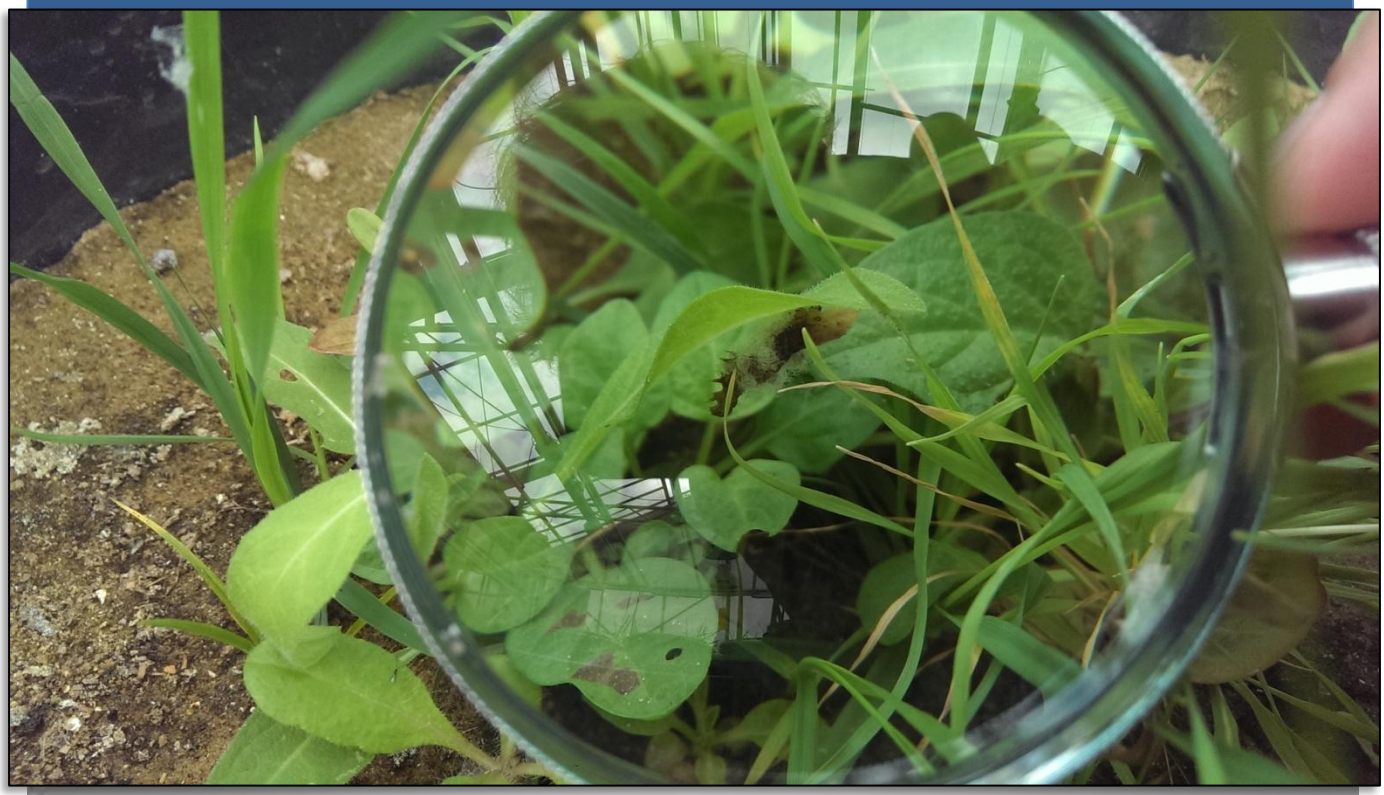
Nutrient availability and plant community composition of N-limited grasslands

A greenhouse experiment into the driving factors behind species composition



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To my grandfather

a man who has always challenged me and inspired my quest for knowledge since childhood

Abstract

Species richness is very important for the perseverance of Dutch grasslands. As eutrophication is one of the major threats to grasslands worldwide, more research is needed into the validity of accepted mechanisms and their ability to explain changes in species composition. The primary goal of this experiment was to give insights into the reaction of N-limited plant communities on eutrophication. This was done by measuring competition and productivity traits of three different functional groups, namely N-specialists, P-specialists and opportunists. Secondly was tried to find supporting evidence that these functional groups are useful in explaining plant community composition dynamics. To provide these results, a nine week during greenhouse experiment was set up using 1125 plants in these three specialisms. N-specialists used were *Rumex acetosa, Crepis capillaris, Trisetum flavescens* and *Alopecurus pratensis*. *Succisa pratensis, Centaurea jacea, Briza media* and *N. stricta* were used as P-specialists and finally *Prunella vulgaris, Knautia arvensis, Carex oederi* and *Agrostis capillaris* compiled the list of opportunists. All species were germinated and put into equal communities, which were divided over low and high nutrient levels under strong N-limitation (N:P < 5). Measurements show no differences in species richness and abundance levels for the different specialisms came back around 33%. Evenness also showed no sign of uneven distribution among species or specialism. Results for the productivity trait show that N-specialists produce more above ground biomass than P-specialists and opportunists. This was also observed for the light competitions traits (canopy height & stretched leaf length). CSR-strategy was compared with the specialism groups but gave no conclusiveness as to validity of either classification. In general this research shows that eutrophication leads to higher expression in productivity and light competition traits. The relative difference between specialism was more clearly visible under higher nutrient levels. Where N-specialists perform better than P-specialists and opportunists, the differences between the latter two is minimal. However, it is not unlikely that some experimental shortcomings have caused the obscure results regarding P-specialists and opportunists. More evidence for the validity of these functional groups is needed and can be provided by research into these specialisms and their reaction on changing nutrient stoichiometry.

Contents

Introduction

Background

Dutch grasslands are appealing and can contain a high biodiversity level for flora and fauna. Grassland communities in the Netherlands host a lot of now nationally endangered species (Bobbink & Willems, 1993). Species composition from a conservational point of view is of very high importance, since eco-system resilience is dependent on species composition (Peterson et al., 1997). Indirect effects of grassland composition include the facilitation of habitats for various types of insects (Tscharntke & Greiler, 1995). These grasslands diminished in numbers when agricultural land use changed and artificial fertilisers were introduced (Willems, 2001). At present, grasslands in Europe are mainly threatened by eutrophication & acidification. Part of the eutrophication is caused by phosphorous enrichment and is occurring at rapid pace (Olde Venterink et al., 2003), introducing a shift in grasslands from phosphorus (P) limited systems to Nitrogen (N) limited systems.

Theories & Conceptual model

Eutrophication

The fertiliser use described above initiated changes in nutrient stoichiometry and availability, the driving factors behind species richness (Olde Venterink et al., 2001). In general the shift mainly induces eutrophication which coincides with a reduction in species richness (Harpole & Tilman, 2007; Hautier et al., 2009). The excess of nutrients can have multiple sources, both internal and external. External sources include atmospheric deposition and fertiliser use (Ceulemans et al., 2013; Willems, 2001). Systems can also be internally enriched by making nutrients available to the plants. Smolders et al. (2006) state that the waterlogging of fields with sulphate rich water mobilises phosphorus. Furthermore, drainage also enlarges net nutrient release (Bridgham et al., 1998). Once eutrophication has taken place it is hard to reverse the effects because decrease of niche dimensionality reduces the number co-occurring species. Simplified habitats instated by an increase in limiting nutrients also lead to higher productivity which can have indirect negative effects on biodiversity (Harpole & Tilman, 2007).

Competition & Community composition

Research by Tilman (1982; 1988; 1997) already mentions interspecific competition as an important factor in plant community development. Hautier et al. (2009) show that light is the main primary resource for which competition takes place. By providing light to the understory in artificially eutrophicated systems biodiversity is maintained, showing that without the light some species would have outcompeted other. Other primary resources such as nutrients, in particular phosphorus and nitrogen, limit growth in natural terrestrial plant communities (Elser et al., 2007). Depending on the type of limitation different species are expected to thrive (Ceulemans et al., 2013; Wassen et al., 2005). When N and P are available in extensive amounts as described in the previous paragraph, the enriched system enables competitive growers to dominate, whereas low nutrient availability favours slow growing conservative species. Competitive growers thrive because they gain the upper hand in light competition and are better in the uptake and use of nutrients (Hautier et al., 2009; Tilman, 1997). Driving mechanisms in competition are extensively studied but the role of nutrient availability and stoichiometry remains unclear (Güsewell & Bollens, 2003)

Mechanisms

Cardinale et al. (2009) and Olde Venterink (2011) combined several mechanisms to understand the different roles nutrient stoichiometry and availability, plant traits and the species pool have in the determination of plant community composition. Cardinale et al. (2009) published a study based on three theories: the species energy theory, resource ratio theory and biodiversity & eco-system functioning. These are used to create a multivariate approach to give insights into the productivity-diversity relations within plant communities. The species energy theory (SET) states that the total available energy present determines the biomass production and species diversity. Available energy is defined as the production rate of resources used by the species (Wright, 1983). Secondly, the resource ratio theory (RRT) argues that increase in one nutrient puts constraints on another (Tilman, 1982). Species are regarded to be more specialised in one type of nutrient. This provides that balanced nutrient supply can facilitate coexistence (Cardinale et al., 2009). The third theory is named biodiversity and eco-system functioning (BEF) and focuses on the influence of diversity on biomass production (Tilman et al., 1996). Cardinale et al. (2009) provides significant evidence proving these individual theories are lacking at different points in full coverage of the productivity-diversity relations. However, combined availability of nutrients, stoichiometry of nutrients, summed biomass of competitors and the richness of coexisting species are the determining factors in productivity-diversity relations in plant communities.

Olde Venterink (2011) makes plant traits explicitly important where they are implied in the theories reviewed by Cardinale et al. (2009). Nutrient acquisition traits together with the available nutrients determine the availability of the limiting nutrient and are thus crucial to the SET. Secondly, he states that mechanisms and traits influence species competition and coexistence under N and P limitation (e.g. root length, phosphatase production and root mortality). Thirdly, the size of the regional species pool (larger pools show more differentiation specific to the N or P limited system). And finally, interactions with the productivity of the vegetation provide a check for species richness. Where some communities might be more productive under N-limitation others might produce more biomass under P-limitation. These system states can be altered by input of one or the other nutrient which can have major effects on the diversity.

Thus, there are several mechanisms believed to influence the species composition within a plant community. Major concepts regarded as driving factors are nutrient availability and stoichiometry and the present species itself (represented as A, B and C respectively in figure 1). These processes influence each other by plant traits regarding nutrient acquisition, productivity and light competitions. Rhe relation between traits and mechanisms is given in figure 1.

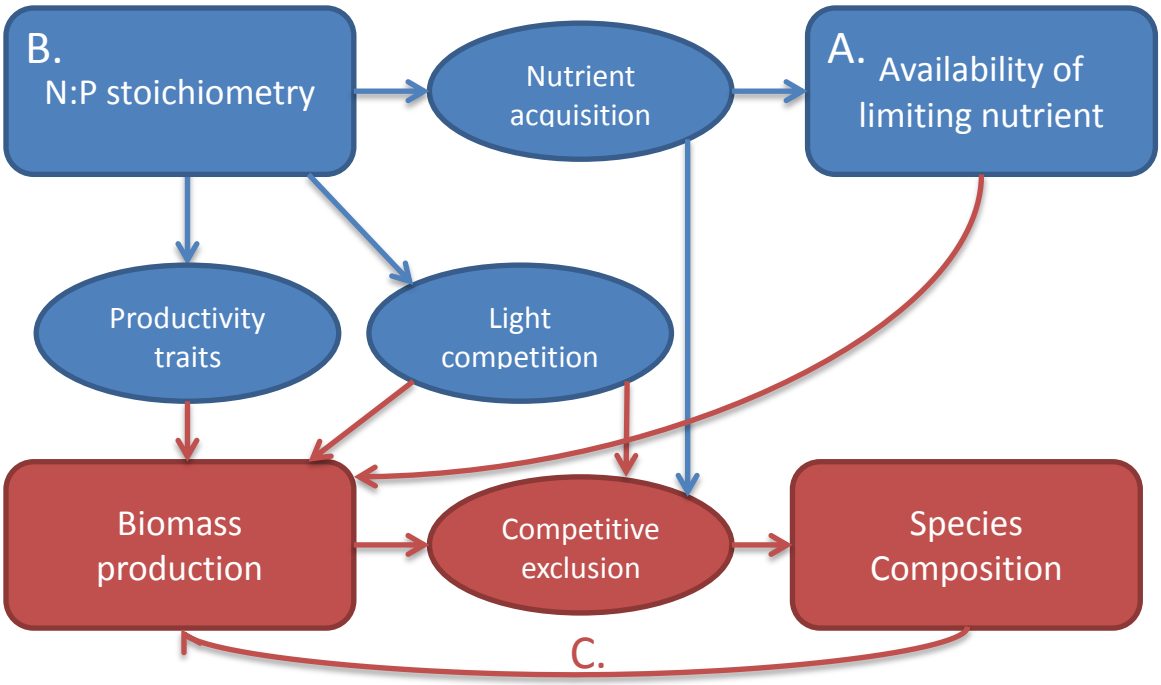

Figure 1. Schematic overview based on Olde Venterink (2011) and Cardinale et al (2009) of direct and indirect relations within the framework, in which evolutionary influences are removed. Nutrient forms and regional species pool are also removed as they are kept and non-limiting and constant respectively in this research. A depicts species energy theory, B shows resource ratio theory and C depicts that biomass production is dependent on the types of species present in the community. The specific pathways measured in this experiment are given in red.

Functional Groups

Plant community composition can be seen as a result of competition outcome. Species composition has been hypothesised to differ between N- and P-limited systems under equal productivity values (Olde Venterink, 2011), implying that there is more to it than competition for light alone. Wardle et al. (2003) hypothesised that functional groups can be made in which plants would react similarly. They tested the influence of forbs, grasses and N-fixing legumes on the different trophic levels of a community, but found interspecific differences to be much bigger and not coherent within groups. However, on the basis of the relations shown in figure 1 differences and similarities in plant traits can still be a way to categorise species.

Relations have been identified between nutrients and the plant traits shown in figure 1. To start with overall nutrient availability is related to the traits regarding light competition (e.g. canopy height). Secondly, N:P stoichiometry and nutrient availability relate to productivity traits (e.g., leaf mass & above ground biomass). Thirdly N:P stoichiometry is related to nutrient acquisition traits (e.g. above ground N & P concentration) (Olde Venterink, 2011). It is thus not unlikely that functional groups of some sort categorise species reactions to environmental conditions and are a driving factor for competitive advantage and community development. Grime first developed his functional groups theory in 1974 (Grime, 1974). His theory distinguishes three groups: competitors (C), stress tolerators, (S) and ruderals (R). Competitors are species that are high in productivity and thrive in habitats of low disturbance. They usually monopolise resource acquisition at a rapid pace by spatially dynamic foraging of roots (competition trait). Stress-tolerators also live in habitats of low disturbance but are associated with low productivity. Their long lived tissues are more resistant to herbivory and environmental extremes. Furthermore, they dominate where growth is restricted by low nutrient levels. Ruderals are associated with

high productivity and large disturbance of habitat. They have a short lifespan and rapid investment in reproduction (Grime, 2006). Although this theory is tested, several species have been identified in two or more of the different groups (Hodgson et al., 1995) which begs the question if there are other, more adequate functional groups.

A research paper by Fujita et al. (2010) states that species have varying growth strategies with typical plant traits that are adjusted to different N:P ratios. For example, species can adjust their P retention or capacity to take up P under P-limited conditions. Furthermore, the three relations defined by Olde Venterink (2011) mentioned above all regard nutrient availability and stoichiometry, an idea that is also acknowledged by Cardinale et al. (2009). This indicates that approaching functional groups with nutrient availability and stoichiometry as determining factor is sensible. However, more research in such a classification is needed (Fujita et al., 2010).

Specialisms

As mentioned above the idea of functional groups can be of high importance in the explanation of plant community dynamics. Considering only N and P, there are three limitation states that occur. Field studies often show P or N as limiting nutrient or an intermediate regime of co-limitation is present (Wassen et al., 2005). Güsewell and Bollens (2003) found that species thrive when they find a way to monopolise the limiting nutrient, conserve it and use the nutrients efficiently. There is a range of traits that can be assigned to plants that thrive in the three different limitation states. They will be discussed below.

N-Specialists (specialists at low N:P)

N-limitation occurs when the nitrogen concentration is limiting the growth of plants. This is unrelated to the absolute quantity of nutrients available to the plant (Vitousek & Howarth, 1991). Fast growing species persist at low N:P ratios because phosphorus is an essential compound of RNA. RNA is used in the cell division and is needed for the growth of the plant. Dominant species under N-limitation are thus light competitors and reproductive oriented species (Fujita et al., 2014). Furthermore, Fujita et al. (2014) found that N-limited sites often host species with a large specific leaf area (SLA) and long roots relating back to the fast growing capabilities. Long roots are also grown to enhance the N uptake and productivity in a N-limited system (Venterink & Güsewell, 2010). Because the sites lack vast quantities of nitrogen, species thriving in N-limited sites have a strong N-fixing ability (Fujita et al., 2014). Endangered species richness in high productive N-limited systems is usually lower than in P-limited or co-limited systems because the competitively strong species outcompete others at high production levels (Wassen et al., 2005).

P-specialists (specialists at high N:P)

In P-limited systems, phosphorus is the limiting nutrient on the growth of plants (Vitousek et al., 2010). Compared to the N-specialists described above, plant species that thrive under P-limited conditions are generally smaller and worse light competitors (can be expressed in canopy height and leaf length). This is hard on more productive sites such as N-limited systems (Fujita et al., 2014). Furthermore, P-specialists have low investment in sexual reproduction because this requires large quantities of P (Fujita et al., 2014). Due to this slow reproduction and conservative way of life these species perform better in the second year of growth (Güsewell & Bollens, 2003). Wassen et al. (2005) found that endangered species also mainly persist under P-limited conditions. The different forms of P could create a niche for trait speciation. Several

species with different traits to acquire these different forms can in this way coexist (Blanck et al., 2011)

Opportunists (non-specific N:P)
Opportunist species are defined by their tolerance to a large variance of abiotic and biotic circumstances. Zedler & Kercher (2004) mention that opportunists have more opportunities for establishing and developing within a plant community than non-opportunists. With respect to nutrient limitation states they are expected not be confined to either N or P limitation to thrive. Different plant traits enable species to exploit the opportunities above. Where endangered species have low canopy height and little investment in sexual reproduction (Fujita et al., 2014) opportunists are likely to excel in these traits. In short, opportunists are not defined by specific phenotypic differences but could express traits parallel to N-specialists in N-limited environments and P-specialists in P-limited systems.

The specialisms described above can function as an indicator as to which species will outcompete others. As mentioned functional groups up to now often place species in multiple groups (e.g. CSR-strategy). Furthermore, experimental research often shows that interspecific differences are big and not coherent with the current forms of classification (e.g. Wardle et al., 2003; Grime, 1974). Since nutrient availability and stoichiometry have been found to be of main importance in mechanisms driving community composition it is to be expected that the different specialists will perform differently under a N-limited nutrient regime. The differences in productivity and competition traits between nutrient levels can provide information about the validity of the classification in N- & P-specialists and opportunists. It is to be expected that N-specialists will show significant larger productivity and competition trait expression than opportunist and P-specialists. Since opportunists are not as strictly bound to environmental conditions as N- or P-specialist they are likely to seize the opportunity and gain advantage over P-specialists.

Relevance

As can be read above, a lot of research has been done regarding nutrients, competition and species composition. Eutrophication by N and P is of high importance, but since most studies have focused on nitrogen, policies tend to be biased towards coping with nitrogen surpluses. Thus, P-availability and enrichment also needs to be taken into account (Ceulemans et al., 2013). The N-limited nutrient regime introduced in this experiment sheds light on a relative P-rich community.

Individual plant traits relevant for species competition have been determined in monoculture experiments (Fujita et al., 2014; Blanck et al., 2003) and field studies (Santiago & Wright, 2007; Al-mufti et al., 1977). Three types of traits of main importance are: nutrient acquisition traits, light competition traits and traits regarding productivity (biomass related, see figure 1) (Olde Venterink, 2011).The effect of nutrient level has been studied on individual species (Güsewell & Bollens, 2003) or in comparison with a limited amount of other species (Venterink & Güsewell, 2010) This said, nutrient level and plant competition traits, plant specialism and their relation to plant community composition have yet to be supported by more evidence (Olde Venterink, 2011).

The studies mentioned above were done on a scale inadequate for extrapolation to field conditions, as natural systems are much more species rich than setups in previous experiments. Stevens et al. (2004) found 7 to 42 species in grasslands and Berendse et al. (1992) found around 20 species on average in species rich meadows in the Netherlands. An inventory of a wide range of Dutch grasslands showed an average of around 13 species (Roeling et al., in prep.). Communities with less than 13 species lose extrapolating strength since species influence each other's competitiveness (Goldberg & Werner, 1983). Hypothetically, species A and B may occur in different ratios to each other in the absence or presence of species C. Increasing the number of species makes even more of such interactions possible. Furthermore, a large amount of species is also needed to balance out interspecific differences shown by Wardle et al. (2003) that may occur within specialism.

Field observations have been done in the past to get insights into species richness, composition and plant traits (Berendse et al., 1992; Fujita et al., 2014; Wassen et al., 2005). But field factors influencing the experiment such as the regional species pool & nutrient forms (Olde Venterink, 2011) weather conditions, grazing and succession can alter species composition (Briske, 1996; Foster & Gross, 1998; Jump & Penuelas, 2005). These factors can be controlled in a greenhouse experiment. Identical community setup, something that is not found in a natural eco-system, provides us with the possibility to give more conclusive evidence as to what extent functional specialisms are driving the community composition. Such research can provide us with vital scientific knowledge about the driving factors of plant community development. This has been a quest among various disciplines (Cardinale et al., 2009). The problem and knowledge gap described above can be synthesised in the following research questions:

Research Questions

This paragraph will introduce the research questions and the corresponding hypotheses, the main research question has been synthesised as follows:

What is the influence of eutrophication on productivity and light competition traits and functional community composition of N-limited grasslands?

1. *What is the influence of nutrient enrichment under controlled conditions on the overall species richness of an N-limited plant community?*
    i. Species richness will be lower at higher nutrient level.

2. *What is the influence of nutrient enrichment under controlled conditions on the community composition of an N-limited community, with respect to N-specialists, P-specialists and opportunists?*
    ii. N-specialists will have higher abundance in a N-limited system than P-specialists and opportunists.
    iii. Opportunists will have higher abundance in a N-limited system than P-specialists.

3. *What is the influence of nutrient enrichment under controlled conditions on the productivity and competition traits of N-specialists, P-specialists and opportunists in a N-limited plant community?*

iv. N-specialists will have higher above ground biomass in N-limited systems than P-specialists and opportunists.
v. Opportunists will have higher above ground biomass in N-limited systems than P-specialists.
vi. N-specialists will have larger leaf length and higher canopy height in N-limited systems than P-specialists and opportunists.
vii. Opportunists will have larger leaf length and higher canopy height in N-limited systems than P-specialists.

By conducting a greenhouse experiment in which different grass and herbaceous species are subject to two nutrient levels under N-limitation, this research aims to give insights into the influence of nutrient level, productivity traits and light competition traits on species composition and specialist dominance in grassland plant communities. It also provides a bridge between small scale individually based plant trait research and real world community analysis.

Methods

This research consists of a greenhouse experiment regarding the mechanisms in figure 1. In the experiment the N:P ratio is kept constant below 5. The availability of nutrients and the communities are divided up into two levels (high and low). Species composition was initially set by placing 4 N-specialists, 4 P-specialists and 4 opportunists in a randomly order. For each nutrient level sufficient replicates were made to get viable statistical results. By measuring light competition and productivity traits and determining species composition this research aims to give insights into functional groups and plant species community composition. The conceptual setup is schematically shown in figure 2 below.

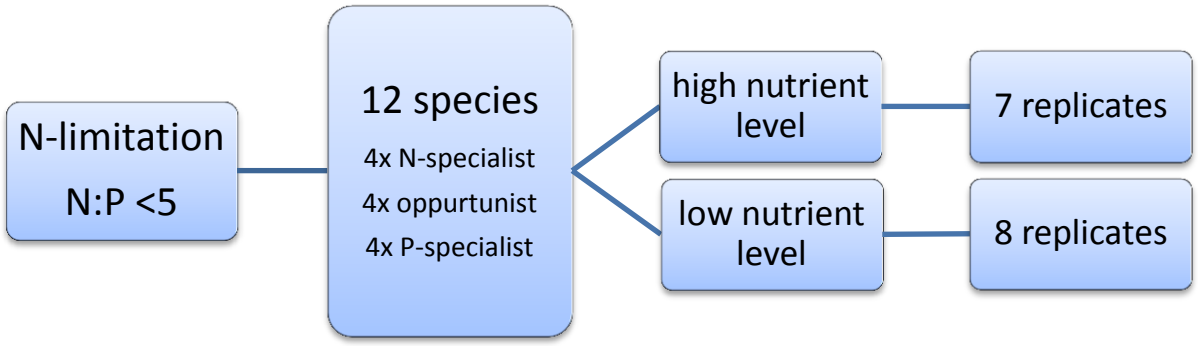

Figure 2 Schematic lay-out of the greenhouse experiment

Demarcation

To ensure some conclusiveness in the results some variables have been made to have no or minimum influence on the species composition. Olde Venterink (2011) mentions regional species pools as one of the controlling factors for species richness in a particular pool. This was countered by ensuring that every replicate consisted of same the type of species (N-specialist, P-specialist and opportunist) and number of individuals per species. Furthermore, to minimise the effect of different interspecific interactions (Bever et al., 2010) on a local scale the spatial setup of the different species was copied throughout every replicate. All other environmental influences were kept at a minimum by conducting the experiment in a greenhouse.

Species

12 species represent an average number for a wide range of Dutch grasslands. To ensure enough of the species germinated, in total 18 Dutch grassland species were sown. Grasslands have varying amounts of grass:herb ratios varying from grass dominated to herb dominated (Wassen et al., 2005). Intermediate values were taken and 2 herb and 2 grass species per strategy were used to assure coverage of both types. This also balances out the effect of morphological differences between grasses and herbs (Anten et al., 1995). Species are chosen in line with research done by Roeling et al. (in prep.). Specialists were categorised out field data published in Fujita et al. (2014). Species were put into groups depending on the limitation type of sites they were found. For example, N-specialists were defined as species that were significantly more often found in N-limited systems, but also significantly less often in P- or co-limited systems. Opportunists are defined as species that had no significant preference. Besides

the field data provided by Fujita et al. (2014), other scientific literature was consulted to give a more definite specialism. The species used can be seen in table 1. More elaborate info on the species used is given in Appendix A.

Table 1. Species sown for the greenhouse experiment with characteristics including the specialism, red list status, CSR-strategy and average height

Latin name	CSR strategy	Specialism	Red list status	Specialism sources	Herb/ grass	canopy height (mm)
Agrostis capillaris*	CSR	O	Common	Venterink & Güsewell, 2010	Grass	100-300
Alopecurus pratensis*	C/CSR	N	Common	Hejcman et al., 2007; Venterink & Güsewell, 2010; Fujita et al., 2014	Grass	100-300
Briza media*	S	P	Red list [a]	Hejcman et al., 2007; Ryser et al., 1997	Grass	<100
Campenula rotundifolia	S	P	Common	Fujita et al., 2014	Herb	100-300
Carex appropinquata			Red list [a]	Fujita et al., 2014	Grass	
Carex flacca	S	P	Common	Fujita et al., 2014	Grass	100-300
Carex oederi*		O	Common	Fujita et al., 2014	Grass	
Carex spicata		N	Common	Fujita et al., 2014; Roeling et al., in prep.	Grass	
Centaurea jacea*		P	Common	Fujita et al., 2014; Roeling et al., in prep.	Herb	
Crepis capillaris*	R/SR	N	Common	Fujita et al., 2014	Herb	100-300
Knautia arvensis*	CSR	O	Red list [a]	Fujita et al., 2014; Roeling et al., in prep.; Roscher et al., 2013	Herb	300-600
Nardus stricta*	S	P	Red list [b]	Hejcman et al., 2007	Grass	300-600
Potentilla erecta	S/CSR	P	Common	Fujita et al., 2014	Herb	100-300
Prunella vulgaris*	CSR	O	Common	Fujita et al., 2014; Roeling et al., in prep.	Herb	<100
Ranunculus repens	CR	N	Common	Fujita et al., 2014	Herb	100-300
Rumex acetosa*	CSR	N	Common	Kirkham et al., 1996; Fujita et al., 2014; Roeling et al., in prep.	Herb	100-300
Succisa pratensis*	S	P	Red list [b]	Fujita et al., 2014	Herb	<100
Trisetum flavescens*	CSR	N	Common	Hejcman et al., 2007	Grass	100-300

*Used in the final experiment
[a] Vulnerable
[b] Sensitive
Average height and CSR was obtained from Hodgson et al. (1995)

All seeds used in the experiment were ordered in from "Cruydthoek" (Groningen), a firm specialised in collecting seeds. Seeds are sown in existing grassland and harvested in different ways such as: mowing, picking, sucking and threshing (after drying). After this the seeds are

cleansed at appropriate temperature and humidity. Finally they are stored at 12-15 ^{0}C with a humidity of 40% to keep the seeds at rest but to prevent them from going into dormancy. No chemical compounds are used in this process. Upon arrival the seeds were kept in the fridge for a period three weeks to simulate a colder winter period and provoke the dormant state, after which they were sown into germination treys.

Germination

Germination trays (40*44*8 cm) were filled to a height with "zaai en stek grond" from the firm Holland. Around 300-500 seeds per species were evenly distributed over the surfaces of two germination trays, to minimise chance of mould or algae damage to a complete species. These trays where covered with transparent plastic and put in a greenhouse under UV lamps (280 Watt), moisture content of the trays was checked daily and watered with rainwater (Appendix B) when necessary. After sufficient individuals had germinated the trays were moved to a colder greenhouse in which they awaited the translocation to the final Set-up buckets.

Mortar tubs & Substrate

Substrate used in the final experiment consists of two layers of sand. This was put into mortar tubs displayed below. The tubs were first cleansed with tap water and then provided with eight drainage holes with a diameter of 15mm to prevent the soil of becoming too wet. As depicted in figure 3, the first layer of sand is 10 litre of industrial grinded dorsolit, consisting of a 31-67 % mixture with 0,8mm and 0,63mm grainsizes. The mortar tubs were inlayed with a perforated plastic to prevent the sand from flowing out. The top layer is 24 litres of dune sand from the "Kennemerduinen" (52°25'0" N and 4°34'60" E in DMS). Dune sand was used whilst it is naturally low in nutrients (Bakker et al., 2005) and still contains microbial life as opposed to the dorsolit. Microbial life can be an influencing factor in the establishment of species (Wardle et al., 2003). To minimise the death by replanting this natural sand was used. The dorsolit was ridded of algae after which it was washed two times with osmosis water to get rid of dust and grinding residue (the concentrations for elements in osmosis water are given in appendix B). The dune sand was first sieved (5mm) to filter out stones, and organic material that could cause inconsistencies between tubs. Tubs where then saturated with water to protect the seedlings from vast quantities of water after relocation. To check for initial N and P content, 2 dorsolit and 4 dune sand samples were taken from the batch and added with demi water after which they were shook for two hours. P and N content was measured using LCK 349 Phosphate (Murphy & Riley, 1962) and LCK 138 Nitrogen analysis (Helder & De Vries, 1979) from the Hach firm. To obtain the dry weight of the sand, samples were dried in an oven for an hour at 100^{0}C. The dorsolit samples contained N and P under the detection limit (<1µg/g for P and <0,02mg/g for N). The N and P concentration of the dune sand was determined at 12,5µg/g and 2,5µg/g respectively.

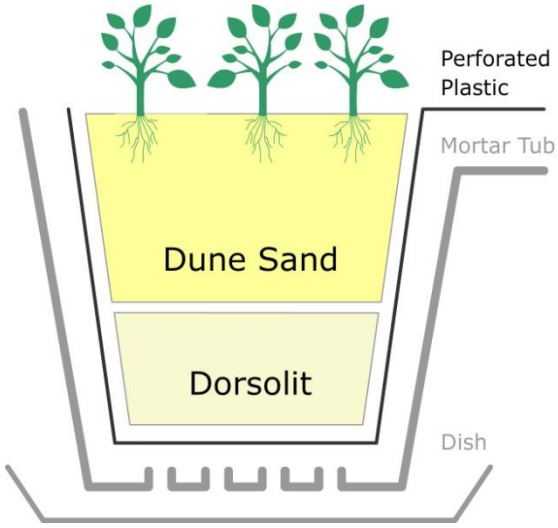

Figure 3. Schematic cross-section of the mortar tub setup

Relocation

The individuals were planted within a tub according to the schematic figure shown in the appendix (C). Plants were chosen randomly but put down in such a manner that no two equal species are neighbours. Also, not all neighbours of one species can consist of one specialism to minimise the effect of specialism "islands". Davis et al. (2012) point out the use of an outer line of plants to provide a buffer. The buffer was created to equal circumstances for all individuals within the dashed line since species on the far ends of the containers could catch more sunlight. It consisted of randomly appointed species in the same ratios as the species within the data-collection. For most species 2 individuals were used in this buffer. To supplement one extra *R. acetosa* (N), *C. jacea* (P) and *A. capillaris* (O) were placed. Since the number of *K. arvensis* that germinated was insufficient to provide for buffer plants, these were replaced by a *P. vulgaris* and *C. oederi*. Different replacement species were selected not to give one species the upper hand. Even if they are not used for the data selection one species could have a different effect on the community than another. All tubs were given equal buffers.

Before relocation the tubs were fully saturated with water to minimize disturbance just after replanting. Four individuals per species were used for the final experiment. The small amount of potting soil that was transported from the monoculture to the final plots was presumed to be negligible on the total amount of nutrients applied during the experiment. A wooden board into which holes were drilled according to the scheme was used (figure 4). This board was placed on the sand and colour-coded sticks were stuck through. These sticks were then one by one replaced with the corresponding species. After relocation the plants were left to settle for 2 weeks and were watered with osmosis water.

Figure 4: three photographs depicting the relocation of species to the experimental setup

Nutrient Regime

Nutrient Levels

The plants were provided with nutrition solutions on a regular basis for 9 weeks, in coherence with research done by Olde Venterink & Güsewell (2010) and Güsewell & Bollens (2003). The nutrients naturally present in the dune sand resulted in an availability of 6,03 mg N and 1,16 mg of P per plant. Two nutrient levels were chosen for this experiment 6.82 (low) and 14,16 (intermediate), these are geometric means of both N and P. The geometric mean gives a value for total nutrient content that transcends the separated concentration for phosphorus and

nitrogen. As stated in Mitchell (2004) to acquire the geometric mean the following formula can be used (formula 1 & 2)

$$\textit{Geometric mean} = [x_1 * x_2 \dots x_n]^{\frac{1}{n}} \qquad (1)$$

This formula in the light of this experiment can be used as follows:

$$\textit{Geometric mean nutrient level} = \sqrt{[N] * [P]} \qquad (2)$$

The nutrient ratio established in the tubs after application was determined at 4,31 for intermediate and 3,62 for low. N was provided as KNO_3 and $Ca(NO_3)_2{\cdot}4H_2O$, P was made available through KH_2PO_4.

Based on quantities used in several papers (Fujita et al., 2010; Güsewell & Bollens, 2003; Güsewell, 2005) the plants were given non-limiting amounts of micro-nutrients. Per plant the following amounts were provided: 12,01 mg Mg/ 0,84 mg Fe/ 0,09 mg Zn/ 0,02 mg Na/ 0,04 mg Mo/ 0,02 mg Cu/ 0,08 mg B/ 1,08 mg Mn. For K and Ca amount for high and low differed since it's partly provided by the molecules supplying N and P and for a large part in KCl and CaCl. This can be seen as an inconsistency between the different treatments, however the difference is minimal and both quantities are non-limiting. This is not assumed to result in discrepancies in the results. Table 2 can be consulted to find the values.

Table 2. Total amounts per plant of micro (purple) and macro (blue) elements per nutrient level during the experiment

Level	P	N	K	Ca	Cl	Mg	Fe	Zn	Na	Mo	Cu	B	Mn	S
Low (mg)	2,01	8,63	206	54,35	228	12,01	0,84	0,09	0,02	0,04	0,02	0,08	1,08	17
High (mg)	6,01	21,76	229	61,74	228	12,01	0,84	0,09	0,02	0,04	0,02	0,08	1,08	17

Table shows added amount without the nutrients present in the dune sand.

Nutrient amounts were multiplied by 78 (individuals per tub) and were applied with 18 litres osmosis water. Application of nutrient quantity was enlarged two times during the experiment to guarantee non-toxic levels for growing seedlings and sufficient supply of nutrients for further developed individuals. Application started with 1 litre solution per tub for the first three weeks, continuing with 2 litres for week 4 to 6 and finishing with 3 litres for week 7-9. A portion of the micro nutrients was already applied in the three weeks before start of the macro treatment to help the plants cope with the iron deficiency. Table 3 shows what was given before the main experiment.

Table 3. Micro nutrients (mg) applied before start of the main experiment

Mg	Fe	Ca	Cl	Zn	Na	Mo	Cu	B	Mn	S
1,46	0,10	6,45	5,70	0,01	0,00	0,01	0,00	0,01	0,13	2,07

Halfway through the experiment (5 weeks) the plots were leached with osmosis water to counter possible toxic accumulation of nutrients (Güsewell & Bollens, 2003). Mortar tubs were filled with to the brim twice and allowed to drain (without dishes underneath).

Nutrient Solution

The nutrient solution used was created out of a stock solution every week. The stock was refrigerated at around 5 ˚C. The compounds of the nutrient solution were based on a modified recipe of the Hoagland solution (Hoagland & Arnon, 1950) which complies with the preambles set by Steiner (1961). The compounds that provided the elements are listed in appendix D.

The stock solutions were created as stated in the appendix D. To make the Fe available to the plants EDTA was added at 1,5 times the weight of $FeSO_4 \cdot 7H_2O$ to osmosis water and heated to 70˚C on a stir flow. After heating the $FeSO_4 \cdot 7H_2O$ was added. Solution 8 was made by carefully adding the compounds to the osmosis water. To prevent precipitation to form they were added in the order listed in the appendix. Every week of applying nutrient solution the ml of stock solution was added to osmosis water. Every amount doubled after three weeks and was then multiplied by 1,5 after another three weeks resulting in gradual increase of nutrient dosage.

Measurements

During the data collection period all data was collected on the form presented in appendix E. The following paragraphs shows the methodology regarding the taking of measurements and the determining of values of community evenness and species diversity

Plant traits

Productivity traits are traits related to the biomass produced by a single plant or a community. Fujita et al. (2014) and Olde Venterink et al. (2001) use above ground biomass of vascular plants as a proxy for productivity of a plot or plant community. Biomass was dried for 48h after collecting at (75˚C) before weighing. Competition traits can be roughly classified in two categories: above and below ground competition (Tilman, 1987). This study focuses on above ground competition since ultimately light completion is the determining factor for plant survival. Traits relevant for above ground competition are linked to light competition (Fujita et al., 2014). These light competition traits include canopy height and crown expansion. Canopy height is defined by Cornelissen et al. (2003) as the shortest distance between upper boundary of photosynthetic tissues and the ground. As some leaves in herbaceous plants can be folded, bended or kinked a second measure was taken, being crown expansion. This is defined by a measure called stretched length (Cornelissen et al., 2003). Stretched length is the distance from the base of the plant to the stretched length of the longest leaf.

Other characteristics

Besides plant traits, herbivory and flowering were also documented. Flowering was noted as the amount of flowers present and the amount of flower buds the plants developed during the entire experiment. The herbivory by *Autographa gamma* was measured on a three point ordinal scale with 1: Slightly damaged, 2: Significantly damaged & 3: Severely damaged.

Species Diversity

Mulder et al. (2004) state that diversity consists of two components, being species richness and evenness. Richness is defined as the number of different species, whereas the evenness is defined as a fraction which gives puts a measure to the relative division of biomass over community members. Evenness can be measured by individuals or by biomass. The equation based on Simpson's dominance index (D) (Simpson, 1949) is given below.

$$E = \frac{D}{S} = \left(1/\sum_{i=1}^{S} P_i^2\right)/S \qquad (3)$$

P_i represents the proportion of biomass in species I and S is the amount of species in the plot. The index is a fraction and will provide a value between all biomass in one species (0) to equal distribution among species (1).

Environmental Characteristics

The total lifespan of the plants can be divided in two phases, the germination period (09-02-2015/20-03-2015) and the experimental phase (21-03-2015/29-07-2015). During germination plants were constantly exposed to UV light of at least 400 W/m² (natural or artificial). Above 600 W/m² sun blocking screens provided for protection. Mean temperature and humidity are given in table 4

Table 4. Day and night averages for temperature and humidity during germination

	Mean Temperature (˚C)			Mean Relative Humidity (%)		
	Total	Day	Night	Total	Day	Night
Mean	20,9	21,7	20,0	49	48	50
St. dev	1,6	1,9	0,3	9	11	6
Min.	18,6	18,6	18,7	22	22	33
Max	27,6	27,6	23	80	80	76

During the experiment, differences in lighting settings were made to ensure more natural conditions. Artificial lighting set in at values below 400 W/m², sun screens were deployed above 700 W/m². At night lighting was switched off. Averages can be found in table 5. As can be seen values fluctuate more than during germination, which is partly due to the night-day cycle. Also differences are far greater in a spring-summer period than in winter.

Table 5. Day and night averages for temperature and humidity during experiment

	Mean Temperature (˚C)			Mean Relative Humidity (%)		
	Total	Day	Night	Total	Day	Night
Mean	19,4	21,2	17,6	55	49	61
St. dev	4,3	4,5	3,1	15	16	13
Min.	14,1	14,3	14,1	15	16	22
Max	39,8	39,8	36,5	88	88	88

Pests

During the experiment there was nuisance of different pests including caterpillars, larvae and plant lice. Pests were dealt with, with the help of biological control, since pesticides can negatively affect the plants. To start with, the Silver Y or *Autographa gamma* is a moth that comes forth out of green caterpillar that forages on plant leaves. Since it mainly forages at night and they can be hard to spot, caterpillars were removed by hand. They were found to affect *C. jacea, C. capillaris* and *P. vulgaris* all to a similar extent, in terms of above ground biomass it is impossible to quantify losses. But *P. vulgaris* was the only species affected significantly more than all non-affected species (α=0,05). No significant differences were found between affected species (α=0,05). Secondly, moss fly larvae can do significant damage to the plants and can even

kill the host. The tubs were treated with a solution containing parasitic nematopods. These nematopods *(Steinernema feltiae)* target the larvae and are harmless for the plant community (Bird & Bird, 1986). Thirdly, Aphidoidea are parasites that live on the fluids from plant stems they infiltrate the phloem cell and parasitise the flow of the plant. The lice were spotted during the final phase and were treated with several species of parasitic wasps, Ervipar (*Aphidus ervi*), Aphipar (*Aphidius colemani)* and Aphidend (*Aphidoletes aphidimyza*) and the infection was kept at a minimum. Before placing the infected plants in bags the lice were removed from the plants. Finally, Thysanoptera are bugs that feed of fluids from plant stems similar to lice, they were countered with a predatory mite Swirskii Plus *(Amblyseius swirskii)*. The larvae of these thrips are also targeted by the nematopods.

Statistics

Methods regarding the statistics results all include the use of SPSS Statistics (IBM). To gain information about treatment effect and the influence analyses of variance were executed (ANOVA's). To execute ANOVA's conditions regarding normality and variance have to be met to guarantee the strength of the test. Where possible variables were transformed to obey normality (α=0,05) by using a Log10 transformation. Although transformed variables did not quite obey normality (Shapiro-Wilk $p<0,05$) plots of the histogram showed a more normal distribution with exception of a lot of zero values given by dead plants. Levene's tests came back with unequal variances for all variables but abundance, but were more equal after log transformation. ANOVA's were used despite the violation of normality and equality of variance since it is required to do a post hoc test for the comparison and quantification of the differences between variables. Furthermore, Field (2009) states that an ANOVA is robust when the homogeneity of variance pretence is violated as long as sample sizes are similar. Post hoc testing was done with the Games-Howell procedure, since this is the most powerful procedure under unequal variance and large sample size (Field, 2009). Under equal variance, Gabriel's post hoc test was used. Where ANOVA's were unnecessary a non-parametric Mann-Whitney test was done. In correlations Pearson's r was used whenever data was distributed normally, Spearmans rho was used for non-normal data over kendall's tau, since tau is for small sample tied ranks which, in this research was not the case (Field, 2009).

Mann-Whitney tests were performed to gain the significance of differences between abundance for the specialisms at different nutrient levels. Evenness scores, for both species and specialisms were tested with the same method. ANOVA's were used to described the differences within nutrient levels for biomass, leaf length and canopy height. Determining significance of differences between nutrient levels for the traits mentioned above was again done by performing a Mann-Whitney test. Post hoc testing was done for all traits across specialism, both for low and high nutrient level, either by Gabriel or Games-Howell procedure depending on homogeneity of variance.

Plants may experience various conditions in different positions in the green house. The position of plants in the greenhouse was introduced as a random factor (pallet number.) in the ANOVA and proven to result in no significant differences (α=0,05) for biomass, leaf length or canopy height.

Furthermore, the effect of competition by direct neighbours has been stressed as an influential factor determining competition outcome (Güsewell & Bollens, 2003). The influence of different species having different effects on the target species has been tested by looking for significant intraspecific differences on the four locations of species within in the tubs. After doing ANOVA's for biomass, leaf length and canopy height only 4 out of 1782 possible comparisons came back significantly different. Effect of direct neighbouring species identity was strongly ruled out for this experiment and will thus not be further elaborated upon in the results section.

For the statistical results, it has to be taken into account that *C. capillaris* was removed from the dataset unless otherwise mentioned. *C. capillaris* had a disproportional high death rate, due to the size and strength of the seedlings. Comparison showed that *C. capillaris* scored significantly lower than all other N-specialists on biomass, canopy height and leaf length.

As mentioned in the methodology the effect of the A. gamma moth was compared between species, to do this an ANOVA's was used with Games-Howell post hoc procedure.

Results

Species Richness & Abundance of Species

As stated in the introduction is the richness of species defined as the total species count. In most communities all 12 species are represented (st. dev. 0), in two cases all individuals of *C. capillaris* died during the experiment. This lower count contributes to a lower mean species richness for the high nutrient level of 11,71 (st. dev. 0,488). However, richness in low and high nutrient level is not significantly different (U=20, asym. sig. p=0,117/exact sig. p=0,397).

Species richness does not differentiate between one or four individuals present in a community. Abundance gives this measure based on the absolute number of individuals per specialism. The relative fractions of specialisms are shown in figure 5. Differences between specialisms are marginal; moreover, differences within specialisms are even smaller. All range around the 33%. Table 6 & 7 show the p values. Within specialism no significant differences are present. On a species level only the abundance of *R. acetosa* is significantly larger at low nutrient levels (*U*=6,500, asymp./exact p=0,012/0,009), but only by 2 percentage points. In the determination of the abundance fraction of N-specialists *C. capillaris* is included. The species was initially excluded because of unnatural high death rate, but removing them from the abundance calculation would lower the score even further (care should be taken regarding the N-specialist abundance it could be lower than it would naturally be).

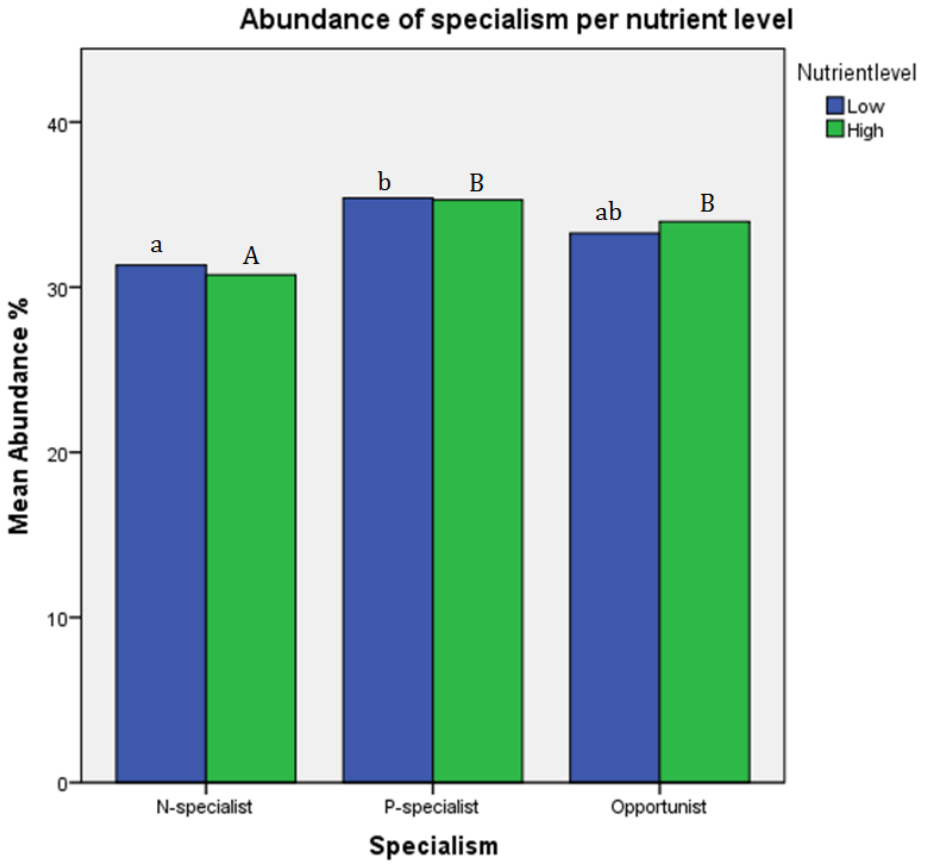

Figure 5. The abundance of species shown in an aggregate term per specialism for the two nutrient levels. *C. capillaris* is used in the compiling of N-specialists. Significantly different means have different letters, capitals are used for high nutrient level and normal letters for low nutrient level

Table 6. Abundance Post hoc Gabriel, p values between specialism at both nutrient levels		
Nutrient level	low	high
N-O	0,000**	0,000**
N-P	0,114	0,010*
P-O	0,072	0,450
*sig values at α=0,05 **sig values at α=0,01		

Table 7. Mean abundance (%) values per specialism for both nutrient levels						
Nutrient level	Low		High		Mann Whitney U	
	Mean	St. dev	Mean	St. dev	U-value	Asymp. Sig (p)
N-specialist	31	2	31	2	423	0,709
P-specialist	35	1	35	1	446	0,976
Opportunist	33	1	34	2	403,5	0,505

Evenness

As was discussed in the previous paragraph specialisms showed similar scores on abundance at different nutrient levels. Evenness, as discussed in the methodology, can be used to weigh the abundance by using the biomass of species in the specialism groups. Specialism evenness values in table 8 show an aggregate value where all N-specialists are regarded as one species. Values within species evenness and specialism between nutrient levels do not vary much. Furthermore, the observed differences in means are not significant (α=0,05). Species evenness is a lot lower than specialism evenness, which shows that the variation in biomass per species differs largely, but interspecific opposites within the specialism groups balance each other. The fact that standard deviation diminishes greatly under high nutrient levels shows that species give a more consistent value for biomass under higher conditions. The relative influence of nutrient regime becomes more important as opposed to other factors in the low nutrient regime.

Table 8. Evenness values per species and specialism for both nutrient levels						
Nutrient level	Low		High		Mann Whitney U	
	Mean	St. dev	Mean	St. dev	U-value	Exact sig./Asymp. sig
Species Evenness	0,557	0,129	0,653	0,06	17	0,232/0,203
Specialism Evenness	0,888	0,121	0,947	0,035	19	0,336/0,298

Above ground biomass

The third research question regards productivity and competition traits. Above ground biomass production is used as a proxy for the productivity of the different species. Mean biomass values per species from the different specialisms are used to discover differences between nutrient levels.

All mean values for biomass production (figure 7 & table 9) are significantly higher under high nutrient level conditions as compared to low levels. This can also clearly be seen in the tubs displayed in figure 6. Differences between specialism are only present in the higher nutrient level, implying that species specialism does not manifest itself in higher above ground biomass at low levels. Under high nutrient level conditions N-specialists have a significantly higher mean above ground biomass than P-specialists and opportunists (table 10). The latter two scores have a similar mean and do no differ significantly from each other. This indicates that neither P-specialists nor opportunists have an advantage over each other when it comes to above ground biomass production in N-limited systems. This is also illustrated by the difference in biomass

between nutrient levels; table 9 shows it is twice as high for N-specialists than the other specialists.

Figure 6. Difference between low and high nutrient level regime was clearly visible and consistent over time, photos were taken on 09-07-2015 (left) and 27-07-2015 (right).

When it comes to differences between nutrient levels and the response of the different specialisms' biomass, driving factors also have a combined effect. The interaction term within the ANOVA of nutrient level and specialism shows a significant effect (nutrient level * specialism, p=0,000). When the specialisms are broken up into the separate species (figure 8) N-specialists all respond similar on the different nutrient levels. Within P-specialists *C. jacea* greatly enlarges the average effect of nutrient level as it attributes to two thirds of the biomass difference between low and high nutrient levels. Despite this, the average for P-specialists is significantly lower than that of N-specialists. Without *C. jacea* P-specialists would score considerably lower than opportunists. Besides absolute difference also relative difference of biomass is higher in all N-specialists than other species (figure 8). Table 9 also shows that average relative difference in biomass is higher for opportunists than for P-specialist.

Table 9. Mean above ground biomass (mg) values per specialism between nutrient levels

Nutrient level	Low		High		Mean difference	Mann Whitney U	
	Mean	St. dev	Mean	St. dev		U-value	Asymp. Sig (p)
N-specialist	77	91	543	455	466	338,5	0,000**
P-specialist	86	102	304	381	218	4241	0,000**
Opportunist	90	117	280	250	190	3089,5	0,000**
**sig. values at α=0,01							

Table 10. Mean above ground biomass (mg), Games-Howell p values for the specialism comparison for both nutrient levels

Nutrient level	Low	High
N-P	0,790	0,000**
N-O	0,634	0,000**
P-O	0,951	0,844
**sig. values at α=0,01		

Linking back to the CSR-strategy shown in table 1, it is hard to show any large differences as almost all species used are of intermediate CSR-strategy. However, the only competitor (*A. pratensis*) shows much more above ground biomass under higher nutrient regime coherent with its strategy. This is also illustrated by the big relative difference of *A. pratensis* (figure 8). The stress tolerators (*B. media, N. stricta and S. pratensis*) show among the lowest relative difference in under more nutrients.

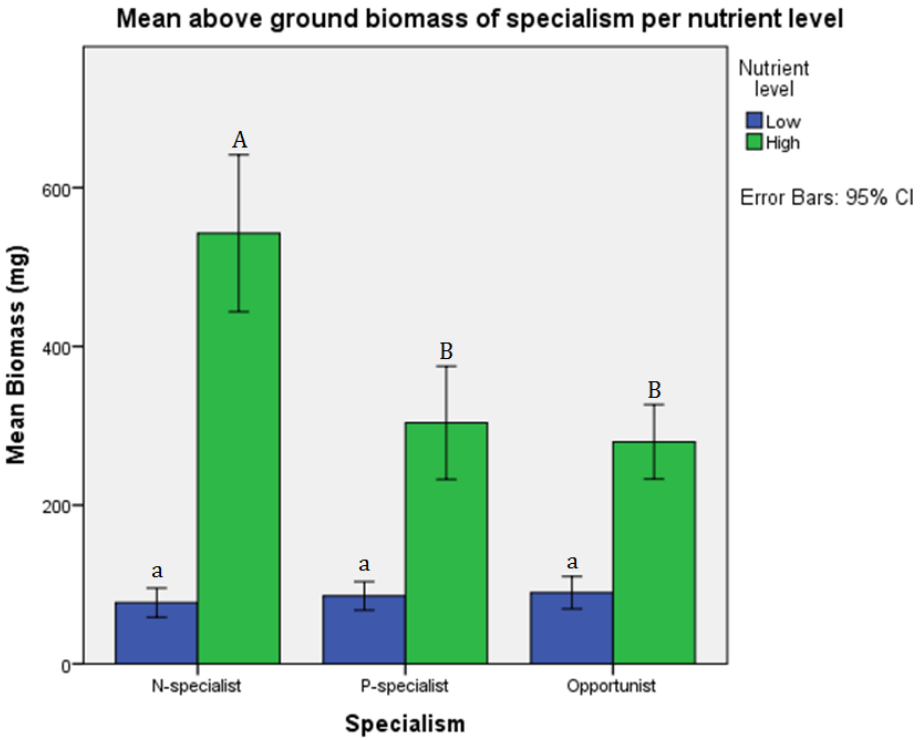

Figure 7. Mean above ground biomass of N-specialists, P-specialists & opportunists per nutrient levels. Significantly different means have different letters, capitals are used for high nutrient level and normal letters for low nutrient level

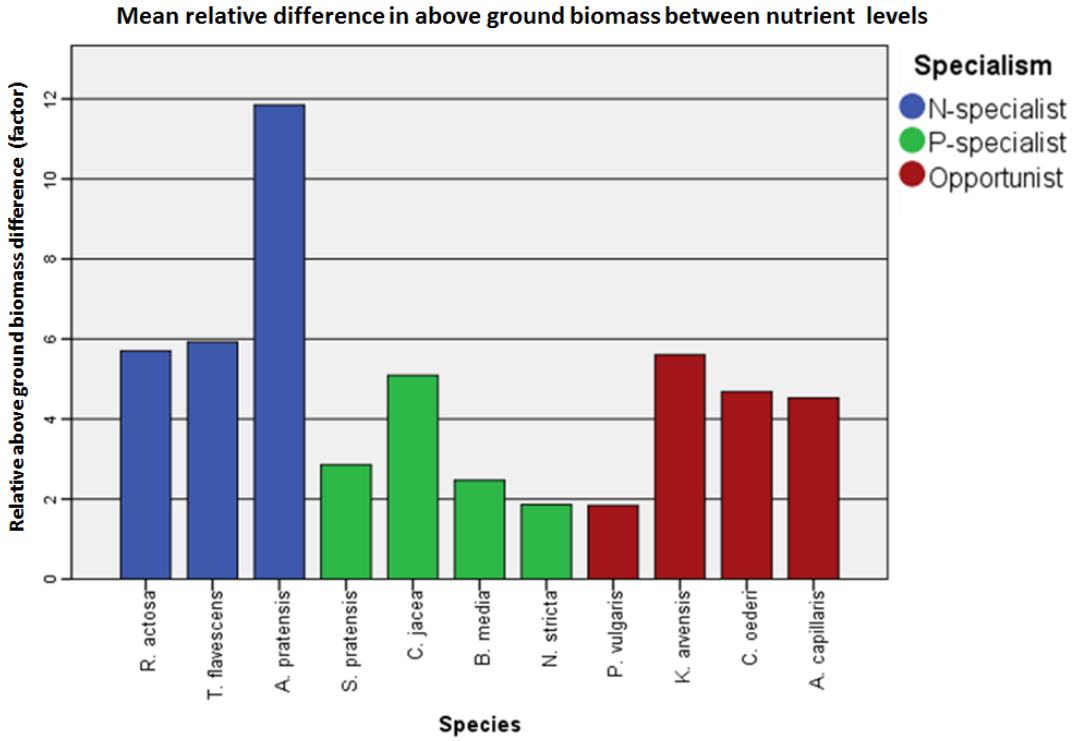

Figure 8 Pooled relative difference in biomass per species, Specialism is shown in different colours. The relative increase is a factor given by high/low

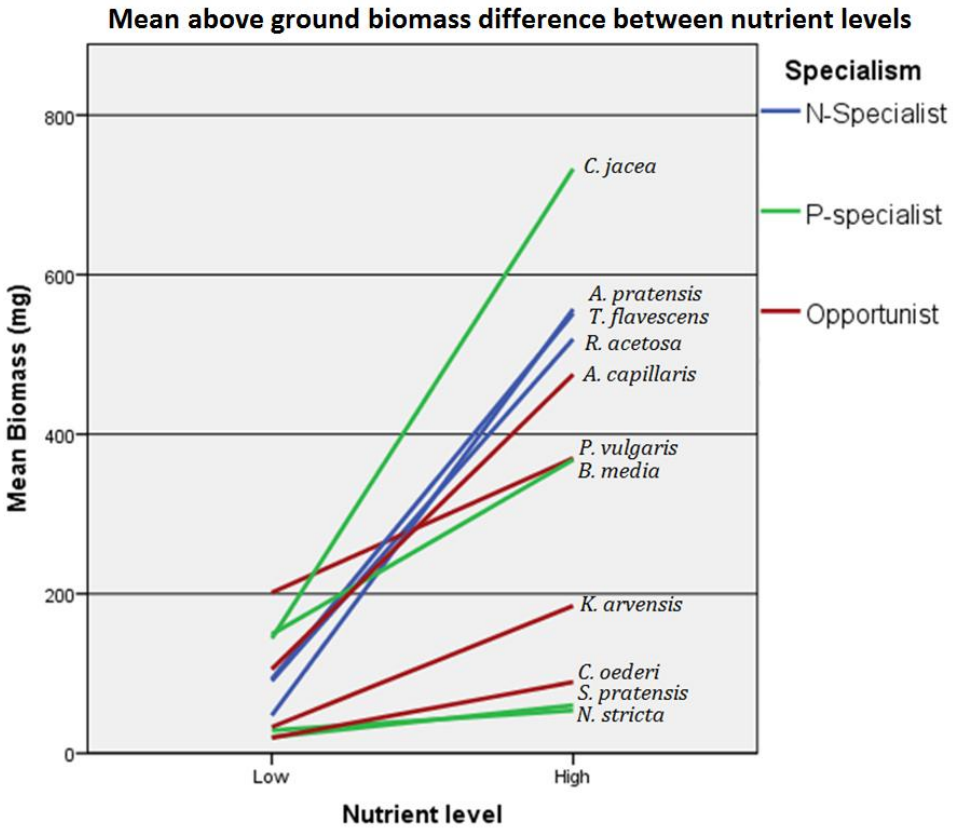

Figure 9. Mean above ground biomass difference for separate species between nutrient levels.

Leaf length

For leaf length the same holds true as for biomass with respect to normality and homogeneity of variances (α=0,05). So the log transformation is used for computing the results of table 11 & 12. P-specialists had significantly (p=0,000) lower leaf lengths than N-specialists under low and high nutrient levels. Opportunist values for leaf length were similar to those of P-specialists and also significantly (p=0,000) lower than N-specialists. Not only total leaf length is larger for N-specialists also the difference in leaf length is twice that of opportunists or P-specialists (table 11). Relative difference (figure 12) of leaf length shows no clear patterns. Similar to the results for biomass, the interaction effect of nutrient level and specialism is also significant for leaf length (p=0,030).

The previous biomass results show no significant differences between specialism under low nutrient conditions. Combining this with the significant difference in mean leaf length under low nutrient levels implies that N-specialists invest a relatively large portion of their biomass in producing long leaves.

Table 11. Mean Leaf length (mm) values per specialism for both nutrient levels

Nutrient level	Low		High		Mean increase	Mann Whitney U	
	Mean	St. dev	Mean	St. dev		U-value	Asymp. Sig (p)
N-specialist	168	102	372	147	204	1097,5	0,000**
P-specialist	87	61	182	109	93	3404,5	0,000**
Opportunist	71	59	193	105	122	1919,5	0,000**

**sig. values at α=0,01

Table 12. Leaf length, Games-Howell p values for the specialism comparison between both nutrient levels

Nutrient level	Low	High
N-P	0,000**	0,000**
N-O	0,000*	0,000**
P-O	0,0,553	0,039*

*sig. values at α=0,05 **sig. values at α=0,01

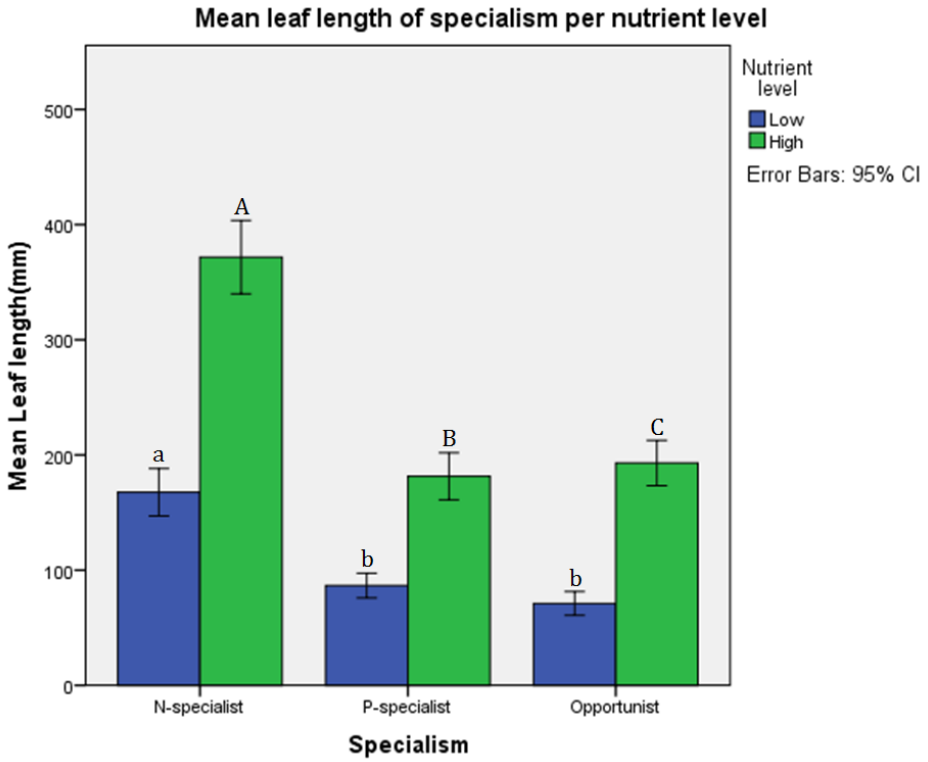

Figure 10. Mean stretched leaf length of N-specialists, P-specialists & opportunists per nutrient level. Significantly different means have different letters, capitals are used for high nutrient level and normal letters for low nutrient level

Figure 11 shows the leaf length reaction of separate species on both nutrient levels. It clearly shows the morphological factor of grasses in leaf length since they are structurally taller than herbs. The top four of species are all members of the *Poaceae*, even with the considerably lower *N. stricta* and *C. oederi* (*Cyperaceae*) grasses are significantly taller than herbs for both nutrient levels (table 13). Grasses show also 1,5 times greater mean difference than herbs. The fact that there were no significant differences between grasses and herbs for biomass puts more strength to the morphology argument.

Table 13. Leaf length of herbs and grasses for both nutrient levels

Nutrient level	Low		High		Mean increase	Mann Whitney U	
	Mean	St. dev	Mean	St. dev		U-value	Asymp. Sig (p)
Herbs	51	43	150	83	99	4975	0,000**
Grasses	147	85	311	145	164	5469	0,000**
**sig. values at α=0,01							

For leaf length differences between CSR-strategy are not very clear besides the fact that *A. pratensis* is one of the species with the longest leaves. Stress tolerators *N. stricta* and *C. oederi* are among lowest in leaf length, but *B. media* has relatively long leaves. In general CSR-links to leaf length are less straightforward than in biomass.

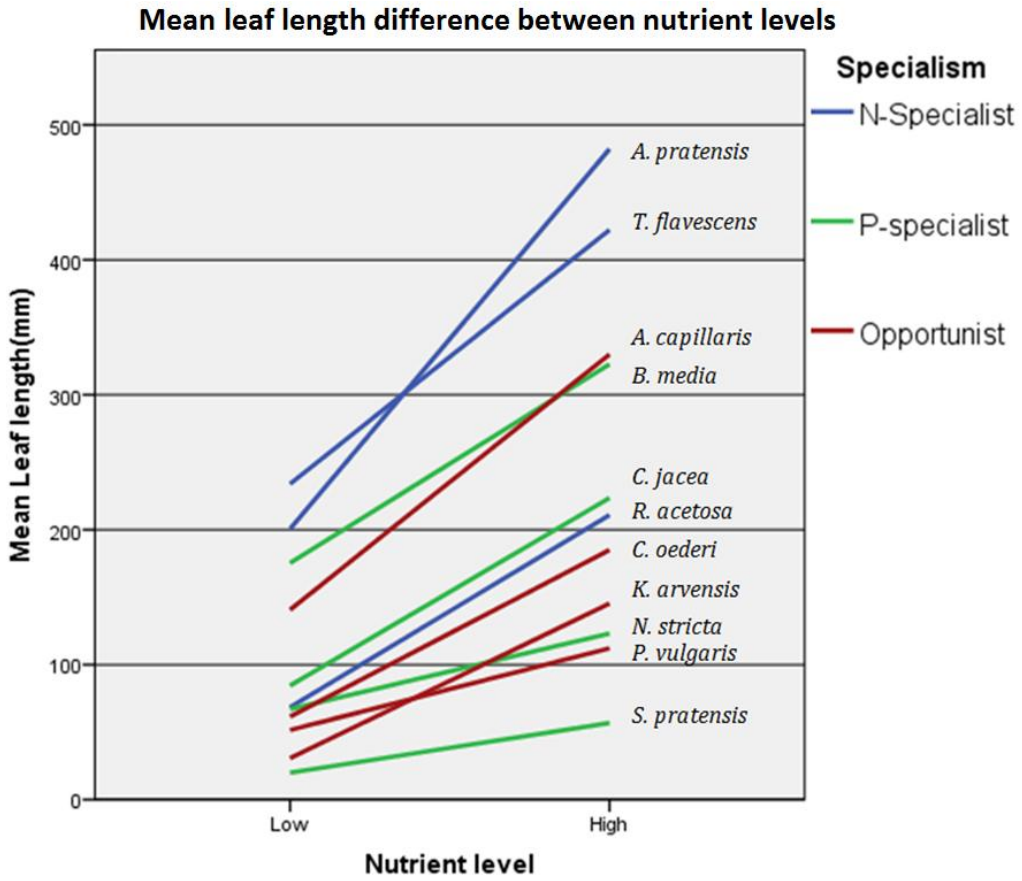

Figure 11. Mean stretched leaf length difference between separate species under different nutrient levels.

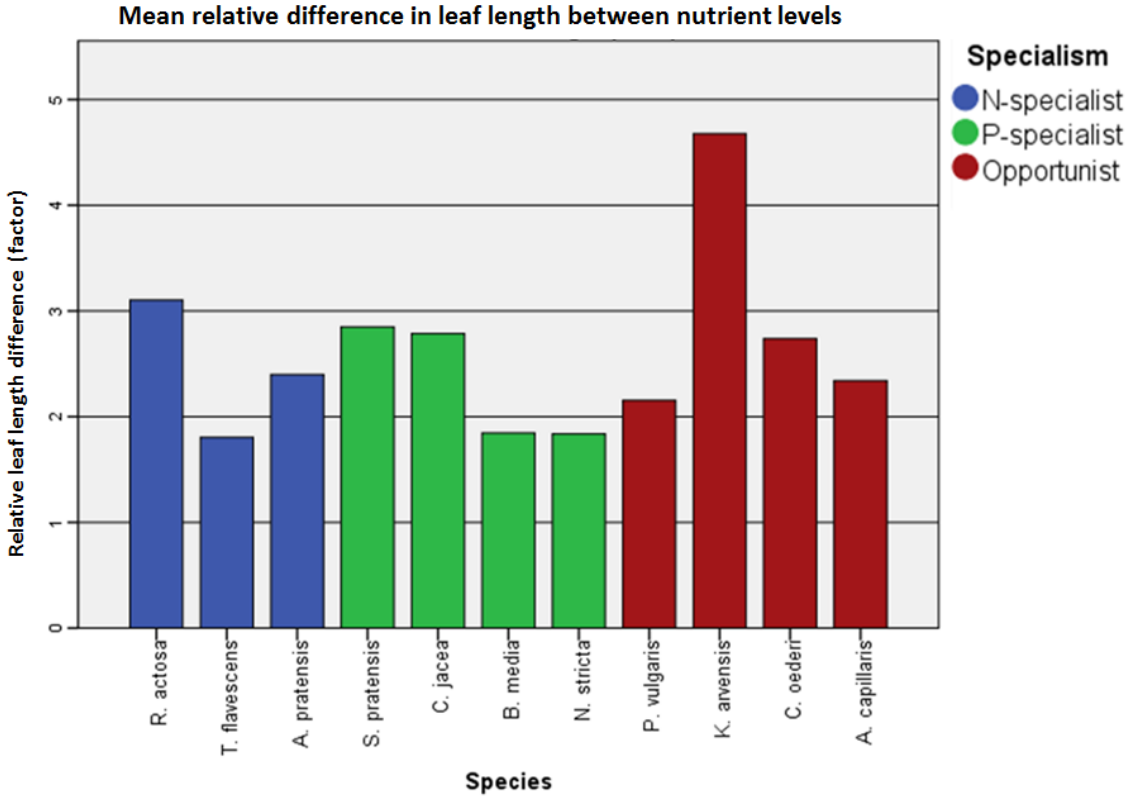

Figure 12. Pooled relative difference in stretched leaf length per species, specialism is shown in different colours. The relative increase is a factor high/low.

Canopy height

Canopy height and leaf length are strongly correlated (r=0,903, p=0,000), as a high canopy cannot be established without long leaves. However, the bending of leaves results in a difference between actual canopy height and leaf length. Canopy height is the highest reach of a plant in normal situation, vital for the competition for light (Hautier et al., 2009).

For canopy height the same relations are present in low nutrient levels as for leaf length, including the interaction effect of nutrient level and specialism (p=0,012). Under high nutrient conditions, apart from N-specialists having a significantly larger canopy height, opportunists are also significantly larger than P-specialists (figure 13, table 14 & 15). These differences are also very clear in figure 14, which shows the canopy height difference for separate species between low and high nutrient levels. The N-specialists form the upper section of the graph, opportunists are clustered in the middle. P-specialists take the bottom ranks, apart from *C. jacea* which as in biomass and leaf length scores relatively high. Falster and Westoby (2003) put together an overview that showed that biomass investment in leaf area, competitive canopies and hereby shading capacity is if not (in)directly beneficial via a competitive advantage over neighbours. The higher canopy for opportunists can be a sign that biomass investment went to making a taller canopy.

Table 14. Mean canopy height (mm) values per specialism for both nutrient levels

Nutrient level	Low		High		Mean difference	Mann Whitney U	
	Mean	St. dev	Mean	St. dev		U-value	Asymp. Sig (p)
N-specialist	134	85	252	108	118	1594	0,000**
P-specialist	69	52	121	60	52	3620,5	0,000**
Opportunist	60	56	142	75	82	2455	0,000*

*sig. values at α=0,05 **sig. values at α=0,01

Table 15. Canopy height, Games-Howell p values for the specialism comparison between nutrient levels

Nutrient level	Low	High
N-P	0,000**	0,000**
N-O	0,000**	0,000**
P-O	0,596	0,006**

**sig. values at α=0,01

Since canopy height is correlated to leaf length it is to be expected that grasses and herbs show similar relations. Figure 14 shows the same four grasses holding the top four positions regarding canopy height. The grasses tested significantly larger than herbs for both nutrient levels (p=0,000). Mean difference for both herbs are more or less similar indicating that they respond similar to higher nutrient levels with respect to canopy height (table 16).

As with leaf length the only CSR-competitor (*A. pratensis*) scores high and benefits among highest from higher nutrient levels. Stress tolerators (*B. media, N. stricta and S. pratensis*) do not show results within the same magnitude regarding canopy height. They do have similar mean difference in canopy height, along with *P. vulgaris* canopy height is lower than all others, again this could be caused by the reduced nutrient stress with which N-specialists and opportunists are expected to cope better.

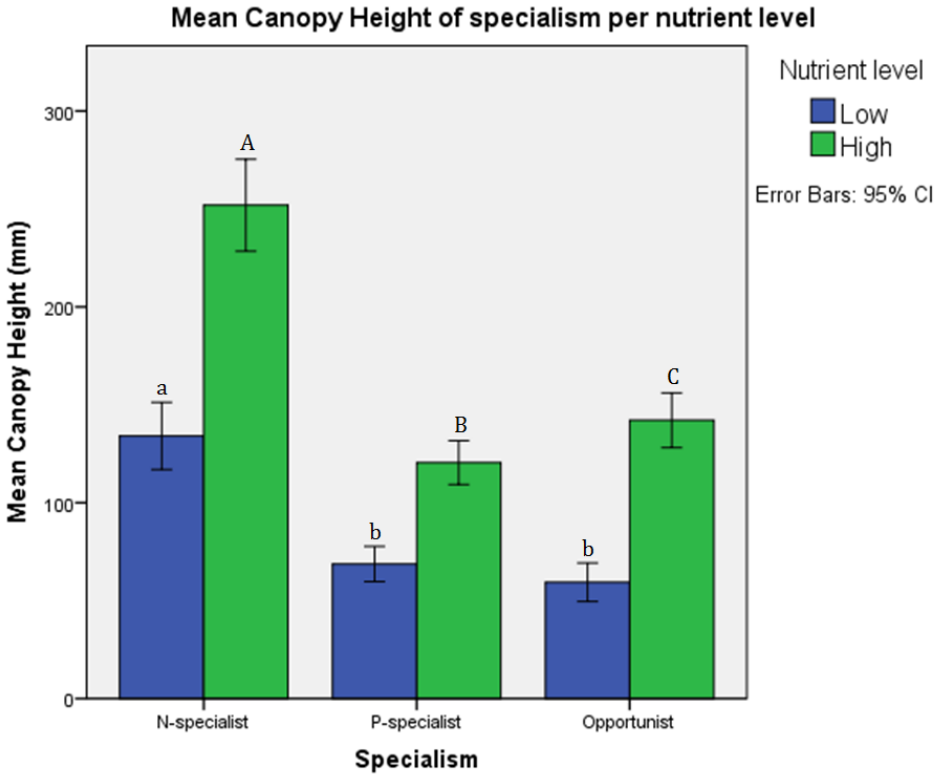

Figure 13. Mean canopy height of N-specialists, P-specialists & opportunists per nutrient level. Significantly different means have different letters, capitals are used for high nutrient level and normal letters for low nutrient level.

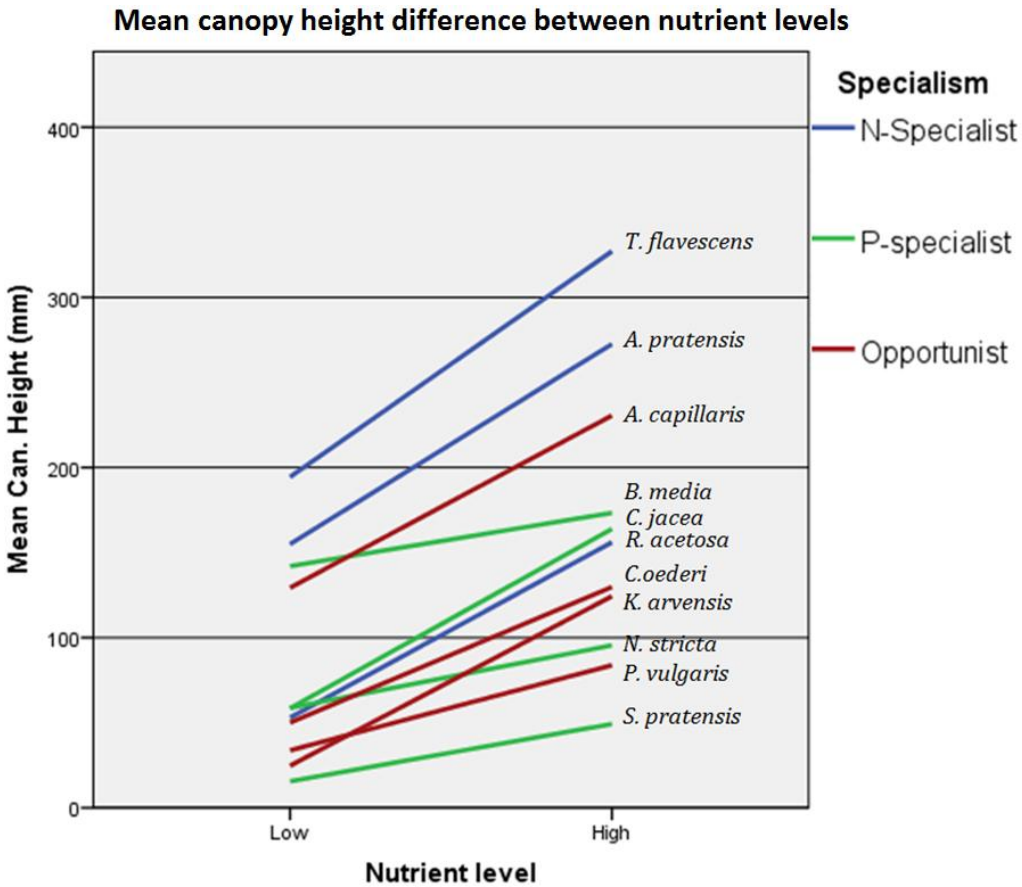

Figure 14. Mean canopy height difference between separate species under different nutrient levels.

Table 16. Leaf length of herbs and grasses for both nutrient levels							
Nutrient level	Low		High		Mean difference	Mann Whitney U	
	Mean	St. dev	Mean	St. dev		U-value	Asymp. Sig (p)
Herbs	37	33	115	65	78	3311	0,000**
Grasses	122	71	205	101	83	7882	0,000**
**sig. values at α=0,01							

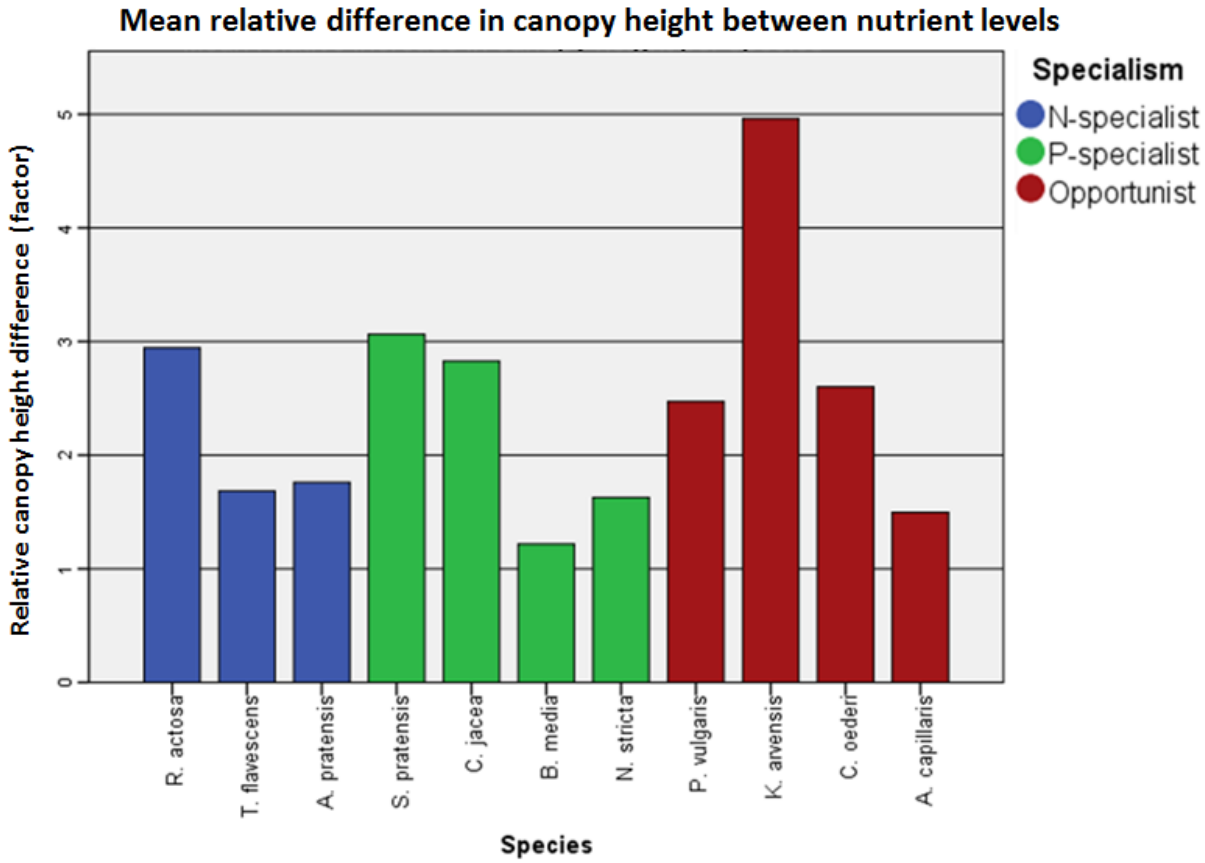

Figure 15. Pooled relative difference in canopy height per species, Specialism is shown in different colours. The relative increase is a factor high/low

Discussion

The primary goal of this experiment was to identify the reaction of different specialisms, namely N-specialists, P-specialists and opportunists on eutrophication in terms of community composition and trait expression.

Species composition & Abundance

The increase in N and P gift between the nutrient levels did not have significant effects on total species richness; species numbers for both groups were still similar to the starting mixtures. The only difference observed was caused by the death of the *C. capillaris* in some tubs. *C. capillaris* was in a relatively fragile early development stage during relocation. Since the dead *C. capillaris* individuals all died within a few weeks after relocation and rather evenly distributed over nutrient level it is unlikely to be caused by nutrient treatment. The equal species richness seems to be in contradiction with established research regarding eutrophication, where eutrophication resulted in species loss (Ceulemans et al., 2013; Hautier et al., 2009; Olde Venterink et al., 2001). But nutrient levels that resulted in species loss are often higher than the high level used in this experiment. Nutrient appliance by Hautier et al. (2009) and Olde Venterink et al. (2001) that showed effects on species richness was considerably higher at 10 as opposed to 6,52 g/m^2 in this experiment (geometric mean). Furthermore, Hautier et al. (2009) found no effects on species richness at 7.25 g/m^2. This support the notion that high nutrient levels used in this research were not high enough to differences regarding species richness and possibly also evenness and abundance. On the basis of the results the first hypothesis (i), stating that species richness will be lower at high nutrient level, has to be rejected. However, most research is done on established communities in the fields or on long term set up experiments. Furthermore, the differences in nutrient levels with established research gives direction to an experiment with a third (higher) nutrient level. Results from such a study could significantly differ from results presented in this research, since this was a developing community and a relatively short term experiment.

More evidence contradicting established research comes from weighted differences in richness in forms of evenness and abundance. These also show no effect of nutrient level on the species composition (abundance) or the biomass division over these species (evenness). With respect to the second research question evenness per specialism is relatively high indicating that biomass per specialism is evenly distributed over the different groups. Hypothesis ii and iii have to be rejected as N-specialists do not have higher abundance than opportunist and the two are also not significantly larger than P-specialists. In general has to be taken into account that low and high nutrient level vary a bit in N:P ratio (4,31 and 3,62 respectively), due to a calculation error in the preparation of the nutrient solutions. Since both are still strongly N-limited (Koerselman & Meuleman, 1996) it is not very likely that this has improved the performance of N-specialists under high nutrient level significantly.

Biomass

Total above ground biomass productivity increased significantly with higher nutrient levels. As was concluded in the previous paragraph community composition itself was not affected much by the change in nutrient level. However, changes in biomass were obviously visible. Regarding

the above ground biomass production (productivity trait) this experiment shows that where biomass under low nutrient level conditions does not significantly differ between specialism. N-specialists have higher biomass at high nutrient levels (figure 7) than other species. Furthermore, the absolute (figure 9) and relative (figure 8) differences between nutrient levels of N-specialists are also higher than the other specialists in the experiment. This confirms hypothesis iv for high nutrient levels, but rejects it for low nutrient levels. That this dominance by N-specialists only occurs under higher nutrient levels could be caused by the capability of N-specialists to use the surplus of nutrients in a more efficient way than P-specialists and opportunists. Several traits that could contribute to this differentiation at higher nutrient levels include N-fixation and nitrogen use efficiency. Higher N-fixing ability (Fujita et al., 2010) can through N-fixing bacteria or mycorrhizal fungi account for more than 50 % of N-uptake depending on N-concentrations (Aerts & Chapin, 2000). Apart from specialised uptake, Aerts & Chapin (2000) also name nitrogen use efficiency as an important trait. High nitrogen use efficiency possibly enables N-specialists to be more productive at higher nutrient levels. As limitation state under high nutrient levels remains equal, N-specialists are still in their supposed beneficial nutrient regime. Previous studies show that infertile grounds promote the allocation of nutrient to root as opposed to shoot biomass (Grime, 2006; Tilman & Wedin, 1991; Tilman, 1988). Also Güsewell & Bollens (2003) found that for a N:P ratio of 5 under low nutrient conditions root biomass was considerably higher than medium or high levels. Since root biomass was not measured in this research it cannot be verified for the currently used species. But the results for above ground can fit these findings, as it makes sense that higher allocation to roots under low nutrient levels leads to lower allocation to shoots. A review study by Aerts and Chapin (1999) shows fast growing species such as N-specialist allocate more biomass to root systems. This can attribute to non-significant differences at lower nutrient levels. Above ground biomass is similar between specialism whereas it is likely that N-specialists have a larger root system. Previous section stresses the importance of roots over shoots, though of significance Wilson (1988) provides important evidence that above ground biomass production and competition is more important in the first year.

Figure 8 clearly shows minor differences between the change in above ground biomass for P-specialists and opportunists. On basis of the results the hypothesis that P-specialist would be more productive than opportunist should be rejected (hypothesis v). However, *C. jacea* has a high biomass production compared to other P-specialists or even N-specialists. There is no strong reason to remove *C. jacea* from the data set. Two subspecies within the *C. jacea* taxa (*jacea & angustifolia*) are considered very similar based on the nutrient status of their habitat (Landolt, 1978). Güsewell and Bollens (2003) showed that *C. angustifolia* produces more biomass at N-limitation as opposed to other limitation states, consistent with our findings on *C. jacea*. The high biomass in *C. jacea* can be caused by its high resource use as documented by Ruijven et al. (2003). As characteristic the high resource use would be expected at both nutrient levels, which is confirmed by the data of this research (figure 9). Having noted the outlier, other P-specialists clearly show lower biomass than opportunists and the relative increase of opportunists is higher. In the aggregate biomass production term for P-specialists *C. jacea* balances this out. If total production would have been larger, as result of for example longer duration of the experiment or a higher nutrient level, differences might have appeared. This gives some nuances to the rejection of the hypothesis (v).

Leaf Length & Canopy Height

Both leaf length and canopy height are light competition traits and ultimately light competition is the determining factor in grassland community competition (Cardinale et al., 2009). Although investing in tall structures is costly, advantages regarding light competition outweigh the high nutrient cost (Falster & Westoby, 2003). Where leaf length is a more absolute measure, canopy height has more of a competition component. Shading of plants by other plants gives them a competitive advantage (Falster & Westoby, 2003). To achieve this, long leaves are not enough, they should also stand higher than that of their neighbours to gain advantage. As for biomass, usually root competition (70 %) is more important than shoot competition (30 %) making below ground competition determining in interspecific competition (Wilson, 1988). However, as mentioned before if experiments are run for shorter time periods than a year species have too little time to interact extensively belowground and competition mainly takes place above ground (Wilson, 1988). This was also observed in this experiment as roots were not severely entangled upon braking up the experimental setup.

In contrast to results in biomass, the canopy height and leaf length of shoots are significantly higher for N-specialists in comparison with P-specialists and opportunists, already under low nutrient regime (figure 10 & 13). These results fully confirm hypothesis vi. That significant differences already show under low nutrient levels could be caused by the investment in higher leaf length and canopies before growing more leaves (biomass). The difference between the P-specialists and opportunists are of a lower magnitude compared to N-specialists. Opportunists have significantly higher canopy height and leaf length than P-specialists, but this only holds true for high nutrient levels. Differences are small and although significant, not of great explanatory value due to the large overlap in error value. The fact that this only manifests at high nutrient levels can be explained by the same lack of above ground competition under low nutrient levels mentioned above. The lack of large differences between opportunists and P-specialists regarding leaf length as opposed to canopy height can be explained by the more competitive nature of canopy height. The hypothesis (vii) stating that opportunists would have higher leaf length and canopy height than P-specialists only holds true at higher nutrient levels. And although strongly correlated with each other (r=0,903) can be more strongly confirmed for canopy height than stretched leaf length. As with biomass a longer experiment or a third nutrient level could increase effects and give more conclusive results.

The low relative differences in canopy height and leaf length (figure 12 & 15) between nutrient levels by N-specialists as opposed to P-specialists and opportunist can also be explained by this reduced competition component. Species first gain a foothold by producing underground biomass, as nutrients are low, after which they often invest in creating a single high shoot. Reduced biomass production provides a light penetrating system where nutrient supply is the main limiting resource (Aerts et al., 1991). Producing more shoot biomass is unnecessary since no direct above ground competition is present. Take note that one tall stem gives a same measurement as ten stems of the same length.

Morphological differences between grasses and herbs show in length and grow orientation of plants (Anten et al., 1995). Differences between structural groups are obviously present in length measurements, figure 12 & 15 show that on average grasses are considerably longer and have a higher canopy height than herbaceous species. These results are backed by the data

presented by Craine et al. (1999). For N-specialists has to be taken into account that after the removal of *C. capillaris* the group consists of two grasses and one herb. This could have caused the averages for canopy height and leaf length to be slightly higher than without the removal of *C. capillaris*. However, as leaf length and canopy are both twice as high under high nutrient levels general trends observed are still valid. Also because one other N-specialists could not lower results to the extent that differences would become insignificant. For biomass there are no problems as results show no clear difference between grasses and herbs.

Synthesis

This research aimed to give insights into the effect of eutrophication on different groups of plants in N-limited environments by answering the following research question:

What is the influence of eutrophication on productivity and light competition traits and functional community composition of N-limited grasslands?

In general eutrophication resulted in heightened productivity and larger expression in light competition traits. The relative differences between canopy height, leaf length and biomass increase with higher nutrient levels. In these differences species defined as N-specialists show larger trait expression than P-specialists and opportunists. In terms of species composition this research has not proven that eutrophication leads to a significant change in the amount of species, the abundance of species or the evenness of species and specialism. However, results for species composition might be different had the experiment run longer or a third nutrient level had been present to show a trend. Focussing on two nutrient levels, supposed differences could not be confirmed. Differences between opportunists and P-specialists were small and the only relevant significant difference between the two was found in canopy height under high nutrient levels. This difference is in line with the expected outcome, a single confirmation though in the right direction is not enough to draw any strong conclusion towards opportunists performing better than P-specialists under N-limited conditions.

Combining results regarding diversity and productivity, specialism can be used to predict the performance of community and weighted biomass in the field. Results from Chapin et al. (2000) and Loreau et al. (2002) show the relation between diversity and productivity with population diversity as a driving factor for biomass. This research put strength to these findings by showing that the specialisms produce different amounts of above ground biomass. In N-limited systems a larger proportion of N-specialists in the population would result in more biomass, given that the total amount of resources is not limiting. This provides indirect evidence to support the idea that species adjusted to nutrient regime perform better. More research into this, preferably at different nutrient ratios is needed to confirm this for P-specialists and opportunists.

Furthermore, this research shows that the differences in specialism are linked to what species invest in which trait at a given moment in time. This is narrowly linked to the concept of plant trait plasticity discussed in Aerts and Chapin (2000), as a plant's need to invest nutrients in certain traits is dependent on the species it is in competition with (Aerts & Chapin, 2000). Results from this research make it likely that N-specialists are more efficient in their trait plasticity to ensure a competitive advantage over others during eutrophication in an N-limited system.

When putting the results in the perspective of mechanisms and pathways presented in figure 1, this research can start to fill the gap defined by Olde Venterink (2011). It starts to do so by using species specialism on nutrient limitation to tie together the three factors: nutrient availability, nutrient stoichiometry and competition traits. Narrowing the gap but definitely not closing it, since this still needs to be proven for P-specialists and opportunists. In trying to put strength to the statement that availability of limiting resources is the proximate cause of species diversity (Cardinale et al., 2009) by conducting a controlled experiment, this research has failed to clarify any differences possibly as result of height of nutrient levels.

Finally, some recommendations for further research include a more synchronised germination to help in strengthening the case by evening development state of species in the plant community at the start of the experiment. But more important: an experiment with a similar setup to the one conducted here with differentiation in nutrient stoichiometry (N-, P-, and Co-limitation state) and at least three nutrient levels. This would provide the information likely to offer conclusive results regarding specialism and reaction on eutrophication.

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Appendices

Appendix A: Used species overview

Rumex acetosa

Garden Sorrel (figure 16) is a widely spread herb, that prefers a sunny to lightly shaded habitat. Nutrient wise it thrives in medium nutrient rich areas without great fertilisation. It has no distinct preference for soil type. *Rumex acetosa* was found to be better represented in a N-limited system (Hejcman et al., 2007). Furthermore, Kirkham et al. (1996) state *R. acetosa* dominates a grassland area after fertilisation with P. The same field did not show such great numbers after N addition showing that *R. acetosa* is likely to be a N-specialist. Finally, Fujita et al. (2014) shows that Garden Sorrel is better represented in N-limited systems.

Figure 16 *Rumex acetosa* (Garden Sorrel) *(*Flora van Nederland, n.d.)

Crepis capillaris

Crepis capillaris or Smooth Hawksbeard is a species that does not demand very special environmental conditions. It lives on dry to moist soils and prefers to be in a sunny spot (Dijkstra, 2015). Field observations show that *C. capillaris* was mainly found in P rich environments. Since no very special environmental conditions are demanded for its growth it could indicate that it is due to its specialism to cope with relative low N that *C. capillaris* grows here. It is regarded as an N-specialist (Fujita et al., 2014).

Figure 17. *Crepis capillaris* (Smooth Hawksbeard) (Flora van Nederland, n.d.)

Trisetum flavescens

T. flavescens or in common tongue: Golden or Yellow Oat Grass is a perennial grass species. It thrives in sunny areas on a dry to lightly moist soil preferably of a calcareous nature. Furthermore, it prefers medium nutrient levels and no fertilisation (Dijkstra, 2015). *T. flavescens* is a dominating species in a mixture after the addition of P in the study by Hejcman et al. (2007). And is thus classified as a N-specialist in this study.

Figure 18. *Trisetum flavescens* (Golden Oat Grass) (Floron & NDFF, 2015)

Alopecurus pratensis

The Meadow Foxtail is a perennial grass species which is commonly found in meadows and grasslands. It prefers sunny, nutrient rich areas with generally clayish soil (Dijkstra, 2015). Olde Venterink & Gusewell showed that *A. pratensis* performs better under N-limitation. Also Hejcman et al. (2007) found A. pratensis dominating a community which was enriched with P asopposed by a treatment of just N. To conclude the database made by Fujita et al. (2014) also shows that it is mostly found in N-limited systems.

Figure 19. *Alopecurus pratensis* (meadow foxtail) in a field (Flora van Nederland, n.d.)

Succisa pratensis

Devilsbit is a perennial herb mainly growing in dry to moist, sunny areas. Medium nutrient levels and low to no fertilisation is needed for this species to thrive (Dijkstra, 2015). Hejcman et al. (2007) found these species only present in a field study where no P fertilisation had taken place indicating that it thrives under low P. Together with the occurrence data from (Fujita et al., 2014) this species is classified as a P-specialist.

Figure 20. *Succisa pratensis* (Devilsbit) (Dijkstra, 2015)

Centaurea jacea

C. jacea or Brown Knapweed is an herbaceous species widely spread through Europe. It thrives in sunny areas on dry ground containing medium nutrient levels. Soils of a calcareous origin are best suited for the Brown Knapweed (Dijkstra, 2015). *C. jacea* was one of the species used in the greenhouse experiment of Roeling (n.d.) and was thus chosen to be a part of this experiment to compare the results. Also Fujita et al. (2014 & 2010) mentions it mostly found in P-limited systems.

Figure 21. *Centaurea jacea* (Brown Knapweed) (Flora van Nederland, n.d.)

Briza media

Quaking Grass is a perennial grass which's habitat consist of a open sunny place with a medium dry to wet soil with a mediocre nutrient regime (Dijkstra, 2015). Hejcman et al. (2007) found *B. media*'s numbers and productivity to increase with the addition of N, indicating that it can cope with relative low P. Alongside this the data from the database (Fujita et al., 2014) makes that it is seen a P specialist in this study.

Figure 22. *Briza media* (Quacking grass) (Floron & NDFF, 2015)

Nardus Stricta

Matgrass is a grass species that thrives on a lightly acid to acid, sandy soil. The soil preferably has a dry to medium moisture content and a low nutrient level. Furthermore, a considerable amount of sun is for the optimum situation (Dijkstra, 2015). (Hejcman et al., 2007) and (Venterink & Güsewell, 2010) both state that *N. stricta* is highly adapted and dominates (extremely) P-limited systems. *N. stricta* is the last P-specialist in this experiment

Figure 23. *Nardus stricta* (Matgrass) (Flora van Nederland, n.d.)

Prunella vulgaris

This perennial herb grows in sunny to lightly shaded places on moist, medium nutrient rich soils of a calcareous nature. Selfheal is found on loam, (sabulous) clay, sand and peat soil (Dijkstra, 2015). P. vulgaris was found to be common in either N- or P-limited systems with no particular preference and was classified as an opportunist (Fujita et al., 2014).

Figure 24. *Prunella vulgaris* (Selfheal) (Flora van Nederland, n.d.)

Knautia arvensis

K. arvensis or Field Scabious is a perennial herb from the Caprifoliaceae family. It mainly grows in sunny spots on medium dry to often moist substrate. The soil is preferably (highly) calcareous with medium nutrient levels (Dijkstra, 2015). It has been classified as an opportunist species by (Fujita et al., 2014) and is regarded as such in this study.

Figure 25. Knautia arvensis (Field Scabious) (Flora van Nederland, n.d.)

Carex oederi

Little green sedge is a perennial sedge species from the Cyperaceae family. It is often found in pioneer vegetation in sunny open fields under wet conditions. It prefers sandy/loamy calcareous soils which are slightly acidic to alkaline. A medium nutrient level with little to no fertilisation is preferred (Dijkstra, 2015). Field study has shown that this species is common in most nutrient limitation states (Fujita et al., 2014). However, little is known about its response to nutrient additions making it harder to be more conclusive about the specialism. This said, it is regarded as an opportunist species in this research.

Figure 26. *Carex oederi* (Little green sedge) (Dijkstra, 2015)

Agrostis capillaris

Agrostis capillaris or Common Bent is a perennial grass species from the Gramineae family. It grows in sunny to lightly shaded areas on dry to moist soils. In the ideal situation the slightly acidic soils should contain medium nutrient levels. It can grow on sand, loam, clay, dried peat, marl and gravel (Dijkstra, 2015). *A. capillaris* has been found in P- and N- limited systems under a wide range of nutrient ratios (Venterink & Güsewell, 2010). Also Kirkham et al. (1996) found *A. capillaris* to contribute largely to the biomass in either N or P addition treatments and is thus regarded as an opportunists.

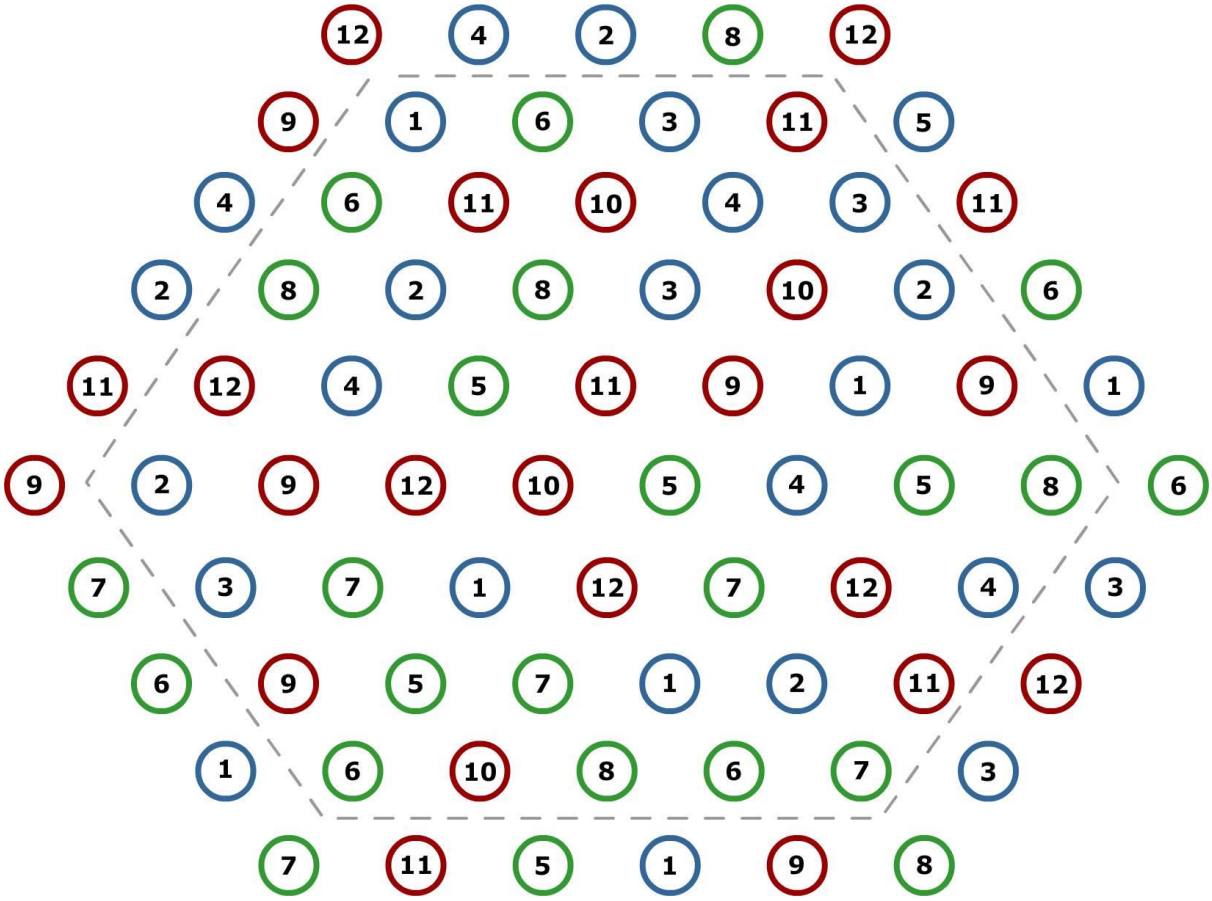
Figure 27. *Agrostis capillaris* (Common bent) (Flora van Nederland, n.d.)

Appendix B: Irrigation water characteristics

Total amounts per plant of micro (purple) and macro (blue) present in the osmosis water (mg/l)														
Level	P	N	K	Ca	Cl	Mg	Fe	Zn	Na	Mo	Cu	B	Mn	S
Mean	0	*	0,09	0,22	0	0	0,03	0,11	0	0	0,20	*	0	0
St. dev	0	*	0,01	0,01	0	0	0,01	0	0	0	0,01	*	0	0
Table shows values extracted from a sample taken 01-08-2014 *not measured														

Total amounts per plant of micro (purple) and macro (blue) present in the rain water collection pond(mg/l)														
Level	P	N	K	Ca	Cl	Mg	Fe	Zn	Na	Mo	Cu	B	Mn	S
Mean	0	*	0,36	4,95	0,04	0	37934,38	20760,33	0	0	27978,73	*	0	0,17
St. dev	0	*	0,21	2,86	0,03	0	65770,61	8989,43	0	0	12114,14	*	0	0,11
Table shows values extracted from a sample taken 01-08-2014 *not measured														

Appendix C: Planting scheme for relocation



Appendix D: Nutrient solution concentration and protocol

Concentrations of nutrient solutions for high and low					
Compound	Element	Stock solution	[Stock] mg/l	ml stock per litre final solution	
				Low	High
KH2PO4	K, P	1	3081	5,2	30
KNO3	K, N	2	9945	5	30
$Ca(NO_3)_2.4H_2O$	Ca, N	3	7532	5	30
KCl	K, Cl	4	99809	16,6	16,6
$MgSO_4 \cdot 7H_2O$	Mg, S	5	46316	10	10
$FeSO_4 \cdot 7H_2O$	Fe, S	6	1582	10	10
$CaCl \cdot 7H_2O$	Ca	7	54999	10	10
$ZnSO_4 \cdot 7H_2O$	Zn, S	8[1]	144	10	10
$MnSO_4 \cdot H_2O$	Mn, S	8[2]	39		
$CuSO_4 \cdot 5H_2O$	Cu, S	8[3]	30		
H_3BO_3	B	8[4]	171		
$Na_2MoO_4 \cdot 2H_2O$	Na, Mo	8[5]	1266		
[1,2,3,4,5] Substances 1 t/m 5 for the 8th stock solution					

Creating the stock solutions
For the total experiment 3,5 litre stock solution was created per stock solution.

Stock Solution 1 - Phosphorous/ Potassium			
Compound	Element	[Stock] mg/l	mg/3,5l
KH_2PO_4	K, P	3081	10783,5
Step 1.	Take 500 ml. osmosis water		
Step 2.	Weigh of the substances in the fourth column		
Step 3.	Mix it with the osmosis water		
Step 4.	Top of to 3,5 litres		

Stock Solution 2 - Nitrogen / Potassium			
Compound	Element	[Stock] mg/l	mg/3,5l
KNO_3	K, N	9945	34807
Step 1.	Take 500 ml. osmosis water		
Step 2.	Weigh of the substances in the fourth column		
Step 3.	Mix it with the osmosis water		
Step 4.	Top of to 3,5 litres		

Stock Solution 3 - Nitrogen / Calcium			
Compound	Element	[Stock] mg/l	mg/3,5l
$Ca(NO_3)_2.4H_2O$	Ca, N	7532	26362
Step 1.	Take 500 ml. osmosis water		
Step 2.	Weigh of the substances in the fourth column		
Step 3.	Mix it with the osmosis water		
Step 4.	Top of to 3,5 litres		

Stock Solution 4 - Potassium / Chloride			
Compound	Element	[Stock] mg/l	mg/3,5l
KCl	K, Cl	99809	349332
Step 1.	Take 500 ml. osmosis water		
Step 2.	Weigh of the substances in the fourth column		
Step 3.	Mix it with the osmosis water		
Step 4.	Top of to 3,5 litres		

Stock Solution 5 - Magnesium / Sulphate			
Compound	Element	[Stock] mg/l	mg/3,5l
$MgSO_4 \cdot 7H_2O$	Mg, S	46316	162106
Step 1.	Take 500 ml. osmosis water		
Step 2.	Weigh of the substances in the fourth column		
Step 3.	Mix it with the osmosis water		
Step 4.	Top of to 3,5 litres		

Stock Solution 6 - Iron / EDTA			
Compound	Element	[Stock] mg/l	mg/3,5l
$FeSO_4 \cdot 7H_2O$	Fe, S	1582	5537
Na_2EDTA		1044	3654
Step 1.	Take 1000 ml. osmosis water		
Step 2.	Weigh of the substances in the fourth column		
Step 3.	Add the Na_2EDTA and heat on a stir flow to 70 ˚C		
Step 4.	Add the $FeSO_4 \cdot 7H_2O$ and wait until it turns brown (rust colour)		
Step 5.	Let cool down to decrease the volume		
Step 6.	Top of to 3,5 litres		

Stock Solution 7 - Calcium			
Compound	Element	[Stock] mg/l	mg/3,5l
$CaCl \cdot 7H_2O$	Ca	54999	192497
Step 1.	Take 500 ml. osmosis water		
Step 2.	Weigh of the substances in the fourth column		
Step 3.	Mix it with the osmosis water		
Step 4.	Top of to 3,5 litres		

Stock Solution 8 - Nitrogen / Potassium			
Compound	Element	[Stock] mg/l	mg/3,5l
$ZnSO_4 \cdot 7H_2O$	Zn, S	144	504
$MnSO_4 \cdot H_2O$	Mn, S	39	137
$CuSO_4 \cdot 5H_2O$	Cu, S	30	105
H_3BO_3	B	171	599
$Na_2MoO_4 \cdot 2H_2O$	Na, Mo	1266	4431
Step 1.	Take 5x500 ml. osmosis water		
Step 2.	Weigh of the substances in the fourth column		
Step 3.	Add $ZnSO_4 \cdot 7H_2O$ to 500 ml osmosis water		
Step 4.	Add $MnSO_4 \cdot H_2O$ to 500 ml the osmosis water		
Step 5.	Add $CuSO_4 \cdot 5H_2O$ to 500 ml the osmosis water		
Step 6.	Add H_3BO_3 to 500 ml the osmosis water		
Step 7.	Add $Na_2MoO_4 \cdot 2H_2O$ to 500 ml the osmosis water		
Step 8.	Add the substances above in the same order to 1000ml of osmosis water		

Final Solution for 1 week– Stock solution 1 t/m 8			
		Low (8 litre)	High (7 litre)
Step 1.	Start with osmosis water	6000ml	5000ml
Step 2.	Measure of Solution 1	41,8ml	210ml
Step 3.	Top off to 250ml		
Step 4.	Measure of Solution 2	40ml	210ml
Step 5.	Top off to 250ml		
Step 6.	Measure of Solution 3	40ml	210ml
Step 7.	Top off to 250ml		
Step 8.	Measure of Solution 4	132,5ml	116ml
Step 9.	Top off to 250ml		
Step 10.	Measure of Solution 5	80ml	70ml
Step 11.	Top off to 250ml		
Step 12.	Measure of Solution 6	80ml	70ml
Step 13.	Top off to 250ml		
Step 14.	Measure of Solution 7	80ml	70ml
Step 15.	Top off to 250ml		
Step 16.	Measure of Solution 8	80ml	70ml
Step 17.	Top off to 250ml		
Step 18.	Add all solution to the starting amount of osmosis water in the order described in step 2 to 17		

Appendix E: Measurement form

Sheet No.				Pallet no.:		Date	
researcher				Nutrient Level:	High / Low	Bucket no.:	
No.	Name	Rep/†	Can. Height (mm)	Leaf length(mm)	Herbivory(1/2/3)	Flowers/buds	Biomass (mg)
buf01	*A. capillaris*						
buf02	*A. pratensis*						
buf03	*C. capillaris*						
buf04	*N. stricta*						
buf05	*A. capillaris*						
buf06	*S. pratensis*						
buf07	*C. oederi*						
buf08	*C. jacea*						
buf09	*R. acetosa*						
buf10	*C. jacea*						
buf11	*T. flavescens*						
buf12	*A. capillaris*						
buf13	*T. flavescens*						
buf14	*N. stricta*						
buf15	*P. vulgaris*						
buf16	*R. acetosa*						
buf17	*S. pratensis*						
buf18	*C. oederi*						
buf19	*B. media*						
buf20	*R. acetosa*						
buf21	*C. jacea*						
buf22	*B. media*						
buf23	*P. vulgaris*						
buf24	*C. oederi*						
buf25	*C. capillaris*						
buf26	*A. pratensis*						
buf27	*P. vulgaris*						
pos01	*R. acetosa*						
pos02	*C. jacea*						
pos03	*T. flavescens*						
pos04	*C. oederi*						
pos05	*C. jacea*						
pos06	*C. oederi*						

Sheet No.				Pallet no.:		Date	
researcher				Nutrient Level	High / Low	Bucket no.:	
No.	*Name*	Rep/†	Can. Height (mm)	Leaf length(mm)	Herbivory(1/2/3)	Flowers/buds	Biomass (mg)
pos07	*K. arvensis*						
pos08	*A. pratensis*						
pos09	*T. flavescens*						
pos10	*N. stricta*						
pos11	*C. capillaris*						
pos12	*N. stricta*						
pos13	*T. flavescens*						
pos14	*K. arvensis*						
pos15	*C. capillaris*						
pos16	*A. capillaris*						
pos17	*A. pratensis*						
pos18	*S. pratensis*						
pos19	*C. oederi*						
pos20	*P. vulgaris*						
pos21	*R. acetosa*						
pos22	*P. vulgaris*						
pos23	*C. capillaris*						
pos24	*P. vulgaris*						
pos25	*A. capillaris*						
pos26	*K. arvensis*						
pos27	*S. pratensis*						
pos28	*A. pratensis*						
pos29	*S. pratensis*						
pos30	*N. stricta*						
pos31	*T. flavescens*						
pos32	*B. media*						
pos33	*R. acetosa*						
pos34	*A. capillaris*						
pos35	*B. media*						
pos36	*A. capillaris*						
pos37	*A. pratensis*						
pos38	*P. vulgaris*						
pos39	*S. pratensis*						
pos40	*B. media*						

Sheet No.				Pallet no.:		Date:	
researcher				Nutrient Level	High / Low	Bucket no.:	
No.	*Name*	Rep/†	Can. Height (mm)	Leaf length(mm)	Herbivory(1/2/3)	Flowers/buds	Biomass (mg)
pos41	*R. acetosa*						
pos42	*C. capillaris*						
pos43	*C. oederi*						
pos44	*C. jacea*						
pos45	*K. arvensis*						
pos46	*N. stricta*						
pos47	*C. jacea*						
pos48	*B. media*						