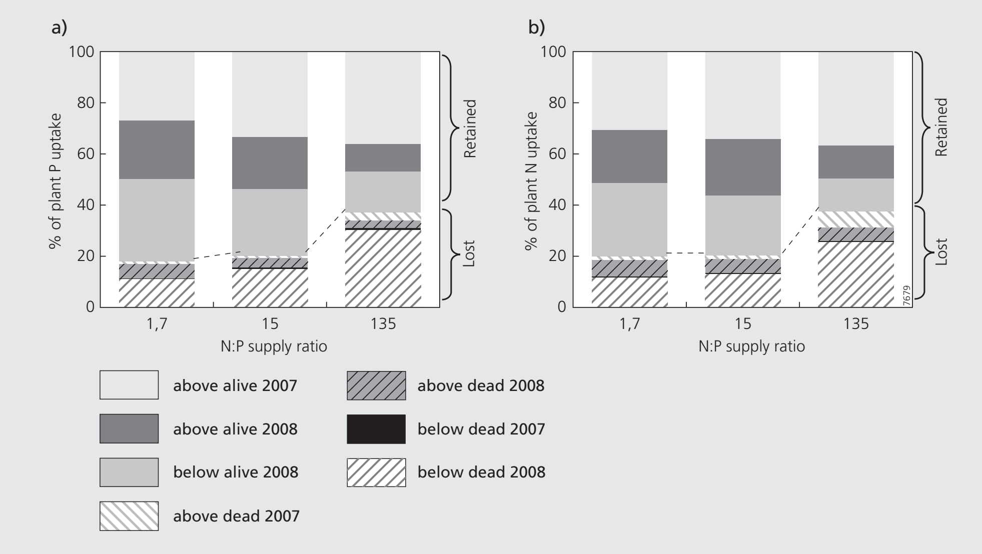

Figure 3.2 Loss or retention of P (a) and N (b) of plants grown with different N:P supply ratios, shown as percentages of total uptake of P or N in two years. Means of high and low supply level across seven species (except *L. flos-cuculi*) are shown. Each compartment represents a part of the biomass (above-ground alive, below-ground alive, above-ground dead, below-ground dead) of each year. Dotted lines show the distinction between nutrient lost from plants and nutrient retained in plants

supply ratio 1.7, 20.2% for N:P 15, and 37.1% for N:P 135. Of the total P loss, the loss from below-ground dead biomass in 2008 was larger than that from above-ground biomass. Moreover, that loss from below ground increased along with the N:P ratio: the loss was 61.0% for N:P supply ratio 1.7, 74.0% for N:P 15, and 81.2% for N:P 135. The same patterns were observed for N loss. That is, we found an increasing N loss and increasing proportion of N loss from below-ground dead biomass in 2008 as the N:P supply ratio increased (Figure 3.2b).

The total P loss over two years largely differed among species, especially at N:P supply ratio 135. *F. ovina* had the lowest P loss rate whereas *A. pratensis* and *S. pratensis* had a higher P loss rate than the others (Table 3.2, Figure 3.1d). For all species, P was lost mainly from below-ground dead biomass in 2008. The contribution of P loss from below-ground biomass to the total P loss in 2008 was especially large for *S. pratensis* (93%), *M. caerulea* (92%), and *A. pratensis* (87%).

The N resorption rate from senescing leaves in 2007 was influenced by the N:P supply ratio ($F_{2,14} = 14.7$, $p < 0.01$). The N resorption rate was higher for an N:P supply ratio of 15 than of 135 ($p < 0.05$). The P resorption rate was not influenced by the N:P supply ratio ($F_{2,14} = 1.1$, $p = 0.35$). Species variation in the resorption rates was statistically significant for both N resorption ($F_{7,14} = 57.3$, $p < 0.001$) and P resorption ($F_{7,14} = 11.4$, $p < 0.001$). *M. caerulea* and *F. ovina* had the highest and the second highest resorption rates for both N and P.

Discussion

General trends in N and P supply effects on plant growth and nutrient economy

The present study shows that the availability of N and P influences plant growth and nutrient economy in terms of supply levels as well as of the N:P supply ratio. Moreover, the effects of N:P supply ratio are shown to depend on time. Our results regarding plant biomass productivity are comparable to those of previous similar experiments (Güsewell 2005a, Güsewell 2005c). Notably, in the first year, both high and low N:P supply ratios resulted in a relatively low biomass compared to the intermediate N:P supply ratio, whereas in the second year it was especially the high N:P supply ratio that resulted in a low biomass. As the present experiment traced the major nutrient flows to and from plants over a period of two years, we were able to relate the negative effects of high N:P supply ratio, especially in the long term, to high nutrient losses via the death of roots.

The high death rate in the second year was influenced by a high N:P supply ratio, but not by the nutrient supply level alone. Our experimental design included, besides the different N:P supply ratios and nutrient supply levels, some identical supplies of N and of P. By comparing these identical supplies we could see that the increased death rate at the high N:P ratio was due to a *relative* scarcity of P to N, rather than to N toxicity or P limitation per se. First, the two treatments with the same high N supply but at a contrasting P supply (i.e., High & N:P=15 and Low & N:P=135) showed that a high N supply led to high death rates only when the P supply was relatively low. This implies that N toxicity is not the main cause of high N:P ratio effects. Moreover, the two treatments with the same low P supply but a contrasting N supply (i.e., High & N:P=135 and Low & N:P=15) showed that a low P supply led to a high death rate only when the N supply was relatively high. This implies that P limitation per se is not the main cause either. Together, these results suggest an independent effect of N:P supply ratios on plant death rate apart from the effects of N or P supply levels.

The effects of N:P supply ratio on plant death rate might be explained by differences in the way plants are able to adapt the N and P concentration in biomass to their environment (Aerts & Chapin 2000, Güsewell & Koerselman 2002). We found that the plants adjusted their P concentrations more flexibly than their N concentration. That is, the P concentration in living biomass decreased considerably as the N:P supply ratio increased (82% decrease from N:P supply ratio 1.7 to 135), whereas the N concentration decreased only slightly as the N:P supply ratio decreased (26% decrease from N:P supply ratio 135 to 1.7) in 2007 (see Appendix II Table 3.A1 for the average nutrient concentrations of each treatment for each part of the biomass). Considering that the reduction of P concentration below a minimum requirement will cause senescence of tissues (Batten & Wardlaw 1987), higher flexibility of P than N concentration in biomass could explain the increased death rate at high N:P supply ratios, but not at low N:P supply ratios. The importance of P concentration to the death rate is also underpinned by our observation that plant death rates were more strongly correlated to P than to N concentration in biomass.

In addition, poor P uptake (in terms of both the absolute amount and the percentage relative to supplied P) at a high N:P supply ratio could be another reason why plants kept such low P concentrations. Our additional laboratory experiment showed that part of the supplied P could be adsorbed to the substrate (ca. 30 % of the orthophosphate was adsorbed to the quartz sand within a week when the P concentration in the solution was comparable to that in our Low, N:P=15 treatment). We are also aware of a possible underestimation of nutrient uptake because of our discrete (once a year) rather than continuous measurements of the nutrient content in biomass. With this method, we were unable to trace nutrient losses via root exudates, which are often stimulated by P deficiency (Vance *et al.* 2003). These artefacts of our experiment, however, are still not enough to explain the extremely low percentage of P taken up by plants (15%) at a high N:P ratio. Although a high rate of P uptake by P-deficient plants is commonly observed in short-term experiments (e.g. Shaver & Melillo 1984, Güsewell 2005c), another two-year growth experiment also revealed poor nutrient recovery (i.e., percentage of nutrient accumulated in plants relative to its supply) under conditions of P- rather than N-limitation (Güsewell 2005a). Furthermore, Craine and Jackson (2009) reported a trend whereby plants grown on low-P soil do not increase biomass upon P fertilization alone, whereas those on low-N soil do increase biomass upon N fertilization, indicating a stronger inhibition of nutrient acquisition under P rather than N stress.

In reality, however, drastic nutrient losses and poor P uptake at high N:P supply ratios, as those observed in our experiment, are less likely to happen. Under natural conditions, unlike in our experiment, there exist various forms of P bound to soil that plants can effectively take up by several mechanisms such as excretion of root exudates (Vance *et al.* 2003, Raghothama & Karthikeyan 2005, Lambers *et al.* 2006). A low mycorrhizal infection rate on the plant roots in our experiment (on average 14% in 2007) compared to their field status (Weishampel & Bedford 2006) may have also led to the observed low P uptake by plants. Still, rapid increase of atmospheric nitrogen deposition (Galloway 1995, Holland *et al.* 1999) could invoke a strong (relative) P limitation in plants and influence the nutrient budget of the ecosystems by enhancing nutrient loss from the plant rhizosphere.

Interspecific difference in response to high N:P supply ratios

By including eight different grassland species in the experiment, we were able to identify possible mechanisms that cause species-specific responses to varying N:P supply ratios. Species variation in impaired P uptake and increased P loss in the second year, which were the main negative effects of the high N:P supply ratio (see previous section), seemed to determine the differences in the growth of the species. The top three fastest growing species in the second year at the high N:P supply ratio (*L. europaeus*, *F. ovina*, and *F. rubra* in rank order) suffered less from P losses than the other species, and the fastest growing species (*L. europaeus*) had the highest P uptake compared to all other species.

Interspecific differences in P loss could be caused by several factors. First, species that had poor P uptake inevitably had a lowered P concentration, which resulted in high death rates. Also the sensitivity of death rate to low P concentration seemed species specific: *M. caerulea* and *F. ovina* had relatively low death rates even at low P concentrations, possibly due to interspecific differences in optimum nutrient concentrations in biomass (Agren 2008). Secondly, variation in leaf resorption rates could have influenced the species differences in P losses. The higher resorption rate by *M. caerulea* and *F. ovina* counteracted the negative effects of increased death rates. Such interspecific differences in resorption efficiency are also reported by other studies (Aerts & Chapin 2000, Güsewell 2005b). Thirdly, variation in root mass ratio (RMR) (results not shown) could also have contributed to the species difference in P loss. Since loss from below-ground dead biomass was the major path of P loss, higher allocation to roots may ultimately have resulted in a higher loss rate. The much lower nutrient resorption rate from senescing roots compared to that from leaves (Gordon & Jackson 2000; this experiment) may have accelerated this effect. Indeed, the contribution of below-ground P loss to total P loss was very high for species that had high RMR (*M. caerulea, S. pratensis,* and *A. pratensis*).

Contrary to our hypothesis (4), those species that commonly occur in P-limited grasslands, i.e., *M. caerulea* and *S. pratensis*, did not perform better at a high N:P supply ratio than the other species. They actually had the worst performance in terms of P uptake (*M. caerulea*) or P loss (*S. pratensis*). A study on phosphatase activity with the same set of species (Chapter 2) shows that *M. caerulea* and *S. pratensis* have efficient phosphatase activity (i.e., high phosphatase activity per unit of biomass). Because phosphatase excretion requires large quantities of N (Treseder & Vitousek 2001), the intensive investment of these two species in phosphatase seemed to turn out to be ‘wasted’ in our experimental setting, as we did not supply organic P, the P form that phosphatase mobilizes. In contrast, *L. europaeus,* which had the highest P uptake in our experiment, has poor phosphatase activity (Chapter 2). *L. europaeus* has less dependency on phosphatase but may have other traits to promote P acquisition, e.g., nutrient carrier enzyme in roots, which might have worked advantageously in our experiment.

Concluding, we have quantitatively shown for the first time that severe P limitation (i.e., low availability of P relative to N) causes high root death and thus results in poor nutrient retention in plants. These results indicate that unbalanced anthropogenic impacts on N and P availability to grassland ecosystems will influence their nutrient budget in the long term, for instance by stimulating nutrient loss from the rhizosphere under a high level of N eutrophication relative to P eutrophication. This might consequently alter the plant community structure by favoring plants with an adequate growth strategy that circumvents unbalanced N:P availability, e.g., an efficient P uptake or an enhanced P retention under conditions of P limitation. To further understand the significance and consequence of the increased root death under P stress, future research needs to examine the underlying physiological mechanisms that trigger the high death rate. Future studies should also test the effects of P stress in experimental conditions that mimic the field nutrient status in soil more realistically (i.e., by including various forms of phosphorus).

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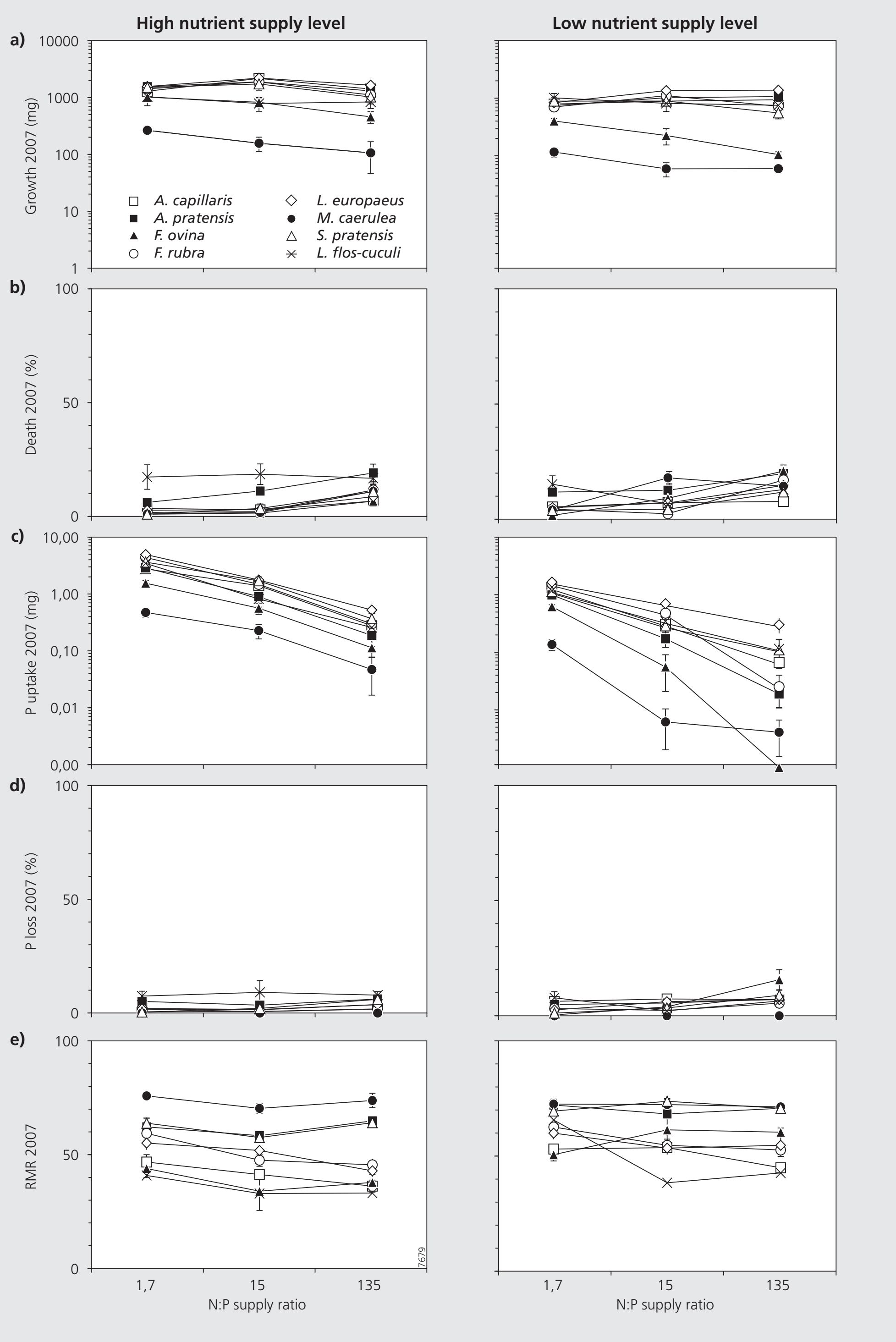
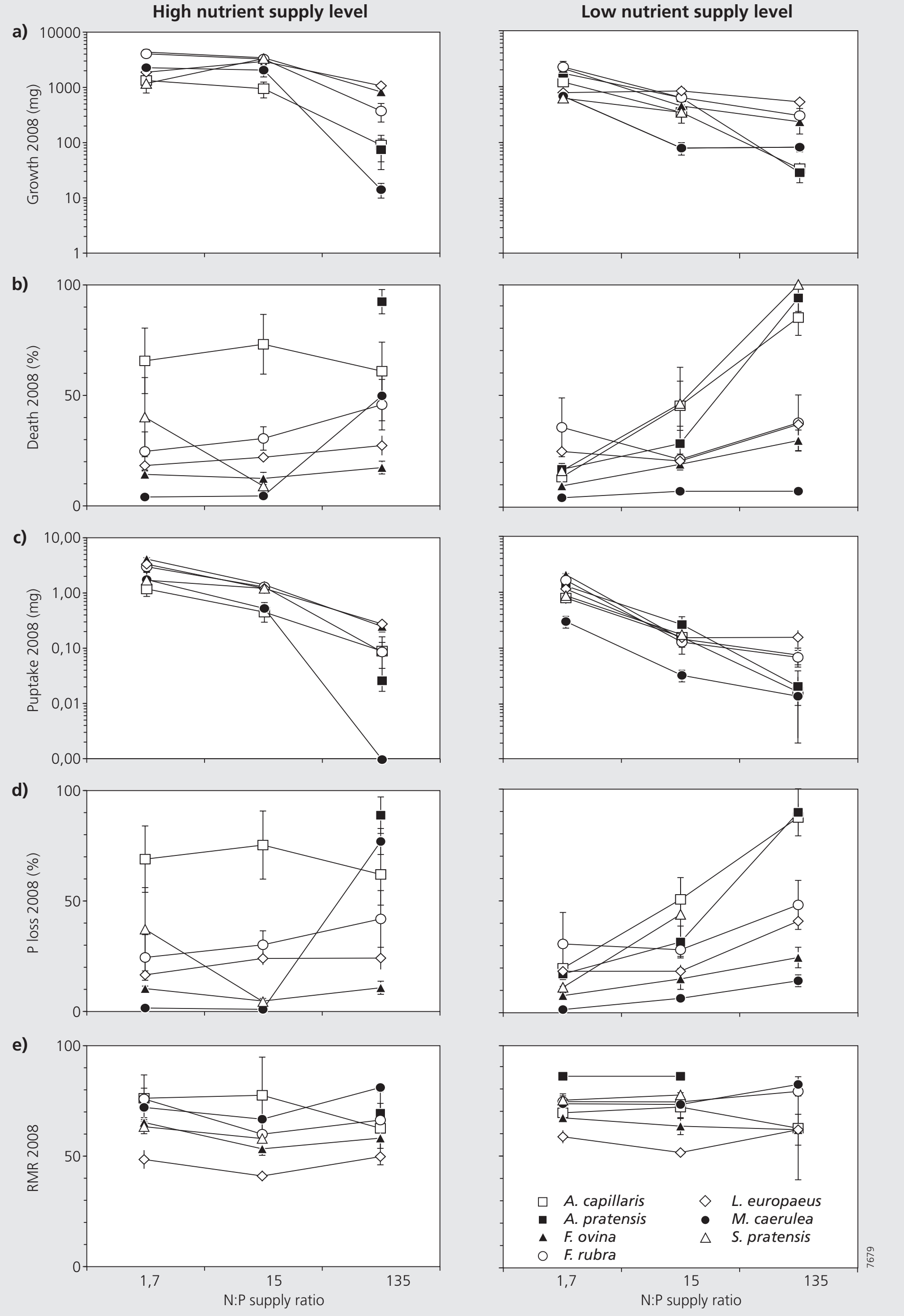
Appendix I. Responses of each species to N:P supply ratios

⇨ **Figure** 3.A1 Species difference in growth (a), death rate (b), P uptake (c), P loss (d), and root mass ratio (e) in 2007 in response to different N:P ratios for high (left) and low (right) nutrient supply levels. Means and SE are shown


High nutrient supply level
Low nutrient supply level
a)
10000
1000
100
10
1
Growth 2007 (mg)
□ A. capillaris
■ A. pratensis
▲ F. ovina
○ F. rubra
◇ L. europaeus
● M. caerulea
△ S. pratensis
✳ L. flos-cuculi
b)
100
50
0
Death 2007 (%)
c)
10,00
1,00
0,10
0,01
0,00
P uptake 2007 (mg)
d)
100
50
0
P loss 2007 (%)
e)
100
50
0
RMR 2007
7679
1,7
15
135
N:P supply ratio
1,7
15
135
N:P supply ratio

⇨ **Figure 3.A2** Species difference in growth (a), death rate (b), P uptake (c), P loss (d), and root mass ratio (e) in 2008 in response to different N:P ratios for high (left) and low (right) nutrient supply levels. Treatments that had fewer than three regenerated pots were omitted. Means and SE are shown


High nutrient supply level
Low nutrient supply level
a)
b)
c)
d)
e)
Growth 2008 (mg)
10000
1000
100
10
1
Death 2008 (%)
100
50
0
Puptake 2008 (mg)
10,00
1,00
0,10
0,01
0,00
P loss 2008 (%)
RMR 2008
N:P supply ratio
1,7
15
135
□ A. capillaris
■ A. pratensis
▲ F. ovina
○ F. rubra
◇ L. europaeus
● M. caerulea
△ S. pratensis
7679

Appendix II. Nutrient concentrations in plant biomass

Table 3.A1 Concentrations of N and P in plant biomass for each treatment. Treatment means and SE (mg/g) are shown for alive and dead parts for below-ground and above-ground biomass. Note that not all individuals had a complete set of these four parts each year. Number of samples measured is shown in brackets.

	P above-ground alive	P below-ground alive	P above-ground dead	P below-ground dead	N above-ground alive	N below-ground alive	N above-ground dead	N below-ground dead
2007								
Low & N:P= 1.7	2.19±0.12	2.19±0.12	0.70±0.11	0.68±0.09	14.0±0.7	6.7±0.5	5.4±0.6	10.0±1.2
	(40)	(40)	(40)	(23)	(40)	(40)	(40)	(23)
15	0.53±0.03	0.53±0.03	0.21±0.03	0.53±0.07	14.8±0.9	9.2±0.4	5.6±0.4	8.9±1.0
	(40)	(40)	(40)	(25)	(40)	(40)	(40)	(25)
135	0.28±0.02	0.28±0.02	0.10±0.01	0.44±0.06	15.0±0.8	11.2±0.4	7.4±0.6	8.5±1.0
	(40)	(40)	(40)	(26)	(40)	(40)	(40)	(26)
High & N:P=1.7	2.98±0.20	2.98±0.20	1.17±0.14	0.72±0.12	16.9±0.7	8.6±0.6	5.8±0.6	9.5±1.1
	(40)	(40)	(40)	(28)	(40)	(40)	(40)	(28)
15	1.15±0.07	1.15±0.07	0.22±0.03	0.81±0.07	22.1±0.9	13.2±0.7	6.5±0.7	12.6±0.4
	(40)	(40)	(39)	(25)	(40)	(40)	(39)	(25)
135	0.46±0.02	0.46±0.02	0.12±0.01	0.42±0.07	19.6±0.9	15.1±0.6	10.2±0.8	9.0±1.2
	(40)	(40)	(40)	(24)	(40)	(40)	(40)	(24)
2008								
Low & N:P= 1.7	1.36±0.09	1.36±0.09	0.58±0.06	0.94±0.04	7.5±0.4	4.4±0.2	5.6±0.5	9.5±0.8
	(38)	(38)	(39)	(41)	(38)	(38)	(39)	(41)
15	0.48±0.03	0.48±0.03	0.30±0.04	0.57±0.03	12.8±0.6	8.5±0.3	11.8±0.8	12.0±0.7
	(38)	(38)	(38)	(42)	(38)	(38)	(38)	(42)
135	0.39±0.06	0.39±0.06	0.30±0.03	0.42±0.02	16.2±0.8	9.5±0.6	15.4±1.0	13.2±0.6
	(25)	(27)	(34)	(42)	(25)	(27)	(34)	(42)
High & N:P=1.7	1.57±0.08	1.57±0.08	1.12±0.12	1.28±0.12	7.5±0.3	5.0±0.2	6.1±0.6	8.4±0.5
	(33)	(34)	(37)	(41)	(33)	(34)	(37)	(41)
15	0.50±0.02	0.50±0.02	0.23±0.03	0.63±0.03	10.8±0.3	8.0±0.3	7.3±0.6	11.6±0.5
	(31)	(32)	(32)	(42)	(31)	(32)	(32)	(42)
135	0.39±0.04	0.39±0.04	0.38±0.06	0.54±0.03	20.1±1.1	12.0±0.7	21.2±1.5	11.8±0.5
	(25)	(28)	(34)	(42)	(25)	(28)	(34)	(42)

4 High richness of threatened species in P-limited vegetation is linked to their reproduction strategy

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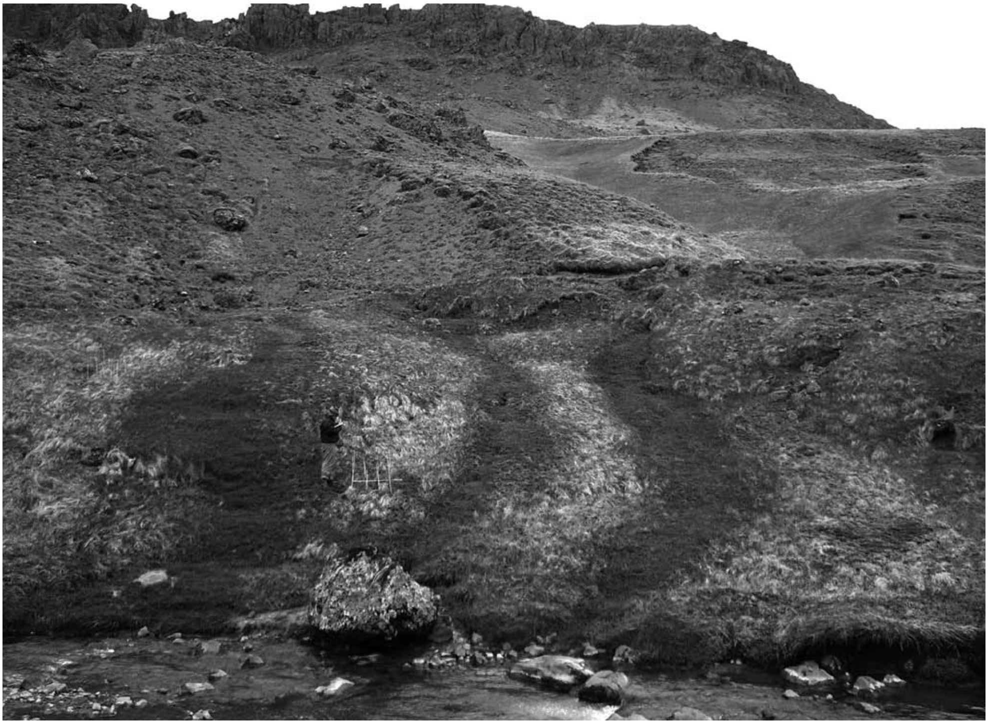


Abstract

Eutrophication is a major threat to the biodiversity of herbaceous ecosystems. The diversity decline may not only be caused by increased nutrient availability, but also by altered stoichiometry, such as the ratio between nitrogen and phosphorus (N:P). We hypothesized that species with particular functional traits would survive better in imbalanced nutrient conditions, eventually altering species composition and decreasing species diversity.

We conducted a meta-analysis of species distributions along a gradient of N:P ratios in Eurasian herbaceous ecosystems. Functional traits of occurring species, e.g. growth and reproduction parameters, were related to their distribution patterns.

Species richness had a weak hump-shaped relationship with N:P ratio. The number and percentage of threatened species were strongly and positively related to N:P ratio. Although association between high N:P ratio and low productivity partly explained high richness of threatened species under high N:P ratios, we found that unique effects of N:P ratio on threatened species were much larger than that of productivity.

Species occurrence along a N:P gradient was associated with reproduction strategy: i.e. species with a less dependency on seed reproduction (e.g. small seed number, short flowering period, perennials) occurred more often in P-limited grasslands, irrespective of the confounding biomass effects. Threatened species tended to have less dependency on seed reproduction, which could account for their high richness in P-limited plots.

Our comparison between species pool size and current distribution of herbaceous vegetation along a N:P gradient showed that the vegetation did not shift to be more P-limited in regions with high N deposition rate; they rather shifted to be more N-limited. This counterintuitive pattern may be due to promoted P foraging by increased N availability, resulting in dominance of fast-growing species which have inherited low N:P ratios. Increased N deposition, therefore, may not benefit threatened species by providing P-limited conditions, but it may rather cause a negative impact on species diversity due to increased productivity and concomitant change in species composition.

Keywords
Functional traits, meta analysis, N:P stoichiometry, herbaceous plant community, species diversity, growth strategies, reproduction strategies, threatened species, Nitrogen deposition

Introduction

The negative impacts of eutrophication on species diversity in herbaceous ecosystems have been intensively studied, but still remain topical (Stevens *et al.* 2004, Clark & Tilman 2008) since the underlying mechanisms have not been completely clarified. A prevailing explanation of the diversity loss due to eutrophication is that eutrophication increases biomass productivity. The concomitant loss in species diversity can be explained by trait-based mechanisms: i.e. species which have advantageous traits under productive conditions exclude others (Grime *et al.* 2007).

A changed balance of resources due to eutrophication might also account for species diversity loss. The resource ratio hypothesis states that an intermediate balance of resources should support the highest species diversity (Braakhekke & Hooftman 1999), because individual species may be limited by different resources, allowing multiple species to coexist. This view was supported by results of a fertilization experiment (Harpole & Tilman 2007), as well as by analyses of field data (Güsewell *et al.* 2005, Cardinale *et al.* 2009). In search for a trait-based mechanism to explain biodiversity loss, one could say that species that survive better under a certain type of resource imbalance would have better adapted traits for such conditions. Although there have been multi-species comparative studies analyzing functional traits in relation to nutrient *availability* (Diekmann & Falkengren-Grerup 2002, Suding *et al.* 2005, Xia & Wan 2008), we are not aware of any such a study in relation to nutrient *balance*.

In terrestrial ecosystems, nitrogen (N) and phosphorus (P) are the primary growth limiting nutrients (Elser *et al.* 2007). The balance between N and P affects plant physiological processes and growth, yet in a species-specific manner (Güsewell 2005a), which causes inter-specific variation in plant N:P ratios (Güsewell 2004). For example, fast growing species generally have a low N:P ratio, because they require a relatively high amount of P to intensively invest in rRNA (Sterner & Elser 2002, Wright *et al.* 2005, but see Matzek & Vitousek 2009). As a consequence of this inter-specific variation, some species may persist better in either N- or P-limited conditions than others. This may eventually result in different species composition of plant communities depending on whether the vegetation is N- or P-limited (Verhoeven & Schmitz 1991, Olde Venterink *et al.* 2003, Güsewell 2004), and more frequent occurrence of endangered species in P- than in N-limited grasslands and wetlands (Olde Venterink *et al.* 2003, Wassen *et al.* 2005). Clarifying whether and how species functional traits influence such shifts in species composition under varying relative availability of N and P will provide us a mechanistic explanation for the observed relationships between species diversity and balance between N and P availability (Roem & Berendse 2000, Aerts *et al.* 2003, Güsewell *et al.* 2005). This will subsequently improve our understanding of the impacts of nutrient enrichment (e.g. atmospheric N deposition, which possibly increases N:P ratio of ecosystems) on community structure and species diversity of herbaceous plant communities.

In order to examine the effects of N:P ratio on plant species diversity and composition, we conducted a meta-analysis of vascular plant distribution from field studies in Eurasian herbaceous plant communities. These sites encompass a wide geographical range and cover a gradient from very low to high atmospheric N deposition. First, we tested if increased N deposition could have shifted N:P ratios of vegetation. Although there is no direct way to measure pre-industrial N:P ratios of vegetation, it could be approximated by species pool size, which is believed to reflect past biogeographical and historical events (Wisheu & Keddy 1996, Schamp *et al.* 2002). We, therefore, compared the species pool size (as a proxy of historical abundance of the habitat) with the current frequency distribution of plots along a N:P gradient. Second, we correlated N:P ratio of plant communities to their species diversity and functional composition. Functional traits of occurring species, such as growth and reproduction parameters, were examined in relation to their distribution patterns along a N:P gradient.

Our hypotheses were; 1) in regions where N deposition rate is currently high, vegetation have shifted to be more P-limited than before; 2) species richness is highest at intermediate N:P ratio; 3) threatened species persist more in communities with higher N:P ratios; 4) threatened species have particular functional traits in common, and those traits are positively associated with occurrence in P-limited sites.

Methods

Relevee data of herbaceous vegetation

We used vegetation data of 647 plots from wet to moist herbaceous ecosystems. The plots were selected from a wide geographical range of Eurasian countries; the Netherlands (287 plots), Belgium (20), Poland (155), Germany (43), Russia (83), Iceland (17), Sweden (16), Scotland (12), Belarus (10), France (2), and Italy (2). The Polish plots are located in two regions: central (Kampinos) and north-east (Rospuda and Biebrza) Poland. The Belgium and Dutch plots are treated as within the same region. Thus, we distinguish 11 geographically distinct regions in the analysis (also see Appendix I Table 4.A1).

Vegetation types include grasslands, fens, bogs, marshes, and dune slacks. 102 plots are exposed to periodical river flooding. For each plot above-ground standing crop of vascular plants was harvested at the height of the growing season, between June and August. The harvested surface area ranged from 0.06 m^2 to 1 m^2. Concentration of N, P and K in the biomass was analyzed after Kjeldahl digestion (Bremner & Mulvaney 1982). Vascular plant species composition were recorded in or around the harvested plots for a size of 0.06, 1, 4, 10, or 25 m^2 (See Appendix I for justification of using different plot sizes). The plant communities are similar among the countries: averagely 86% (and minimum 70%) of the species recorded in a country was also observed in other countries.

Characteristics of species

A number of functional traits were selected to distinguish functional strategies of species. For 537 species recorded, trait values were retrieved from several databases: Biobase (CBS 1997), LEDA traitbase (Kleyer *et al.* 2008), BiolFlor (Klotz *et al.* 2002), and CLOPLA (Klimesova & Klimes 2006). For each species, categorical-scale trait data were obtained for functional group (woody/herbaceous, dicots/monocots), life span (annual/biennial/perennial), N-fixing legumes (nodulated legumes/other herbs) (Sprent 2001), pollen vector (by wind or water/by animal/by selfing/multiple vectors), and type of reproduction (mostly by seed/by seed and vegetatively/mostly vegetatively). Ordinal-scale trait data were obtained for starting month of flowering (month), duration of flowering (month), and lateral spread (<0.01/0.01-0.25/>0.25m per year). Continuous-scale trait data were obtained for canopy height (CH; m), specific leaf area (SLA; mm^2/mg), leaf dry matter content (LDMC; %), leaf mass (LM; mg), seed mass (mg), and number of seeds (number per shoot). Furthermore, C-S-R strategy (Grime *et al.* 2007) was attributed to each species by use of seven traits (CH, LDMC, flowering period, flowering start, lateral spread, LM, and SLA) according to the method of Hodgson *et al.* (1999). For each species, scores of each primary element (C: competitor, S: stress tolerator, R: ruderal) were assigned simply from its proportional contributions (e.g., C scores of C, CS, CSR, and C/CR strategies are 1, 0.5, 0.33, and 0.75, respectively (cf. Willby *et al.* 2001)).

Threatened species were distinguished using the red lists of the Netherlands (van der Meijden *et al.* 2000, Tamis *et al.* 2004), Germany (Schnittler & Ludwig 1996), and Poland (Mirek *et al.* 2006), because plots from these countries consisted of a majority (77%) of our dataset. Species composition of the other countries were similar to theirs: in our dataset, overlaps of species with these three countries were 97% for Belgium and France, 95% for Iceland, 92% for Belarus, 83% for Scotland, 76% for Sweden, 74% for Italy and Russia. Species which are categorized in "critically endangered", "endangered" or "vulnerable" (but not "rare") in at least one of the three countries were classified as threatened. Therefore, these are species that are actually disappearing (due to habitat or environmental change) and not those that have always been rare.

Data analysis

The N:P ratio of the total above-ground herbaceous vegetation is taken as the indicator of the type and strength of nutrient limitation of the plant community as a whole (Güsewell & Koerselman 2002, Olde Venterink *et al.* 2003). Since the consideration of N:P ratio is relevant only when a plot is (co-)limited by N or P, we excluded K-limited plots from the analysis for N:P ratio. According to Wassen *et al.* (2005), we considered a plot as K-limited if N:K ratio is more than 2.1 and K:P ratio is less than 3.4. We had 61 K-limited plots.

Firstly, we compared species pool size and current frequency distribution of plots along a N:P gradient. Species pool size (see below for calculation) was used as a proxy of historical frequency distribution of vegetation, assuming that species pool size is larger for a abundant habitat type than a rare habitat type in a evolutionally time scale (Zobel 1992). To test if current conditions are more P-limited than historical conditions in regions with high atmospheric N deposition, we examined the discrepancy of species pool size and current frequency distribution of vegetation for three regions which have the largest number of plots (i.e. the Netherlands and Belgium, NE Poland, and Siberia). These regions are exposed to distinct levels of atmospheric N deposition, with estimated deposition levels of 30 for the Netherlands, 10 for NE Poland, and 3 for Siberia (kgN ha^{-1} $year^{-1}$, sum of oxidized and reduced nitrogen in 2002) (Tarrasön *et al.* 2004). Species pool size was calculated as follows, a comparable method to Wisheu & Keddy (1996). All non-K-limited plots were grouped into 15 categories of N:P ratio with the same interval (*0.2*) on a log-scale. We allowed overlap of *ln0.1* between categories to avoid improper influence of the group boundaries which we arbitrarily chose. Within each category, subsets of 10 plots were randomly chosen, for which the total number of species (= species pool size) was determined. The same procedure was repeated 10 times, and average and SE of the total species number were calculated.

Secondly, we compared species diversity of vascular plants with biomass and N:P ratio of vegetation. We used three indices to assess species diversity: number of species, number of threatened species, and percentage of threatened species over total number of species. Sequential regression analysis (Graham 2003) was conducted to distinguish unique and shared effects of biomass and N:P ratio on each diversity index. Data were log- or square-root-transformed to obtain normal distribution of the residuals. For the number of species and number of threatened species, both linear and quadratic effects of biomass and N:P ratio were considered. For the percentage of threatened species, a logistic regression analysis was applied in order to restrict the prediction between 0 and 100. K-limited plots were excluded from the analysis.

Thirdly, functional traits of each species were related to the habitat conditions (biomass and N:P ratio of above-ground vegetation) of their occurrence. For all the herbaceous species which occurred at least in three plots, we calculated average N:P ratio and biomass of the plots where they occurred. K-limited plots were excluded from the calculation of average N:P ratio. CH, SLA, seed number, and average biomass were square-root-transformed, and LDW, seed mass, and average N:P ratio was log-transformed, to obtain normal distributions. First, we tested the correlations between habitat conditions (i.e. average N:P ratio and average biomass) and six continuous-scale traits (CH, SLA, LDMC, LM, seed mass, seed number) by Pearson's correlation analysis, as well as correlations between the habitat conditions and six ordinal-scale traits (flowering start, flowering period, lateral spread, and C-, S-, R-score) by Spearman's correlation analysis. Furthermore, we tested the relationships between five categorical traits (functional group, life span, N-fixing legumes, pollen vector, and type of reproduction) and the habitat conditions by ANOVA. Since N:P ratio and biomass of plots were negatively correlated ($r=-0.47$, $p<0.001$ with Pearson's correlation analysis), we conducted additional analyses to remove the confounding effects of biomass on the relations between traits and N:P ratio, and vice versa, in the following ways. For the continuous-scale traits, partial correlation analysis with average N:P ratio or average biomass was conducted to control the effect of the other. For the ordinal- and categorical-data, Spearman's correlation or ANOVA analysis were repeated with corrected average N:P ratio and biomass (corrected for the effects of the other, by using residual values of regression models).

Finally, functional traits were compared between threatened species and the other species. For the quantitative and categorical traits, we analyzed with Mann-Whitney's U-test and chi-square test, respectively.

Results

Species pool size and current frequency distribution of plots along a N:P gradient under different levels of N deposition

Species pool size (as a proxy of historical frequency distribution of plots) was highest at intermediate N:P ratio (12-15) and decreased drastically toward high N:P ratio (Figure 4.1a). The current frequency distribution of plots had almost the same shape as species pool size (Figure 4.1a).

For all of three regions, the peaks of species pool size were at intermediate N:P ratio (Figure 4.1b-d). For Siberia and NE Poland subsets, the shape of the current frequency distribution of plots matched with that of species pool size (Figure 4.1b & 1c). In contrast, for the subset of the Netherland and Belgium, the shape of the current frequency distribution of plots were skewed toward lower N:P ratio (ca. 7-8) compared to that of species pool size (Figure 4.1d).

Patterns of species richness of plant communities with N:P ratio and biomass

Number of species had hump-shaped relationships with both N:P ratio and biomass (Figure 4.2a). Sequential regression analysis showed that the unique effect of N:P ratio explained only a minor variation, whereas biomass counted for a larger variation (Table 4.1).

Both number and percentage of threatened species increased with N:P ratio, whereas they decreased with biomass (Figure 4.2b, 2c). Sequential regression analysis showed that the unique effect of N:P ratio was much larger than that of biomass (Table 4.1).

Correlations between functional traits and species occurrence patterns along a N:P and biomass gradient

Several significant correlations were found between species functional traits and the average N:P ratio of vegetation in the sites where they occurred (Figure 4.3; see Appendix II Table 4.A2a for statistical values for the relations). Average N:P ratio was positively correlated with LDMC (correlation coefficient *+0.23, $p<0.001$*; Figure 4.3c), whereas it was negatively correlated with CH (*-0.21, $p<0.01$*; Figure 4.3a), SLA (*-0.14, $p<0.05$*; Figure 4.3b), seed number (*-0,35, $p<0.001$*; Figure 4.3f), flowering period (*-0.33, $p<0.001$*; Figure 4.3h), and lateral spread (*-0.14, $p<0.05$*; Figure 4.3i). Average N:P ratio was higher for monocots than dicots ($p<0.01$; Figure 4.3m), and higher

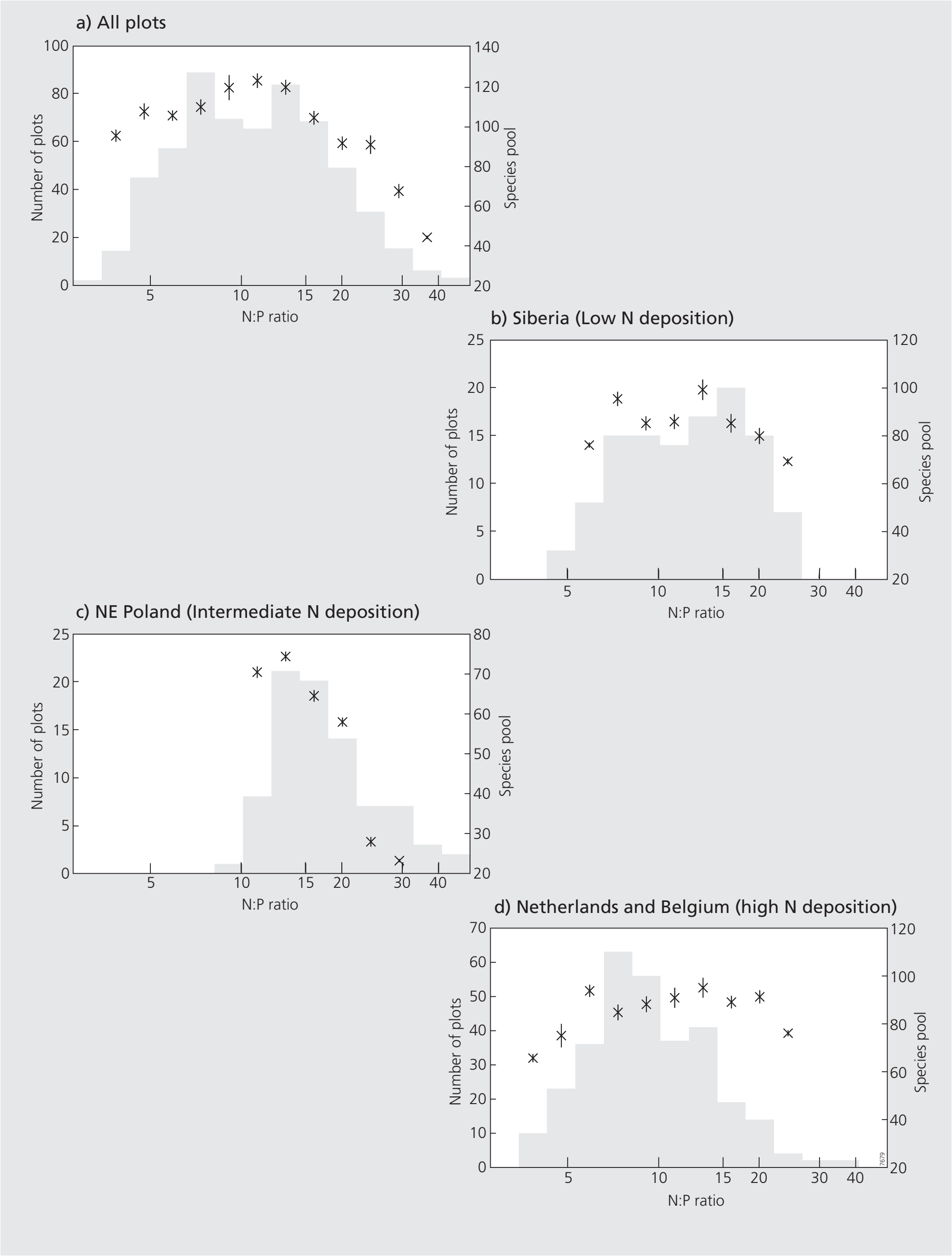


Figure 4.1 Species pool size (crosses) and current frequency distribution of plots (bar graphs) along a gradient of N:P ratios for all plots (a), Siberian plots only (b), NE Poland plots only (c), and Netherlands and Belgium plots only (d). Species pool size, assumed to represent historical frequency distribution of vegetation, was calculated as the total number of species of randomly selected 10 plots within each N:P ratio category. The calculation was repeated 10 times, and its average and SE are shown in the figures. K-limited plots were excluded from the analysis.

Table 4.1 Percentage of variation explained by sequential regression analyses for number of species per plot, number of threatened species per plot, and percentage of threatened species per plot. The explanatory variables are N:P ratio and biomass of above-ground plant material. Unique and shared effects of N:P ratio and biomass are separately shown. K-limited plots were excluded from the analysis.

	Response variables		
	Number of species	**Number of threatened species**	**% of threatened species**
Explanatory variables			
N:P ratio	2.1	20.1	21.1
Biomass	9.9	6.6	9.2
N:P & Biomass	3.4	14.4	25.1
Total explained	15.4	41.1	55.4

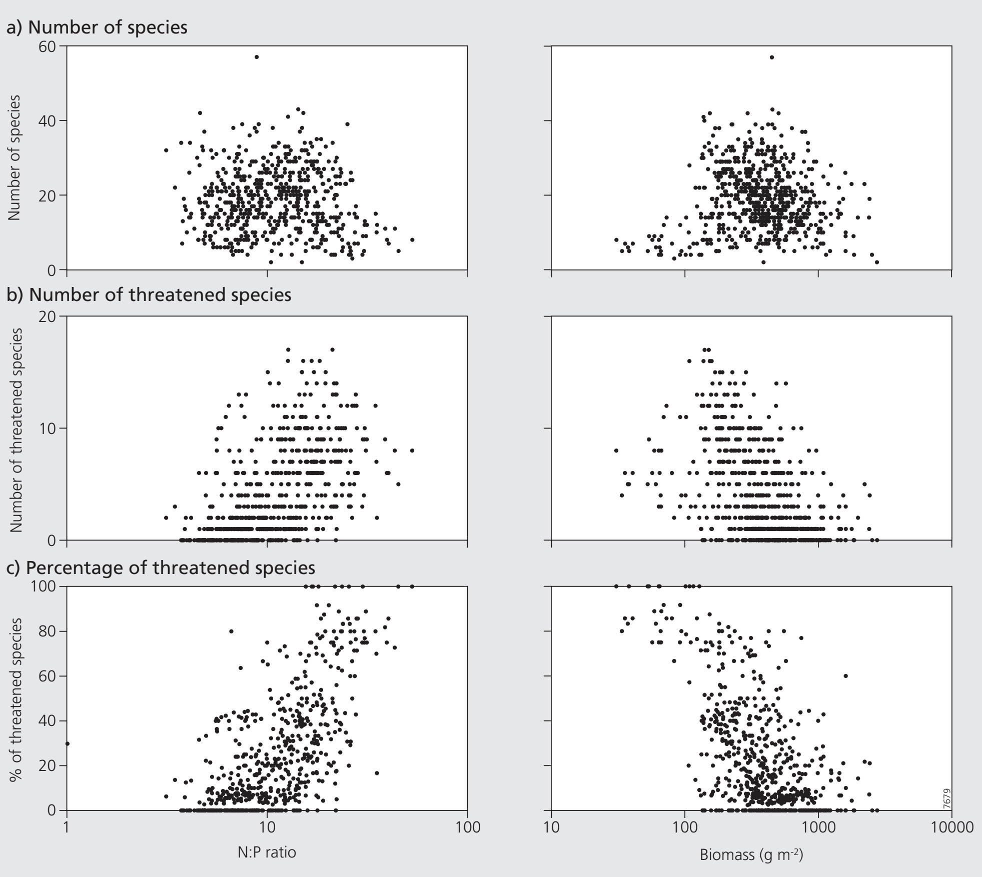


Figure 4.2 Relationship of N:P ratio (left) and biomass (right) of above-ground plant material with a) number of vascular species per plot, b) number of threatened species per plot, and c) percentage of threatened species per plot in herbaceous plant communities. Although several plot sizes were used, the relationships shown here do not seem to change with the plot size (see Appendix I). K-limited sites were excluded from the figures of N:P ratio.

for perennials than annuals ($p<0.001$; Figure 4.3n). Legumes were not related to the N:P ratio (Figure 4.3o). Average N:P ratio differed almost significantly ($p=0.055$) between different types of reproduction (Figure 4.3q); it was higher for species which reproduce mostly vegetatively than for those which reproduce both by seeds and vegetatively ($p<0.05$). Even when the effects of biomass on N:P ratio were corrected, negative correlation with average N:P ratio remained for seed number (-0.30, $p<0.001$), flowering period (-0.33, $p<0.001$), and lateral spread (-0.14, $p<0.05$) (see Appendix II Table 4.A2b for statistical values of all traits).

We also observed several significant correlations between species functional traits and the average biomass of the sites where they occurred (Figure 4.4; see Appendix II Table 4.A2c for statistical values). Average biomass was positively correlated with CH (correlation coefficient $+0.35$, $p<0.001$; Figure 4.4a), SLA ($+0.18$, $p<0.01$; Figure 4.4b), LM ($+0.21$, $p<0.01$; Figure 4.4d), seed number ($+0.19$, $p<0.01$; Figure 4.4f), and flowering period ($+0.17$, $p<0.01$; Figure 4.4h), whereas it was negatively correlated with LDMC (-0.28, $p<0.001$; Figure 4.4c). Reflecting the negative correlation between average biomass and average N:P ratio ($r=-0.54$, $p<0.001$ with Pearson's correlation analysis), signs of the correlations with average N:P ratio were opposite to that with average biomass (compare the relationships in Figure 4.4 to that in Figure 4.3; also see Appendix II Table 4.A2). Average biomass was higher for dicots than monocots ($p<0.01$; Figure 4.4m). Even when we corrected for the effects of N:P ratio on biomass, the positive correlation with average biomass remained for CH ($+0.29$, $p<0.001$), SLA ($+0.12$, $p<0.05$), LM ($+0.19$, $p<0.01$), and flowering start ($+0.14$, $p<0.05$), while negative correlation remained for LDMC (-0.19, $p<0.01$) (see Appendix II Table 4.A2d for statistical values of all traits).

Grime's CSR strategy types were also related to species distribution patterns along gradients of biomass and N:P ratio (Figure 4.3j-l, Figure 4.4 j-l). Even when the effects of the other was corrected, correlation analysis showed that species which have stronger S characteristics occurred more often in sites with low biomass and with high N:P ratio (correlation coefficient -0.21, $p<0.001$ with biomass; $+0.17$, $p<0.01$ with N:P ratio). On the other hand, species which have stronger R characteristics occurred more often in sites with low N:P ratio (-0.24, $p<0.001$), and species which have stronger C characteristics occurred more often in sites with high biomass ($+0.22$, $p<0.001$).

Functional traits of threatened species

Threatened species had lower CH ($p<0.001$), shorter flowering period ($p<0.001$), smaller SLA ($p<0.05$), fewer seeds per shoot ($p<0.001$), and smaller lateral spread ($p<0.001$) than non-threatened species. Threatened species were less likely to be N-fixing legumes ($p<0.05$) and more likely to be perennials ($p<0.01$) than non-threatened species. There were no differences between threatened and non-threatened species in C-, S-, or R-scores, flowering start, LDMC, LM, seed mass, functional group, pollen vector, and type of reproduction ($p>0.05$).

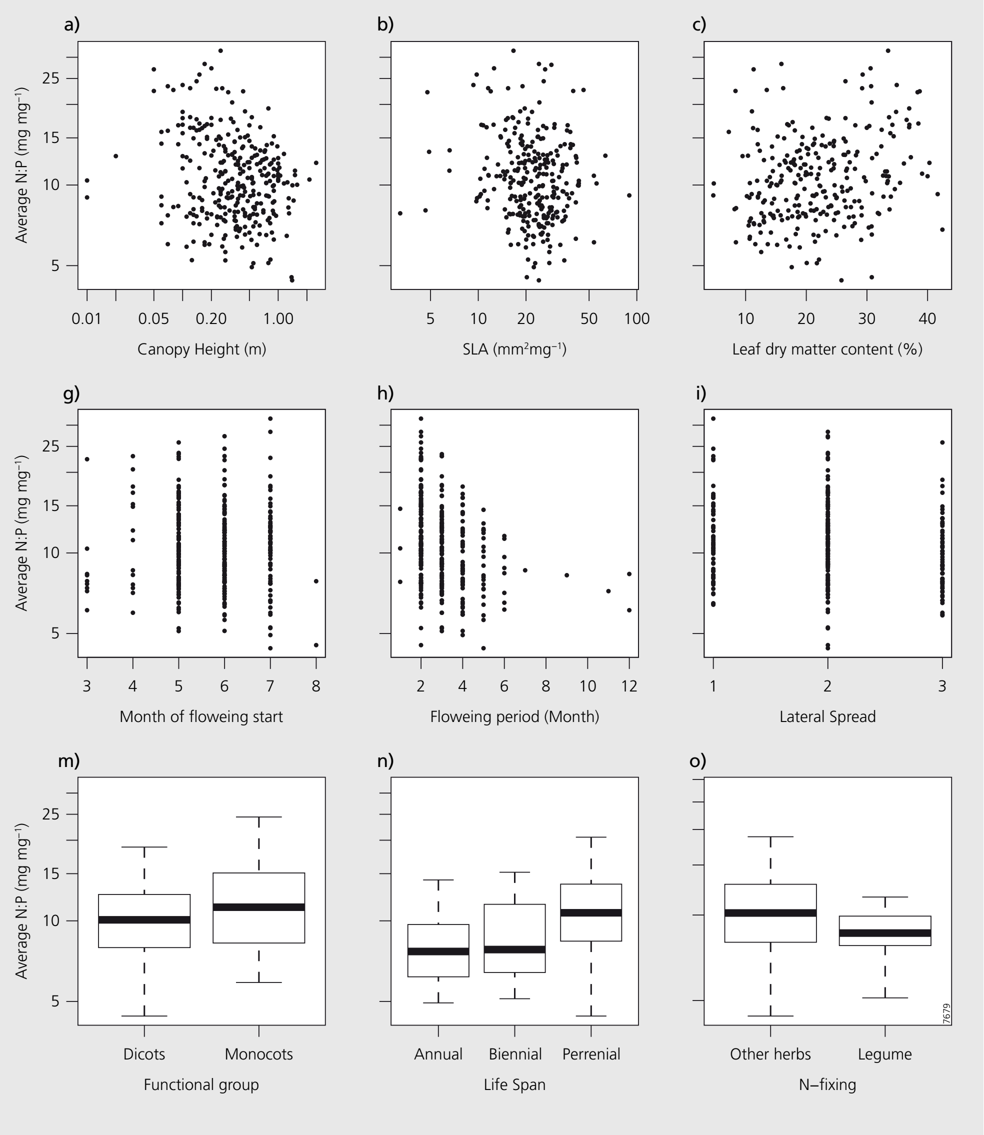
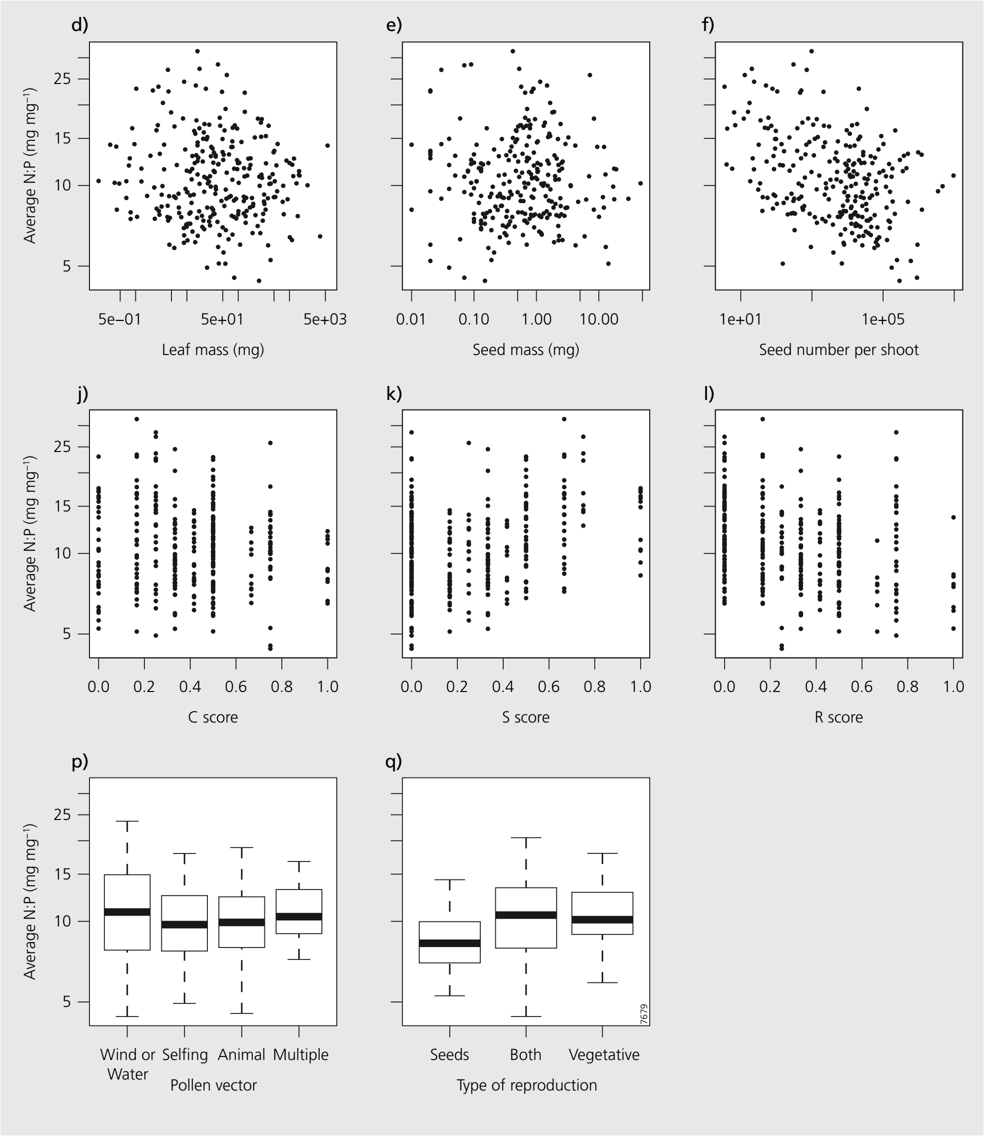


Figure 4.3 Relationships between species functional traits and their occurrence along a gradient of N:P ratio. The occurrence of a species was expressed as the averages of N:P ratio in above-ground vegetation of the plot where they occurred. Only species which was recorded in at least 3 plots were considered. The relationships were shown as scattered graphs for the continuous-scale traits (a: Canopy height, b: Specific leaf area, c: Leaf dry matter content, d: Leaf mass, e: Seed mass, f: Seed number per shoot), for the ordinal-scale traits (g: Flowering start, h: Flowering period, i: Lateral spread), and for the element scores of Grime's CSR strategy (j: C-score, k: S-score, l:R-score). The relationships are shown as box plots for the categorical traits (m: Functional group, n: Life span, o: N-fixing legumes, p: Pollen vector, and q: Type of reproduction). Lateral spread and N-fixing are evaluated only for non-annual species and for herbs, respectively. The box plots show the range from 25th to 75th percentiles and the median value, accompanied with whiskers from minimum to maximum. For the continuous- and ordinal-scale



traits, correlations with average N:P ratio was tested with Pearson's and Spearman's correlation analysis, respectively. For the categorical-scale traits, difference in average N:P ratio among categories were tested with ANOVA. Because N:P ratio and biomass were negatively correlated, we did additional analyses to remove the possible confounding effects of biomass on the relations between traits and N:P ratio, and vice versa (see Methods). See Appendix II Table 4.A2 for the results of these statistical analyses.

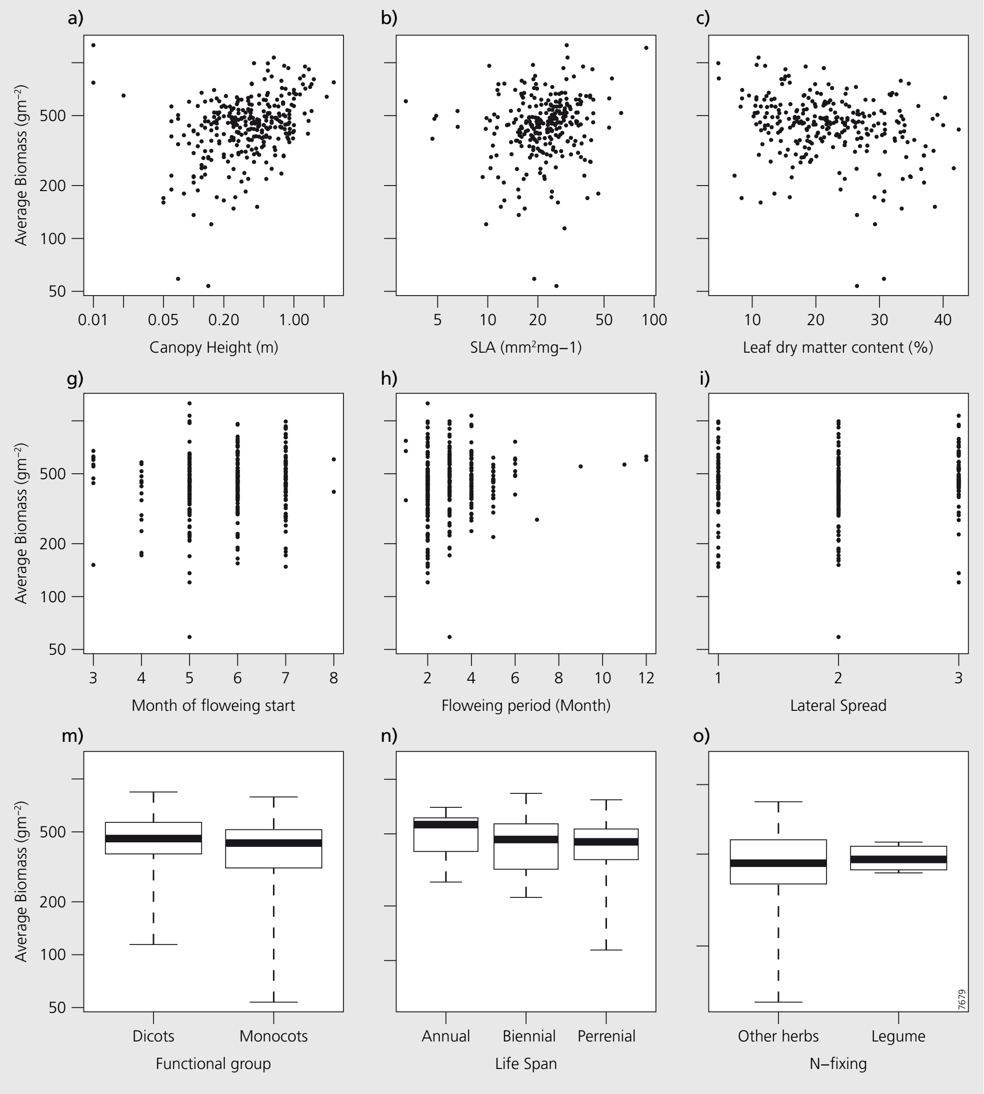
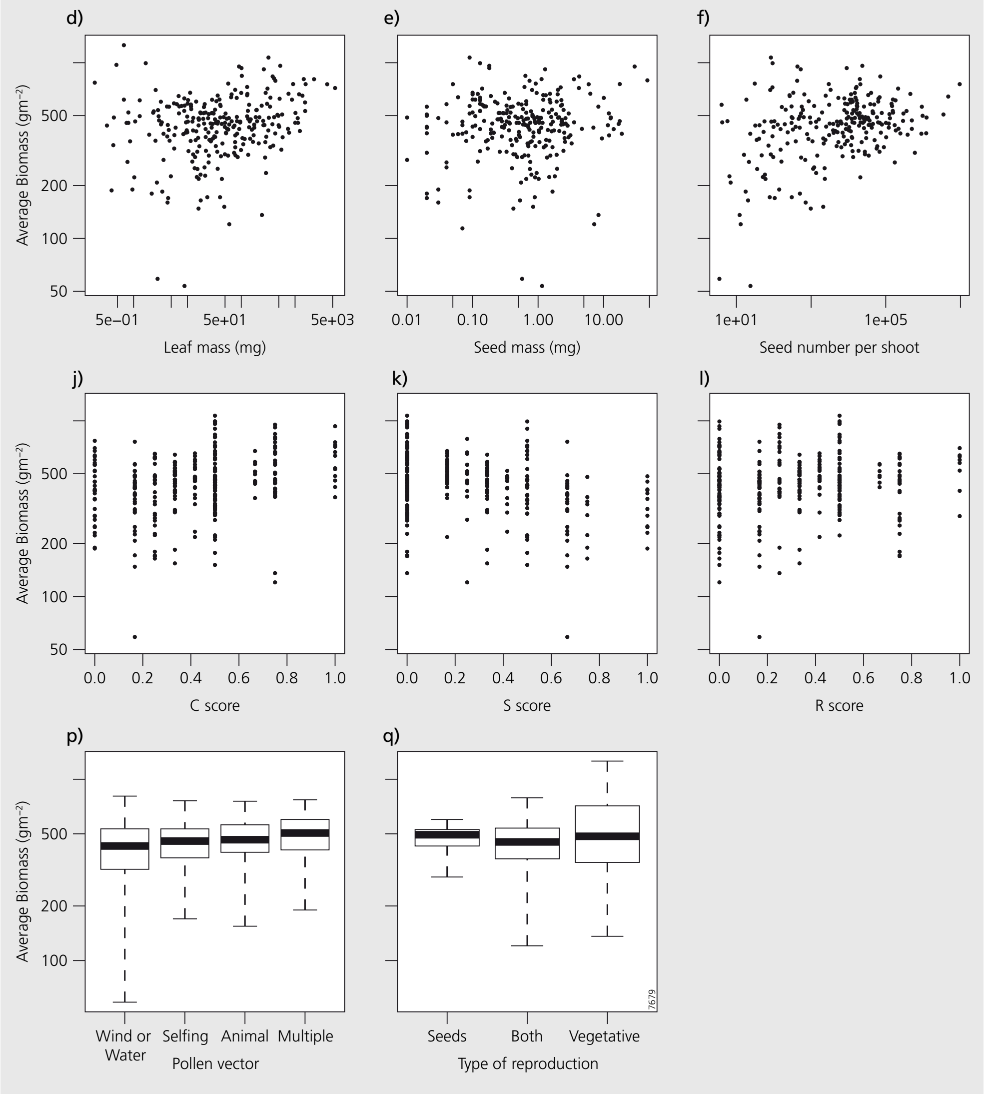


Figure 4.4 Relationships between species functional traits and their occurrence along a gradient of biomass. The methods of graphical presentations and the statistical analyses are identical to those in Figure 4.3.


d)
e)
f)
Average Biomass (gm⁻²)
50
100
200
500
5e−01
5e+01
5e+03
Leaf mass (mg)
0.01
0.10
1.00
10.00
Seed mass (mg)
1e+01
1e+05
Seed number per shoot
j)
k)
l)
0.0
0.2
0.4
0.6
0.8
1.0
C score
S score
R score
p)
q)
Wind or Water
Selfing
Animal
Multiple
Pollen vector
Seeds
Both
Vegetative
Type of reproduction
7679

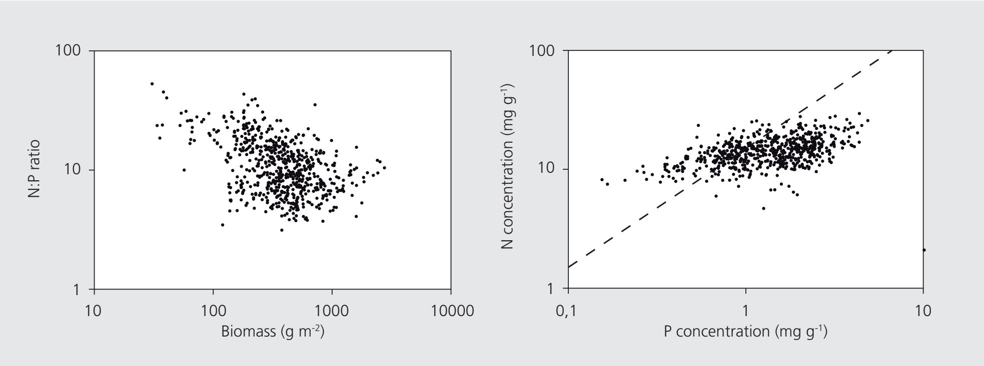


Figure 4.5 Relationship between (a) N:P ratio and biomass, and (b) N and P concentrations, in above-ground plant material for each plot. K-limited plots were excluded. The dotted line shows that the ratio between N and P concentrations is 15.

Discussion

Effects of N:P ratio on species diversity of vascular plants

Species richness of vascular plants was weakly related to N:P ratio, and was highest at intermediate (ca. 15) N:P ratio. This means that balanced availability of N and P is favored by plants to co-exist, supporting the resource ratio hypothesis. The unique effect of N:P ratio, however, explained only a small percentage of variation in species richness. This concurs with the analysis of Güsewell *et al.* (2005) in which N:P ratio accounted for a small, though higher than our study, variation (11-17%) of species richness. Other local conditions such as biomass, pH and disturbance, as well as regional processes like dispersal limitation (Ozinga *et al.* 2005), likely play far more important role in determining species diversity of herbaceous plant communities (Grace 1999).

The number and percentage of threatened species were much more strongly influenced by N:P ratio than overall species number. This confirms the results of previous studies with a smaller number of plots (Olde Venterink *et al.* 2003, Wassen *et al.* 2005) in which more endangered species persisted under P-limited than N-limited conditions. Moreover, our study showed that the unique effect of N:P ratio on richness of threatened species was much stronger than that of biomass. This is consistent with Olde Venterink *et al.* (2001) who observed that the occurrence of threatened species in Dutch meadows and fens (46 sites also included in our dataset) was negatively related to soil extractable P, regardless whether soil-P was related to biomass or not. Furthermore, High pH, a stress factor that may affect persistence of threatened species, had only minor influence on richness of threatened species (results not shown).

Two different mechanisms may explain higher richness of threatened species in P- than N-limited plots. The first mechanism is the possible indirect effect of N:P ratio on species composition via productivity. P-limited conditions are related to the low productivity of the site (this study (Figure 4.5a), Mcjannet *et al.* 1995, Olde Venterink *et al.* 2003), although the association is weak and not always consistent (Willby *et al.* 2001, Güsewell *et al.* 2005). The low productivity would benefit threatened species that are typically inferior in competition for light in highly productive vegetations. The causal relationship between high N:P ratio and low productivity is not evident. It is speculated that P-limited conditions are, at least partly, the consequence rather than the cause of low productivity: low productivity attract slow-growing species which tend to have lower N:P ratio than fast-growing species (Sterner & Elser 2002, Wright *et al.* 2005) because of their different physiological requirement. This underlies the well established scaling pattern of N and P concentration in plants: N and P concentrations are positively related but with a scaling slope smaller than 1 (i.e. species which have high nutrient concentrations tend to have a low N:P ratios) on individual level (Wright *et al.* 2004, Niklas *et al.* 2005, Westoby & Wright 2006) as well as community level (this study (Figure 4.5b), Bedford *et al.* 1999, He *et al.* 2008).

The second mechanism may be direct effects of N:P ratio on species composition due to inter-specific difference in functional traits. Our study supported such direct effects by showing several correlations between species functional traits and their distribution patterns along N- to P-limited grasslands even when confounding effects of biomass on N:P ratio were removed. For example, we found that traits related to reproduction accounted for occurrence in N- or P-limited conditions; species which have a shorter flowering period and a smaller number of seeds, those which rely on vegetative rather than seed reproduction, and perennial rather than annual species, occurred more often in P-limited (high N:P ratio) than N-limited (low N:P ratio) plots. A relatively high requirement of P in reproductive structures compared to vegetative structures (Kerkhoff *et al.* 2006) could contribute to a better persistence of those species under P-limited conditions. On the other hand, species with high canopy height, high SLA, low LDMC and high LM, which are all characteristics of fast-growing species, were positively related to biomass even when the effect of N:P ratio was controlled. This indicates that, although productivity and N:P ratio are partly auto-correlated, N:P stoichiometry and productivity also partly influence species composition independently. In this respect, productivity affects species composition via growth strategies whereas N:P stoichiometry could influence species composition via reproductive strategies.

The trait-based direct effect of N:P ratio on species composition was further supported by our analysis on species functional strategy. CSR strategies and distribution patterns along a N:P gradient were related irrespective of confounding biomass effects; stress tolerators occurred more often in P-limited plots whereas ruderals occurred more often in N-limited plots. This links to the above-mentioned discussion on each functional trait, as ruderals tend to produce seeds more intensively than the others (Grime *et al.* 2007). A similar pattern has been suggested in previous studies: ruderal species have a lower N:P ratio in biomass than other types of wetland species (Mcjannet *et al.* 1995, Güsewell 2004); plant communities characterized by stress tolerators had a higher N:P ratio in biomass than those by competitors or ruderals (Willby *et al.* 2001). The occurrence of another functional type of species, legumes, was surprisingly not related to N:P ratio, although symbiotic N_2 fixation seems to be of advantage under N limitation. However, legumes appear to produce high rates of phosphatase and hence could be adapted to P limitation as well (Houlton *et al.* 2008).

The relationship between N:P gradient and richness of threatened species poses the question whether threatened species have particular functional traits in common which are favored in P-limited conditions. Our study and a number of previous studies shared the idea that difference in characteristics between threatened and common species mainly lies in reproduction strategy: i.e. threatened species have a shorter flowering period (Lahti *et al.* 1991, this study), smaller seed mass (Murray *et al.* 2002), fewer number of seeds (this study), and poorer dispersal ability (Kunin & Gaston 1993, Farnsworth & Ogurcak 2008). Therefore, the typical reproductive strategies of threatened species could, at least partly, explain why they occur more often in P-limited than N-limited vegetation.

Atmospheric N deposition shift ecosystems toward P-limitation and thus change species composition of plant communities?

Whether increased nitrogen deposition alters the type of nutrient limitation and thus species composition of herbaceous plant communities is an important concern for ecosystem conservation. Our analysis on species pool size and current distribution of vegetation along a N:P gradient indicated that regions with a high N deposition rate did not shift to more P-limited, but rather to more N-limited conditions. This counterintuitive pattern may be due to difference in biogeochemical forms between N and P. P exists mainly in compounds which are not-readily available for plants. Plants have a variety of traits to forage P from compounds, such as root exudate, cluster root, or mycorrhizal infection (Lambers *et al.* 2006), which could be flexibly adjusted according to their demand (e.g., stimulated phosphatase activity following P scarcity (Johnson *et al.* 1999)). Increased N deposition, therefore, may promote P availability for plants and thus increase productivity, which concurs with the finding of a direct, long-term field measurement (Malmer & Wallen 2005). A higher P eutrophication level in the Netherlands than in Poland and Siberia (via P-enriched surface water as well as internal eutrophication) could also have stimulated P foraging by plants. Furthermore, the resulting increased productivity would have changed plant communities to be dominated by faster growing species in the long term. This, as discussed earlier, could have caused a lowered N:P ratio in vegetation.

One may think that increased N deposition might be preferable for ecosystem conservation because it shifts N-limited grassland ecosystems to more balanced N:P ratios which support high species diversity (Aerts *et al.* 2003, Güsewell *et al.* 2005), or even to P-limited where more threatened species could persist (Wassen *et al.* 2005). Our findings, however, indicated an opposite possibility: increased atmospheric N deposition could increase bio-availability of P (Johnson *et al.* 1999) as well as alter species composition toward dominance of fast growing species and loss of threatened species. In order to predict the effects of eutrophication on ecosystems, a more mechanistic (and modeling) approach is required which includes physiological responses of plants to varying N:P stoichiometry and its consequence on species competition and community structure.

Acknowledgements

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Appendix I: Justification of using different plot sizes

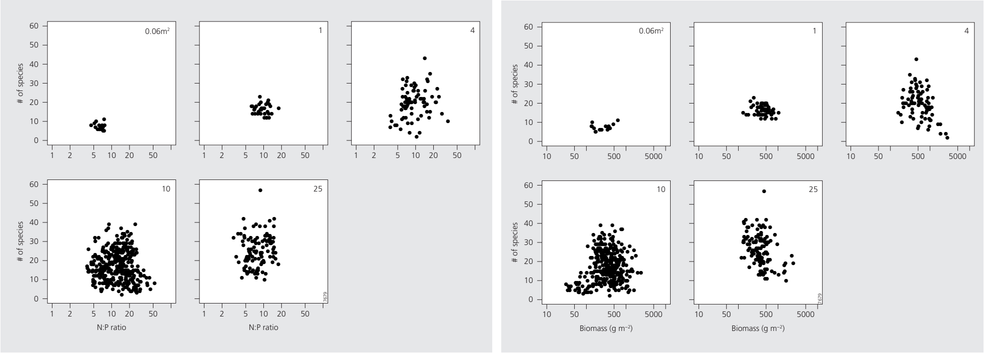
Since data were collected by various researchers, the plot size for species identification varied from 0.06 to 25 m^2 (Table 4.A1). The plot size was chosen by each researcher according to his/her experience with the vegetation of the research area, so that the plot represents well the diversity of the investigated vegetation. For Iceland, an exceptionally small plot size (25 cm x 25 cm) was used. Although the number of species was small in Iceland plots (ranging from 5 to 11, average 7.3), the species pool size of Iceland was very limited too: there were only 20 species in total from 17 plots. Therefore, the effect of the small plot size of Iceland would be minor.

The relationships between species diversity and plot properties (as in Figure 4.2) are shown for each plot size in Figure 4.A1. The shapes of the relationships are similar among different plot sizes, suggesting no major consequence of using various plot sizes on our conclusions.

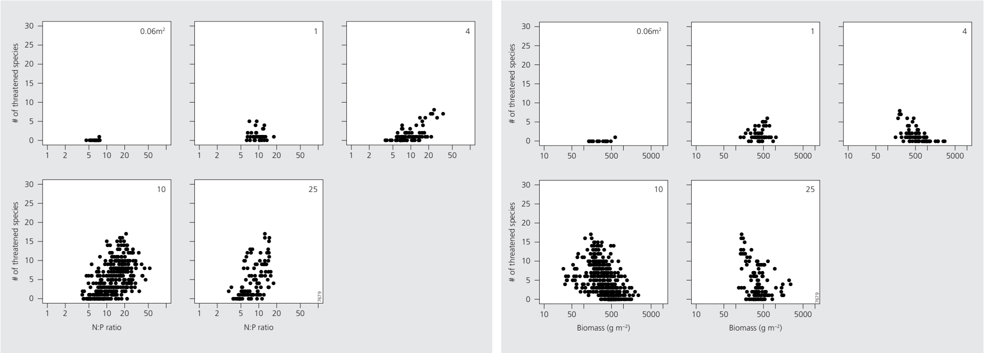
Table 4.A1 Plot size for species identification for each region

Country	Region	Plot size (m^2)	Number of plots
The Netherlands, Belgium	The Netherlands & N. Belgium	1	55
		4	85
		10	167
Germany	Northern upper Rhine	25	43
Poland	NE Poland (Biebrza & Rospuda)	10	76
		25	41
	Central Poland (Kampinos)	25	38
Scotland	Gairloch	10	12
Sweden	Umeå	10	16
Belarus	Ruzhany	10	10
France	Jura	10	2
Italy	Grand Paradiso	10	2
Russia	Novosibirsk	10	83
Iceland	Hengill	0.06	17

a) Number of species



b) Number of threatened species



c) Percentage of threatened species

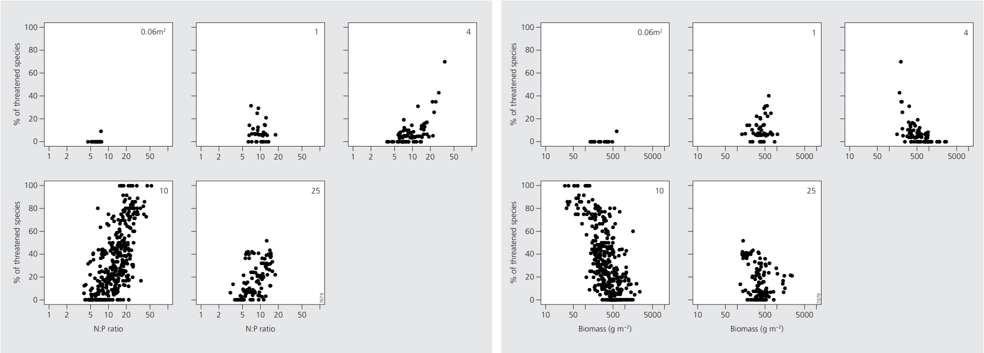


Figure 4.A1 Relationship of N:P ratio (left) and biomass (right) of vegetation with a) number of vascular species, b) number of threatened species, and c) percentage of threatened species in herbaceous plant communities for each plot size of species identification. K-limited sites were excluded from the figures of N:P ratio.

Appendix II. Relationships between species functional traits and their occurrence patterns on gradients of N:P ratio and biomass

See next page

Table 4.A2 Relations between species traits and average N:P ratio (a) or average biomass (c) of above-ground plant materials in plots where they occurred. Only herbaceous species which occurred at least in three plots were used. Because not all species were examined due to incompleteness of trait information, percentages of examined species are shown for each trait. Continuous- and ordinal-scale trait data were analyzed by Pearson's and Spearman's correlation analysis, respectively. Categorical-scale trait data were analyzed by ANOVA. Since there was a negative correlation between N:P ratio and biomass, we did additional analyses to remove the possible confounding effects of biomass on the relations between traits and N:P ratio (b), and vice versa (d), in the following ways. For the continuous-scale traits, partial correlation analysis with average N:P ratio or average biomass was conducted to control the effect of the other. For the ordinal- and categorical-data, Spearman's correlation or ANOVA analysis were repeated with corrected average N:P ratio and biomass (corrected for the effects of the other, by use of residual values of regression models). Correlation coefficients and P values are shown for continuous- and ordinal-scale traits. F values and P values of ANOVA and the results of multiple comparison are shown for categorical-scale traits. P values are: ***, *p<0.001*; **, *p<0.01*; *, *p<0.05*; ^{ns}, not significant.

Species traits	**Average N:P ratio**		**Average biomass**		^{§1} **% of species examined**
	(a)	**(b)**	**(c)**	**(d)**	
		Corrected for biomass		*Corrected for N:P*	
Continuous-scale (Pearson's and partial correlation analysis)					
CH	- 0.21**	ns	+ 0.35***	+ 0.29***	95%
SLA	- 0.14*	ns	+ 0.18**	+ 0.12*	88%
LDMC	+ 0.23***	ns	- 0.28***	- 0.19**	84%
LM	ns	ns	+ 0.21**	+ 0.19**	84%
Seed Mass	ns	ns	ns	ns	82%
Seed Number	- 0.35***	- 0.30***	+ 0.19**	ns	80%
Ordinal-scale (Spearman's correlation analysis)					
Flowering Start	ns	ns	ns	+ 0.14*	96%
Flowering Period	- 0.33***	- 0.33***	+ 0.17**	ns	96%
Lateral spread	- 0.14*	- 0.14*	ns	ns	78%
C-score	ns	ns	+ 0.24***	+ 0.22***	93%
S-score	+ 0.28***	+ 0.17**	- 0.31**	- 0.21***	93%
R-score	- 0.27***	- 0.24***	+ 0.13*	ns	93%
Categoricall-scale (ANOVA)					
Dicot/Monocot	$F_{1,320}$=8.4 ** D < M$^{**\ §2}$	3.5^{ns}	9.7** M < D$^{**\ §2}$	4.4* M < D$^{*\ §2}$	100%
Life span	$F_{2,309}$=12.9 *** AP < PP$^{***\ §3}$	10.3*** AP < PP$^{**\ §3}$	0.9^{ns}	0.4^{ns}	97%
Legumes	$F_{1,210}$=2.7 ^{ns}	2.8^{ns}	0.0^{ns}	2.1^{ns}	100%
Pollen Vector	$F_{3,284}$=1.6 ^{ns}	0.9^{ns}	3.0*	2.4^{ns}	92%
Type of Reproduction	$F_{2.294}$=2.9 ^{ns} S < B$^{*\ §4}$	2.9^{ns} S <V$^{*\ §4}$	2.8^{ns}	1.9^{ns}	89%

^{§1}: out of total 212 species (herbs only) for the analysis of legumes, out of total 322 species for the rest, ^{§2}: D (dicots), M (monocots), ^{§3}: AP (annual plants), PP (perennial plants), ^{§4}: S (mainly by seeds), B (both by seeds and vegetatively), V (mainly vegetatively)

CH: Canopy Height, SLA: Specific Leaf Area, LDMC: Leaf Dry Matter Content, LM: Leaf Mass, C-, S-, R-score: proportional contribution of primary elements of Grime's CSR strategy

5 Effects of increased N supply on competition of plant species with different phosphatase activity levels: a modeling study

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In revision with Theoretical Ecology

Abstract

Eutrophication affects terrestrial ecosystems not only by increasing overall nutrient availability but also by changing the balance between necessary nutrients, such as nitrogen (N) and phosphorus (P). Plastic responses (e.g. flexible nutrient concentrations in biomass) and adaptive traits (e.g. phosphatase activity) of plants to varying N:P availability may influence the way in which N eutrophication affects ecosystems. With a plant competition model coupled in N and P cycles, we examined how increased N input at different P input levels affects species growth, competition, and ecosystem P retention. In the model, plant species flexibly adjust their nutrient concentrations, and have different magnitudes of phosphatase activity. The model results showed that, in N-limited conditions, species with low phosphatase activity always win, and increased N input hardly changes ecosystem P retention. In P-limited conditions, increased N input benefits species with high phosphatase activity, unless under eutrophic conditions (i.e. high N and P input). Here, phosphatase activity increases P retention only slightly because of its high cost in N which hampers biomass production. The model results also showed the possibility that, if there is a trade-off between phosphatase activity and growth rate, increased N input can rapidly increase ecosystem P retention via altering species composition. Our results suggest that N eutrophication benefits species with high phosphatase activity, but only under low P input conditions. Moreover, the changed species composition due to N eutrophication might result in increased P retention. Explicit consideration of N:P stoichiometry is needed when predicting the eutrophication effects on ecosystems.

Keywords
N:P ratio; plant competition model; N supply; root-surface phosphatase activity; soil phosphorus adsorption; P retention

Introduction

Nitrogen (N) and phosphorus (P) are the nutrients that most frequently limit plant productivity in terrestrial ecosystems (Elser *et al.* 2007). Since N and P have different but mutually dependent physiological roles in plants (Kerkhoff *et al.* 2006), plants should maintain a stoichiometric balance of N and P to function properly. Experimental studies showed that plants respond differently to N or P additions (e.g. Shaver & Melillo 1984) as well as to different relative supply of N and P (Güsewell 2005a), supporting the idea that not only the overall nutrient *level* but also the nutrient *balance* between N and P are important to plant growth.

Plants have various strategies to maintain stoichiometric balances under varying (relative) availabilities of N and P (Lambers *et al.* 2008), which include both plastic responses to changing nutrient availabilities (e.g. adjusting nutrient concentrations in plant biomass) and adaptive traits developed on an evolutionary time scale. Several of such adaptive traits of plants are identified with which plants can survive better in N or P limited conditions, such as a high resorption rate of limiting nutrients (Aerts & Chapin 2000, Güsewell 2005b), phosphatase activity in P-limited conditions (Johnson *et al.* 1999), or N fixing symbiosis in N-limited conditions (Sprent 2001). The role of phosphatase activity in P-limited conditions is especially well studied, because of its large contribution to plant P uptake (e.g., P uptake via hydrolyses of organic P by phosphatase enzymes makes up as much as 65 % of the annual P intake of a plant (Kroehler & Linkins 1988)) as well as its high sensitivity to the N and P availability in the soil (Johnson *et al.* 1999). Although the positive effects of these adaptive traits on individual plant growth are well recognized, there is little understanding about their long-term effects on plant species competition and community composition. Fujita *et al.* (Chapter 3) found, in a greenhouse experiment with eight grassland species, that typical species from P-limited grasslands tend to have a higher efficiency in phosphatase activity than non-typical species. This indicates that an adaptive plant trait plays a role in determining the species distribution patterns along a gradient from N to P limited conditions. Moreover, the plant adaptive traits to nutrient imbalances may have consequences for nutrient cycling on ecosystem level. It is expected that adaptive traits of plants which enhance the uptake or conservation of limiting nutrients will improve the retention of these nutrients in the ecosystem, because it will increase residence time of the nutrients in plants and thus reduce loss by leaching. Such increased nutrient retention will feed back to bio-availability of nutrients, which may also affect plant species competition.

Understanding the N:P stoichiometry effects on plant growth, competition and nutrient cycles of ecosystems may provide crucial implications for ecosystem conservation. Given that N and P processes in plants interact with each other, N eutrophication might cause not only an increase in N availability, but also an increase in P availability in the ecosystem via plant-mediated processes (Perring *et al.* 2008). This may lead to an increase in ecosystem productivity, resulting in the loss of particular species. It is, therefore, important to expand our knowledge about whether and how eutrophication of a single nutrient affects retention of other nutrients, as well as how the prevailing adaptive traits of plants to stoichiometric imbalances, such as phosphatase activity, play a role in determining community structure.

Modeling studies may help examining the effects of the plant adaptive traits on species competition as well as nutrient retention on an ecosystem level. Perring *et al* (2008) showed with a multiple nutrient cycling model that an increased N supply may promote P retention in ecosystems by increasing plant growth, although only under particular soil conditions (i.e. when loss of plant available P is greater than that of organic P). This model, however, might have underestimated P retention due to increased N input, because neither the flexibility of the nutrient concentrations in plant biomass nor the adaptive traits of the plants, which both may results in increasing P contents in plants under P limited conditions, were considered. By explicitly including stoichiometric dynamics of N and P in plant growth, as proposed by Rastetter and colleagues (e.g. Rastetter & Shaver 1992, Herbert *et al.* 2004), we will be able to better examine the effects of eutrophication on plant growth and competition, as well as plant-mediated effects on nutrient retention in the ecosystem.

In this study we present a plant competition model incorporated in N and P cycles. Our model includes flexible nutrient concentrations in the plant biomass as well as phosphatase activity. First, we examined how flexibility in plant nutrient concentrations affected plant growth by comparing two models: with flexible and with fixed nutrient concentrations in plant biomass. Second, we studied how the additional inclusion of phosphatase activity affected plant growth in the flexible nutrient concentration model. Subsequently, we tested how N input level influenced competition between plant species of different magnitudes of phosphatase activity, and more specifically if increased N input favored species with high phosphatase activity at different levels of P input. Finally, we tested if increased N input increased P retention in the ecosystem.

Model description

Our model simulates plant growth, with plants that are able to regulate their phosphatase activity along varying N and P input levels depending on N and P concentrations in their biomass. The model of Perring *et al.* (2008) simulates plant growth coupled within N and P cycles. We adapted this model in such a way that flexible concentrations of N and P in plant biomass as well as P uptake from organic P compounds by means of phosphatase activity are included. We also followed Perring *et al.* (2008) for the equations and parameter values of nutrient dynamics in the soil (Appendix I). See Figure 5.1 for the model structure. The model equations of plant growth of two species (i = 1 or 2) are described as follows.

Nutrient concentrations in plant biomass fluctuate depending on nutrient availability in the soil, yet within constraints of homeostasis regulation (Sterner & Elser 2002). It is also known that in plants P concentrations are more variable than N concentrations (de Groot *et al.* 2003, Güsewell 2004). To include nutrient-specific flexibility in nutrient concentrations, we calculated the biomass production rate of species i (dB_i/dt) dually from N and P uptake rate ($dB_{N,i}/dt$ and $dB_{P,i}/dt$) with a larger influence from N uptake (i.e. $w_i < 1$; also see Figure 5.A1a in Appendix I):

$$\frac{dB_i}{dt} = \frac{\dfrac{dB_{N,i}/dt}{q_{N,i}} + w_i \dfrac{dB_{P,i}/dt}{q_{P,i}}}{1+w_i} \tag{eq. 1}$$

where w_i is the plasticity parameter of P concentration of species i, $q_{n,i}$ is the optimum concentration of the nutrient n (n = N or P) in biomass for plant growth of species i.

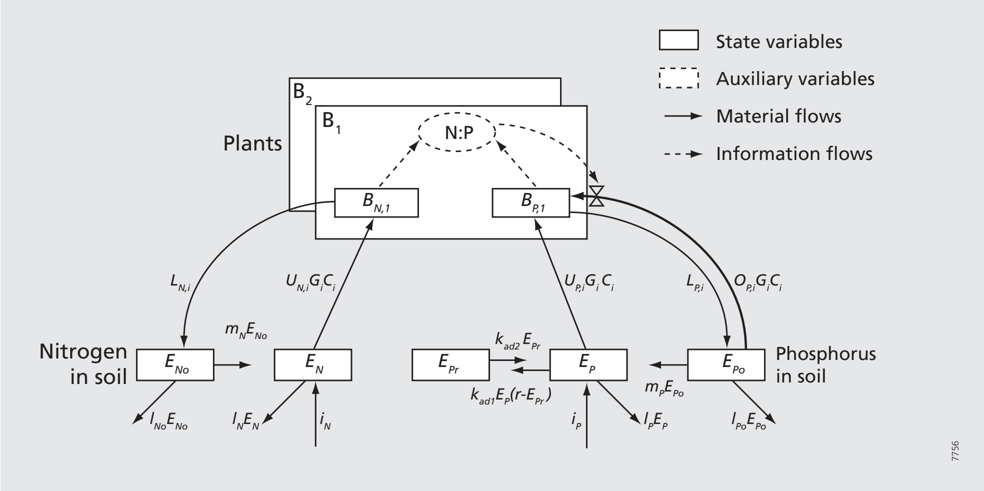

Figure 5.1 Model of two plant species incorporated in nitrogen and phosphorus cycles. The model comprises two nitrogen pools (bio-available dissolved N, E_N, and organic N, E_{NO}) and three phosphorus pools (bio-available dissolved P, E_P, organic P, E_{PO}, and reactive inorganic P, E_{Pr}) in soil. Each species i (i=1 or 2) has N and P pools in biomass (B_{Ni} and B_{Pi}, respectively), which determine the plant biomass (B_i). Plant P uptake from organic P is controlled by phosphatase activity, which depends on the N:P ratio of plant biomass. Filled arrows represent material flows (flows between pools as well as inputs to and leaching from the system), while dotted arrows represent information flows. See Table 1 for description of each parameter.

The plant uptake of nutrient n from dissolved forms in the soil depends on its concentrations:

$$U_{n,i} = a_{n,i} \frac{E_n}{E_n + k_n} B_i \qquad \text{(eq. 2)}$$

where $a_{n,i}$ is the uptake rate of nutrient n per biomass for species i, E_n is the dissolved nutrient n in the soil, k_n is the half saturation parameter of nutrient n.

Furthermore, we assume that plants take up P from the organic P pool in soil via phosphatase activity. The extracellular phosphatase enzyme is assumed to be excreted from plant roots only, although in reality soil microbial communities also contribute to phosphatase activity in the rhizosphere (Vance *et al.* 2003). Phosphatase activity is stimulated as biomass N:P ratio increases (Chapter 2). Furthermore, we assumed that a low concentration of organic P in the soil hampers the reaction of the enzyme with a half saturation concentration of 3.3 gP m^{-2} (calculated with the median Km value of Duff *et al.* (1994), 0.22 mmol l^{-1}, with an assumption of 50cm depth root zone). The P uptake from organic P pool of species i is then modeled as:

$$O_{P,i} = a_{Po,i} \frac{\exp\left(u + v \dfrac{B_{N,i}}{B_{P,i}}\right)}{1 + \exp\left(u + v \dfrac{B_{N,i}}{B_{P,i}}\right)} B_i \frac{E_{Po}}{E_{Po} + k_m} \qquad \text{(eq. 3)}$$

where $a_{Po,i}$ is the maximum phosphatase activity per biomass for species i, u and v are the parameters for phosphatase activity regulation depending on biomass N:P ratio (Figure 5.A1b in Appendix I), E_{Po} is the organic P in soil, k_m is the half saturation parameter of organic P.

To reflect that the growth rate is reduced when nutrient concentrations are below an optimum level for growth (Sterner & Elser 2002), we assumed that the growth rate is maximum when the nutrient concentrations are optimum, and their growth rate reduced to zero when one of the nutrient concentrations is less than half of the optimum concentration. The growth limitation due to low nutrient concentrations for species i is formulated as:

$$G_i = MIN\left(2 - \frac{q_{N,i}}{B_{N,i}/B_i}, 2 - \frac{q_{P,i}}{B_{P,i}/B_i}\right) \qquad \text{(eq. 4)}$$

Here $G_i = 1$ when both N and P concentration equals to their optimum concentrations, while $G_i = 0$ when either N or P concentration reaches at half of their optimum concentration. G_i is reflected in the nutrient uptake rate (see eq. 7).

Species competition for light was also explicitly included, so that the total biomass of two species does not exceed the carrying capacity. We assumed that the species do not differ in competition strength for light. The competition for light of species i is formulated as:

$$C_i = 1 - \frac{B_i + \alpha_{ij} B_j}{c} \qquad \text{(eq. 5)}$$

where α_{ij} is the competition strength of species j on species i, c is the carrying capacity of plant biomass. C_i is reflected in the nutrient uptake rate (see eq. 7).

Plants are assumed to lose nutrients with a constant rate. In addition, we assumed cost of 15 mg N needed for excretion of an amount of phosphatase enzyme which hydrolyzes 1mg P (Treseder & Vitousek 2001), because phosphatase enzyme is rich in N (ca. 16% N). The loss of nutrient n for species i is formulated as:

$$L_{N,i} = d_{N,i} B_{N,i} + s\, a_{Po,i} \frac{\exp\left(u + v \frac{B_N}{B_P}\right)}{1 + \exp\left(u + v \frac{B_N}{B_P}\right)} B_i \qquad \text{(eq. 6)}$$

$$L_{P,i} = d_{P,i} B_{P,i}$$

where $d_{n,i}$ is the loss rate of nutrient n for species i, and s is the cost parameter in N for phosphatase activity. Note that the reduction of P uptake from organic P due to low organic P concentration, $E_{Po,i}/(E_{Po,i}+k_m)$ in eq. 3, is not reflected in the N cost, so that the phosphatase excretion in conditions poor in organic P results in high cost but poor P uptake.

Finally, the rate of change of nutrient content of n for species i is given as:

$$\frac{dB_{n,i}}{dt} = (U_{n,i} + O_{n,i})\, G_i\, C_i - L_{n,i} \qquad \text{(eq. 7)}$$

Analysis

The model equations were analyzed using an ODE solver in Matlab (ode45). First we compared our model with the model in which nutrient concentrations in plant biomass were fixed. In the model with fixed nutrient concentrations, N dynamics in plants were formulated in the same way as in the model with flexible nutrient concentrations (see Model Description), while P dynamics in plants were formulated as depending on the N dynamics:

$$\frac{dB_{P,i}}{dt} = \frac{q_P}{q_N} \frac{dB_{N,i}}{dt} \qquad \text{(eq. 8)}$$

Here the rate of change in P content in biomass is simply proportional to that of N content (thus not explicitly modeled), so that N and P concentrations in biomass are always optimal for growth. For the fixed concentration model, the phosphatase activity is turned off by putting the parameter $a_{Po,i}$ to zero.

Next, we compared the results of the flexible concentration model with and without phosphatase activity, i.e. $a_{Po,i} = 0.0001$ or $a_{Po,i} = 0$, respectively.

Furthermore, with the flexible concentration model, we let two species with different magnitudes of phosphatase activity, $a_{Po,i}$, compete. The two species were simulated with either the same growth rates or different growth rates (i.e. the species with higher phosphatase activity has a lower growth rate). Finally, P retention of the ecosystem was compared for different competition simulations along increasing N input levels. P retention was expressed in two ways: as soil P (i.e. dissolved P plus organic P) and as total P (i.e. soil P plus P in plants).

All the simulations were executed under varying N and P input levels for 2000 time steps.

Results

Effects of introducing flexible nutrient concentrations in plant biomass

We first checked, without inclusion of phosphatase activity, how plants which can adjust nutrient concentrations in their biomass (flexible concentration model) grow along a gradient of increasing N input in the ecosystem. Here we show the result for an intermediate P level only, since the model behaved qualitatively the same for the other P levels (results not shown).

Along a gradient of increasing N input, if the nutrient concentrations are fixed in the plant biomass (fixed concentration model), growth is first N-limited at low N input and then P-limited after a threshold value of N input is reached. As a consequence, plant growth first increases with N-increase and then saturates (dashed line in Figure 5.2a). When flexibility in nutrient concentrations in biomass is introduced (flexible concentration model), plant growth is higher as soon as the system is not N-limited anymore and saturates at a higher value (filled line in Figure 5.2a).

This happens because the flexible plants take up more N when more N is available in the environment, as reflected in the amount of N in their biomass (Figure 5.2c). Note that the concentration of N in plant biomass hardly changes along the N input gradient (Figure 5.2d) reflecting that N intake and growth simultaneously increase along the gradient, cancelling out each other. However, the amount of P input remains constant along the gradient, so plants cannot take up more P (figure 5.2e, amount of P in biomass). Thus, the increase in growth and N uptake along the N input gradient results in a decreasing P concentration in plant biomass (figure 5.2f). These variations of N and P in plant biomass along the N input gradient explain the pattern in

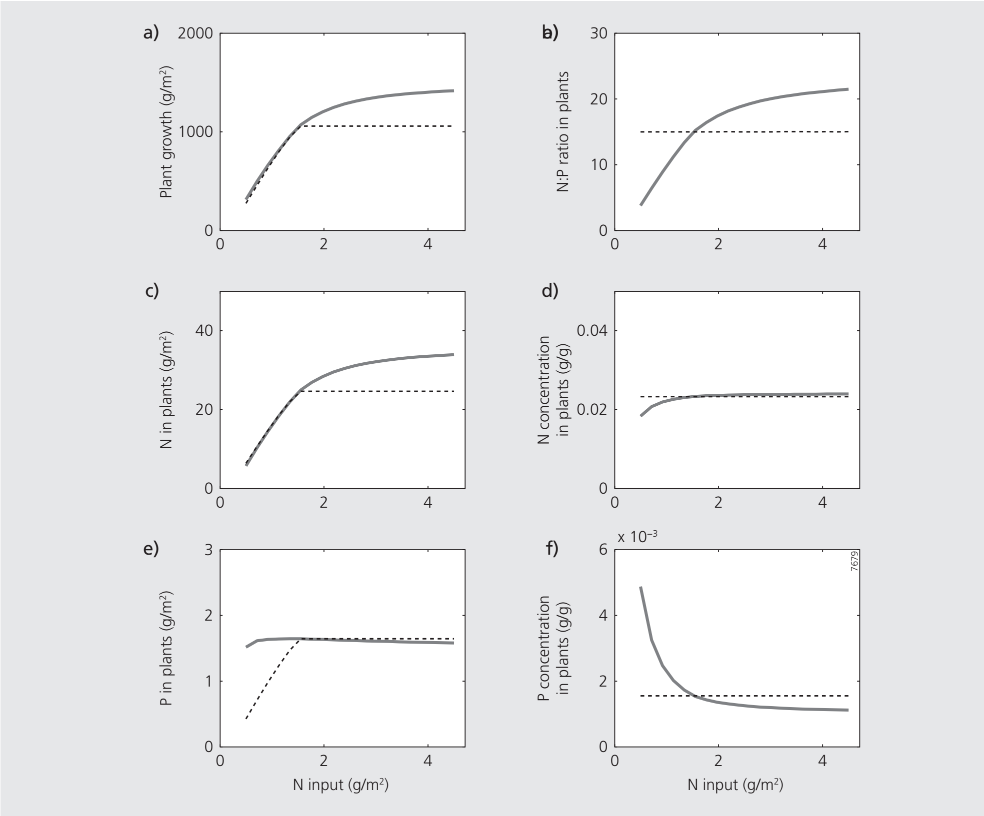

Figure 5.2 Difference between models with fixed (dashed lines) and flexible (filled lines) nutrient concentrations in biomass. Model responses to increasing N input levels at an intermediate P input level (0.15 mg m^{-2} $year^{-1}$) are shown for plant growth (a), N:P ratio in plants (b), N content in plants (c), N concentration in plants (d), P content in plants (e), and P concentrations in plants (f). The simulation results after 2000 time steps are used.

the N:P ratio in the plant biomass (Figure 5.2b), which increases with increasing N input in the flexible plants.

Note also that, with the flexible concentration model, the amount of P in plant biomass slightly decreases when the N input level becomes very high (Figure 5.2e). This is because a too low P concentration in plant biomass causes a growth limitation (eq. 4), resulting in a smaller net P uptake rate per unit biomass.

Effects of introducing phosphatase activity on plant growth

When phosphatase activity is introduced, in addition to flexibility in nutrient concentrations in biomass, growth is slightly lower than without phosphatase activity (Figure 5.3a).

Because of the phosphatase activity, which is controlled by the N:P ratio in biomass (eq. 3), the plants have access to an additional P pool (i.e. organic P) especially when P content in their biomass is relatively low compared to N content. This enables plants to store more P in their biomass when N input increases (Figure 5.3c). However, because the phosphatase activity occurs at the cost of N, the N content in plant biomass decreases (Figure 5.3b). The decrease in N content in biomass results in a decrease in plant growth, since biomass production is more strongly determined by N than by P content in biomass (eq. 1).

Effect of flexible nutrient concentrations and phosphatase activity on plant competition

We now look at two competing species with different magnitudes of phosphatase activity. We first assume that the two species have the same growth rate. The outcome of the competition between the two species depends not only on the N input to the ecosystem, but also on the P input (Figure 5.4a).

The species with low phosphatase activity always wins from the one with high phosphatase activity under N-limited input conditions (i.e. when the ratio of N input to P input is lower than 15, which equals to the optimum N:P ratio in biomass for plant growth ($q_{N,i}/q_{P,i}$); top left region of Figure 5.4a). On the other hand, the species with high phosphatase activity wins under P-limited input conditions (bottom right region of Figure 5.4a) except when both P and N are simultaneously high (i.e. N and P inputs exceed ca. 3.0 and 0.2 g/m^2, respectively; top right region of Figure 5.4a). The mechanism responsible for the uncompetitiveness of the species with higher phosphatase activity under P-limited but eutrophic conditions (i.e. both N and P are higher than a certain level) is the competition for light. In other words, species with high phosphatase activity could win under P-limited, eutrophic conditions if no light competition was included in the model (results not shown). Due to intensive light competition caused by high biomass production under eutrophic conditions (see Figure 5.4b), not all released P by phosphatase is used to produce biomass, despite of the cost in N to excrete phosphatase.

We now consider that there is a trade-off between phosphatase activity and growth rate. So we look at two plant species, one with high phosphatase activity and slow growth and conversely for the other.

In this case, the results are similar to when no trade-off was considered, but the range of survival of the species with high phosphatase activity is restricted to severely P-limited input conditions (i.e. the ratio of N input to P input is higher than ca. 30; Figure 5.4e). Moreover, coexistence of the two species is possible under moderately P-limited input conditions (i.e. the ratio of N input to P input is between ca. 15 and 30; Figure 5.4e). Under eutrophic conditions where biomass production is high (i.e. N and P inputs exceed ca. 2.5 and 0.15 g/m^2, respectively; see also Figure 5.4f), the species with the lower phosphatase activity and the faster growth rate wins (Figure 5.4e).

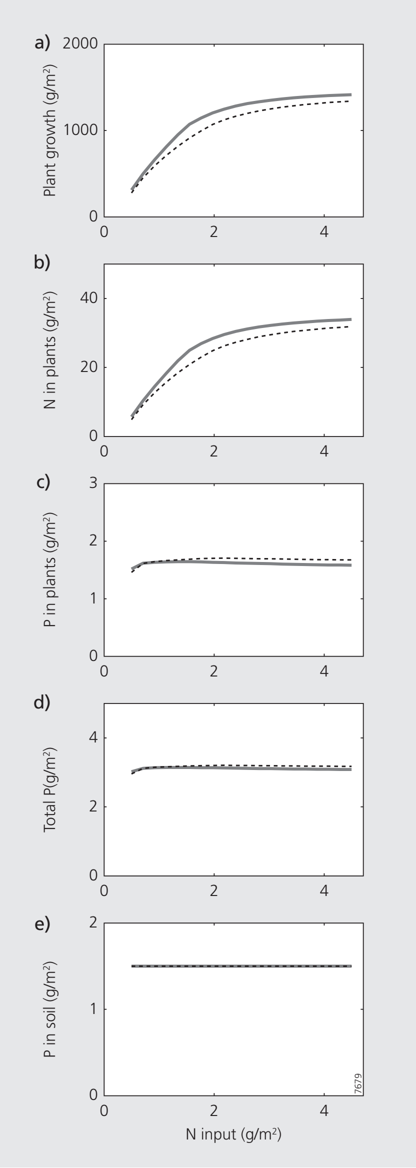

Figure 5.3 Difference between models with phosphatase activity ($a_{Po,i} = 0.0001$: dashed lines) and without phosphatase activity ($a_{Po,i} = 0$: filled lines). Model responses to increasing N input levels at an intermediate P input level (0.15 mg m^{-2} year^{-1}) are shown for plant growth (a), N contents in plants (b), P content in plants (c), ecosystem total P (d: all P pools except reactive P), and soil P (e: dissolved and organic P). The simulation results after 2000 time steps are used.

The initial biomass of the two species does not change the competition outputs (results not shown).

Effects of phosphatase activity on ecosystem P retention

We now investigate the consequences of increasing N input on P retention in the ecosystem where two species with different magnitudes of phosphatase activity compete. P retention was assessed in terms of “soil P” (dissolved and organic P in soil) as well as ecosystem “total P” (soil P plus P in biomass). Reactive P is not considered here because it is not available for plant uptake.

Table 5.1 Description of state variables and parameters in the model. Parameter values for soil nutrient dynamics are from Perring *et al.* (2008). For other parameters, biologically reasonable values are chosen.

	Description	**Dimension**	**Value**
State Variable			
B_i	Biomass of species *i*	biomass-g m^{-2}	
$B_{n,i}$	Content of nutrient *n* of species *i*	*n*-g m^{-2}	
E_n	Concentration of nutrient *n* in dissolved, bio-available inorganic forms in soil	*n*-g m^{-2}	
E_{no}	Concentration of nutrient *n* in organic forms in soil	*n*-g m^{-2}	
E_{Pr}	Concentration of P in reactive forms in soil	*n*-g m^{-2}	
Parameters			
$U_{n,i}$	Uptake rate of nutrient *n* (dissolved form) of species *i*	*n*-g m^{-2} yr^{-1}	
$O_{n,i}$	Uptake rate of nutrient *n* (organic form) of species *i*	*n*-g m^{-2} yr^{-1}	
G_i	growth limitation due to low nutrient concentrations in biomass for species *i*	(fraction)	
C_i	Competition for light for species *i*	(fraction)	
$L_{n,i}$	Loss rate of nutrient *n* of species *i*	*n*-g m^{-2} yr^{-1}	
$q_{n,i}$	Optimum concentration of nutrient *n* in biomass for plant growth of species *i*	*n*-g biomass-g^{-1} m^{-2}	$q_{N,i}$=0.0233, $q_{P,i}$=0.0016
w_i	Plasticity parameter of P concentration of species *i*	(fraction)	0.1
$a_{n,i}$	Uptake rate of nutrient *n* per biomass for species *i*	*n*-g biomass-g $^{-1}$ yr^{-1}	$a_{N,i}$=0.025 or 0.01, $a_{P,i}= a_{N,i}/15$
$a_{Po,i}$	Maximum phosphatase activity (in unit of P released) per biomass for species *i*	P-g biomass-g $^{-1}$ yr^{-1}	0.0001 or 0.00002
u,v	Parameters for phosphatase activity regulation depending on biomass N:P ratio	(fraction)	*u*= -3, *v*=0.2
k_n	Half-saturation parameter of nutrient *n*	*n*-g m^{-2}	$k_{N,}$=1.5, k_P=0.1
k_m	Half-saturation parameter of organic P	*n*-g m^{-2}	3.3
a_{ij}	Competition strength of species *i* on species *j*	(fraction)	1
c	Carrying capacity of biomass	biomass-g m^{-2}	2000
s	Cost parameter in N for phosphatase activity	*N*-g *P*-g^{-1}	15
$d_{n,i}$	Death rate of nutrient *n* of species *i*	yr^{-1}	5 $a_{N,i}$
i_n	Input rate of nutrient *n*	*n*-g m^{-2} yr^{-1}	variable
l_n	Leaching rate of nutrient *n*	yr^{-1}	l_N =1, l_P=0.1
l_{no}	Leaching rate of nutrient *n* in organic form	yr^{-1}	l_{No} =0.1, l_{Po}=0.1
m_n	Mineralization rate of nutrient *n*	yr^{-1}	m_N =0.2, m_P=0.04
r	Density of adsorption sites in soil	*P*-g m^{-2}	2
k_{ad1}	Adsorption rate	m^2 *P*-g^{-1} yr^{-1}	1
k_{ad2}	Desorption rate	yr^{-1}	0.01

We found that soil P remains constant along a gradient in N input for each P input level (Figure 5.4c). Similarly, ecosystem total P hardly changes along the N gradient except when P input is extremely low (Figure 5.4d), because P in plant biomass hardly changes with increasing N input due to the flexible nutrient concentrations in biomass (see Results chapter 1, Figure 5.2e). Although increased N input makes species with high phosphatase activity to replace those with low phosphatase activity (see Results chapter 3), this replacement of dominant species hardly affected the ecosystem total P. This is because high phosphatase activity increases P content in plant biomass only slightly (see Results chapter 2, Figure 5.3c).

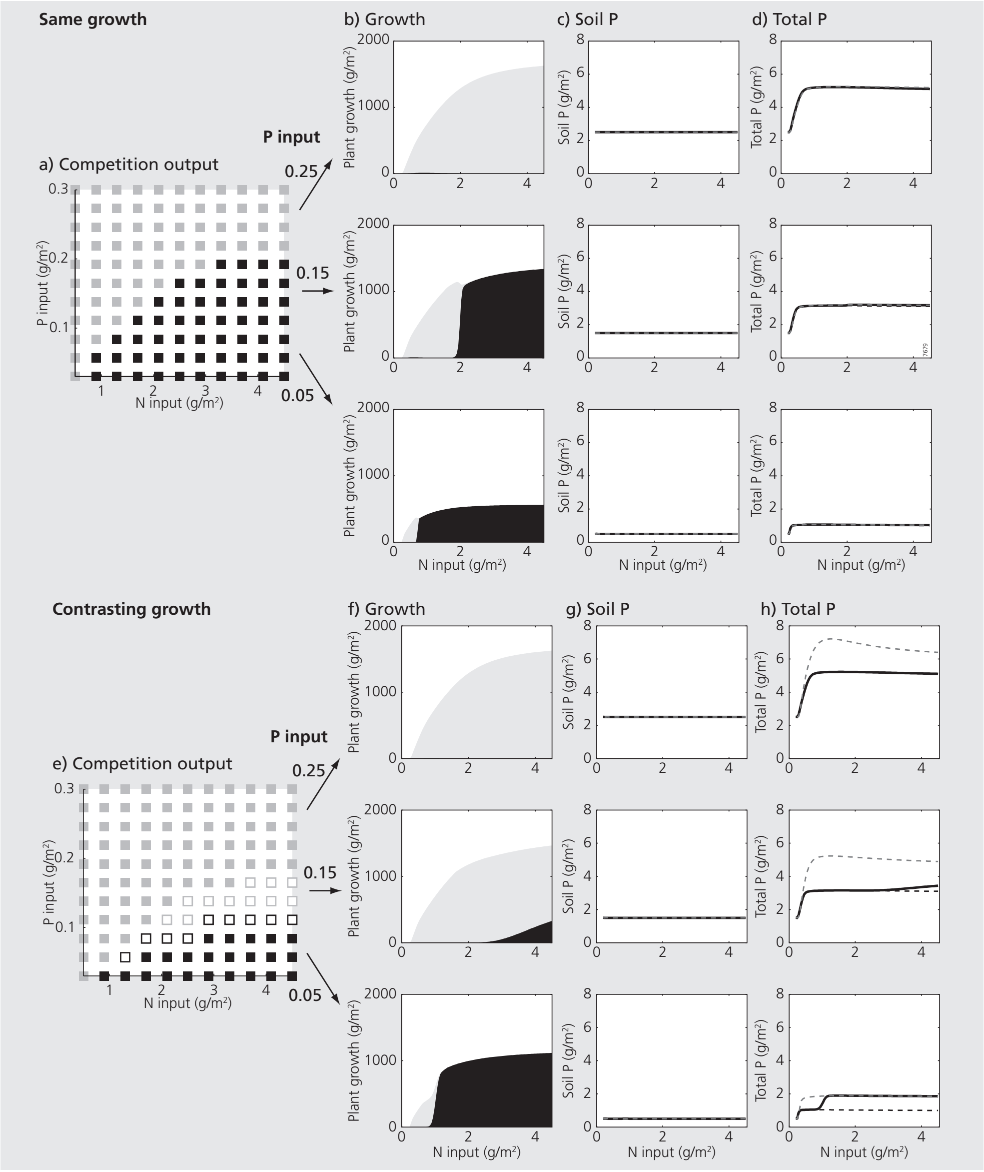

Figure 5.4 Simulation outputs of competition between species 1 (with high phosphatase activity: $a_{Po,1}$ = 0.0001) and species 2 (with low phosphatase activity: $a_{Po,2}$ = 0.00002) when growth rates are the same for two species ($a_{N,1}$ = $a_{N,2}$ = 0.25) (a-d), and when species with high phosphatase activity has a slower growth rate ($a_{N,1}$ = 0.01) than that with low phosphatase activity ($a_{N,2}$ = 0.025) (e-h). Competition output after 2000 years are presented as species 1 only (filled black squares), species 1 more than species 2 (open black squares), species 2 more than species 1 (open gray squares), or species 2 only (filled gray squares) (a & d). For three levels of P input (0.05, 0.15, 0.25 mg m^{-2} $year^{-1}$), growth of species 1 (black) and species 2 (gray) (b & f), soil P (c & g: dissolved and organic P), and ecosystem total P (d & h: all P pools except reactive P) are shown along increasing N input level. For c, d, g, and h, outputs of single species simulation are also shown with dashed lines (dashed black: species 1 only, dashed gray: species 2 only).

When a trade-off between phosphatase activity and growth rate was considered, soil P did not change along a gradient of N input (Figure 5.4g). We, however, observed an abrupt increase in ecosystem total P with increasing N input at a low P supply level (Figure 5.4h). This abrupt increase coincides with the species replacements from those with low phosphatase activity (and with fast growth rate) to those with high phosphatase activity (and with slow growth rate). Since slower growing species can better conserve nutrients in their biomass at the equilibrium stage (see sensitivity analysis: Appendix II Figure 5.A2e), a dominance of the species with lower phosphatase activity and slow growth rate, promoted by increased N input, eventually results in an increased plant P content.

Discussion

Increased N inputs may influence terrestrial ecosystems by promoting dominance of nitrophilic species (Bobbink *et al.* 1998), as well as by altering P dynamics of ecosystems via plant growth that couple dynamics of multiple nutrients (Perring *et al.* 2008). Here we propose additional mechanisms of N effects on ecosystem properties, by explicitly including flexible responses of plants to varying N:P stoichiometry in a model. With plasticity in plant nutrient concentrations as well as an adaptive trait (i.e. phosphatase activity), our model showed that increased N input may change species composition as well as ecosystem P retention.

Our model predicts that species with a high phosphatase activity wins from those with low phosphatase activity in P-limited conditions, even if the former is a slower growing species, unless under very eutrophic conditions. The advantage of species with efficient phosphatase activity in P-limited conditions is indicated by a short-term experiment (Chapter 2). However, whether the advantage remains for the longer term is in question, because competitive hierarchies can change due to nutrient dynamics in the soil as well as feedbacks of plants to the nutrient cycles (Clark *et al.* 2005, Daufresne & Hedin 2005). By use of our model, we showed that the dominance of species with high phosphatase activity in extremely P-limited conditions is robust at any combination of soil parameters, although the threshold of N:P input ratio where the species shift occurs is subject to subtle changes in soil parameter values (see Appendix III for the extra analyses on the effects of several soil parameters on species competition).

Another finding from our model simulations is that, when the system is N-limited, ecosystem P retention is rather constant along a gradient of N input level. This contrasts with the results of Perring's model, which, with fixed nutrient concentrations in plants, predicts that N addition to N-limited ecosystems can conserve P in ecosystems. This difference can be explained by the flexible nutrient concentrations in our model, which enabled luxury consumption of the excessive nutrients by the plants. In that way the amounts of P in plants is stabilized even when the N input level fluctuates. When the system becomes P-limited, our model showed that phosphatase activity does, though only slightly, increase P retention. Furthermore, our model predicted that under a very high N input level, P retention even decreases due to a lowered growth rate caused by too low P concentration in plants. Increased plant death rate under extreme P-limitation, but not under N-limitation, is observed in greenhouse (Güsewell 2005a) and field (El-Kahloun *et al.* 2003) experiments. In this regard, phosphatase activity may have an extra positive effect in adjusting plant N:P ratio to prevent N toxic effects by N eutrophication.

Although the single effect of phosphatase activity on P retention was relatively small, we showed a possibility that increased N input may rapidly increase P retention of the ecosystem via shifting species composition toward the domination of species with a high phosphatase activity, if there is a trade-off between phosphatase activity and growth rate. The trade-off is indeed likely, since the maintenance for a trait to acquire a specific fraction of P needs investment (Turner 2008). This provides an example of a potential mechanism by which the functional composition of plant communities drives a change in ecosystem properties such as nutrient retention (Hooper *et al.* 2005). To improve our understanding on the N effects on P retention, further validation by long-term field measurements is necessary.

There are several unexplored traits which may affect species composition under varying N:P availability. Optimum biomass N:P ratio for growth can be different among species with different functional strategies (Güsewell 2004), such as lower N:P ratios for annual species (Mcjannet *et al.* 1995), species typically from fertile habitats (Güsewell *et al.* 2003), or species with high dependency in seed reproduction (Chapter 4). As the resource ratio theory predicts (Tilman 1982), a species with lower optimum N:P ratio may have advantage when N:P input ratio is low. Resorption efficiency from senescing leaves is another trait which may play an important role under a varying availability of N and P (Aerts & Chapin 2000). Indeed, species adapted to P-limited conditions tend to have a high P resorption efficiency (Güsewell 2005b). Plastic adjustments of resorption rates to nutrient concentrations in plants, although not always observed (Aerts 1996), would lead to similar effects of resorption to that of phosphatase activity on species competition under varying N:P availability. N fixation by plant symbioses, an important mechanism for plants to gain N from the environment (Sprent 2001), may also affect species competition under varying N:P availability. Houlton *et al.* (2008) showed with their model that N-fixing plants survive in P-limited conditions by investing N into P acquisition by phosphatase activity. Inclusion of N-fixation in our model, thus, will even accelerate the advantage of species with high phosphatase activity under P-limited conditions and may increase P retention of the ecosystem.

Our model results may have implications for ecosystem conservation. The effects of N eutrophication on terrestrial ecosystems regard not only the loss of species richness (Clark & Tilman 2008), but also shifts in the (functional) composition of species (Suding *et al.* 2005, Xia & Wan 2008). Our model results supported the latter effect on species composition, by showing that N eutrophication favors species with a better adaptive trait to N:P stoichiometry. Most of the conservation measures common in European grasslands (e.g., harvesting, rewetting, and increasing groundwater seepage) primarily aim at decreasing nutrient availability. These measures, however, have different pathways to influence N and P dynamics and thus affect N:P stoichiometry differently. The prediction of effects would be more reliable when the stoichiometric effects are explicitly considered.

This study provided a simple but stoichiometrically explicit framework to examine the effects of increased N input on the (functional) composition of species and on the P retention of terrestrial ecosystems. This model enabled us to disentangle the mutually affecting roles of plant growth, a species adaptive trait, and nutrient cycles in soil, which are not easily examined by a short term experiment or a field observation alone. Based on our results we conclude that N eutrophication will benefit species with high phosphatase activity, but only under low P input conditions. Moreover, the changed species composition due to N eutrophication might result in increased P retention, although this result still needs verification from field studies. Further theoretical and experimental studies may clarify how N eutrophication affect community structure of plant communities, as well as associated changes in ecosystem properties such as nutrient retention.

Acknowledgements

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Appendix I. Model specifications

Model equations of nutrient dynamics in soil

In soil, we consider two pools of nitrogen and three pools of phosphorus, following Perring *et al.* (2008). For both nitrogen and phosphorus, those bound to soil organic matter (E_{no}) and those dissolved in solution (i.e. readily available for plants to uptake: E_n) are included. Organic and dissolved nutrients are lost from the system via leaching with a constant rate. Nutrients are assumed to enter the system via atmospheric deposition or inflow from surrounding systems as dissolved forms. For phosphorus, we also included reactive P, i.e., adsorbed P to soil particles via chemical processes. This pool exchanges P with dissolved P pool via adsorption and desorption kinetics. The rates of change for each pool of N and P are:

$$\frac{dE_N}{dt} = i_N - l_N E_N - \sum_{i=1}^{2}\left(U_{N,i}\, G_i\, C_i\right) + m_N E_{No}$$

$$\frac{dE_{No}}{dt} = \sum_{i=1}^{2}\left(L_{N,i}\right) - l_{No} E_{No} - m_N E_{No}$$

$$\frac{dE_P}{dt} = i_P - l_P E_P - \sum_{i=1}^{2}\left(U_{P,i}\, G_i\, C_i\right) + m_P E_{Po} - k_{ad1} E_P\left(r - E_{\mathrm{Pr}}\right) + k_{ad2}\, E_{\mathrm{Pr}}$$

$$\frac{dE_{Po}}{dt} = \sum_{i=1}^{2}\left(L_{P,i}\right) - l_{Po} E_{Po} - \sum_{i=1}^{2}\left(O_{P,i}\, G_i\, C_i\right) - m_P E_{Po}$$

$$\frac{dE_{Pr}}{dt} = k_{ad1} E_P\left(r - E_{\mathrm{Pr}}\right) - k_{ad2}\, E_{\mathrm{Pr}}$$

where i_n is the input rate of n, l_n is the leaching rate of n, m_n is the mineralization rate of n, E_{Pr} is the inorganic reactive P in soil, k_{ad1} is the adsorption rate of P, r is the density of P adsorption sites in soil, and k_{ad2} is the desorption rate of P.

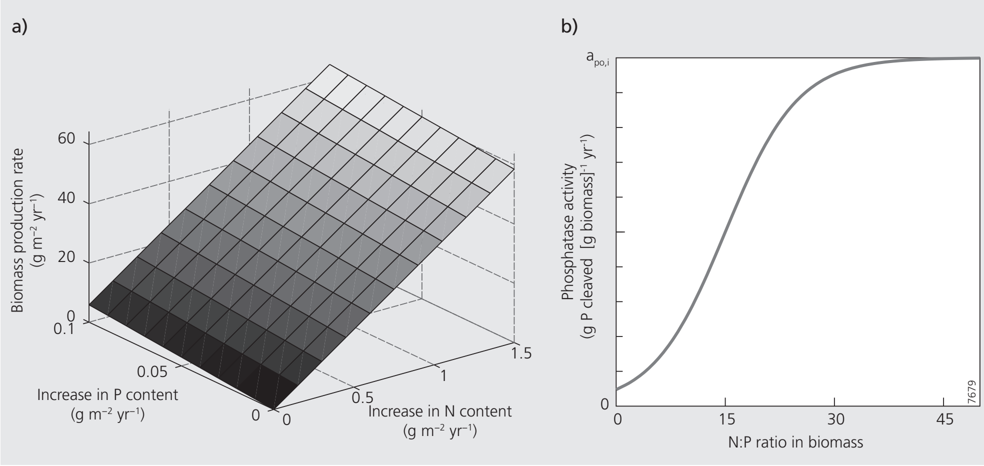


Figure 5.A1 (a) Modeled biomass production as a function of N and P uptake by plants. (b) Modeled phosphatase activity in the rhizosphere as a function of N:P ratio in biomass.

Appendix II. Sensitivity analysis for soil and plant parameters

We examined the model sensitivity to several key parameters (soil adsorption capacity, leaching rate of dissolved P, and leaching rate of organic P, P mineralization rate, plant growth rate, maximum phosphatase activity rate) for the fixed and flexible concentration models (Figure 5.A2). The model output was assessed in terms of ecosystem total P (amount of P in all P pools excluding reactive P) along increasing N input level. Note that sensitivity to the maximum phosphatase activity rate could not be examined for the model with fixed nutrient concentrations, because P uptake was not explicitly modeled in this model. In the figures the simulation results after 2000 time steps are compared.

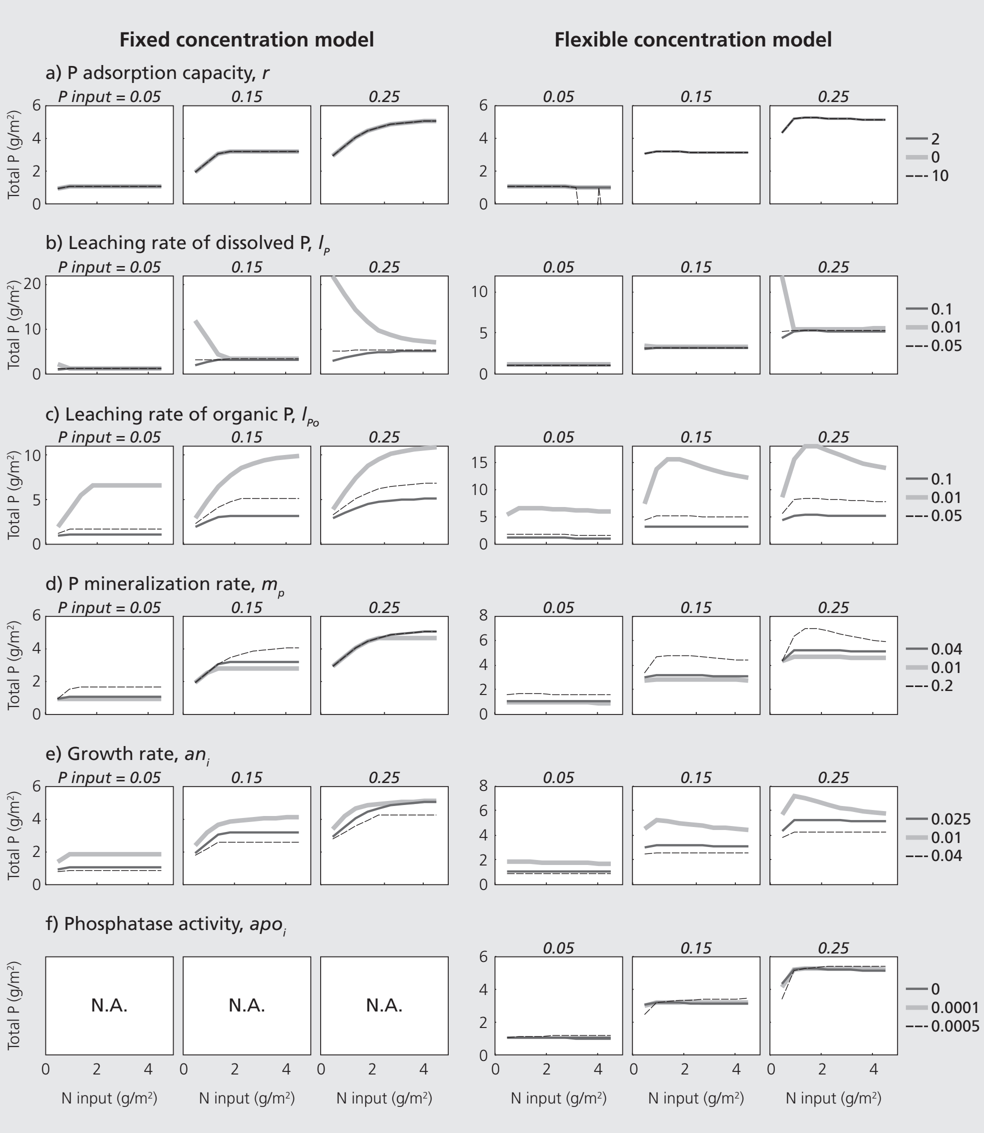


Figure 5.A2 Sensitivity analysis of several parameters on ecosystem total P. Parameters assessed were P adsorption capacity of soil (a), leaching rate of dissolved P (b) and organic P (c), P mineralization rate (d), growth rate (e), and phosphatase activity (f). Results are shown for the model with fixed (left) and flexible (right) nutrient concentrations in plant biomass for three P input levels, 0.05, 0.15, and 0.25 g m^{-2} $year^{-1}$. Filled black lines are the simulation results with the base line value of the parameters.

Appendix III. Effects of soil parameters on competition outputs

We tested if soil parameters affect the competition between species with different magnitude of phosphatase activity. It is expected that species with high phosphatase activity get less advantage if a change in a soil parameter makes P more available for plants. Therefore, we selected four soil parameters which may increase P availability for plants (i.e. lower leaching rate of organic P, lower leaching rate of dissolved P, higher P mineralization rate, and higher P adsorption rate), and compared their competition outputs with those simulated with the default parameter values (as in Figure 5.4a).

We found that a low leaching rate of organic P counteracts the advantage of species with higher phosphatase activity under P limited conditions (Figure 5.A3a), because both dissolved and organic P in soil increased which made the condition easier for the species with lower phosphatase activity. In contrast, low leaching rate of dissolved P does not change the competition outputs (Figure 5.A3b). This is because the leaching rate of dissolved P hardly change the size of P pools in soil under the condition that plants flexibly adjust P concentration in their biomass (see sensitivity analysis: Figure 5.A2b right). High mineralization rate of P has negative impacts on species with higher phosphatase activity under P limited conditions (Figure 5.A3c). This is because higher turnover of organic P decreases advantage of these species to take up more P from organic P than the other species. A high P adsorption capacity of soil benefit species with a higher phosphatase activity on the short term, because dissolved P in soil decreases in the first years which has more negative effects on species with lower phosphatase activity. On the long term (i.e. at the equilibrium states) however, the low P adsorption capacity does not affect the competition outputs (Figure 5.A3d).

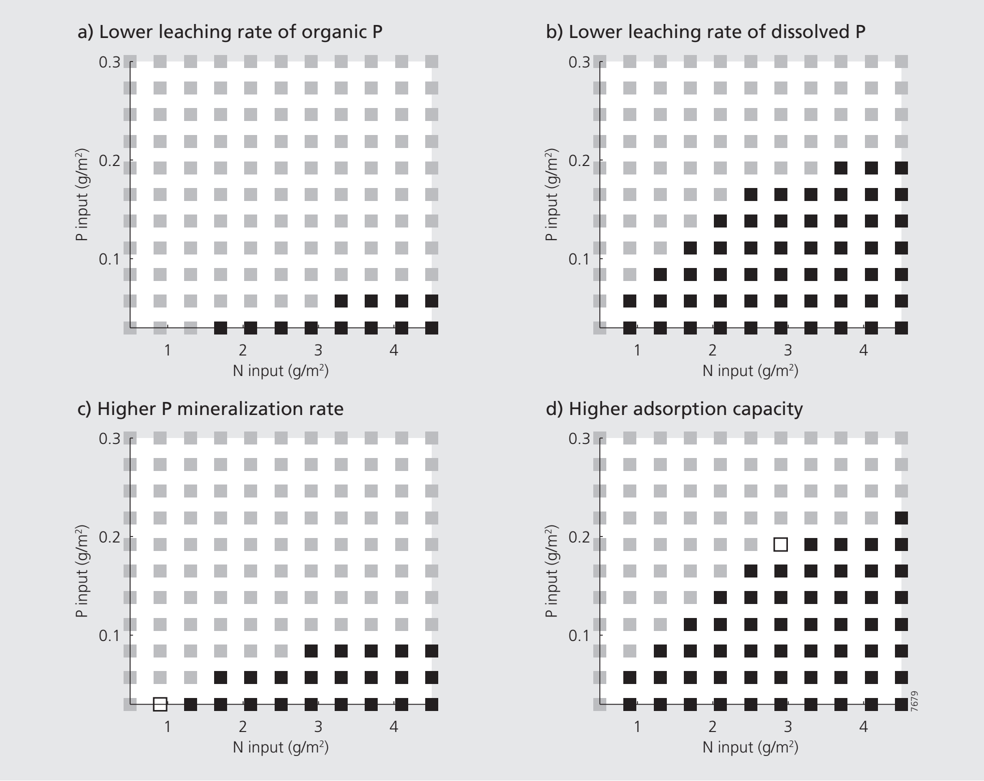


Figure 5.A3 Effects of four soil parameters on plant species competition. Simulations were performed between species with high ($a_{Po,1}$ = 0.0001) and low ($a_{Po,2}$ = 0.00002) phosphatase activity. Competition output after 2000 years are presented as species 1 only (filled black squares) species 1 more than species 2 (open black squares), species 2 more than species 1 (open gray squares), or species 2 only (filled gray squares). The soil parameters values are: a) lower leaching rate of dissolved P (l_P=0.01), b) lower leaching rate of organic P (l_{Po}=0.01), c) higher mineralization rate (m_P=0.2), and d) higher P adsorption capacity of soil (r=10). All other parameter values are the same as Figure 5.4a.

6 Synthesis

Introduction

Anthropogenic eutrophication is threatening the biodiversity of ecosystems worldwide (Vitousek *et al.* 1997, Rockstrom *et al.* 2009). To conserve ecosystems, it is necessary to properly assess and predict the impacts of eutrophication. For that, we need to understand, not only the patterns of the impacts, but also the underlying mechanisms which cause the patterns in response to eutrophication.

Unraveling the relationships between nutrient availability and biodiversity in ecosystems has been a central focus of ecologists for many decades (e.g. Rosenzweig 1971, Grime 1973). Besides, there is a growing interest in how *balance* between nutrients affects functioning and biodiversity of ecosystems (Sterner & Elser 2002). In terrestrial ecosystems, it is especially the availability of nitrogen (N) and phosphorus (P) that affects plant communities (Aerts & Chapin 2000, Güsewell 2004).

In this thesis, I study the mechanisms by which eutrophication affects grassland plant communities with special attention to the effects via N:P stoichiometry (i.e. the balance between N and P in terms of availability and requirement in ecological processes). Regarding the effects, I look at the responses of plants on different organization levels, such as: species functional traits, individual growth, species interactions (i.e. interspecific competition), and plant community structure (i.e. species composition and diversity) (see Figure 6.1). With the greenhouse experiments with eight grassland species I examine the effects of N:P stoichiometry on species traits and individual growth for the time scale of one to two years (chapter 2 and 3). With the field data analyses I look at the effects of N:P stoichiometry on plant community structure (chapter 4) which I see as an outcome of species interaction at the time scale of decades. A modeling study connects these distinct time scales by using the trait-level responses of plant individuals to predict the effects on the plant community structure (chapter 5).

In this chapter, I integrate the results engaged on different organization levels and time scales in order to gain a comprehensive understanding on the N:P stoichiometric effects on grassland ecosystems. I start with outlining the key findings from our greenhouse experiments about the N:P stoichiometric effects on individual plant traits and growth (chapter 2 and 3). Next, I discuss about several trait-based mechanisms which potentially explain species composition of plant communities in a gradient from N- to P-limited grasslands. This is done by relating the inter-specific differences in plant traits observed in the greenhouse experiments to the field distribution patterns of these species (chapter 2 and 3), as well as by discussing the implication from the metadata analysis (chapter 4). Here the mechanisms indicated by the modeling study (chapter 5) help to connect the two organization levels on different time scales. Subsequently, the impact of N:P stoichiometry on species diversity is discussed with an emphasis on threatened species. Additionally, the potential impact of N:P stoichiometry on ecosystem nutrient retention is discussed. Finally, based on the preceding discussion, I provide implications for ecosystem conservation and perspectives for future research.

N:P stoichiometric effects on individual plant species: key findings from the greenhouse experiments

Our greenhouse experiments advance our knowledge on individual-level plant responses to N:P stoichiometry in several points. We found that P concentration in plant biomass rapidly

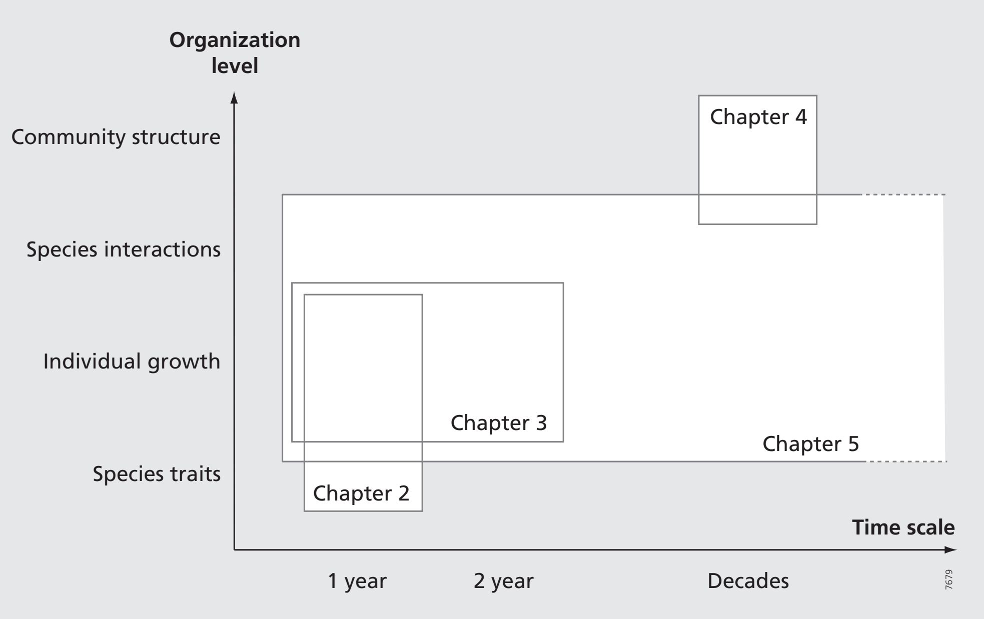


Figure 6.1 The main coverage of each chapter of this thesis depicted in a domain of time scale and organization level.

decreases with increasing N:P supply ratio, whereas N concentration increases only moderately with increasing N:P supply ratio (chapter 3). This is consistent with results from another fertilization experiment that high N supply (i.e. high N:P ratio) promotes biomass production immediately, leading to a decrease in P concentration in biomass, whereas high P supply (i.e. low N:P ratio) does not rapidly promote biomass production and therefore N concentration remains rather stable (Güsewell *et al.* 2003). The physiological mechanism behind these contrasting responses is that cytokinin, which stimulates plant growth, is regulated more strongly by N than P concentrations in biomass (de Groot *et al.* 2003). As a consequence, P concentration in biomass is determined by the *relative* availability of N to P and not by single P availability. The lowered P concentration caused by high N:P supply ratios triggers high death rates in the second year (chapter 3, Fisher *et al.* 2002). This explains why high root death in the second year under P limited conditions was dominantly influenced by N:P supply ratio, rather than by overall nutrient level (chapter 3).

Furthermore, we observed that root-surface phosphatase activity was related to high N:P supply ratio rather than to the availability of single nutrients. In contrast, other uptake traits (root mass ratio and specific root length) were not dominantly influenced by N:P supply ratios (chapter 2). This reflects that regulation mechanisms are trait specific. For example, phosphatase is stimulated by P starvation in plant cells (Duff *et al.* 1994) and thus related to N:P stoichiometry, while allocation to the roots is thought to be regulated by cytokinin (e.g. van der Werf & Nagel 1996) that is influenced by N availability rather than N:P stoichiometry.

Our greenhouse experiments also showed inter-specific differences of plants in response to N- and P-limitation. In short, the plant responses in trait plasticity and growth to N:P stoichiometry were similar in patterns but different in magnitudes among species. The level of phosphatase activity differs significantly among species (chapter 2, Rejmankova & Macek 2008), which partly explained interspecific differences in their use of P in not readily bio-available forms (chapter 2, Perez-Corona *et al.* 1996, Hayes *et al.* 2000). Species variation is also evident in resorption efficiency (chapter 3, Killingbeck 1996, Güsewell 2005b) and in plasticity of nutrient concentrations in biomass in response to increasing nutrient input (chapter 2, Garnier 1998), which may influence the magnitude of negative impacts of nutrient limitation. The potential consequences of these species variations in plant traits and individual growth on plant community structure are discussed in the following paragraphs.

N:P stoichiometry and species composition: trait-based mechanisms explain the field distribution patterns of plant species

Trade-offs between species traits are important in shaping community structure under different environmental conditions (Suding *et al.* 2003). The trade-off between traits may explain the different species composition in N- and P-limited environments, because having an adaptive trait to a particular type of nutrient limitation, such as P uptake traits in P limitation, might be too costly to be successful under another type of nutrient limitation. The role of species functional traits in shaping community structure has been intensively tested for a gradient from low- to high-productive environments (e.g. Campbell & Grime 1992, Fynn *et al.* 2005), but never, to my knowledge, explored for a gradient of N:P. Below I discuss about three trait-based mechanisms which potentially explain the shift in species composition along a N:P gradient.

Trade-off between immediate growth and long-term nutrient retention

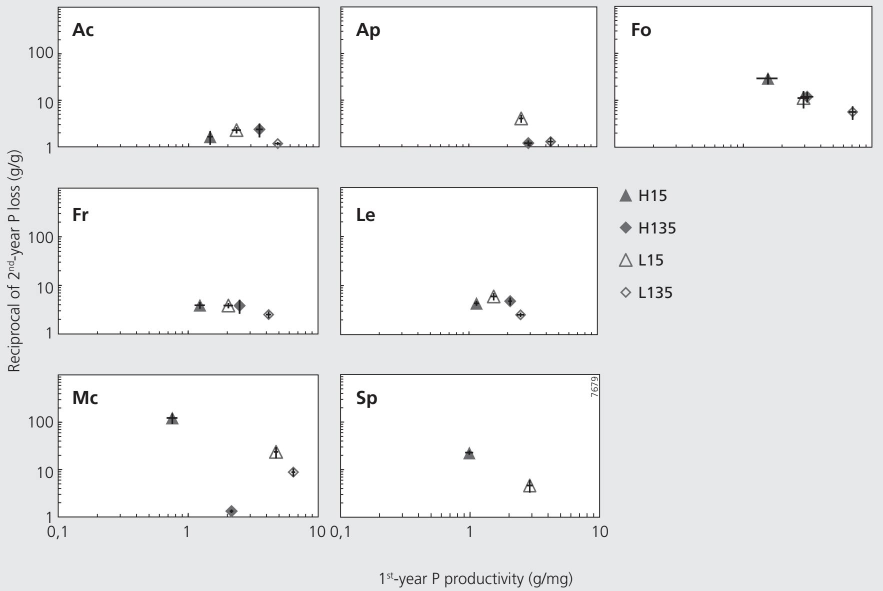
An important factor of plant survival in nutrient limiting environments is their nutrient use efficiency, which is a function of nutrient productivity (i.e. biomass growth per unit nutrient) and nutrient conservation (i.e. mean residence time of the nutrient in biomass, which is a reciprocal of the nutrient loss rate) (Aerts & Chapin 2000). Results of previous studies with

⇨ **Figure 6.2** Testing trade-offs between first-year P productivity (as a proxy of immediate growth) and the reciprocal of second-year P loss (as a proxy of long-term nutrient retention) between treatments of each species (a) and between species (b) by use of the experimental results from chapter 3. Here only the treatments with N:P ratio 15 and 135 were used, because plant responses to P limitation are of our concern. The immediate growth was approximated by the biomass productivity of P in 2007 (i.e. biomass production per unit P, g mg^{-1}, during 15 weeks of fertilization period) because P was the limiting nutrient in all these treatments, judged from their biomass N:P ratios larger than 15. The long-term nutrient retention was approximated by the reciprocal of P loss in 2008 (i.e. fraction of lost P from biomass over total P content in biomass during 14 weeks of fertilization period plus the preceding winter). For the within-species comparison (a), the means and SE of each treatment are shown. The treatments which had less than two regenerated pots in 2008 were excluded. Treatment abbreviations are; H15: high supply level and N:P supply ratio 15, H135: high and N:P 135, L15: low and N:P 15, L135: low and N:P 135. For the between-species comparison (b), I show the relative changes of the two variable, first-year P productivity and reciprocal of the second-year P loss, from N:P supply ratio 15 to 135 for each species. The relative change was calculated as the difference between the natural logarithms of these variables at N:P supply ratio 135 and that at 15. Thus, the positive values on the x-axis mean that the species have a better immediate growth at N:P ratio 135 than at 15, and on the y-axis a better long-term nutrient retention at N:P ratio 135 than at 15. High and low nutrient supply levels are combined. Species abbreviations are; Ac: *A. capillaris*, Ap: *A. pratensis,* Fo: *F. ovina*, Fr: *F. rubra*, Le: *L. europaeus,* Mc: *M. caerulea*, Sp: *S. pratensis*.

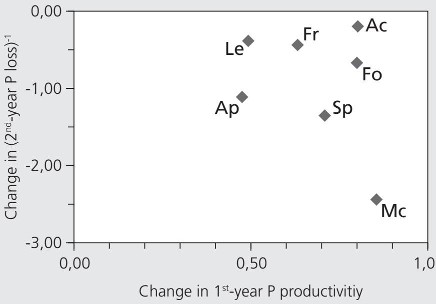
similar greenhouse experiments (Güsewell *et al.* 2003, Güsewell 2005a) suggested that there is a trade-off between immediate growth and long-term (i.e. after second year) nutrient retention under P-limited conditions. Such a trade-off can also be observed in our experiments. In our case, increased N supply, or high N:P supply ratio, promoted plant growth in the first year and decreased P concentration in their biomass (i.e. high immediate growth), which in turn resulted in severe root death and associated nutrient loss in the second year (i.e. poor long-term nutrient retention) (chapter 3). Inversely, if the first-year growth is stimulated less (i.e. poor immediate growth), the second-year nutrient loss may be smaller (i.e. high long-term nutrient retention).

The hypothesized trade-off could be just a temporal consequence of plastic responses of species to a changing environment, or alternatively could be an evolutionary consequence of species adaptation to a particular environment (West-Eberhard 2003) which may explain the distribution pattern of species along a N:P gradient. If the former is the case, it is expected that

a) Within-species comparison



b) Between-species comparison



the trade-off is observed among treatments within each species. If the latter is the case, the trade-off is expected between species. More specifically, species typical from P-limited grasslands are expected to have a lower immediate growth but also a higher long-term nutrient retention under P limitation. To test these two hypotheses, I compare the relations between the immediate growth (approximated by first-year productivity) and the long-term nutrient retention (approximated by the reciprocal of second-year nutrient loss) on both within-species and between-species levels by use of the experimental results of chapter 3 (Figure 6.2; see figure caption for the method of the analysis). The within-species comparison provides a hint that the trade-off seems to prevail (Figure 6.2a): for more than half of the species a negative relation between immediate growth (x-axis) and long-term retention (y-axis) is observed. In the between-species level, however, the expected relation is not observed (Figure 6.2b): species which drastically increased immediate growth upon P limitation do not necessarily reduce long-term nutrient retention. Furthermore, the species which typically occur in P-limited grasslands in the field (*M. caerulea* and *S. pratensis*) do not show the expected trade-offs (i.e. poor immediate growth but high long-term nutrient retention) neither. It seems, therefore, that no particular species have evolved to adapt to P-limited conditions in terms of their nutrient use efficiency. Rather, high nutrient loss in the second year seems to be a consequence of the forced plasticity in nutrient concentration under extreme P limitation.

Variations in P uptake traits

Well established and diverse mechanisms of many species to promote P uptake, such as mychorrizal association, cluster roots and root exudates (Vance *et al.* 2003, Raghothama & Karthikeyan 2005, Lambers *et al.* 2006) imply that species adaptation to P-limited conditions mainly owe to their P uptake ability (Lambers *et al.* 2008). A competition experiment under different N:P supply ratio showed that competition strength of species under N limitation could be explained by their root length and pre-emption of nutrients, but not under P limitation (Olde Venterink & Güsewell 2010). This also indicates that not just root size, but other specialized traits for P uptake, would play an important role in species interactions under P limited conditions. Our greenhouse experiments indeed show that species that have higher efficiency in phosphatase activity occur typically in P-limited grasslands in the field (chapter 2). The dominant effects of N:P supply ratio on phosphatase activity, rather than solo availability of N or P, further support the importance of this trait in adaptation to P-limited conditions. Furthermore, the modeling study (chapter 5) also agrees with the better survival of species associated with higher phosphatase activity in P-limited conditions. The model results, however, indicated that high productivity, which leads to intensive light competition, diminishes the advantage of these species because the benefit of phosphatase activity (i.e. P uptake) is not large enough to pay off its cost (i.e. N loss) in such conditions. Although not examined in this thesis, other P uptake traits, which also involve costs, will probably have similar consequences. Thus, adaptive traits for P uptake are expected to play a role in determining species composition in P-limited conditions as far as the ecosystem remains low productive.

Different types of reproduction strategy

Reproduction strategy of plant species, such as dispersal traits, is another factor to assemble plant community structure under varying environmental conditions (e.g. Ozinga *et al.* 2005). Our database analysis showed that species with less investment in reproduction by seeds occur more often in P-limited grasslands (chapter 4), suggesting that reproductive strategy also matters for the species distribution patterns along a N:P gradient. Additionally, negative impacts of P limitation or high N supply on seed reproduction were reported by other experiments (Brouwer *et al.* 2001, Vergeer *et al.* 2003). These patterns can be explained by the fact that reproductive organs, such as seeds, contain relatively much P compared to other organs because P-rich rRNA constitutes a high proportion of reproductive organs (Kerkhoff *et al.* 2006). Species which have a large investment in the P-demanding seed production, therefore, may be less competitive in P-limited conditions.

N:P stoichiometry and species diversity

Whether biodiversity is related to the type of nutrient limitation is of particular concern for ecosystem conservation (Bobbink *et al.* 1998, Aerts *et al.* 2003, Güsewell *et al.* 2005). In accordance with previous studies (Olde Venterink *et al.* 2003, Güsewell *et al.* 2005, Wassen *et al.* 2005), I found a weak hump-shaped relationship between plant species diversity and N:P ratio in the above-ground plant material, although the contribution of the N:P ratio to the species diversity is much smaller than that of productivity (chapter 4). Richness of threatened species, on the other hand, is much more strongly related to high N:P ratio, rather than to productivity (chapter 4). Understanding why threatened species are associated with P limitation is important for biodiversity conservation, because the decline of species diversity is expected to be initiated by the disappearance of threatened species. Moreover, threatened species are assumed to have some functional characteristics in common which make them fragile (Kunin & Gaston 1993, Kleijn *et al.* 2008). Thus, unraveling why those species persist in a particular type of nutrient limitation will help us understand the mechanism of N:P stoichiometric effects on plant communities.

Two possible explanations go along with the higher frequency of threatened species in P-limited than in N-limited grasslands. First, P limitation is often associated with low productivity (chapter 4, Mcjannet *et al.* 1995, Olde Venterink *et al.* 2003). Adaptive traits in P limited conditions are costly, because P uptake traits often involve a large quantity of root exudates (e.g. Lambers *et al.* 2006). The high costs may limit the above-ground biomass production and thus diminishes the ability of such species to compete for light. Threatened species tend to have a lower SLA and, though not statistically significant, be a stress-tolerator type rather than a competitor type (chapter 4). These species, therefore, are more likely to survive in P-limited conditions because competitive species do even less well in such stressful conditions. Thus, it is misleading to say that threatened species survive better in P-limited conditions. Rather, it is more proper to say that *only* in P-limited and low-productive conditions threatened species are *not* outcompeted by other (more competitive) species.

Another mechanism to explain the association between threatened species and P limitation could be their reproduction strategy which tends to depend less on seed reproduction (chapter 4). As explained in the previous paragraph, this type of reproduction requires less P, making them less affected by P scarcity. Such a reproduction strategy with less dependency on seed reproduction, however, may have the disadvantages of poor dispersal ability on a regional scale. If that is the case, the threatened species are under a higher risk of local extinction and more vulnerable in fragmented landscapes.

Effects of N:P stoichiometry on ecosystem nutrient retention

Nitrogen eutrophication has interactive effects on other major biogeochemical cycles (Gruber & Galloway 2008) such as P (Hedin *et al.* 2003). Its effects on ecosystem nutrient retention (i.e. how much nutrient is kept in the ecosystem) is of particular concern (Perring *et al.* 2008), because the enhanced retention will increase site productivity and thus affect other ecosystem functions. Our 2-year greenhouse experiment shows that severe P limitation promotes nutrient loss from dead roots of plants in the second year (chapter 3), indicating reduced nutrient retention after N eutrophication. Similar results were obtained by a field experiment where N fertilization resulted in much higher root death rate than P fertilization seven years after the fertilization treatment (El-Kahloun *et al.* 2003). The modeling study (chapter 5), however, indicated that P retention remains rather stable under increasing N input on a much longer time scale (more than decades). Also, increasing N input may benefit species with adaptive traits to P limitation, such as high phosphatase activity, which can lead to an increase in ecosystem P retention (chapter 5). Furthermore, our database analysis indicates that high atmospheric N deposition does not necessarily shift ecosystems to be P-limited, but rather to be N-limited (chapter 4, Figure 4.1). This can be explained by a mechanism that high N input on the long term promotes P foraging of plants and subsequently results in increased productivity, which is also indicated by a decades-long field measurement (Malmer & Wallen 2005). The increased productivity may lead to dominance of fast-growing species that have inherited low N:P ratios, further enhancing plant P forage. I suspect, however, that such plant-mediated positive feedback of high N input on P retention occurs only when (potential) P availability of the ecosystem is abundant, because otherwise the high cost associated with P uptake may counteract the feedback effects since the gain of extra P obtained is too little (see an earlier discussion in this chapter).

Implication for ecosystem conservation

The higher frequency of threatened species in P-limited grasslands raises the question whether atmospheric N deposition stimulates P limitation of ecosystems and therefore has a positive impact on biodiversity conservation. As the database analysis (chapter 4) indicated, however, the positive feedback of N eutrophication on P availability will remain N limited ecosystems N limited, but shift previously P-limited ecosystem to be high productive and not P limited anymore. Indeed, low productive wetlands with low P availability are disappearing (Wassen *et al.* 1996). Disappearances of P-limited and low-productive ecosystems will cause a loss of threatened species (see the paragraph on species diversity). Therefore, it is not advisable to expect positive effects of eutrophication on species diversity as well as conservation of threatened species (this study; Güsewell *et al.* 2005).

Conservation measures can interfere N:P stoichiometry of ecosystems. It is evident that changes in abiotic habitat conditions caused by anthropogenic activities lead to degradation of ecosystems (e.g. van Loon *et al.* 2009). Therefore, major conservation measures in moist to wet grasslands in Europe (e.g. mowing, re-wetting, or sod cutting) aim at conserving suitable habitat conditions of ecosystems, in particular low nutrient availability. Each of these measures, however, has its own effects on biogeochemical processes (Lamers *et al.* 2002). For example, re-wetting generally results in lowering N availability via increased denitrification but increasing P release from compounds (Olde Venterink *et al.* 2002). Mowing removes both N and P from above-ground plant biomass (Koerselman *et al.* 1990b), which may shift the ecosystems under high N eutrophication to be P-limited in the long term (Verhoeven *et al.* 1996). These asymmetric impacts of conservation measures on N and P cycles highlight the necessity to consider their effects not only on the availability of each nutrient but also on the kind of nutrient limitation (Olde Venterink 2000) and associated stoichiometric effects on ecosystem functioning (this thesis).

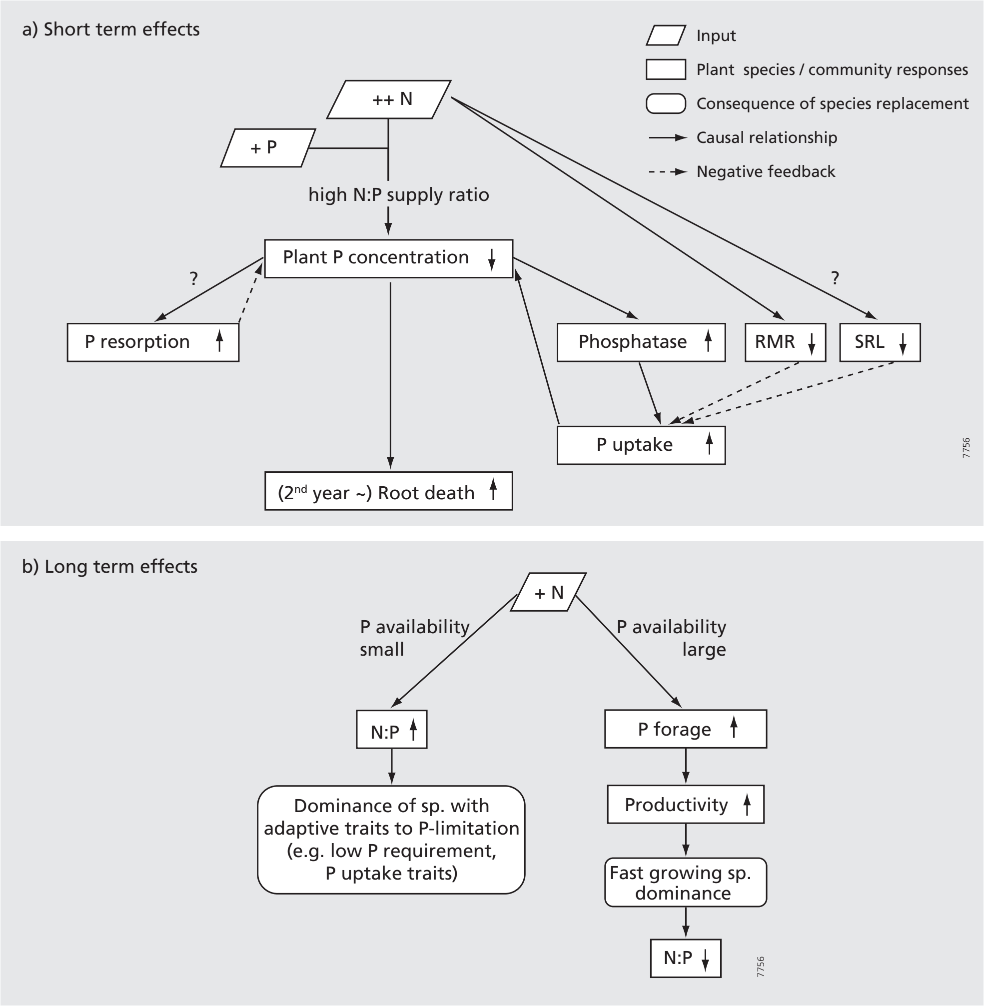


Figure 6.3 Hypothesized short-term (a) and long-term (b) effects of increased N input on plant communities. In the short term (< a few years), increased N input affects several traits of plant species, such as decreasing RMR (root mass ratio) or, though not clearly shown in our study, decreasing SRL (specific root length). Increased N input also decreases P concentration in biomass, although this happens only when N input is *relatively* higher than P input. The decreased P concentration in biomass leads to increased phosphatase activity, resulting in higher P uptake. The increase in P uptake can be mitigated by decreased RMR or SRL. The stimulated P uptake and P resorption from senescing biomass may eventually compensate for the decrease in P concentration. If the P concentration remains very low, it will lead to an increased death rate, especially of roots, in the following years. In the long term (> decades), increased N input may lead to species replacement, but the direction of the species shift depends on potential P availability. If increased N leads to enhanced P forage of plants and thus increased productivity, the community will be dominated by fast growing species. This may even result in a decreased N:P ratio of the community because of an inherited low N:P ratio of fast growing species. When P availability is low and thus productivity does not increase by increased N input, the enhanced P limitation may lead to dominance of species which have advantageous characteristics in P-limited conditions.

Perspectives

The balance between nutrients has consequences for ecological interactions and processes (Sterner & Elser 2002). This thesis showed that the relative availability of N and P affects ecosystems on several organization levels of plants and on different time scales. In the short term (< a few years; Figure 6.3a), high N supply relative to P supply causes a decrease in P concentration in plant biomass, that results in high root death in the following years. The lowered P concentration in biomass can be mediated by other plant traits, such as phosphatase activity or nutrient resorption, yet in a species-specific manner. In the longer term (> decades; Figure 6.3b), plant composition can change to adjust to the stoichiometric status of the environment (Danger *et al.* 2008). Species which have advantageous characteristics under P limited conditions, such as higher phosphatase activity or less P-requiring reproduction strategies, may benefit after N eutrophication. The direction of the species replacement upon N eutrophication, however, will depend on P availability. The species replacement may even lead to higher P retention of the system via the positive feedback of plants to nutrient availability.

Thus, *balance matters*. When P availability is relatively low, N eutrophication may have an extra impact on grassland ecosystems by increasing root death and nutrient loss from the rhizosphere in the relatively short term. N eutrophication may, in the longer term, alter species composition of plant communities, but the direction of the species shift may depend on the relative availability of P.

This brings up suggestions for future research to advance our knowledge on eutrophication effects on grassland ecosystems. We have suggested that inter-specific variations in functional traits play a role in determining plant community structure from P- to N-limited ecosystems. Here the question arises how the selection pressure on an evolutionary time scale has caused the observed variations in species traits. More specific questions include:

- Have trade-offs between functional traits resulted in a certain strategy of species adapted to different types of nutrient limitation?
- Is the direction of the trade-offs different between regions with relatively young, post-glacial soil (where P limitation is prevailing, e.g. temperate Europe) and those with older soil (e.g. tropics)?
- How do reproductive strategy and growth strategy, which are likely in a trade-off relationship, shapes community structure in spatially heterogeneous environment in terms of N:P stoichiometry?

Linking the evolutionary mechanisms to the observed ecological patterns and processes will help us to better understand the N:P stoichiometric effects on ecosystems.

Summary

Human interference in Nitrogen (N) and Phosphorus (P) cycles is a major impact on the Earth's environment. In Europe it is well recognized that increased atmospheric N deposition causes deterioration of grassland biodiversity. Increased P availability is also assumed to impose negative impacts on grasslands, especially on the presence of threatened species.

A well known effect of eutrophication on grassland ecosystems is an increase of above-ground productivity, which intensifies the competition for light and thus benefits only a few competitive species, resulting in loss of species diversity. Besides, eutrophication may also have additional impacts on grassland ecosystems by changing the balance between the availability of multiple nutrients, such as N and P. It is known that plant individuals grow differently in response to a changing balance between N and P. Furthermore, there are a number of field observations which indicate different effects of N- and P-limitation on plant community structure and biodiversity. However, our knowledge is still too incomplete to link the individual- and community-level responses of plants to N:P stoichiometry.

This thesis aims at unraveling the effects of N:P stoichiometry (i.e. the balance between N and P in terms of availability and requirement) on the functioning of grassland plants at the individual plant species level as well as at the plant community level. How do different plant species respond to a changing N:P stoichiometry? Which functional traits influence the N:P stoichiometric effects on plant growth? Do inter-specific differences in these traits play a role in shaping the species distribution patterns along a gradient from N- to P-limited grasslands? How does N:P stoichiometry influence community- and ecosystem-level properties, such as plant species competition, species composition, species diversity, and ecosystem nutrient retention? Answering these questions will help us understand the mechanisms with which N eutrophication affects functioning of grassland ecosystems via N:P stoichiometry effects, which provide necessary information for ecosystem conservation.

First, the individual-level responses of plants to changing N:P stoichiometry were studied (chapter 2 and 3). For this, eight grassland species (five grasses and three herbs), including those common in N- and P-limited grasslands, were grown in greenhouse fertilization experiments. In chapter 2, I examined interspecific differences in plant P uptake from not readily available P sources (bound-P) under different levels of N supply. Only a few species could take up a similar amount of P from bound-P to that from the readily available P source (orthophosphate). These species had higher relative phosphatase activity (i.e. amount of phosphatase activity relative to biomass size) than other species, and tend to occur in P-limited grasslands. This indicated that phosphatase activity plays a role in shaping species occurrence patterns from N- to P-limited grasslands. Additionally, I found that increased N supply stimulated phosphatase activity due to the N:P stoichiometric effect, rather than due to the effect of single N availability. The stimulated phosphatase activity by high N supply, however, did not lead to increased P uptake of plants,

possibly because of decreased root mass ratio and decreased specific root length triggered by high N supply.

Furthermore, in chapter 3, the physiological and growth responses of plant species to changing N:P stoichiometry were examined for two years. Here the same eight plants were grown with readily available forms of N and P, with three N:P supply ratios under two overall nutrient supply levels. In the first year plant growth was similarly reduced at high and low N:P supply ratios, whereas in the second year the growth was reduced especially at high N:P supply ratio. The low growth of the second year was due to increased death rate of below-ground biomass, which was influenced by high N:P ratio rather than overall nutrient supply level. Inter-specific differences were evident especially in the nutrient loss and P uptake under high N:P supply ratio. The species common in P-limited grasslands, however, had neither less P loss nor higher P uptake under high N:P supply ratio.

Subsequently, in chapter 4, the associations between N:P stoichiometry and plant community structure were explored, by analyzing a large field dataset of plant species distribution and functional traits of these species for Eurasian herbaceous ecosystems. Species richness had a weak hump-shaped relationship with N:P ratios of above-ground plant biomass, whereas the number and percentage of threatened species was strongly and positively related to the N:P ratios. Furthermore, species occurrence along a N:P gradient was associated with their reproduction strategies: i.e. species with less dependency on seed reproduction (i.e. small seed number, short flowering period, perennials) occurred more often in P-limited grasslands. The richness of threatened species in P limited grasslands was partly explained by their common reproduction strategy which depends less on seed reproduction.

To link the individual- and community-level responses of plants to N:P stoichiometry, a modeling study was conducted (chapter 5). The model assumes that species have an adaptive trait to P-limitation (i.e. phosphatase activity) as well as plastic responses to changing nutrient availability (i.e. flexible nutrient concentrations in biomass). With the model I examined how interspecific differences in the adaptive trait affect species competition as well as ecosystem P retention under increasing N input at different P input levels. It was found that, in N-limited conditions species with low phosphatase activity always win, whereas in P-limited conditions species with high phosphatase activity win unless under very eutrophic conditions (i.e. inputs of both N and P are above a certain level). Moreover, a possibility was shown that increased N input may increase ecosystem P retention via altering species composition, if there is a trade-off between their phosphatase activity and growth rate.

This thesis provides several implications for ecosystem conservation. Threatened species are observed to occur more frequently in P-limited than in N-limited grasslands. This indicates that maintaining (relatively) low availability of P is important for biodiversity conservation. Does this also mean that N eutrophication can benefit grassland biodiversity via stimulating P limitation? This possibility was not supported by our analysis on current and historical frequency distributions of herbaceous ecosystems along a gradient of N:P ratio (chapter 4). The analysis suggests that in regions with high atmospheric N deposition ecosystems did not shift to be more P-limited than before, but rather be more N-limited and productive. Thus, it is not advisable to expect a positive effect of N eutrophication on richness of threatening species.

Conservation measures for grassland ecosystems often have asymmetric effects on biogeochemical cycles of N and P. The N:P stoichiometric effects on plants, therefore, should be explicitly considered when the effectiveness of these measures is assessed.

This thesis shows the effects of changed N:P stoichiometry on grassland ecosystems in terms of plant responses at several organization levels as well as on different time scales. In the short term (< a few years), plants show contrasting responses to P-limitation and N-limitation, which can be attributed to higher plasticity of P in plant biomass than that of N. More specifically, high N:P ratio in supply induces a drastic decrease in P concentrations in plant biomass, which leads to severe nutrient loss from dead roots. Note that feedback of plants to enhance P uptake under P limited conditions, such as that via phosphatase activity, may mitigate those negative effects of strong P limitation. In the longer term (> decades), the changed balance between N and P availability may influence the competitive hierarchy among species which have different functional traits, and therefore alter species composition. For example, relatively high N availability may benefit species with advantageous characteristics under P -limitation, such as efficient P uptake traits or reproductive strategies with low P requirements. The shift in species composition caused by relatively high N availability may eventually lead to an increase in P retention of the ecosystems.

In conclusion: balance matters. When P availability is relatively low, N eutrophication may have an extra impact on grassland ecosystems by increasing nutrient loss from the rhizosphere in the short term. N eutrophication may, in the long term, alter species composition of plant communities, but the direction of the species shift may depend on the relative availability of P.

Samenvatting

Menselijk ingrijpen in stikstof- (N) en fosforcycli (P) heeft grote gevolgen voor het milieu van de aarde. Zo leidt verhoogde atmosferische N-depositie in Europa tot een afname van de diversiteit aan plantesoorten in graslanden. Er zijn ook sterke aanwijzingen dat verhoogde P-beschikbaarheid een negatieve invloed heeft op graslanden, met name op de aanwezigheid van bedreigde plantesoorten.

Een bekend effect van eutrofiëring van grasland ecosystemen is een verhoging van de bovengrondse plantengroei (primaire productie). Dat versterkt de concurrentie om zonlicht, wat vervolgens resulteert in een afname van het aantal soorten. Daarnaast kan eutrofiëring gevolgen hebben voor de soortsamenstelling in grasland ecosystemen doordat de balans tussen de beschikbaarheid van verschillende nutriënten, zoals stikstof en fosfor, verandert. Plantengroei hangt namelijk ni*et all*een af van de totale beschikbaarheid van nutriënten, maar ook van de balans tussen N en P. Bovendien is in het veld waargenomen dat schaarstes in N en P verschillende effecten hebben op de structuur en biodiversiteit van plantengemeenschappen. Onze kennis is echter nog onvolledig om de reacties van individuele planten of plantengemeenschappen ten opzichte van deze zogenaamde N:P stoichiometrie goed te kunnen begrijpen.

Dit proefschrift richt zich op het ontrafelen van de effecten van N:P stoichiometrie (dwz de balans tussen N en P in relatie tot hun beschikbaarheid en behoefte) op het functioneren van graslandplanten op zowel soorts- als gemeenschapsniveau. Vragen die aan de orde komen zijn: Hoe reageren verschillende plantesoorten op een veranderende N:P stoichiometrie? Welke eigenschappen van planten worden beïnvloed door N:P stoichiometrische effecten? Welke verschillen tussen plantesoorten bepalen de soortensamenstelling langs een gradiënt van N- naar P-gelimiteerde graslanden? Hoe beïnvloedt N:P stoichiometrie de concurrentie tussen plantesoorten, soortensamenstelling, soortendiversiteit en nutriëntenretentie in een ecosysteem? Door het zoeken naar antwoorden op deze vragen heb ik geprobeerd de mechanismen te begrijpen waarmee N-eutrofiëring de soortenrijkdom in graslanden beïnvloedt. Dit begrip is nodig om grasland -ecosystemen beter te kunnen beschermen en de biodiversiteit te behouden.

In de eerste hoofdstukken heb ik de reacties van planten op individuniveau op een veranderende N:P stoichiometrie bestudeerd (hoofdstuk 2 en 3). Hiervoor zijn acht graslandsoorten (vijf grassen en drie kruiden), inclusief veel voorkomende soorten in N- en P-gelimiteerde graslanden, bestudeerd door middel van bemestingsexperimenten in de kas. In hoofstuk 2 heb ik de interspecifieke verschillen bestudeerd in P-opname van planten uit niet direct beschikbare P bronnen (gebonden -P) met verschillende niveaus van N-toevoer. Slechts enkele soorten konden eenzelfde hoeveelheid P uit gebonden-P opnemen in vergelijking met de opname uit een direct beschikbare P-bron (orthofosfaat). Deze soorten hadden een hoge fosfatase activiteit en komen gewoonlijk voor in P-gelimiteerde graslanden. Dat wijst op de belangrijke rol die het enzym fosfatase speelt in het aanboren van moeilijk beschikbare P-bronnen en de veranderingen in soortensamenstelling in de overgang van N- naar P-limitatie in graslanden die daarmee gepaard gaat. Daarnaast vond ik dat een verhoging van de N-toevoer de fosfatase activiteit stimuleert via een verhoging van de N:P verhouding, en niet door het enkelvoudige effect van N-beschikbaarheid. De door hoge N-toevoer gestimuleerde fosfatase activiteit leidde echter niet tot een verhoging in P-opname door planten. Dat is het gevolg van een verlaagde wortel:massa ratio en een verlaagde specifieke wortellengte. In hoofdstuk 3 beschrijf ik mijn studie naar de effecten van een veranderende N:P stoichiometrie gedurende twee jaar op de fysiologie en de groei van plantesoorten. Hiervoor gebruikte ik dezelfde acht plantesoorten en varieerde ik het aanbod van direct beschikbare vormen van N en P, met drie N:P toevoer ratios en twee totale nutriëntenniveaus. In het eerste jaar nam de groei af bij zowel een verhoging als verlaging van de N:P toevoer ratio, terwijl in het tweede jaar de groei met name gereduceerd werd bij een hoge N:P toevoer ratio. De lage groei in het tweede jaar kwam door een verhoogde sterfte van ondergrondse biomassa. Deze ondergrondse biomassa wordt sterker beïnvloed door een hoge N:P ratio dan door het absolute niveau van de nutriëntentoevoer. Interspecifieke verschillen tussen de plantesoorten kwamen met name naar voren bij nutriëntverlies en P-opname onder een hoge N:P toevoer ratio. De algemeen voorkomende soorten in P-gelimiteerde graslanden hadden echter noch minder P-verlies noch hogere P-opname onder een hogere N:P toevoer ratio.

In hoofdstuk 4 beschrijf ik de relaties tussen N:P stoichiometrie en de structuur van plantengemeenschappen op basis van een analyse van een groot veldgegevensbestand van soortenverspreiding en functionele eigenschappen van deze soorten in Euraziatische planten ecosystemen. Soortenrijkdom vertoont een zwakke, *hump*-vormige relatie met N:P ratios van bovengrondse plant biomassa, terwijl het aantal en het percentage bedreigde soorten sterk toenam met N:P ratios. Bovendien bleek dat het voorkomen van soorten langs een N:P gradiënt afhankelijk is van hun reproductiestrategieën: dwz soorten die minder afhankelijk zijn van zaadreproductie (laag aantal zaden, korte bloeiperiode, meerjarig) komen vaker voor in P-gelimiteerde graslanden dan soorten die meer afhankelijk zijn van zaadproductie. De rijkdom van bedreigde soorten in P-gelimiteerde graslanden kan verklaard worden uit hun vergelijkbare reproductiestrategie, d.w.z. deze is minder afhankelijk van reproductie dmv zaden. Dat deze strategie in P-gelimiteerde ecosystemen voordelig is, zou samen kunnen hangen met de relatief grote hoeveelheden P die nodig zijn voor de vorming van reproductieve plantenorganen zoals bloemen en zaden.

Om de respons op individueel en gemeenschapniveau te koppelen aan N:P stoichiometrie, heb ik een modelstudie uitgevoerd (hoofstuk 5). In de modelformulering is aangenomen dat soorten een adaptieve eigenschap hebben ten opzichte van P-limitatie (dwz fosfatase activiteit) en ook een plastische respons ten opzichte van veranderende nutriëntenbeschikbaarheid via flexibele nutriëntenconcentraties in hun biomassa. Met het model onderzocht ik hoe interspecifieke verschillen in deze eigenschappen de concurrentie tussen soorten beïnvloeden als ook de mate van ecosysteem P-retentie onder verhoogde N-input en verschillende niveaus van P-input. De modelresulaten laten zien dat, onder N-gelimiteerde condities, soorten met een lage fosfatase activiteit altijd winnen, terwijl in P-gelimiteerde condities soorten met een hoge fosfatase activiteit winnen mits onder zeer eutrofe condities (zowel N als P boven een bepaald niveau). Bovendien gaf het model aan dat een verhoogde N-input de P-retentie in het ecosysteem zou kunnen verhogen door een veranderde soortensamenstelling. Dat laatste gebeurt alleen als er een trade-off is tussen fosfatase activiteit en groei.

De resultaten van mijn studie hebben een aantal implicaties voor het behoud van soortenrijke graslanden. Dat bedreigde soorten vaker voorkwamen in P-gelimiteerde dan in N-gelimiteerde graslanden, betekent niet dat N-eutrofiëring (wat immers in principe kan leiden tot P-limitatie) een positief effect op de biodiversiteit van graslanden zal hebben. De analyse van de huidige soortensamenstelling langs een N:P gradiënt, in combinatie met de geschatte grootte van de soorten*pool* langs dezelfde gradiënt, suggereert namelijk dat een hoge atmosferische N-depositie deze ecosystemen niet heeft verschoven naar P-limitatie (hoofdstuk 4). Dat laatste wijst er op dat de positieve terugkoppeling van een verhoogde N-input op P-beschikbaarheid via gestimuleerde P-opname door planten, resulteert in een verhoogde productiviteit, wat er vervolgens weer toe kan leiden dat snelgroeiende soorten minder competitieve soorten, zoals bedreigde soorten, kunnen wegconcurreren.

Beheersmaatregelen voor grasland ecosystemen hebben vaak asymmetrische effecten op biogeochemische cycli van N en P. De N:P stoichiometrische effecten op planten moeten daarom expliciet meegenomen worden wanneer de effectiviteit van zulke maatregelen wordt beoordeeld.

Dit proefschrift laat de effecten van een veranderde N:P stoichiometrie zien van grasland ecosystemen in termen van plantrespons op verschillende organisatieniveaus als ook op verschillende tijdschalen. Op de korte termijn (dwz < een paar jaar) zal een hoge N-beschikbaarheid ten opzichte van P-beschikbaarheid een snelle verlaging in P-concentraties in plant biomassa veroorzaken, wat vervolgens leidt tot ernstig nutrientverlies uit dode wortels. Op de lange termijn (> tientallen jaren) zou de veranderde *balans* tussen N- en P-beschikbaarheid de soortensamenstelling kunnen beïnvloeden. Bijvoorbeeld, een relatief hoge N-beschikbaarheid zou soorten met gunstige eigenschappen onder P-limitatie, zoals eigenschappen voor efficiënte P-opname of reproductieve strategieën met een lage P-behoefte, kunnen bevoordelen. De verschuiving in soortensamenstelling veroorzaakt door een relatief hoge N-beschikbaarheid kan uiteindelijk leiden tot een verhoging van de P-retentie in ecosystemen.

Samenvattend: evenwicht doet er toe (balance matters). Als P-beschikbaarheid relatief laag is, kan N-eutrofiëring een extra effect hebben op grasland ecosystemen door een verhoogd nutriëntenverlies uit de rhizosfeer op de korte termijn. N-eutrofiëring kan op de lange termijn de soortensamenstelling van plantgemeenschappen veranderen. Maar de richting van de soortenverschuiving hangt af van de relatieve beschikbaarheid van P.

バランスの重要性
– 草原生態系における窒素対リン比と植物群集の生物多様性 –

人為的撹乱による窒素やリン循環の変化は、地球環境に重大な影響を与える。ヨーロッパでは、大気中の窒素蓄積による草原の生物多様性の低下が知られている。さらに、リン供給量の増加が草原へもたらす負の影響（絶滅危惧種の減少など）についても懸念されている。

草原生態系に対する富栄養化の影響については、以下のメカニズムが知られている。富栄養化による地上部生産量の増加に伴い、光競争が激化し、競争に有利な少数の種のみが生き残り、結果として生物多様性が低下する。また富栄養化は、栄養素の相対的な化学量—たとえば窒素対リン比—を変化させることからも草原生態系に影響を与える。例えば、植物個体は変動する窒素対リン相対量に対して特異的な反応を示すことが知られている。さらに、窒素制限とリン制限が生物多様性や種間競争といった植物群集の特性に対照的な影響を与えることも野外観察から示されている。しかしながら、草本種の窒素対リン比に対する反応が個体レベルと植物群集レベルでどのように関連しているかについては十分に明らかにされていない。

本研究では窒素とリンの相対量が草原性草本種の機能に与える影響について植物個体レベルおよび群集レベルで議論する。植物個体は異なる窒素対リン比にどのように反応するのか、そしてその反応に種間差はあるのか。これら個体レベルでの窒素対リン比への反応は種の機能的特性によって説明できるのか、また、その機能的特性は窒素制限からリン制限への栄養塩勾配に沿った種分布パターンの規定要因となりうるのか。窒素対リン比は種間競争、種構成、種多様性などの群集レベルの特性や栄養塩類の滞留時間などの生態系レベルでの特性にどのように影響するのか。これらの疑問を検証することに

より、窒素を主体とした富栄養化が窒素対リン比の変動を介して草原生態系に与える影響についての理解を深め、生態系保全への提言を目指す。

第二章及び第三章では、窒素制限及びリン制限の草原に典型的な種を含む８種の草原種を対象にした複数の温室実験により、窒素対リン比に対する植物個体レベルでの反応を検証した。

第二章では、異なる窒素供給レベル下の不可給態リン及び可給態リンからのリン摂取およびその種間差について検証した。大半の種では、不可給態リンよりも可給態リンからのリン摂取量が多い傾向がみられたが、特定の数種ではリン摂取量に差が見られなかった。これらの種はバイオマス量に対するホスフォターゼ活性が高いという特徴をもち、また、リン制限の草原に典型的な種であった。よって、ホスフォターゼ活性は、窒素制限草原からリン制限草原への種出現パターンを規定する一因であることが推測される。さらに、異なる窒素供給レベルで比較すると、窒素供給量の増加とともにホスフォターゼ活性に増加が見られ、これは窒素単体の供給量レベルよりむしろ窒素対リン供給量比の上昇によって引き起こされたことが示された。一方、窒素供給量増加に伴い根の重量比および比根長は減少した。よって、結果として、窒素供給量増加に伴うホスフォターゼ活性の上昇が植物のリン摂取の増加を引き起こすことはなかった。

第三章では、同８種の窒素対リン供給量比に対する生理学的反応および成長反応について２年間の温室栽培実験で検証した。本実験では可給態の窒素とリンを用い、二段階の供給量レベル（低・高）に対し三段階の窒素対リン供給量比（低・中・高）の計六処理区を設定した。植物の成長は、一年目には高供給量比処理区と低供給量比処理区で同様に減少したが、二年目には、高供給量比処理区で著しく減少した。二年目の成長の減少は地下部バイオマスの死亡率の増加に起因しており、この死亡率の増加は供給量レベルでなく供給量比に影響されていた。高供給量比下では、栄養損失率およびリン摂取量において特に顕著な種間差が見られた。リン制限草原の典型的な種に対し高

供給比下における低い栄養損失と高いリン摂取を期待したが、そのような傾向はみられなかった。

第四章では、窒素対リン比と植物群集構成の関係ついて検討した。ここではユーラシア大陸の草本性生態系の大規模野外データを用い、窒素対リン比勾配に沿った種分布パターンと種の機能的特性との関連性について解析した。種多様性と群集バイオマス内の窒素対リン比との関係は弱い単峰型パタンを示した。一方、絶滅危惧種の種数と優占率は、窒素対リン比と強い正の相関関係を示した。窒素対リン比勾配に沿った種出現傾向は、種の繁殖戦略と関連していた。つまり、種子繁殖に対する依存性が弱い種（少ない種子生産量、短い開花期間、多年草）はリン制限の草原により多く出現することが示された。絶滅危惧種は種子繁殖への依存性が弱い傾向が見られ、このことがリン制限の草原における絶滅危惧種の豊富さを説明する一因であると考えられる。

第五章では、個体レベルと群集レベルでの窒素対リン比に対する反応を関連付けるために、モデル研究を行った。本モデルでは、リン制限に対する適応的特性としてホスフォターゼを仮定し、さらに、変動する栄養塩供給量に対する種の可塑的な反応としてバイオマス内の窒素とリン濃度の柔軟性を仮定した。このモデルを用い、ホスフォターゼ活性の種間差が種間競争および生態系のリンの滞留にどう影響するかについて、異なる窒素供給量及びリン供給量下で検証した。窒素制限下では、ホスフォターゼ活性の低い種が常に優占した。一方、富栄養条件下以外のリン制限下では、ホスフォターゼ活性の高い種が優占した。さらに、ホスフォターゼ活性と成長速度の間にトレードオフ関係を想定した場合、窒素供給量の増加が、種構成を変化させることにより、生態系のリンの滞留を増加させる可能性があることが示唆された。

本研究によって、生態系保全に対して重要な知見が得られた。まず、絶滅危惧種はリン制限の草原により多く出現することが示され、草本種の多様性維

持にとってリン供給量を（相対的に）低く抑えることの重要性が示唆された。これにより、窒素富栄養化はリン制限を促進することによって草原の生物多様性を高めていると言えるのだろうか？窒素対リン比勾配に沿った現在と過去の草原の頻度分布を比較したところ、この可能性は支持されなかった。すなわち、大気中の窒素蓄積量が多い地域ではリン制限の生態系は過去に比べて増加しておらず、むしろ、窒素制限かつ高生産性の生態系が増加していることが示唆されたのである（第四章）。したがって、窒素富栄養化は競争力の弱い絶滅危惧種のような種には有利な状況をもたらさないと考えられる。草原生態系における保全策の多くは、窒素とリンの生物地化学的動態に非対称な影響を与えることが報告されている。したがって、保全策を評価する際に、窒素対リン比を変化させることで生じる付随的な影響についても十分に留意する必要がある。

本研究では、機能的特性、個体、種間相互作用及び群集レベルといった異なるレベルにおける窒素対リン比が草原生態系に与える影響を、時系列変化を含めて検証した。短期的（数年以内）には、植物は窒素制限とリン制限に対して対照的な反応を示したが、この対照的な反応は体内のリン濃度の可塑性が窒素濃度に比べて高いことから説明できた。すなわち、窒素供給量がリン供給量より相対的に多い場合、植物体内のリン濃度が急激に低下し、枯死根からの甚大な栄養塩損失につながる。ただし、リン制限下の植物はホスフォターゼなどによってリン摂取を促進させることができることから、上記のリン制限による甚大なダメージは植物のフィードバック作用によってある程度は緩和されるであろう。長期的（数十年以上）には、窒素とリンの相対的な供給量が異なる機能的特性を持つ種間の競争関係に影響を与え、種構成が変化する可能性が示唆された。例えば、相対的に窒素供給量が高い場合、リン制限下で有利な特性を持つ種（効率的なリン摂取やリン要求量の少ない繁殖戦略を持つ種など）に有利に働くことが考えられる。このような種構成の変

化の結果として、窒素富栄養化が生態系のリンの滞留の増加を促す可能性もある。

バランスは重要である。リン供給量が相対的に低い時、窒素富栄養化は短期的な根圏からの栄養塩損失を促すなど、草原生態系にさらなる悪影響を与えかねない。長期的には、窒素富栄養化は植物群集の種構成を変化させる可能性があるが、その変化の方向性は相対的なリンの供給量に左右されるであろう。

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Curriculum Vitae

Yuki Fujita was born on July 13th 1976 in Fujinomiya, Japan. She grew up in Fuji city, at the foot of Mt. Fuji, and graduated from Fuji high school. In 1995 she started to study Forestry in the Department of Agriculture in Kyoto University, from which she obtained a Bachelor degree of Agriculture in Agro-Environmental Science in 1999. After graduation she started to study tropical forest ecology in the graduate school of Agriculture, Kyoto University. During her study she stayed for ca. 1.5 years in Jambi, Indonesia, as a guest student of Gadjah Mada University. There she conducted her master thesis research on habitat preference of small mammals in a tropical lowland forest. She obtained a M.Sc. degree of Agriculture in Forest and Biomaterials Science in 2001. In 2002 she received the ambassador scholarship from Rotary International, which enabled her to do her second master study in Forest and Nature Conservation in Wageningen University. During that study she learnt ecological, sociological and economical aspects of forest conservation. Also, she conducted a research project on spatial modeling of dispersal of small mammals in fragmented landscapes. She obtained her second M.Sc. degree *cum laude* in 2004. In 2005 she started her PhD research in Landscape Ecology group and in Environmental Science group of Utrecht University, funded by Utrecht Center of GeoSciences. The results of this PhD study are presented in this thesis. During the PhD study she supervised several bachelor and master students for their research projects with varying duration. From April 2010 she works as an Ecology Researcher at KWR Watercycle Research Institute, Nieuwegein.

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