UNIVERSITY OF KWAZULU-NATAL

# Responses of a South African Mesic Grassland to Long-term Nutrient Enrichment and Cessation of Nutrient Enrichment

**by**

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A dissertation submitted in fulfilment of the requirements for the degree of Doctor of Philosophy,

in the School of Life Sciences,

College of Agriculture, Engineering and Science,

University of KwaZulu-Natal

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**February 2023**

***Special dedication:***

*To my late grandmother,*

*Mrs Lindiwe “MaBhengu” Zama*
*(1945 -2022)*
*You just missed this moment, thank you for everything!*

# ABSTRACT

Nitrogen and phosphorus are two of the most important limiting nutrients required for plant growth and production within grasslands. South African ecosystems are generally considered to be nutrient poor with acidic soils. Increased soil acidity exacerbated by nutrient enrichment are expected to transform South African grasslands over a long period. This PhD research project investigated the cumulative effect of 70 years of nitrogen (N) – in the form of limestone ammonium nitrate (LAN) and ammonium sulphate (ASU), phosphorus (P) – in the form of superphosphate and dolomitic lime application on the Ukulinga Grassland Nutrient Experiment (UGNE). The UGNE has been in operation since 1951 and is located on the plateau at the Ukulinga Research Farm, KwaZulu-Natal, South Africa. Here, the purpose was to evaluate potential changes in above-ground net primary productivity (ANPP), species composition, soil variables and species diversity. In the first experiment (Chapter 2), nutrient-enriched plots were abundantly dominated by taller grass species like *Megathyrsus maximus* rather than shorter species like *Themeda triandra* and *Tristachya leucothrix*. Limestone ammonium nitrate and P did not affect species richness as strongly as ASU, suggesting nutrient identity to be an important factor to consider. Ammonium sulphate enrichment was also associated with low soil pH, enhancing these plots' elevated aluminium (Al) concentration. Considering this key result, careful monitoring of soil pH and Al concentration is required moving forward as Al toxicity may cause a threat to sensitive plant species. To further emphasize the negative effects of soil acidification induced by N on the UGNE, the greenhouse pot-trial experiment (Chapter 5) revealed that the N-enriched soils are extremely acidic and P- deficient, further stunting nodulation development in *Vachellia sieberiana* saplings. *Vachellia sieberiana* is a common nitrogen-fixing species that encroaches mesic environments in South Africa, but its competitive ability may be reduced within grasslands under extremely acidic conditions. Phosphorus enrichment can replenish important soil nutrients and further improve soil fertility. The effects of long-term P enrichment (70 years) and short-term cessation of P enrichment (3 years) has received little to no attention in South African grasslands, therefore this was investigated in the second experiment (Chapter 3). Phosphorus is an important macro-nutrient and essential for biological nitrogen fixation (BNF). Therefore, it is expected that long-term P enrichment would modify soil properties and indirectly influence plant cover percentage and composition. What was unknown was if the cessation of the P enrichment would revert plant cover and composition towards untreated/control conditions. In chapter 3, the results revealed that changes in plant cover among the three treatments of control, P enrichment and cessation of P enrichment were consistent. Furthermore, no difference was observed among the treatments for the ANPP, species diversity indices and richness. However, the Indicator Species Analysis proved that *Setaria nigrirostris* and *Bidens pilosa* were strongly associated with the cessation of P enrichment treatment. The third experiment (Chapter 4) provided the opportunity to assess the best models that predict species compositional changes and species richness declines. Here, it was clear that the enrichment of more nutrients shifted both grass and forb species composition. For grass species composition, the shift in cover-abundance was from the shorter *Tristachya leucothrix* (no nutrients added) species to *Aristida junciformis* (only LAN added) and *Megathyrsus maximus* (LAN + P added). For forb species composition, *Cephalaria pungens* occurred in a high cover-abundance in control plots and *Bidens pilosa* occurred in LAN + P enriched plots. Interestingly, LAN-only and LAN + P enriched plots showed the greatest shift in both grass and forbs when compared to ASU-only, ASU + P and P-only enriched plots. In terms of overall species declines, the models presented identified the number of nutrients added and ANPP as the best predictors. Light was not identified as a significant predictor. The results provided partial evidence for the nitrogen detriment hypothesis and biomass-driven hypothesis over the niche dimension hypothesis.

The key findings from all the experiments highlight that the following factors: 1) nutrient-poor soils, 2) type of nitrogen used for enrichment, 3) soil acidification and 4) high soil aluminium concentrations are important in the observed changes in the soil chemical properties, species composition, species diversity, species richness and species growth dynamics on the UGNE. The results of this research project also emphasize the importance of long-term studies in assessing if the cessation of nutrient enrichment is a strategy for ecosystem rehabilitation. It was identified here that long-term nutrient enrichment heavily modifies a mesic grassland community and alternative rehabilitation methods may need to be implemented.

# PREFACE

The research contained in this thesis was completed by the candidate while based in the Discipline of Grassland Science, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg, South Africa. The research was financially supported by the Agricultural Research Council of South Africa.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

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## DECLARATION 1 - PLAGIARISM

I, Naledi Zola Zama, declare that

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## DECLARATION 2 – PUBLICATIONS

*Indicates the corresponding author

**Chapter 2**

**Publication 1 – published in the African Journal of Range and Forage Science**

Zama, N*., Magadlela, A., Mkhize, N., Tedder, M., Kirkman, K., 2022. Assessing long-term nutrient and lime enrichment effects on a subtropical South African grassland. African Journal of Range and Forage Science. https://doi.org/10.2989/10220119.2021.2014964.

The data for this chapter was collected at the Ukulinga Grassland Nutrient Experiment, KwaZulu-Natal, South Africa. The research was conceived by Prof. Kevin Kirkman, Dr Anathi Magadlela, Dr Ntuthuko Mkhize, Dr Michelle Tedder and I. I performed the data analyses and wrote the manuscript. The abovementioned supervisors contributed greatly to the data analyses, structuring and final manuscript for the journal.

**Chapter 5**

**Publication 2 – published in the Plants journal**

Zama, N*., Kirkman, K., Mkhize, N., Tedder, M., Magadlela, A., 2022. Soil acidification in nutrient enriched soils reduces the growth, nutrient concentrations, and nitrogen-use efficiencies of *Vachellia sieberiana* (DC.) Kyal. & Boatwr saplings. Plants. doi: 10.3390/plants11243564.

The soil data for this chapter was collected at the Ukulinga Grassland Nutrient Experiment, KwaZulu-Natal, South Africa. The greenhouse experiment was conducted at the NM Tainton Arboretum at the University of KwaZulu-Natal. The research was conceived by Dr Anathi Magadlela and I. I performed the data analyses and wrote the manuscript. Prof. Kevin Kirkman, Dr Anathi Magadlela**,** Dr Ntuthuko Mkhize and Dr Michelle Tedder all contributed greatly to the data analyses, structuring and final manuscript for the journal.

Signed: Naledi Zola Zama

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Date: 1 February 2023

# ACKNOWLEDGEMENTS

First and foremost, I would like to thank my esteemed supervisors, Prof. Kevin Kirkman, Dr Anathi Magadlela, Dr Michelle Tedder, and Dr Ntuthuko Mkhize for their invaluable supervision and encouragement throughout the duration of my PhD degree.

Secondly, I would like to express my gratitude to the following individuals:

- J.D Scott for initiating the Ukulinga Grassland Nutrient Experiment (UGNE) in 1951.
- Mrs Alison Young and the UKZN staff who have maintained the UGNE since its establishment.
- Mr Welcome Ngcobo for his assistance in planting and watering my plants in the Neil Tainton Arboretum greenhouse.
- Fieldwork assistance from my lab colleagues, Ms. Yonela Maziko, Mrs. Theresa Ojo and Mr Stuart Demmer.
- Lab assistance from Ms. Zinhle Lembede, Ms. Nonkululeko Sithole and Ms. Khululiwe Ndabankulu during sample preparation and their advice in data preparation.
- The R Core Team and R community who have created a wonderfully advanced and free programming language for statistical outputs. I cannot accurately describe how useful this software has been in all my research chapters and personal development.

Thirdly, I greatly acknowledge the funding received towards my PhD from the Agricultural Research Council (ARC). I also appreciate the travelling opportunities and professional training that have been possible because of the ARC. I would like to offer my special thanks to the ARC- Animal Production unit including Dr. Julius Tjelele, Dr. Kedinnetse Monegi and Mr. Ngoako Letsoalo for creating an encouraging environment for me to thrive.

Fourthly, I would like to thank my friends, Dr Zabentungwa Hlongwane, Ms Nqobile Ndhlovu, Ms Nobuhle Mweli, Mrs Simangele Msweli-Ratsoana, Ms Rerani Ramaano, Ms Thandwayo Mbambo and Ms Abongile Mbande who have in their unique ways kept me motivated, cheerful and accountable. In addition, Mr Lindokuhle Dlamini for consistently convincing me that I am capable of pursuing and completing a PhD and offering great insights and useful comments during our statistical analysis meetings.

My appreciation also goes out to my aunt (Mrs Bongiwe Mathe), uncles (Mr Zamani Zama and Mr Sithembiso Zama), cousins (Ms Khwezi Mathe and Mr Wandile Zama) and my late cousin

(Ms Sinegugu Zama), who have been patient with me throughout my entire postgraduate journey. I know it was difficult and your support through my studies has not gone unnoticed. Thank you for your unwavering support and complete faith in me.

Finally, I would like to thank my mother, Ms Lungisile Zama and brother, Mr Nqobile Zama. I love you and thank you for everything!

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# Chapter 1

# General introduction and literature review

## Introduction

The growing human population has increased agricultural production and this has increased dependence on fertilizer usage (Kidd et al., 2017). In Sub-Saharan Africa (SSA), smallholder farmers acknowledge the role of fertilizers in crop production  but fertilizer use remains low because of high costs and poor market networks (Chianu et al., 2012).  It is well established that fertilizer addition increases agricultural yields and plant productivity in natural grasslands used for experimental purposes (Aydin and Uzun, 2005; Kidd et al., 2017; Ward et al., 2017b). The annual growth in agricultural productivity is persistently low in SSA, therefore increasing food insecurity and dependency on exports from elsewhere (Fuglie, 2015). Findings reveal that increasing fertilizer use in SSA is necessary to intensify crop production (Tsujimoto et al., 2019). Understanding which factors affect species diversity and productivity within ecosystems has been studied for decades (van der Wal et al., 2009). Most of the focus has primarily been on understanding the responses of aboveground variables, however, belowground communities are capable of  supporting a substantial amount of biodiversity and provide ecosystem functions (van der Wal et al., 2009).  For example, research suggests that soil biota diversity is enhanced after nutrient addition and this is explained by increased amounts of organic matter in the soil (van der Wal et al., 2009). Furthermore, nitrogen addition can increase predatory mites, increasing soil biota diversity (Dyer and Letourneau, 2003).  The changes in soil properties caused by fertilizers and nutrient deposition and pollution is important (Kidd et al., 2017). This is because when nitrogen is in excess it can suppress antagonistic microorganisms and enhance the presence of pathogens in the soil (Söchting and Verreet, 2004; Wei et al., 2018). Soil acidification is known to directly impact microbial diversity and even caue an increase in soil-borne diseases (Li et al., 2017).  Studies reveal that soil organisms are capable of influencing vegetation (van der Wal et al., 2009) and that the interaction between above- and below-ground plant and soil communities affects biomass production and biodiversity (van der Heijden, Boller, Wiemken, & Sanders, 1998).

Ecosystem shifts influenced by nutrient enrichment include an increase on above-ground net primary productivity (ANPP) with N and P enrichment  (Vitousek et al., 2010), whereas N enrichment (Cleland and Harpole, 2010) and P enrichment (Kirkham et al., 1996) decrease plant species diversity and richness. These noticeable changes and shifts in ecosystem functioning and structure induced by nutrient addition ignites questions about their impact in mesic grasslands (Kidd et al., 2017). There is increasing uncertainty regarding the long-term impact of nutrient addition on grasslands caused by the reliance of results from short-term experimental studies (Guerrero-Ramírez et al., 2019). The abovementioned statement does not disregard the importance of short-term studies as these may be important in management situations whereby practices are changing constantly (Kidd et al., 2017). However, short-term studies may potentially underrepresent long term processes or fail to detect certain environmental thresholds including species adaptation (Kidd et al., 2017).

The main limiting nutrients in grasslands are nitrogen and phosphorus (hereafter N and P) (Elser et al., 2000). Phosphorus is an essential ingredient of cell membranes as well as nucleic acids and N is an important compound for protein (DeMalach, 2018). Naturally, N inputs are derived from biological fixation processes and P inputs are derived through processes related to rock erosion (Vitousek et al., 2010). Nitrogen and P also differ in rates of diffusion, decomposition of microbes, uptake mechanisms and the amount of  time the nutrient is present in the soil (Chapin, 1980; Elser et al., 2000; Lambers et al., 2008). A meta-analysis study of nutrient addition in various grasslands revealed that P additions increased biomass production in 19 %  of the sites, N increased biomass in 24 % of the sites and the combined effects increased biomass in 60 % of the sites (Fay et al., 2015). Contrary to these findings (Soons et al., 2017), showed that more grasslands responded positively when fertilized by P or N alone.

The anthropogenic activities occurring globally produce more than half of N output (Fowler et al., 2013; Kanakidou et al., 2016). The increase in food demand and therefore production has further increased N production and utilisation (Fowler et al., 2013).  The Haber-Bosch process and biological nitrogen fixation in agriculture all account for approximately 80 % of anthropogenic N fixation (Anderson et al., 2018). The rest of the  anthropogenic N fixation is from internal combustion engines and power plants (Fowler et al., 2013). Atmospheric deposition of N has increased from 1.9 Tg per year in the 1900s to approximately 3.8 Tg N per year in the 2000s (Sutton, 2011). The  high  levels of N deposition are observed in regions with high populations such as Northern America and Asia (Stevens et al., 2018). It is predicted that areas with high deposition rates are likely to spread into other parts of Asia, South America and Africa (Galloway et al., 2004). The excess N has effects on the functioning and processes occurring in the overall environment (Stevens et al., 2018). The past three decades have shown that N deposition poses huge threats to species biodiversity and how ecosystems function (Bobbink et al., 2010). Most of the current knowledge and research is focused primarily on how N inputs affect plant life (Stevens et al., 2018). The detrimental effects of N and P pollution include a greater susceptibility to pathogens, a change in symbiotic relationships and increased toxicity (Bobbink et al., 2010; Wassen et al., 2005).

Soil acidification has effects on the composition of species and abundance (Silvertown et al., 2006). Acidification in the soil is caused by the application of ammonium sulphate and through atmospheric N addition (Silvertown et al., 2006). The addition of N fertilizer has remained constant over the years but atmospheric N inputs have varied within and between years and this has depended largely on rainfall and emissions (Silvertown et al., 2006). Calculations show that the annual atmospheric N additions totalled approximately 10 kg ha $^{-1}$ initially, increased in the 1960s to approximately 30 kg ha $^{-1}$ before reaching its peak at 45 kg ha $^{-1}$ in t mid-1980s and then declining to 35 kg ha $^{-1}$ (Goulding et al., 1998). Sulphur dioxide inputs from emissions from industrial sources also results in acidification (McGrath et al. 2003).

### Models and hypotheses

The addition of nutrients in grasslands is generally linked to the competitive exclusion of certain species and a decrease in biodiversity (Crawley et al., 2005; DiTommaso and Aarssen, 1989; Harpole et al., 2016) although (Soons et al., 2017) highlighted the limitation of this concept by indicating that N input decreased species richness by 16 % but the effect of P input had no effect.

Anthropogenic activities can result in environmental changes (Tilman et al., 2014; Turnbull et al., 2016). This includes increased used of N and P fertilizers, which can influence species coexistence. Hutchinson (1957) was the first researcher to describe one of the most common explanations for how species are able to coexists in different environments. This explanation was termed the *niche dimension hypothesis*. Support of this hypothesis is described in recent times showing species richness with reductions in the number of limiting niche dimensions or in this case, number of nutrients applied in a specific environment (Harpole et al., 2017; Palpurina et al., 2019). Meanwhile, other researchers have attributed plant species loss to limitations in light more than the niche dimension hypothesis (DeMalach and Kadmon, 2017; Hautier et al., 2009). This leads to the *light asymmetry hypothesis* (Newman, 1973). This hypothesis suggests that an increase in biomass after nutrient input results in a drop in available light reaching the plants thus enhancing competitive exclusion (Newman, 1973). Further support of this indicates that taller plant species are able to receive more light than shorter plant species, increasing competition and then accommodating less species (Borer et al., 2014; DeMalach et al., 2017; Lamb et al., 2009; Lepš, 1999). However, there are debates regarding which of the two above-mentioned hypotheses better explains plant species loss in nutrient enriched environments (DeMalach and Kadmon, 2017; Harpole et al., 2017). Alternatively, the *total competition hypothesis* suggests that nutrient addition will increase biomass production, and this has additional effects on species diversity that do not relate to light competition, particularly because of an associated increase in belowground competition (Ranjeameni, 2003). Another hypothesis is related largely to litter and plant seed establishment as this referred to as *the litter hypothesis* states that an increase in litter after nutrient input may result in the loss of species by lowering the proportion of seeds that establish successfully (Foster and Gross, 1998; Grace, 2001; Tilman, 1993). Lastly, the *nitrogen detriment hypothesis* that is closely linked with N addition suggests that high N addition rates reduces plant species performance. Plant performance is believed to be reduced because high N application rates decrease soil pH and enhance the toxicity of aluminium in soils (Britto and Kronzucker, 2002; Crawley et al., 2005). This hypothesis emphasizes that N has a stronger potential than P to reduce plant species richness (Ceulemans et al., 2013) and very recent research conducted on 99 sites worldwide provides evidence to support the nitrogen detriment hypothesis over the niche dimension hypothesis (Band et al., 2022).

Many studies conducted in the early 70s till present, describe a typical “humped back curve” relationship between nutrients and species richness (Grime, 1973; Huston, 1979). This curve indicates that species richness will be at its lowest at low nutrient levels, increase to its peak at an intermediate level and begin to decline at high levels of nutrients (Grime, 1973). The reasoning given for this relationship is that very few species can tolerate extremely low nutrient levels and as nutrients increase, more species can survive thus increasing species richness (Grime, 1973). However, at extremely high nutrient levels, the most competitive species will start to dominate and suppress the less dominant species, resulting in a decline in species richness through competitive exclusion (Grime, 1973). The ‘humped-back curve” relationship is supported by numerous studies (Milton and Davies, 1947; Zhao et al., 2019). However, one could argue that studies may use different measures of nutrient availability and species richness, making comparisons difficult to assess. For example, N input lowered species diversity  but increased evenness at the Songen grassland experiment site in China (Zhao et al., 2019). Recent studies have also emphasized findings that the type of nutrient is also important in determining species diversity changes. One example is how the addition of P had no effect on species diversity or richness but instead increased species evenness (Zhao et al., 2019).

Ecologists have debated the relationships and processes that occur in grasslands as a result of nutrient addition for decades, resulting in the formulation of numerous theories, hypotheses, models and paradigms.  The most common is the relationship between primary productivity and plant species richness. The *productivity-richness relationship* (PRR) is "humped shaped" as explained above (Grime, 1973). The mechanisms used to describe this relationship have attracted considerable controversy. These mechanisms include dispersal limitation (Zobel and Pärtel, 2008), disturbance (Huston, 1979), evolutionary history (Zobel and Pärtel, 2008) and competitive exclusion (Tilman, 1993). The theoretical justification for PRR has been challenged, mainly because the empirical evidence used in its support is mixed (Adler et al., 2011). For example, studies that used meta-analyses revealed that the evidence used to support the "humped shape curve" pattern was weak (Gillman et al., 2006; Mittelbach et al., 2001; Waide et al., 1999). In addition, the majority of these studies demonstrated a U-shaped, negative and non-significant PRRs and various patterns depended on the spatial pattern used and taxon (Adler et al., 2011). These meta- analyses were also criticized as authors indicated that the lack of generality in PRRs is explained by methodological inconsistencies among studies (Gillman et al., 2006; Whittaker, 2010). Also, including studies in meta-analyses that did not measure primary productivity directly but used surrogates such as temperature or latitude instead will make it even more difficult to assess PRR (Mittelbach et al., 2001).

## Nitrogen (N)

### ***Direct toxicity***

Most of the mechanisms impacting on primary producers are caused by changes in soil properties and interactions (abiotic and biotic) (Stevens et al., 2018). Toxicity  occurs when electron transport tis disturbed in chloroplast causing leaves in plants to turn yellow, low root growth and necrosis (Pearson and Stewart, 1993; Van der Eerden et al., 1991). In addition toxicity from either ammonia or ammonium is possible through N build up in the soil resulting in under developed shoots and roots (Roelofs et al., 1996).

### *N enrichment*

Nitrogen is an important limiting resource present in the soil (Vitousek and Howarth, 1991), N input via atmospheric deposition will ultimately increase productivity. In addition, it alters species composition, owing to the fact that species that demand nutrients increase in abundance over those that cannot compete under high levels of N and are more adapted to low nutrient levels, out competing them for resources such as light (Ceulemans et al., 2014). High levels of N levels can cause reductions in plant species richness (Clark and Tilman, 2008). The probable causes for this reduction in plant species richness are eutrophication and acidification (Stevens et al., 2010). The most intense loss in species richness occurs in communities with low levels of nutrient availability (Al-Mufti et al., 1977) and not so intensely in communities that are considered N-saturated (Stevens et al., 2010). Various experiments have highlighted the impacts caused by N enrichments in grasslands (e.g.) (Bobbink and Willems, 1987; Clark and Tilman, 2008; Horswill et al., 2008). Majority of the findings reveal that species that are small in structure are most likely to decline in abundance. Seedling recruitment is also altered in some way (Ceulemans et al., 2017). Also enriched soils tend to allow for invasive species to creep into the vegetation structure (Corbin and D'Antonio, 2004) and these species are associated with higher growth rates and can usually tolerate a variety of weather conditions, including frost (Caporn et al., 2000), wind (Grulke et al., 1998) and drought (Friedrich et al., 2012).

### *Plant biochemistry*

Increased soil N and uptake causes drastic changes to plant's biochemistry (Stevens et al., 2018). Tissue N content changes in plants is widely documented (e.g.) (Bobbink et al., 2010; Pitcairn et al., 1998). A review of long-term studies revealed that trees that grew in high N input areas had increased N concentrations in foliar litter compared to those that grew in low N input areas (Berg and Meentemeyer, 2002). This leads to some plants becoming more vulnerable to herbivory than others (Stevens et al., 2018). This impacts greatly on secondary consumers but also has implications for those species deemed more palatable than others as forage (Stevens et al., 2018). Other changes in plants include alterations in the concentrations of photosynthetic pigments (Arróniz-Crespo et al., 2008) and enzyme activity (Hogan et al., 2010).

### *Nitrogen cycling*

Grasslands N inputs have increased during the last decade due to increased anthropogenic activities (Vitousek et al., 1997, 2010). This is also through increased fertilizer applications

(Peñuelas et al., 2013). The use of fertilizer increases the amount of N that is available in the soil for plants to use, resulting in an increase in productivity (ANPP) (Lebauer and Treseder, 2008) and further affects the composition of species and abundances (Clark and Tilman, 2008; Wedin and Tilman, 1996). The nitrogen cycle is outlined in Figure 1.1.

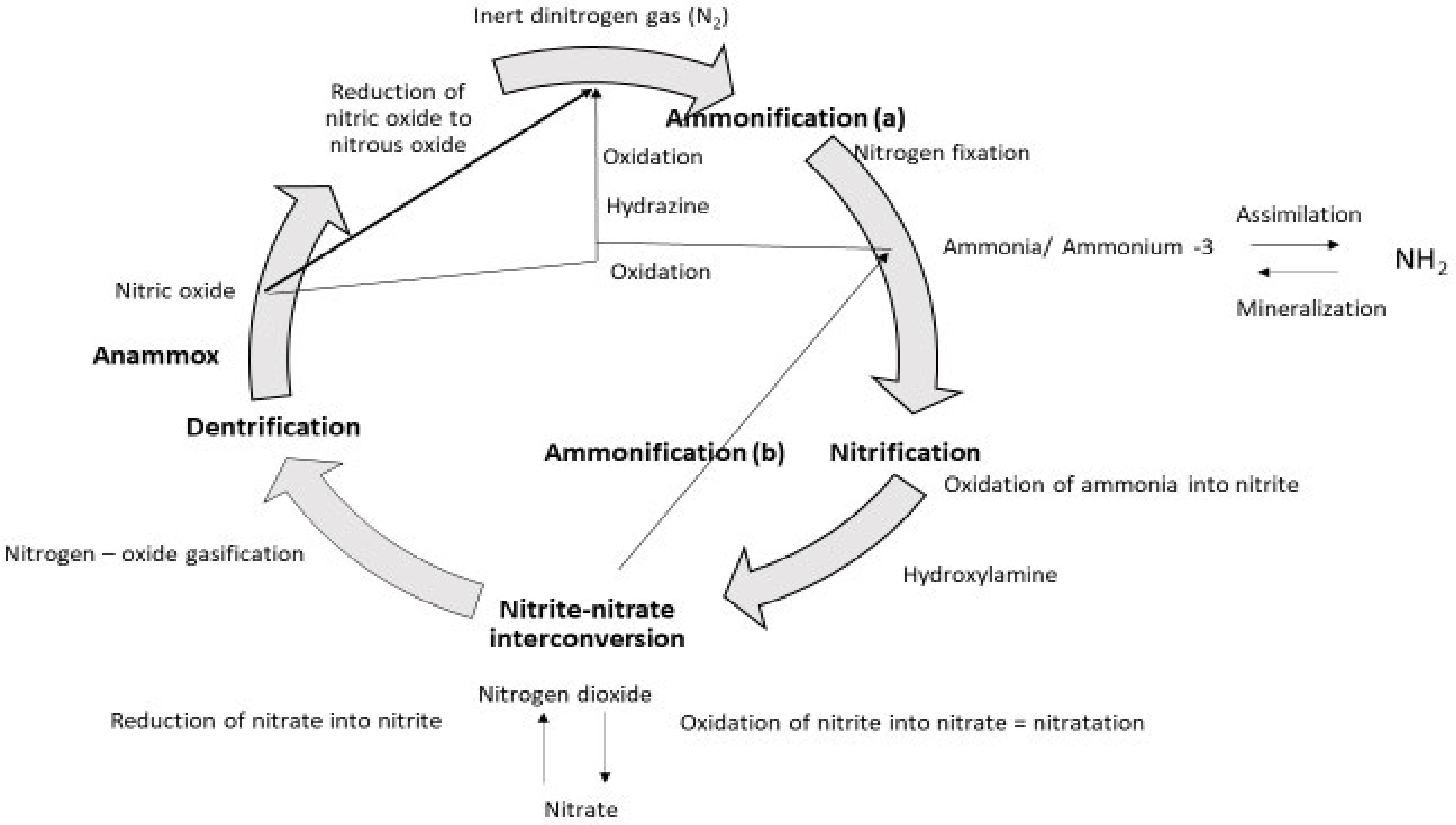


Figure 1. 1. The nitrogen cycle adapted from (Stein and Klotz, 2016).

## Phosphorus (P)

### *P enrichment*

It is important to understand the effects of P in grasslands because P availability influences productivity and abundance of plant species, especially legumes. Phosphorus is required for enhanced legume growth, development of roots and nodule formation (Batterman et al., 2013; Novotny et al., 2007). A global-scale experiment indicated that P enrichment alongside other nutrients such as potassium (K) enhanced legume abundance but did not mitigate the detrimental impacts of nitrogen enrichment (Tognetti et al., 2021). This has consequences for South African ecosystems that are generally acidic and of low nutrient status (Mafongoya et al., 2006). For example, the application of N could cause increased soil acidification and reduce cation exchange capacity, making nutrients such as K and calcium (Ca) less available (Aprile and Lorandi, 2012). In addition, when N is applied with P, the increased soil acidification may further result in the sequestration of P, reducing its availability (Sharma et al., 2013). These combined factors all limit legume performance (Ren et al., 2017), a potential yet overlooked occurrence in Ukulinga Grassland Nutrient Experiment.

From past studies, it is shown that N or P enrichment can positively increase (Grunzweig and Korner, 2003), have no effect on (Avolio et al., 2014) or reduce legume biomass (Sun et al., 2016). A study on an Australian grassy woodland indicated that P enrichment increased total ANPP with certain grass species such as *Cynodon dactylon* (L.) showing increased biomass and height whereas *Eragrostis curvula* (Schrad.) Nees showed decreased biomass (Chieppa et al., 2019). In the previously mentioned study, the authors conclude that P enrichment did not alleviate the detrimental effects of the drought period in the mesic grassland that normally has an annual precipitation of 807 mm (Chieppa et al., 2019). These results do not support findings from a study conducted in North American prairies, reporting positive responses of above-ground production following P enrichment during a period of drought (Pierre et al., 2016). Among the dominant grass species occurring frequently and abundantly on the UGNE is *Eragrostis curvula*, *Themeda triandra*, *Tristachya leucothrix* and *Megathyrsus maximus* (previously known as *Panicum maximum*) (Tsvuura and Kirkman, 2013; Ward et al., 2017b). These grasses showed increased above-ground production with P enrichment (Tsvuura and Kirkman, 2013). This suggests that individual plant functional traits could be providing a competitive advantage for these dominant species over other species on the UGNE.

Increasing P input reduced seedling survival for majority of the species as shown by (Ceulemans et al., 2014). Phosphorus addition in grasslands has also been linked to a decrease of mycorrhizal dependent plant species (Peñuelas et al., 2013). In addition, the loss of symbiotic partners may also occur (Beenhouwer et al., 2015; Helgason et al., 1998). Ceulemans et al. ( 2017) further states that P input may cause a competitive imbalance for species that specialize in access to chemical forms of P and those that access readily available P in the soil.

### *Phosphorus cycling*

Biological cycling is important in grasslands and assists our understanding of the relationship between species composition and P fertility in the soil (Jouany et al., 2011). The successful management of pastures requires for P-limited systems to be properly monitored because continuous and long-term P input may have devasting outcomes in grasslands (Critchley et al., 2002; Janssens et al., 1998; McCrea et al., 2004; Wassen et al., 2005). The reported negative effects of P enrichment include suppressing the activity of C-cycling enzyme at soil depths between 0 and 10 cm (Jing et al., 2016). In European grassland, P contributed to plant biodiversity loss (Ceulemans et al., 2014). This is important in grasslands whereby species diversity is linked to amount of P available in the system (Limpens et al., 2004). A high supply of N input results in an increased production of biomass and this results in the depletion of P reserves in the soil (Fagan et al., 2008; Olde Venterink et al., 2001). Phosphorus is available in different forms including organic and mineral (Jouany et al., 2011). The cycling of P is controlled by biological and geochemical processes, unlike N cycling – mainly controlled by certain biological processes (Jouany et al., 2011).

Nitrogen input management controls the extent in which P flux leaves the system (Stroia et al., 2007; Watson and Matthews, 2008). In the field, P fertilization when over supplied can lead to a surplus in grasslands (Stroia et al., 2007). This causes high P concentration as surface runoff (Schärer et al., 2007). In natural grasslands, animal and plant residues can control P fluxes through decomposition processes in the soil (Jouany et al., 2011). Organic matter inputs cause an uneven availability of nutrients present in the soil (Güsewell, 2005). Essentially this causes fertilized and natural grasslands to build up P in the surface horizons when there is no ploughing occurring and the movement of P ions is limited (Jouany et al., 2011). The phosphorus cycle is illustrated in Figure 1.2.

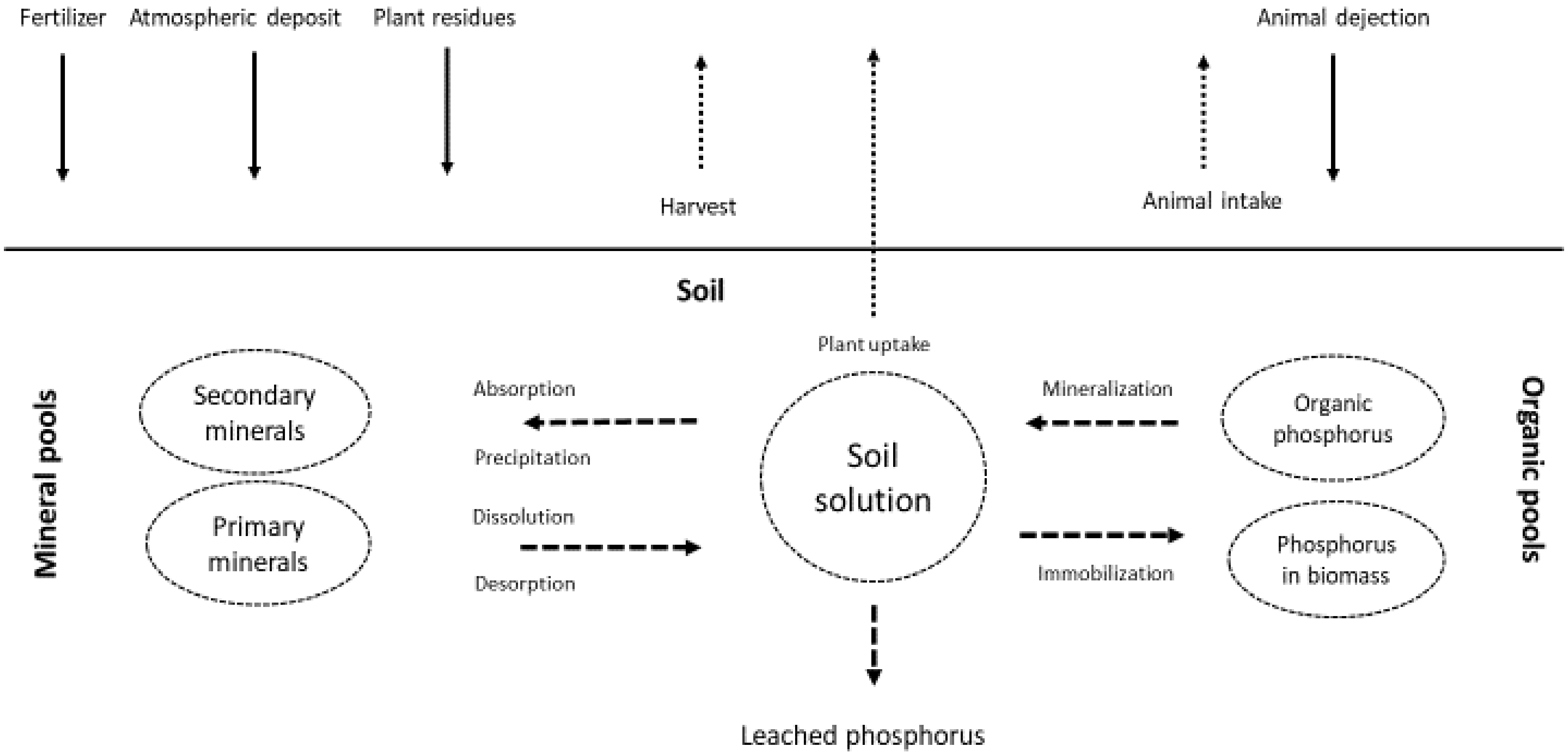


**Figure 1.2**. The phosphorus cycle adapted from (Jouany et al., 2011).

## Soil community

Soil fertility is one important determinant of grassland biomass production and structure (Wardle, 2002). The addition of certain nutrients normally decreases plant biodiversity and ultimately increases productivity (Harpole and Tilman, 2007; Pywell et al., 2007). In particular, the addition of N can increase soil N availability but reduce pH (Chen et al., 2015). Nitrogen addition also decreased the concentration of $Ca^{2+}$, $Na^{+}$ and $Mg^{2+}$ but increased the concentration of $Al^{3+}$ in the soil (Chen et al., 2015; Tian and Niu, 2015).

Nitrogen enrichment can affect the soil microbe community by decreasing total microbial biomass, fungal biomass, bacterial biomass and actinobacteria biomass (Chen et al., 2015). These decreasing effects on microbial biomass are consistent with global scale and local scale studies (Treseder, 2008).

A meta-analysis conducted by (Tian and Niu, 2015), indicated that globally N input reduced soil pH by 0.26. This response in pH was observed only when the rate of N input reached amounts greater than 5 $gm^{-2}$ per year (Tian and Niu, 2015). This indicates that soil acidification responds to N addition , also at low N input levels (Tian and Niu, 2015). Other studies also indicate that ecosystems tend to go through accelerated leaching of $NO_3^-$ from soils if N input is greater than 2.5 $gm^{-2}$ per year (Dise and Wright, 1995).

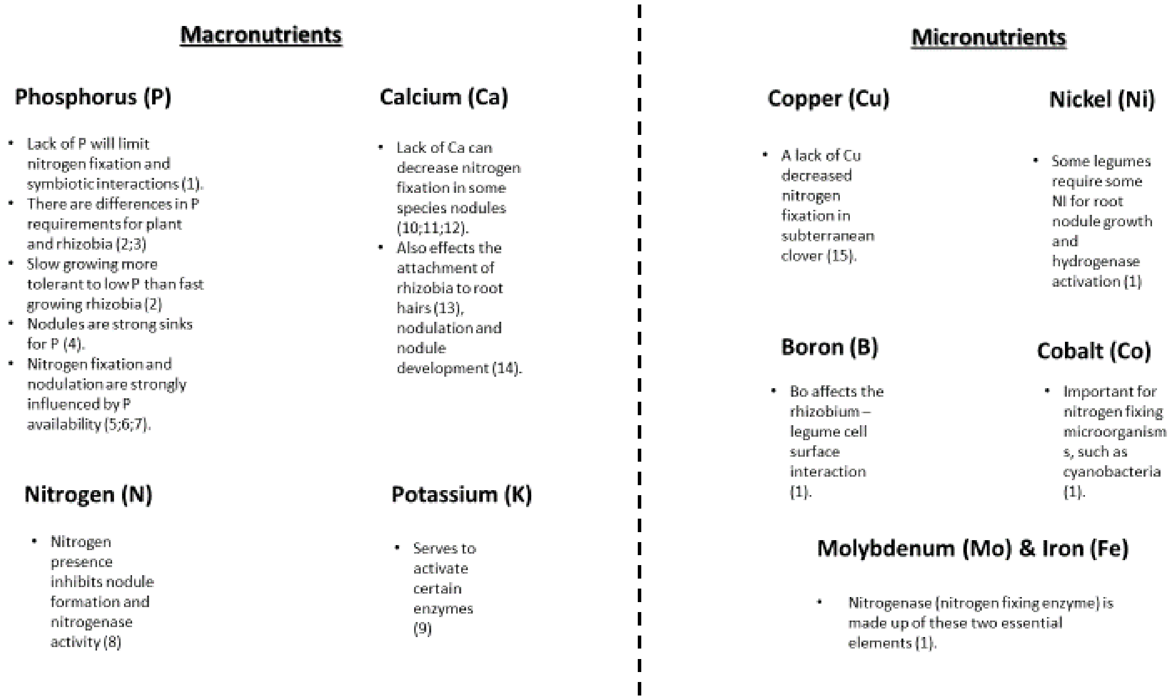
Soil pH changes were also influenced by the number of years that an experiment has been in operation. The soil pH was significantly reduced in experiments that were less than 20 years long but no change for experiments longer than 20 years (Tian and Niu, 2015). Potential reasons for this are firstly, species abundance and richness may change to more N demanding species under high N input rate (Bai et al., 2010) allowing ecosystems to isolate excess N (Tian and Niu, 2015). Secondly, N input causes the loss of base cations and this may accelerate biotic retention coupled with recycling of base cations with the ecosystem (Perakis et al., 2013).

In general, soils in grasslands are sensitive to N inputs in acidification and the complexity of interpreting results is increased by the intertwined interactions between environmental factor and N (Tian and Niu, 2015).

## Biological Nitrogen Fixation

Nitrogen that is present in the atmosphere needs to combine with other molecules to form compound bonds that can be utilised by living organisms (Sharma et al., 2019). Such compounds include ammonia ($NH_3$) and nitrates ($NO_3$) (Sharma et al., 2019). The inert nitrogen in the atmosphere can be converted into a biologically active form through biological nitrogen fixation (BNF), that can be used in the soil with the aid of microorganisms (Sharma et al., 2019).

Fixing nitrogen from the atmosphere is possible through symbiotic relationships that exist rhizobia and legume plants, bacteria present in the soil in which the legume plant supplies energy and other resources such as carbon to the rhizobia and in return the rhizobia provides the legume with N (Howard and Rees, 1996). This form of symbiosis results from the infection of legume roots by the rhizobium forming root nodules where BNF happens (Liu et al., 2011). The symbiosis explained here exists in various legumes such as grains, forages and leguminous trees (Liu et al., 2011). The main effects of certain macronutrients and micronutrients on nitrogen fixation is shown below (Figure 1.3).



**Macronutrients**

**Phosphorus (P)**

- Lack of P will limit nitrogen fixation and symbiotic interactions (1).
- There are differences in P requirements for plant and rhizobia (2;3)
- Slow growing more tolerant to low P than fast growing rhizobia (2)
- Nodules are strong sinks for P (4).
- Nitrogen fixation and nodulation are strongly influenced by P availability (5;6;7).

**Calcium (Ca)**

- Lack of Ca can decrease nitrogen fixation in some species nodules (10;11;12).
- Also effects the attachment of rhizobia to root hairs (13), nodulation and nodule development (14).

**Nitrogen (N)**

- Nitrogen presence inhibits nodule formation and nitrogenase activity (8)

**Potassium (K)**

- Serves to activate certain enzymes (9)

**Micronutrients**

**Copper (Cu)**

- A lack of Cu decreased nitrogen fixation in subterranean clover (15).

**Nickel (Ni)**

- Some legumes require some NI for root nodule growth and hydrogenase activation (1)

**Boron (B)**

- Bo affects the rhizobium – legume cell surface interaction (1).

**Cobalt (Co)**

- Important for nitrogen fixing microorganisms, such as cyanobacteria (1).

**Molybdenum (Mo) & Iron (Fe)**

- Nitrogenase (nitrogen fixing enzyme) is made up of these two essential elements (1).

**Figure 1.3.** The main effects of macronutrients phosphorus, calcium, nitrogen, potassium and micronutrients copper, nickel, boron, cobalt, molybdenum and iron on nitrogen fixation. Information adapted from. 1) (Weisany et al., 2013), 2) (Beck and Munns, 1985) , 3) (Brockwell et al., 2005) , 4) (Hart, 2012), 5) (Leung and Bottomley, 1987), 6) (Singleton et al., 1985), 7) (Saxena and Rewari, 1991), 8) (Sprent and Faria, 1989), 9) Epstein and Bloom, 2005, 10) (Banath et al., 1966) , 11) (Blevins et al., 1977), 12) (Miller and Sirois, 1983), 13) (Smit et al., 1992), 14) Alva et al., 1990 and 15) (Snowball and Robson, 1980).

## Factors affecting legume BNF

### Temperature

It is generally accepted that very high or very low temperatures hinders legume BNF (Roughley and Dart, 1969). One of the controlling factors for nodule establishment is the temperature in the root zone (Liu et al., 2011). For example, *Trifolium vesiculosum* Savi (narrowleaf clover) nodulation was accelerated at a temperature of 25 °C when compared to when growing at 18 °C or 32 °C (Schomberg and Weaver, 1992). This is not the norm as nodule establishment is different between species (Liu et al., 2011; Sharma et al., 2019). Evidence of this is observed for *Trifolium repens* L. (white clover) whereby nodule establishment is enhanced when temperature rises from 10 to 35 °C (Richardson and Syers, 1985).

### Soil water status

The soil water content in roots controls BNF through nodule establishment and gas permeability (Weisz et al., 1985; Weisz and Sinclair, 1987). A reduction in water content hinders nitrogen fixation (Goh and Bruce, 2005) and the effects of this reduction is increased as drought stress increases (Albrecht et al., 1984).

The difficulty in quantifying the relationship between BNF and soil water status accurately is related  to limitations on experimental controls, plant recovery and stress periods (Engin and Sprent, 1973; Ledgard and Steele, 1992). An attempt was made to try and determine the BNF under drought stress conditions using statistical method such as linear regressions, but the "correlation coefficients between the reduction of N fixation under drought compared to under field capacity and the soil water deficit was not statically significant" (Pimratch et al., 2008). However, the responses by *Phaseolus vulgaris L* (common bean)and *Vigna unguiculate L.* (cowpea) to soil stress reveal a sharp reduction in BNF as water stress severity increases (Serraj and Sinclair, 1998; Sinclair et al., 1987).

### Seasonal regulation

Biological nitrogen fixation rates change with physiological stages (Liu et al., 2011).  In the early stages it is low whilst the nodules are developing (Lawrie and Wheeler, 1973) . In the start of the flowering stage  and seed filling stage, it reaches its optimum , depending on various growth conditions and species identity (Jensen, 1987; Klucas, 1974; Lawn and Brun, 1974; Nelson et al., 1984). After this peak phase, BNF decreases drastically (Beverly and Jarrell, 1984; Herridge and Pate, 1977; Sinclair et al., 1987) caused by nodule senescence (Lawrie and

Wheeler, 1973) and a reduction in carbon supply caused by an accumulation of seed dry matter (Herridge and Pate, 1977; Voisin et al., 2007, 2003).

### Effects of nitrogen and phosphorus on BNF

The consensus is that N application will generally favour grasses which possess traits allowing them to be more competitive than legumes under non-limiting N and this reduces legume abundance in grasslands (Loiseau et al., 2001). In contrast, frequent defoliation and a lack of N fertilization will either maintain or increase clover content in mixed swards (Barthram et al., 1992). Conversely, fertilizing with P only coupled with defoliation will assist in increasing the development of legumes (Jouany et al., 2011). These are trends observed for grazed grasslands (Aydin and Uzun, 2005; Martiniello and Berardo, 2007) and grasslands that are used for in hay production (Nevens and Rehuel, 2003). The increase of leguminous plants within grasslands following P addition causes higher plant N concentrations because generally N concentration tends to be higher in legume plants than grass plants (Henkin et al., 1996; Mackay et al., 1995).

### Cessation of nutrient enrichment in grasslands

The withdrawal of nutrient addition in grasslands (hereon referred to as nutrient enrichment cessation) has been studied mainly in America (Clark and Tilman, 2010; Isbell et al., 2013) and Europe (Kralovec et al., 2009) where a decrease in standing crop was observed (Olff and Bakker, 1991). This resulted in the increase in species richness, however this increase tended to follow when the standing crop was less than ±400 gm $^{-2}$ (Olff and Bakker, 1991). In terms of nitrogen mineralization, the lowest values were recorded in the field with the longest period of nutrient cessation (Olff et al., 1994).

Nutrient cessation in a grassland also resulted in species replacement in the Netherlands (Olff and Bakker, 1991), but this was not observed in Czech Republic, but changes in resident species cover and the establishment of new species (low cover) was observed (Kralovec et al., 2009). Another study conducted in a north-eastern grassland in China, revealed a change in the species composition (Shi et al., 2014). In addition, the species richness showed an increasing trend and plant height and coverage decreased after time since cessation of nutrients (Shi et al., 2014). One of the aims of assessing species compositional change in grasslands following nutrient enrichment cessation is to assess if species recovery is possible. Evidence from a study conducted in Cedar Creek found that the recovery of species diversity following a period of nutrient enrichment cessation of high rates of N addition would require an extended period or may not be feasible by simply lowering N input (Carpenter et al., 1998). A more recent study conducted at the Park grass Experimental site contradicted these statements by indicating that plots where N input has been stopped, started to recover various species that had disappeared (Storkey et al., 2015). Changes in soil characteristics in four successional grasslands following the cessation of nutrient enrichment is shown below (Table 1.1) (Olff et al., 1994). The main effects of nutrient cessation in previously fertilized grasslands are summarised below (Table 1.2). The main research regarding nutrient enrichment cessation is conducted in America and Asia and none has yet been conducted in South Africa.

**Table 1.1**. The significant changes of various soil characteristics in four successional grassland fields on Anloebrdiepjie (53 °05' N, 6° 40' E) following the cessation of nutrient enrichment. Adapted from Olff et al. 1994, pg. 614

| Field | Cessation of nutrients enrichment period (years) | P pool size (g m-$^{2}$) (0-10 cm) | Total P concentration (g 100 g$^{-1}$ DW) | Soil pH (KCl) | Soil pH ($H_20$) | Organic matter (g 0.100 g $^{-1}$ DW) |
|---|---|---|---|---|---|---|
| A | 2 | 511 | 0.124 | 4.69 | 5.9. | 15.40 |
| B | 6 | 362 | 0.093 | 4.80 | 5.90 | 10.30 |
| C | 19 | 365 | 0.055 | 4.60 | 5.70 | 12.60 |
| D | 45 | 246 | 0.030 | 4.20 | 5.40 | 7.30 |

**Table 1.2.** An overview of scientific findings from published journal articles, detailing the main effects following the cessation of fertilizer application in grasslands over a variety of years

| Aim of study | Study location (country) and type | Number of years of nutrient enrichment cessation | Key results | Scientific article reference |
|---|---|---|---|---|
| Assess the changes in standing crop, species richness and composition of 3 different grasslands following cessation of fertilizer application. | Netherlands, field | Changes over 16 years | A decrease in standing crop, increase in species richness and gradual species replacement. Mechanisms behind these changes were not determined. | (Olff and Bakker, 1991) |
| The effect that stopping the application of fertilizer addition would have on nutrient availability. | Netherlands, field | 2,6,19 and 45 | A decrease in N mineralization between 26 to 45 years and all plots had a net N offtake. | (Olff et al., 1994) |
| To determine the state of N pools and fluxes for plots that receiving N treatment for 10 years, none for 12 years and then received N for the entire 22 years. | USA, field | 12 | A decline in plant N pools and biomass after cessation. N mineralization increased by N addition but unaffected by N cessation. Mixed results therefore further research required. | (Clark et al., 2009) |
| To determine if a fertilized grassland recovers: a look at species composition and richness after treatment ending and determining which species contributed to observed changes | Czech Republic, field | 1,5,6,7,9,13 and 16 | An increase in the species richness during the 16 years after fertilization cessation. | (Kralovec et al., 2009) |

| | | | | |
|---|---|---|---|---|
| To determine the effect of cutting frequency and liming on Phosphorus and Potassium (K) availability in the soil, biomass, and plant species composition after cessation of fertilizer application | Germany, field | Changes over 15 years | Results show a decrease in productivity and available P and K in the soil, favouring species richness | (Hejcman et al., 2010) |
| Using isotope exchange kinetics to determine the rate of decline in soil P pools in grazed grassland soils following a halt to P fertilizer application. | New Zealand, field | 21 to 16 | Exchangeable P progressively declined after withholding P fertilizer | (Dodd et al., 2013) |
| To test for alternative states in competitive grassland plant communities that are controlled by soil nutrients | USA, field | 20 | A recovery of species diversity following cessation in low-diversity grasslands | (Isbell et al., 2013) |
| To test the reversion and pattern of a semi-arid grassland in relation to diversity and species composition after cessation | China, field | 3 | An increase in species richness after cessation. A change in species composition and dominance over time. The reversion rate also differed between functional groups in the same community. | (Shi et al., 2014) |
| To investigate soil P forms with fertilizer application and cessation and a compare soil P form with P pools. | Northern Ireland, field | 5 | The concentrations were reduced when P fertilization ceased. Fertilizer P build-up and cessation had little effect on the concentrations of organic P forms. | (Cade-Menun et al., 2017) |
| To determine the legacy effects of N addition on plant N:P stoichiometry | China, field | 3 | N cessation can ameliorate the biological N:P imbalance | (Hu et al., 2020) |
| To examine the responses of seed banks and aboveground changes to resource addition | USA, field | 3 | Highlighted the vulnerability of seed banks in nutrient-poor annual grasslands | (Eskelinen et al., 2021) |

## The state of the Ukulinga Grassland Nutrient Experiment

The data collection for the data chapters within this these were conducted on the Ukulinga Grassland Nutrient Experiment (UGNE). The UGNE, previously known as the Veld Fertilizer Experiment or Veld Fertilizer Trial is located at the Ukulinga Research farm, KwaZulu-Natal province, South Africa. This experiment was initiated 1951 and is the longest running nutrient enrichment experiment in Africa. The most recently published articles (excluding the work published and presented from the present thesis) indicated the following (Ward et al., 2020):

1. Greatest ANPP with N and P enrichment
2. A negative correlation between ANPP and specie richness
3. Ammonium sulphate had a much stronger reducing effect on species richness than limestone ammonium nitrate
4. Applying lime had a positive effect on species richness but did not alleviate the detrimental impacts of N enrichment.

In fact, the authors indicated that the results were consistent with those obtained from other long-term nutrient enrichment experiments in England, commonly known as the Park grass experiment (Crawley et al., 2005; Silvertown et al., 2006; Tilman and Isbell, 2015), and Cedar Creek (Isbell et al., 2013; Tilman and Isbell, 2015).

The earliest scientific record for soil pH was approximately 5.7 on average for the UGNE (Scott and Booysen, 1956). In the late 1990s, soils enriched with ammonium sulphate (ASU) alone or ASU and P had the lowest pH, well below 4 units (Fynn and O’Connor, 2005). More recently, the soil pH on the site ranges from 3 to 6.8 units, with a threshold of approximately 4.5 whereby species richness no longer changes (Ward et al., 2017b). Since the first scientific evidence of soil chemistry changes, advancements in approach and statistical analyses have allowed for more in-depth studies. For example, early studies focussed more on vegetation patterns of change in response to nutrient enrichment treatment, without relating the patterns to soil chemistry (Le Roux and Mentis, 1986; Scott and Booysen, 1956). In the early 2000s, research indicated that soil pH influenced species richness on the UGNE (Fynn and O’Connor, 2005). The authors further indicated that the type of N applied was important in explaining the decline in species richness, but this was the case for forb richness and not grass richness. This finding was later supported by more recent papers, identifying ammonium sulphate (ASU) over limestone ammonium nitrate (LAN) as having a stronger negative impact by decreasing species richness (Ward et al., 2020, 2017b). In addition, there appeared to be a positive correlation between soil respiration and pH.

The first records from the UGNE revealed that LAN was better at increasing yield on the site over ASU and the application of P with either N forms also greatly increased yield (Scott and Booysen, 1956). The most recent research supports the early findings of the greatest ANPP achieved when N is added in combination with P (Ward et al., 2020). A botanical composition analysis in 1953 showed that desirable grasses increased with the combined application of N, P and L, however the authors recommended that meaningful observation and conclusions may be reached over a longer period (Scott and Booysen, 1956). After more than 58 years later since the first botanical analysis, *Themeda triandra* no longer dominated the site (Tsvuura and Kirkman, 2013). The authors further state that, *Megathyrsus maximus* outcompeted shorter species for dominance (Tsvuura and Kirkman, 2013), through shading and *T. triandra* is known to reduce tillering rate when shaded (Ghebrehiwot et al., 2006). Within the N and P enriched plots, *M. maximus* and *E. curvula* were dominant and interestingly *Tristachya leucothrix* was considered less sensitive to increased nutrient addition in comparison to *T. triandra*, explaining its dominance in certain enriched plots (Tsvuura and Kirkman, 2013). The addition of lime has significantly affected the most abundant grass species, suggesting high sensitivity to soil pH on the site (Fynn and O'Connor, 2005; Ward et al., 2017b). The most recent findings indicated that *T. triandra* and *Setaria sphacelata* responded favourably to soils with high pH levels and *E. curvula* and *M. maximus* responded positively to acidic soils (Ward et al., 2017b). Patterns of species composition have been documented for the UGNE, however, the mechanisms behind these changes are lacking. Although, most research suggesting decreased light conditions following nutrient addition and ANPP increase, to be the main mechanism explaining change in species richness and composition (Borer et al., 2014; Harpole et al., 2016).

New developments that have only recently been studied on the UGNE are related to nutrient assimilation rates, biological nitrogen fixation and enzyme activity. For example, a study on *Leucaena leucocephala* which is an alien invasive legume species not found on the UGNE but occurring on the Ukulinga research farm, reported to increase belowground biomass in acidic soils (Ndabankulu et al., 2022).The authors indicated that this invasive relied on free-living nitrogen-fixing bacteria and root structure to persist on site. In addition, *L. leucocephala* was able to utilize soil derived- and atmospheric nitrogen. Another study considering soil enzyme activity and revealed higher soil enzyme (b-Glucosaminidase, acid and alkaline phosphates) activities on soils that had not received P in comparison to soils that had received P (Magadlela et al., 2023).

## Gaps in research

Long-term studies have many practical and biological implications for grasslands. However, many of these experimental studies were initiated at a time when management methods and objectives were different to the approaches that are used today. To date, most of the world's leading, and longest experiments are in the Americas, Europe and Asia, and very little similar research has been conducted in Africa. However, projections suggest that the greatest increases in fertilizer application will take place in developing countries, many of which are situated in Africa, including South Africa. Currently, in South Africa, there is the Ukulinga Grassland Nutrient Experiment, established in 1951 on the Ukulinga Research Farm that allows for the effects of long-term nutrient enrichment and cessation of nutrient enrichment to be assessed. This creates opportunities for responses of mesic grasslands in South Africa to be scientifically tested and compared to findings from research across the globe.

The areas of research that need to be addressed, particularly in South Africa are listed below.

1. **Nitrogen amount -response relationships:** whereby a strongly negative relationship between plant species biodiversity and N enrichment has been observed (Clark and Tilman, 2008; Midolo et al., 2019; Soons et al., 2017).

2. **Nitrogen fertilizer type:** meta-analyses tend to not observe effects of N fertilizer types inducing changes (Midolo et al., 2019). However, the chemical form of fertilizer used is neglected in most experimental designs, with some exceptions (see, (Dias et al., 2011; Ward et al., 2017b). There is evidence suggesting that plants differ their capacity to take up N fertilizer forms, suggesting that species composition and abundance may be altered by the partitioning of available N in the soil (Kahmen et al., 2006; Mckane et al., 2002; Miller and Bowman, 2003).

3. **Phosphorus enrichment importance:** some studies suggest that P input could be more important than N in reducing plant biodiversity (Ceulemans et al., 2014; Wassen et al., 2005). In contrast P lowers plant biodiversity only when combined with N, solely P inputs have very little effect (Soons et al., 2017).

4. **Scale dependent results:** the trends in biodiversity increases or decreases may be linked to the scale of the experiment (Midolo et al., 2019). These trends in local plant diversity may also be true at larger scales, the ongoing debate is related to the mechanisms involved in the resultant change (Mcgill et al., 2015).

5. **Soil properties:** soil acidification has been identified as one process that drives the loss of biodiversity following N input (Stevens, 2016). Whilst some meta-analyses (Humbert et al., 2016; Schrijver et al., 2011) studies support this statement, a more recent meta-analysis did not reveal evidence of soil pH influencing local plant diversity (Midolo et al., 2019).

6. **Plant life forms:** the losses of certain plant forms following N addition is known (Bobbink et al., 2010; Craine et al., 2002). The implications for a loss in legumes in caused by increased N input may be a disadvantage for BNF (Craine et al., 2002). Very little is known about legumes and grass species abundances within the mesic grasslands of South Africa.

7. **Biological nitrogen fixation of *Vachellia* spp:** research relating to the plant performances, (i.e., BNF) of *Vachellia* species that co-exist with grass and forb species within mesic South African grasslands with varying nutritional status is lacking. Further insight on the growth kinetics, carbon construction costs and bacterial diversity associated with the nitrogen fixation of common *Vachellia* spp is necessary.

8. **Nutrient cessation:** global biodiversity conservation assessment focus primarily on sites that are species rich (Soons et al., 2017). Continued N input potentially reduces biodiversity, also altering animal communities and the recovery of ecosystems after cessation from fertilization is slow (Stevens, 2016). The effects after cessation of nutrient addition is an aspect that has not been investigated in South African grasslands and could provide insight on recovery of species and soil conditions.

## Thesis outline

This literature review presented here has shown the main subjects of interest of the succeeding data chapters. A large majority of research related to nutrient enrichment and cessation of nutrients within grasslands has been conducted elsewhere and not in South Africa. Although there has been work conducted on the Ukulinga Grassland Nutrient Experiment (UGNE), most of it was conducted in in the 2000s, with four others conducted later on (Tsvuura and Kirkman, 2013; Ward et al., 2020, 2017a, 2017b). In this thesis I consider other aspects that were not considered in previous research studies and provide alternative statistical methods to assess the changes that have occurred on the UGNE, making use of more recent species composition data than the previous studies.

In chapter two I assessed the cumulative long-term effect of nitrogen, phosphorus, and lime enrichment on the UGNE.  Here I determined how these nutrients and lime have shaped the current species composition and assessed the relationship between soil pH and species richness. The focus here was to show that grass and forb species respond differently to changes in nutrient and lime enrichment and ultimately soil acidity. In chapter three, I studied the effect of short-term P enrichment cessation on plots that had been enriched with P for sixty-seven years. To my knowledge, such a study has not yet been conducted in an African grassland. This chapter gives an opportunity to identify whether a three-year cessation period results in changes to species abundances and composition dynamics and sets a baseline for future monitoring of the reversal component of the experiment. In chapter four, I aimed to determine if multiple nutrients explained the pattern of declining species richness observed on global-scale studies. This chapter provided the opportunity to explore various hypotheses employed to explain the mechanisms behind species richness declines following nutrient enrichment. Lastly, chapter five considered the growth dynamics and nitrogen-use efficiencies of *Vachellia sieberiana* (DC.) Kyal. & Boatwr (*V. sieberiana*) saplings grown in nutrient-enriched soils collected from the UGNE. This chapter highlighted the effect of long-term N and P enrichment on soil properties and emphasized the negative impacts of soil acidification on *V. sieberiana* and raises concerns about how other agriculturally important legumes would respond under similar conditions of long-term N and P addition.

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# Chapter 2

# Assessment of long-term nutrient and lime enrichment effects on a subtropical South African grassland

(Published journal article*)

Zama, N*., Magadlela, A., Mkhize, N., Tedder, M., Kirkman, K., 2022. Assessing long-term nutrient and lime enrichment effects on a subtropical South African grassland. African Journal of Range and Forage Science. https://doi.org/10.2989/10220119.2021.2014964.

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## Abstract

Nutrient enrichment influences grassland ecosystem structure, typically manifested by reduced species richness and increased productivity. Quantifying the long-term impacts of nutrient enrichment on grasslands contributes to understanding eutrophication effects on grassland, particularly for grasslands adapted to low soil nutrient status. Over extended time periods, nutrient enrichment may modify soil fertility. The Ukulinga Grassland Nutrient Experiment situated on a natural $C_4$ grassland was set up in 1951 on the Ukulinga research farm, South Africa. Continuously applied treatments on plots measuring 2.7 × 9 m included combinations of nitrogen at 0, 7, 14 and 21 g $m^{-2}$ per annum, phosphorus at 0 and 33.6 g $m^{-2}$ per annum and lime at 0 and 225 g $m^{-2}$ applied every five years. Nitrogen sources included ammonium sulphate (ASU) (acidifying) and limestone ammonium nitrate (LAN) (less acidifying). Grass species composition was influenced by both nitrogen forms. In contrast, forb species composition was more sensitive to LAN. We found evidence of nitrogen limitation on aboveground net-primary production. However, species richness (for grass and forbs) declined with increasing ammonium sulphate levels, owing to increased soil acidity and high aluminium concentrations. Aluminium toxicity can affect overall species composition by replacing Al-sensitive species with Al-tolerant species on site.

**Keywords:** acidification, biodiversity, biomass, fertilisation, mesic

## Introduction

Human activities result in excess amounts of nutrients reaching natural environments, mainly through fertilization and atmospheric depositions associated with industries (Peñuelas et al., 2013). A continued increase in nutrient enrichment in natural environments threatens biodiversity and ecosystem services (Bobbink et al., 2010; Clark et al., 2017; Sala et al., 2000; Tilman et al., 2001). The main causes of this are the altered competitive relationships in highly productive environments, which exclude species that are incapable of competing for resources such as light (DeMalach et al., 2017; Harpole and Tilman, 2007). Furthermore, increased availability of nutrients also affects belowground growth and competition (Rajaniemi, 2003) and increases litter thus lowering the success of seedling establishment (Bobbink et al., 2010). In addition, studies show that long-term nitrogen addition affects seed germination and soil seed banks (Basto et al., 2015a, 2015b; Xia and Wan, 2013).

It is well known that, apart from water, nitrogen (N) and phosphorus (P) are the two most limiting nutrients in grassland productivity (Fay et al., 2015; Harpole et al., 2017). These nutrients strongly influence productivity, as well as species composition and diversity (Avolio et al., 2014; Ceulemans et al., 2013; DeMalach, 2018; Riesch et al., 2018; Soons et al., 2017). Limitations of N and P affect plant groups differently. For example, N deficiency suppresses the development of grasses while P deficiency curbs that of legume and forb species (Kidd et al., 2017; Lambers et al., 2011; Zarzycki and Kopeć, 2020). N is reported to be the most limiting in terms of productivity in terrestrial ecosystems (Bobbink et al., 2010; Fay et al., 2015; Vitousek and Howarth, 1991). Therefore, understanding the factors that limiting and co-limiting nutrients have on species composition and diversity in grassland ecosystems is important. This will allow the development of strategies to counteract these potential changes caused by nutrient enrichment (Schelfhout et al., 2017; Storkey et al., 2015). Research indicates that high N supply rates negatively affect species diversity whereas P is capable of influencing productivity and species composition (Clark and Tilman, 2008; Duprè et al., 2010; Elser et al., 2007; Soons et al., 2017; Wassen et al., 2005). Also, it is suggested that a decrease in species richness that was mainly attributed to N supply, may be due to excess P (van Dobben et al., 2017).

Although plants may take a long time to respond to nutrient manipulation, their effects tend to persist for a long time (Mašková et al., 2009; Semelová et al., 2008; Silvertown et al., 2006). Therefore, the use of long-term studies such as the Ukulinga Grassland Nutrient Experiment (UGNE), to evaluate these changes is pertinent to understand the relationships that occur.

One of these widely discussed relationships is the plant productivity-plant richness (PRR) relationship. The main findings regarding this relationship are as follows: (1) a monotonic relationship, showing that there is either a negative or positive relationship between the number of species and biomass (Balvanera et al., 2006; Duffy et al., 2017; Roscher et al., 2005), (2) a non-significant hump-shaped/monotonic relationship (Gillman et al., 2006; Mittelbach et al., 2001; Waide et al., 1999) and (3) a hump-shaped relationship, whereby richness increases and then decreases with a rise in productivity (Grace et al., 2014) . To date, research published on the UGNE demonstrates a significantly negative relationship between species richness and biomass (Ward et al., 2017). This is opposite to the most cited pattern of the PRR that shows a positive/unimodal relationship (Ward et al., 2017). Therefore, further clarification on the current relationship between these two variables at the UGNE is necessary.

Previous studies showed that soil pH influences species richness at the UGNE (Fynn and O’Connor, 2005; Ward et al., 2017). This is linked to nitrogen form applied, with ammonium sulphate lowering the soil pH and having a stronger negative effect on forb richness than grass richness (Fynn and O'Connor, 2005). Liming reduced the negative effects of N enrichment, but less so for ammonium sulphate relative to limestone ammonium nitrate (Fynn and O'Connor, 2005; Silvertown et al., 2017). Future studies need to include not only N form but also N application rate, which is less emphasized. Identifying a pH threshold where species richness does not decline any further below a certain threshold is also beneficial in understanding the relationship between soil pH and species richness. A meta-analysis showed a threshold of pH 4.9 for subtropical grasslands and savanna (Azevedo et al., 2013). A previous study at UGNE indicated a threshold pH of about 4.5 (Ward et al., 2017). Soil pH is identified as an indicator of level of acidity in soil and has an important role in predicting plant presence and structure mainly because it is correlated to various soil nutrients and acidifying pollutants such as sulphur and aluminium (Kozlov and Zvereva, 2011; Peppler-Lisbach and Kleyer, 2009; Van Zelm et al., 2007). Acidic soils, also referred to as ultisols/oxisols, are common in the subtropics and tropics and they generally have pH levels lower than 5.5 (Sade et al., 2016). Acidic soils occur naturally or through anthropogenic processes, such as the use of excessive fertilizers and the burning of fossil fuels that release nitrogen and sulphur dioxide that undergo further processes resulting in acid rain (Bojórquez-Quintal et al., 2017). Acid rain contributes towards acidifying the soil and such soils are characterized of consisting of high levels of certain metals such as, iron (Fe) and aluminium (Al) (Gupta et al., 2013). Aluminium is a metal that is known to be toxic to plants (Sade et al., 2016) and has no particular biological role in the soil (Poschenrieder et al., 2008). In highly acidic soil, Al is abundant in its trivalent ($Al^{3+}$) form and can pose a serious threat to production by inhibiting root growth (Kopittke and Blamey, 2016). This supports an investigation into the effect of soil pH and aluminium soil content on species richness at the UGNE, since this may potentially be contributing towards low species richness observed in acidic soils.

In this paper, we present the findings of an experiment initiated in 1951 that involved a combination of nutrient enrichment strategies, with two forms of nitrogen (limestone ammonium nitrate and ammonium sulphate), phosphate and lime at varying application levels and rates. The experiment was first established at a time when applying nutrients to increase forage production from natural grassland was of interest (Morris and Fynn, 2001). Nutrient application to natural grassland is no longer commonly used in South Africa. However, the 70-year-old experiment allows for important ecological questions to be addressed. We asked the following questions:

1) Are there any differences in natural grassland species composition, above-ground biomass, species richness, Shannon-Weiner diversity index and Pielou's Evenness, including comparisons within and between grasses and forbs following 70 years of nutrient and lime enrichment?
2) What is the current relationship between above-ground biomass and total richness?
3) What is the relationship between soil pH and a) grass richness, b) forb richness and c) total richness?
4) Is there a relationship between soil pH and aluminium soil content?

## Methods

### Study site

The UGNE is located at Ukulinga Research Farm, University of KwaZulu-Natal, Pietermaritzburg, South Africa (29° 24 E, 30° 24 S). The long-term experiment is located at an altitude ranging from 838 to 847 m above sea level (Ward et al., 2017). The mean annual precipitation and temperature of the area is approximately 838 mm and 18°C respectively (Ward et al., 2020). The soil is known to be derived from shales under the Westleigh classification (Soil Classification Working Group, 1991). In addition, the soils are relatively infertile and considered to be acidic (Soil Classification Working Group, 1991). The vegetation of the UGNE and surrounding area is classified as KwaZulu-Natal Hinterland Thornveld of Sub-Escarpment Savanna (Mucina and Rutherford, 2006). The area is a dense tall grassland with scattered *Vachellia sieberiana* (D.C) Kyal & Boatwr and an understory of *Hyparrhenia hirta* (L.) Stapf and *Themeda triandra* (Forssk.) with other herbaceous species (Fynn et al., 2005). The grass species that are common in the absence of fire include *Aristida junciformis* Trin. & Rupr. and with regular burning, trees are sparse and the dominant grass species include *T. triandra*, *Tristachya leucothrix* (Trin. ex Nees) and *Heteropogon contortus* (L.) Roem. & Schult (Morris and Fynn, 2001). The most frequently occurring species in the UGNE plots according to previous studies include *Setaria sphacelata* (Schumach.) Stapf & C.E. Hubb. ex M.B, Moss, *Eragrostis curvula* (Schrad.) Nees, *T. leucothrix* (*T.leucothrix)*, *Eragrostis plana* Nees. and *Megathyrsus maximus* Jacq. All the grasses occurring follow the $C_4$ photosynthetic pathway (Morris and Fynn, 2001). All plots are mown at a yearly basis to prevent carry over plant growth from the previous season (Fynn et al., 2005) and the clipping were removed from the study site. Grazing has been excluded since 1951.

## Experimental design and nutrient enrichment

The UGNE involves the manipulation of nitrogen (N), phosphorus (P) and lime. There are 96 plots and each plot is 9.0 x 2.7 m in size with a 1 m spacing between plots. The experiment was replicated in three blocks, each block containing 32 plots, resulting in a 4 x $2^3$ factorial design. Firstly, the two forms of nitrogen applied were limestone ammonium nitrate and ammonium sulphate (henceforth LAN and ASU respectively). Four levels of N fertilizer were applied annually on the plots (0 (control), 7.1, 14.1, and 21.2 gm $^{-2}$) for both LAN and ASU. In addition, the N treatments were either applied alone or in combination with P and L. Both LAN and ASU were applied twice a year in October and December per plot. Secondly, in terms of P addition, the application was in the form of super-phosphate at two levels (0 (control) and 33.6 gm$^{-2}$). Phosphorus was applied once a year in October. Thirdly, the lime treatments were applied every 5 years at two levels (0 (control) and 225 gm $^{-2}$) (Le Roux and Mentis, 1986; Tsvuura and Kirkman, 2013) , with the last application being in 2016.

## Sampling

All the data used in this study were from one growing season (2019). Soil samples were collected from a depth ranging from 0 to 15 cm in each plot. The topsoil region is reported to be the most active site where fine roots absorb nutrients (Basto et al., 2015a; Li et al., 2017; Xia and Wan, 2013). Two samples were collected per plot and sent to the Institute for Commercial Forestry Research in Pietermaritzburg for comprehensive soil analyses, including pH (KCl) and aluminium (µmolg$^{-1}$). Aboveground net-primary production (ANPP) was measured at the end of the growing season in March 2019. We used ANPP here for ease of comparison with previous studies on the same experiment. Above-ground net-primary production was estimated in each plot by mowing a 2.13 m wide strip per plot at the end of the growing season. All plant material present (grasses and forbs) was mown to ground level. The fresh material in each plot was collected and weighed on site. The dry biomass was determined with the use of a grab sample that was dried at 60 °C for 2 days. Species composition was determined using cover abundance, for each plot. Cover abundance is described as the cover proportion of each plant species present in a quadrat as a percentage (Peratoner and Pötsch, 2019). Each plot was sampled in January using four systematically placed quadrats (1m x 1m). The species richness, which is the simplest indicator of species diversity was defined as the number of plant species (Keylock, 2005) in a plot. To further describe plant diversity the two most common measures of plant diversity, the Shannon-Weiner Diversity Index and Pielou's Evenness, were used. The former is a function of relative frequency of the varying plant species and the latter indicates the similarity in relative abundance of different species in the site (Keylock, 2005; Mendes et al., 2008).

**Shannon-Weiner diversity (H') calculation:**

$$H' = SUMpiLog(b)pi$$

Where, pi = proportional abundance of species *i* and *b* = base of logarithm.

**Pielou's evenness calculation:**

$$J' = H'/In(S)$$

Where, *H'* = Shannon-Weiner diversity and *S* = total number of species in a sample, across all samples.

### Statistical analyses

All statistical analyses were performed using R Studio (version 4.0.2), with packages "vegan" for analysis and "ggplot2" for plotting (RStudio team, 2020). Prior to performing the canonical correspondence analyses and diversity analyses, we transformed and manipulated the cover plant cover abundance dataset. We used the "dplyr" and "tidyverse" packages (Wickham et al., 2019) to filter the replicated data and calculate the average values for each species per plot, to avoid pseudo-replication. We used CCA to investigate the relationships between the plant community composition of grasses and forbs separately, with the inclusion of environmental factors. The lengths of the calculated gradients were less than 4. This permitted a further analysis of the species composition using a CCA (Jongman et al., 1987). This form of eigenvalue ordination analysis uses the selected environmental matrix to explain the variation in the plant community matrix. In each CCA we separated the categorical factors into their specific levels using the "ellipses" function in the "vegan" package. Species occurring in less than 10 % of the plots were removed in the forb CCA, to reduce clutter. We used permutation tests to observe if each model CCA (CCA "terms" and CCA "axes") explained more variance of the species abundance matrix than expected by chance. Both grass and forb CCA's had $p < 0.05$. suggesting that the model was indeed significant. The biplot scores for constraining variables and eigenvalues for constrained axes (with p-values) are shown in Table 2.A1 and Table 2.A2 respectively.

The effects of nutrients (LAN, ASU, and P) and lime on ANPP, grass richness, forb richness, Shannon-Weiner’s diversity and Pielou’s evenness were assessed using generalized linear models (GLM). This is similar to methods used in other studies (Storkey et al., 2015; Ward et al., 2017). We used a Poisson distribution for richness, a Gaussian distribution for diversity and evenness and a Gamma distribution for ANPP as the error distribution. For each GLM we tested for overdispersion and used diagnostic model misfits’ plots for model validation. For overdispersion measures, values greater than 1.1 indicated mild overdispersion and therefore transformations were necessary. The model misfit plots assisted in visualizing, 1) constant variance of fitted values against residuals, 2) the normality of residuals, 3) residuals vs leverage for outliers and 4) scale-location plots. We also conducted Breusch-Pagan tests to check for homoscedasticity. No transformations were required for ANPP, grass richness, diversity, and evenness. However, a box-cox transformation was performed on the forb richness dataset to obtain normality and constant variance. We also performed a simple linear regression of firstly ANPP and total species richness and secondly soil pH and aluminium. Polynomial regressions were performed on 1) soil pH and grass richness and 2) forb richness. The function “specnumber” was used to calculate the number of species in the R software.

## Results

### Species composition

Along axis one of the CCA, there is a shift in grass species composition as influenced by N form and levels (Figure 2.1A; Figure 2.1B). The species composition changes along axis two are related to P level (Figure 2.1C) and to a lesser degree, lime (Figure 2.1D). The occurrence of bare ground is more associated with plots with high nutrient application level than lower levels. The N applications affected forb species composition differently to grass composition. Limestone ammonium nitrate shifted forb composition along axis one (Figure 2.2A). This change also resulted in higher variability (Figure 2.2A). Unlike limestone ammonium nitrate, ammonium sulphate shifted forb species composition along axis two (Figure 2.2B). Phosphate and lime had a similar effect on forb species composition change along axis two (Figure 2.2C and Figure 2.2D). For phosphate, the forbs species belonging to the Fabaceae family were closely associated apart from *Vachellia nilotica* that was not associated with the control, phosphate treatment or any other plant species (Figure 2.2C). This is explained by the low cover abundance of *V. nilotica* and its’occurrence in a single plot compared to the other Fabaceae plant species with higher cover abundances and presence in variance plots.

### Above-ground net primary productivity (ANPP)

We tested the effects of LAN, ASU, P, and lime on ANPP. The main effect of LAN significantly affected the ANPP (Table 2.1A). The two-way interaction between limestone ammonium nitrate and lime was significant (Table 2.1A). Limestone ammonium nitrate application resulted in peak increase at the lowest level (level 1) and then decreased as LAN level increased (Figure 2.3A). In addition, the interaction between LAN and lime was similar to that of LAN level two only (Figure 2.3B). Above-ground biomass was separately regressed on total species richness and there was a non-significant relationship ($p = 0.10$) with a correlation coefficient of -0.121 (Figure 2.B1).

### Species richness and diversity

Species richness differed greatly between functional groups of grasses and forbs on the UGNE. For grass richness, the values ranged from 1 to 8, while forb richness varied greatly, with values ranging from 1 to 22. There was a significant main effect of ASU on grass richness (Table 2.1B). We found a significant effect of ASU reducing grass species richness at levels two and three (Figure 2.4A). The GLM indicated significant main effects of LAN, ASU and lime on forb richness (Table 2.1C). Similarly, to ANPP, there was a significant increase of forb richness at level one due to limestone ammonium nitrate (Figure 2.4B). There was a sharp decline in forb richness with increasing ASU level (Figure 2.4C). Adding lime increased forb richness at the UGNE (Figure 2.4D). In the GLM test for the effects of 1) LAN and lime, 2) ASU and lime and 3) P and lime on forb richness, the overall effects were significant (Table 1.1C). Of these effects, the interaction between ASU and lime showed a clear increase in forb richness with addition of lime (Figure 2.5B). Overall, the addition of lime improved the negative effect that LAN, ASU and P had on forb richness. There was also a significant three-way interaction effect of LAN, P and lime on forb richness (Table 1.1C). This interaction caused forb richness to be similar for the following interactions, 1) LAN, P, and no lime and 2) LAN, P and lime. The interaction with LAN, no P and no lime, resulted in a peak in forb richness at LAN level one. Whereas, the last interaction of LAN, no P and lime slightly increased forb richness at LAN level three (Figure 2.5D). This suggests that forb richness at the highest LAN level is increased in the presence of lime (and absence of P).

We tested the effects of limestone ammonium nitrate, ammonium sulphate, phosphate and lime on the grass and forbs species diversity and evenness separately. The overall effects were non-significant for all nutrients and lime for both groups (Table 1.2). However, there was no significant effect of ASU ($p = 0.055$ and the interaction between ASU and lime on forb evenness ($p = 0.075$).

### Soil pH and species richness

There was a significantly positive correlation between soil pH and grass species richness ($r^2 = 0.30$; $p < 0.0001$), and this relationship was best explained by a polynomial regression. A similar trend was observed for the relationship between soil pH and forb species richness, whereby the polynomial regression ($r^2 = 0.50$; $p < 0.0001$) explained the relationship better than a quadratic ($r^2 = 0.46$; $p < 0.001$) or simple linear regression ($r^2 = 0.26$; $p < 0.001$).

The addition of the two N nutrient forms yielded different responses in terms of richness for both grass and forbs. Limestone ammonium nitrate addition level did not show any clear trend (Figure 2.6A; Figure 2.6E). Whereas the highest ASU level strongly reduced the richness on those plots (Figure 2.6B; Figure 2.6F). This trend was also true for total richness. The application of lime contributed to increasing the soil pH and the species richness for both grass and forbs, but this was more prominent for forb species (Figure 2.6D; Figure 2.6H). Nutrient enrichment by P did not show any clear trend (Figure 2.6C; Figure 2.6G).

The grass species occurring in soils with the lowest pH frequently included *Megathyrsus maximus* Jacq. and *Eragrostis curvula* (Schrad.) Nees. In these plots, these were commonly the only grass species present. In contrast to the lowest pH soils, control plots had more grass species including *Aristida junciformis* Trin. & Rupr. Subsp., *Themeda triandra* Forssk., *Tristachya leucothrix* Trin. ex Nees, *Hyparrhenia hirta* (L.) Stapf and *Setaria nigrirostris* (Nees) T. Durand & Schinz. *Cephalaria pungens* Szabó was the most common forb species found in low pH soils. A wide variety of forb species belonging to a range of plant families occurred in control plots.

### Soil pH, total richness, and soil aluminium

A regression on total species richness indicated that 54 % of the variance in total species richness is explained by soil pH at the UGNE. A threshold of approximately 4.6 (pH) was observed (Figure 2.7A). Soil pH was a good predictor of aluminium content in the soil at the UGNE. The correlation coefficient between the two variables was -0.81, suggesting that when soil pH increases, the aluminium content in the soil decreases. A hyperbola best explained the relationship ($r = -0.814$: $p < 0.0001$) (Figure 2.7A).

Aluminium ranged from 7.597 ($\mu molg^{-1}$) to 49.759 ($\mu molg^{-1}$) and pH ranged from 3.205 to 6.840. The two N nutrient forms affected the relationship between pH and aluminium differently. Limestone ammonium nitrate application level did not have a noticeable effect (Figure 2.7B), a similar effect was observed for P (Figure 2.7B). In contrast, a high level application of ASU was clearly associated with plots having low pH soils (pH < 4, with one exception) and high aluminium content (Figure 2.7B). Generally, plots with lime had high pH values (pH > 5, with some exceptions) and lower aluminium content (Figure 2.7B). The exceptions referred to here are associated with plots with interacting nutrients having been applied.

**Table 2.1.** Generalized linear model analyses on the effects of nutrients. These nutrients are in the following form and levels; limestone ammonium nitrate (0 (control), 7.1, 14.1, and 21.2 g m $^{-2}$), ammonium sulphate (0 (control), 7.1, 14.1, and 21.2 gm $^{-2}$), phosphorus (0 (control), 33.6 g m $^{-2}$) and lime (0 (control), 225 g m$^{-2}$) on (a) Above-ground Net primary Productivity (ANPP), (b) Grass species richness (count) and, (c) forb species richness at the UGNE. *= significant difference observed

| **A) Response variable: ANPP** | | | |
|---|---|---|---|
| **Factor** | **Estimate** | **df** | **P** |
| Limestone ammonium nitrate (LAN) | -0.0002 | 3 | **0.016*** |

| | | | |
|---|---|---|---|
| Ammonium sulphate (ASU) | -0.0001 | 3 | 0.345 |
| Phosphorus (P) | -0.0003 | 1 | 0.233 |
| Lime (L) | -0.0003 | 1 | 0.236 |
| LAN *P | 0.0002 | 3 | 0.133 |
| ASU*P | 0.0002 | 3 | 0.303 |
| LAN*L | 0.0003 | 3 | **0.035*** |
| ASU*L | 0.0000 | 3 | 0.580 |
| P*L | 0.0003 | 1 | 0.360 |
| LAN*P*L | -0.0004 | - | 0.063 |
| ASU*P*L | -0.0003 | - | 0.176 |
| Block | -0.0002 | 2 | 0.049 |

**B) Response variable: Grass species richness**

| **Factor** | | **df** | **P** |
|---|---|---|---|
| Limestone ammonium nitrate (LAN) | -0.2135 | 3 | 0.064 |
| Ammonium sulphate (ASU) | -0.3037 | 3 | **0.015*** |
| Phosphorus (P) | 0.0667 | 1 | 0.765 |
| Lime (L) | -0.0250 | 1 | 0.910 |
| LAN *P | -0.0495 | 3 | 0.159 |
| ASU*P | -0.0628 | 3 | 0.773 |
| LAN*L | 0.2051 | 3 | 0.173 |
| ASU*L | 0.1945 | 3 | 0.234 |
| P*L | 0.0750 | 1 | 0.810 |
| LAN*P*L | -0.3120 | - | 0.174 |
| ASU*P*L | -0.0947 | - | 0.690 |
| Block | -0.0908 | 2 | 0.159 |

**C) Response variable: Forb species richness**

| **Factor** | | **df** | **P** |
|---|---|---|---|
| Limestone ammonium nitrate (LAN) | -0.3477 | 3 | **<0.001*** |
| Ammonium sulphate (ASU) | -0.9973 | 3 | **<0.001*** |
| Phosphorus (P) | -0.1693 | 1 | 0.121 |

|  |  |  |  |
|---|---|---|---|
| Lime (L) | -0.3480 | 1 | **<0.001*** |
| LAN *P | 0.1778 | 3 | 0.129 |
| ASU*P | 0.0966 | 3 | 0.555 |
| LAN*L | 0.3268 | 3 | **<0.001*** |
| ASU*L | 0.7448 | 3 | **<0.001*** |
| P*L | 0.3291 | 1 | **0.041*** |
| LAN*P*L | -0.3184 | - | **0.022*** |
| ASU*P*L | -0.0562 | - | 0.650 |
| Block | -0.0129 | 2 | 0.874 |

**Table 2.2.** Generalized linear model analyses on the effects of nutrients diversity and evenness. These nutrients are in the following form and levels; Fstone ammonium nitrate (0 (control), 7.1, 14.1, and 21.2 gm-$^{2}$), ammonium sulphate (0 (control), 7.1, 14.1, and 21.2 gm $^{-2}$), phosphorus (0 (control), 33.6 g m $^{-2}$) and lime (0 (control), 225 g m$^{-2}$) on (a) Shannon-Weiner's diversity indices (H') and (b) Pielou's evenness (J') for grasses and forbs at the UGNE

| | | **Shannon-Weiner (H')** | | **Pielou's Evenness (J')** | |
|---|---|---|---|---|---|
| **Factor** | **df** | **Grasses** | **Forbs** | **Grasses** | **Forbs** |
| Limestone ammonium nitrate (LAN) | 3 | 0.205 | 0.671 | 0.138 | 0.397 |
| Ammonium sulphate (ASU) | 3 | 0.102 | 0.334 | 0.221 | 0.055 |
| Phosphorus (P) | 1 | 0.879 | 0.185 | 0.886 | 0.339 |
| Lime (L) | 1 | 0.674 | 0.517 | 0.954 | 0.513 |
| LAN *P | 3 | 0.167 | 0.877 | 0.145 | 0.998 |
| ASU*P | 3 | 0.651 | 0.714 | 0.100 | 0.252 |
| LAN*L | 3 | 0.420 | 0.996 | 0.679 | 0.664 |
| ASU*L | 3 | 0.455 | 0.440 | 0.609 | 0.075 |
| P*L | 1 | 0.677 | 0.455 | 0.406 | 0.984 |
| LAN*P*L | - | 0.286 | 0.886 | 0.412 | 0.675 |
| ASU*P*L | - | 0.857 | 0.794 | 0.868 | 0.351 |
| Block | 2 | 0.108 | 0.791 | 0.145 | 0.353 |

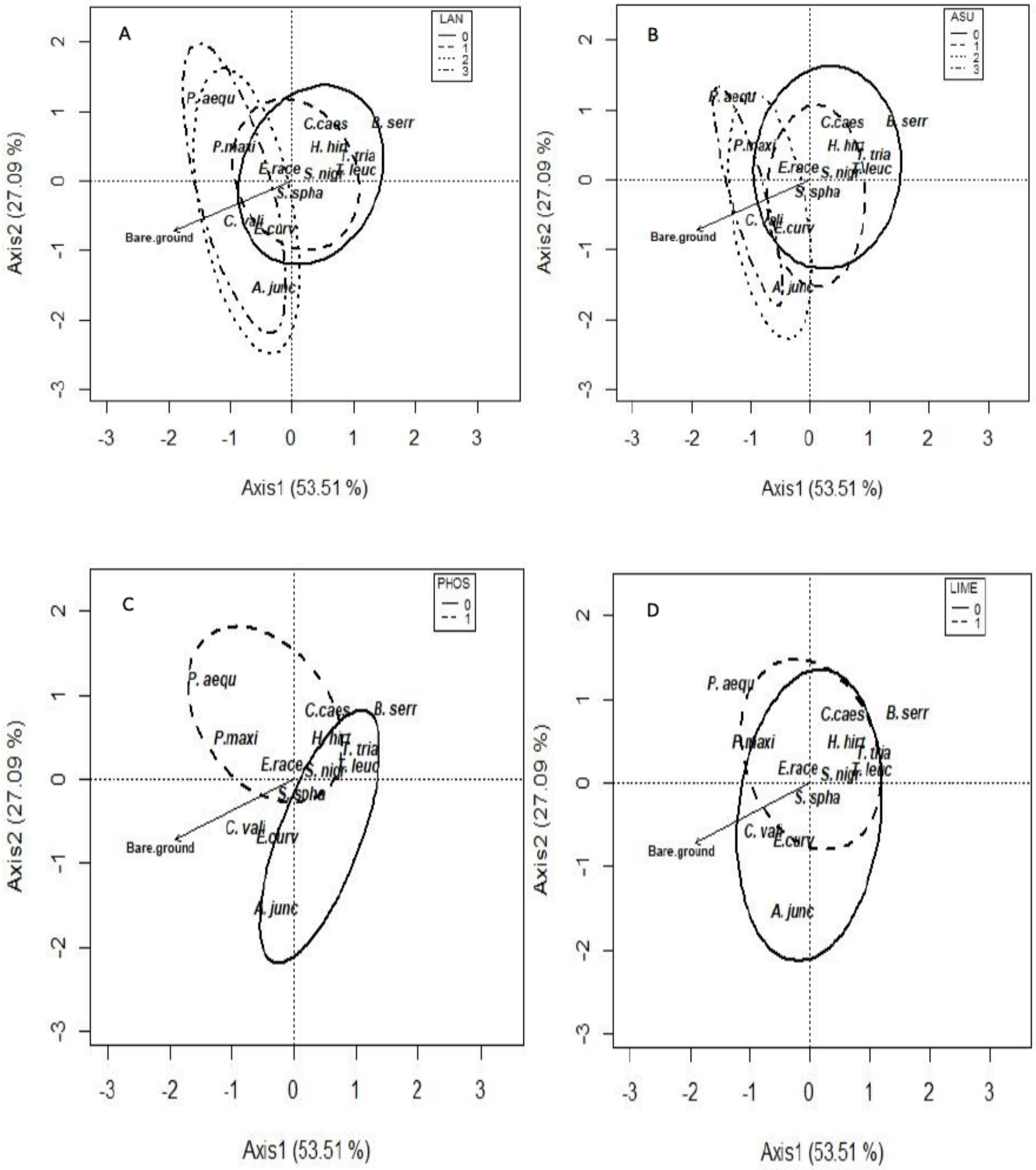


**Figure 2.1.** A CCA of grass species community composition in 2019 at the UGNE. Axes one and two account for 53.51 and 27.09 % of the total variability of the data set and 57.39 and 29.05% of the variability in the environmental data set respectively. The plots shown here are separated into the three nutrient treatments (limestone ammonium nitrate, ammonium sulphate and phosphate) and lime treatment for ease of interpretation. Key: limestone ammonium nitrate and ammonium sulphate: (0 (none); 1= 7.1 $gm^{-2}$; 2 = 14.1 $gm^{-2}$ and 3 = 21.2 $gm^{-2}$), phosphate :(0 (none); 1 = 33.6 $gm^{-2}$) and lime: (0 (none); 1 = 225 $gm^{-2}$). Full species names are shown in Appendix A, Table 2.A3.

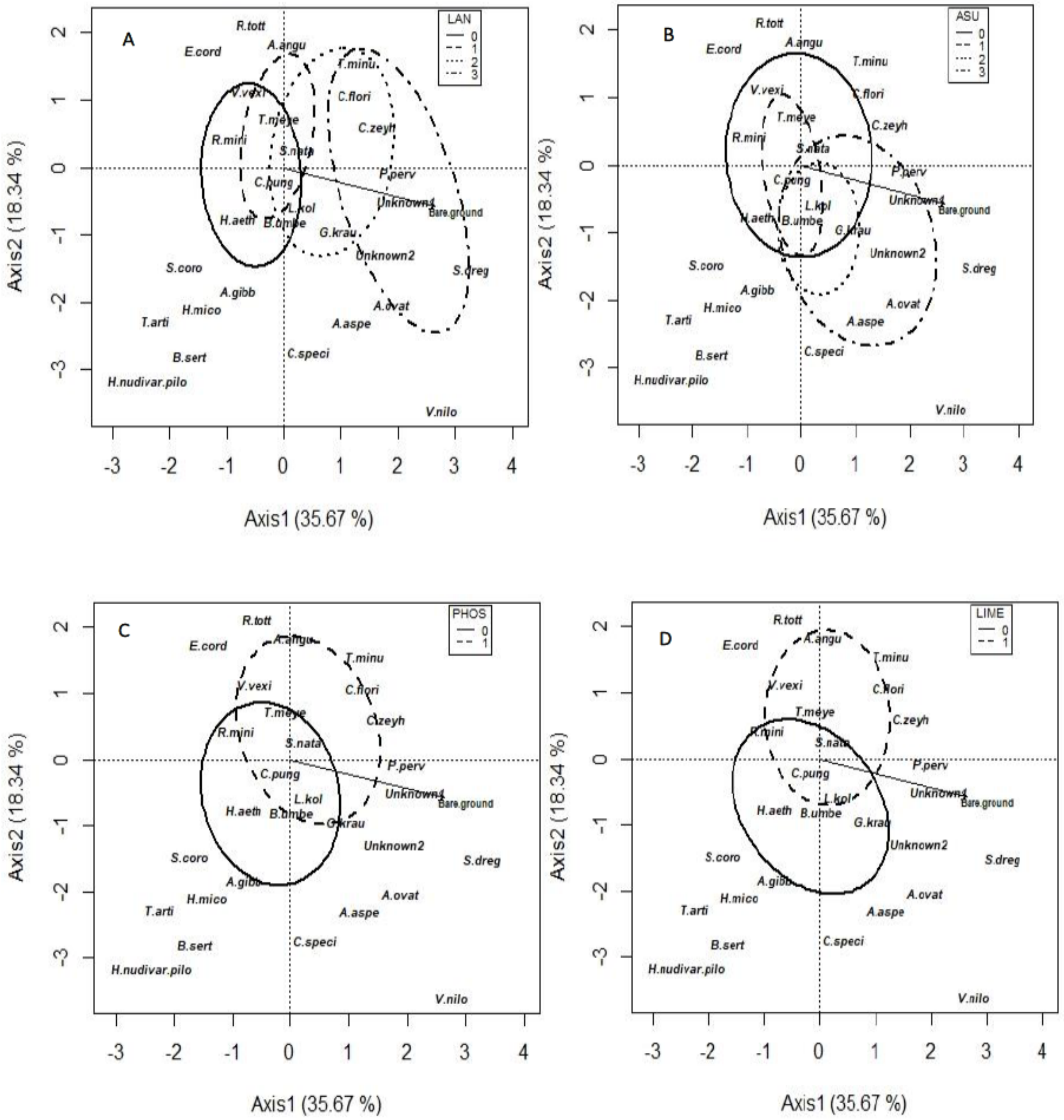


**Figure 2.2.** A CCA of forb species community composition in 2019 at the UGNE. Axes one and two account for 35.67 and 18.34 % of the total variability of the data set and 27.39 and 21.02% of the variability in the environmental data set respectively. Species with an occurrence of <10 % were excluded from the ordination plots to reduce clutter. The plots shown here are separated into the three nutrient treatments (limestone ammonium nitrate, ammonium sulphate and phosphate) and lime treatment for ease of interpretation. The ellipses show the level of application per treatment. Key: limestone ammonium nitrate and ammonium sulphate: (0 (none); 1= 7.1 gm$^{-2}$; 2 = 14.1 gm$^{-2}$ and 3 = 21.2 gm$^{-2}$), phosphate :(0 (none); 1 = 33.6 gm$^{-2}$) and lime: (0 (none); 1 = 225 gm$^{-2}$). Full species names are shown in Table 2.A4.

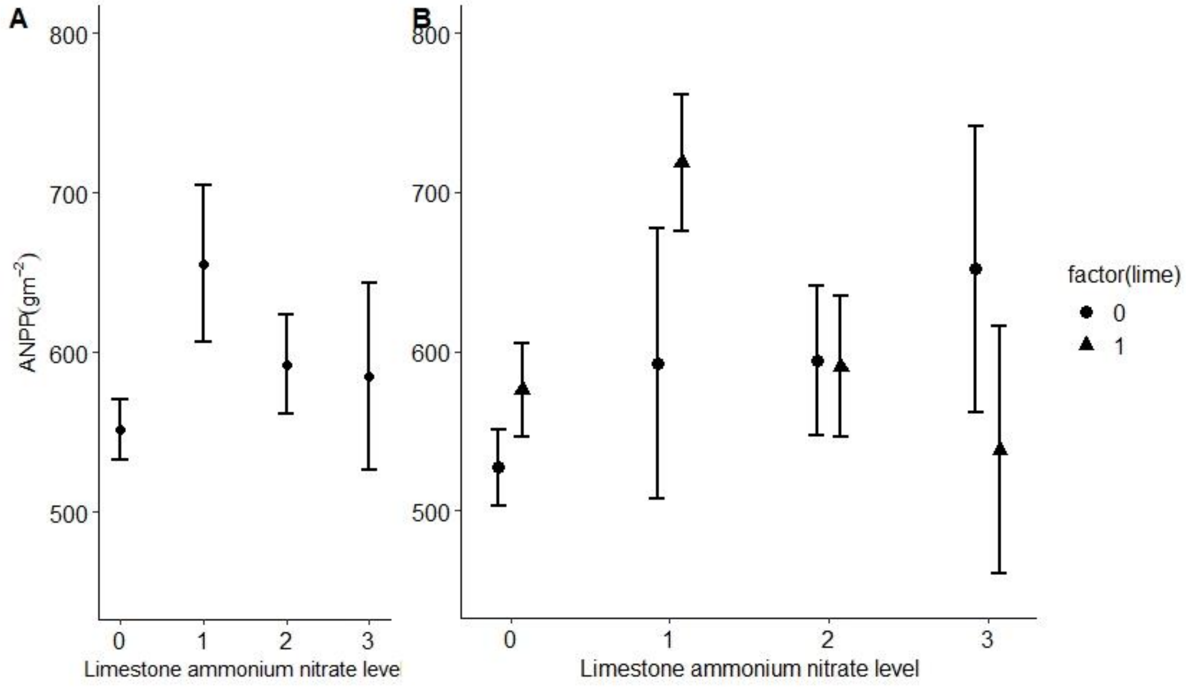


**Figure 2.3.** The main effect of limestone ammonium nitrate (A) and interaction effect of limestone ammonium nitrate and lime (B) on mean (±SE) Aboveground Net-Primary Production (ANPP) at the UGNE. Key: limestone ammonium nitrate (0 (control), 7.1, 14.1, and 21.2 g m $^{-2}$), lime (0 (control), 225 g m$^{-2}$).

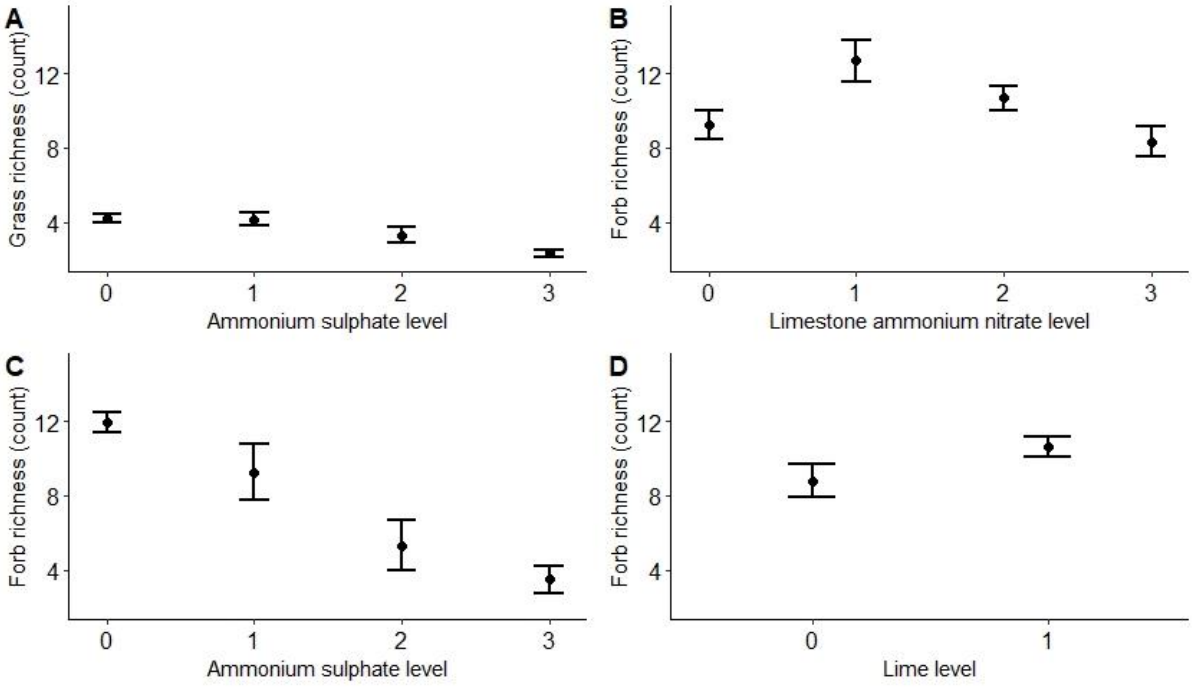


**Figure 2.4.** The main effect of ammonium sulphate on mean (±SE) grass richness(A), limestone ammonium nitrate (B), ammonium sulphate (C) and lime (D) on mean (±SE) forb richness at the UGNE. Key: limestone ammonium nitrate (0(control),7.1, 14.1, and 21.2 g m$^{-2}$), ammonium sulphate (0(control),7.1, 14.1, and 21.2 gm $^{-2}$), lime (0(control), 225 gm$^{-2}$).

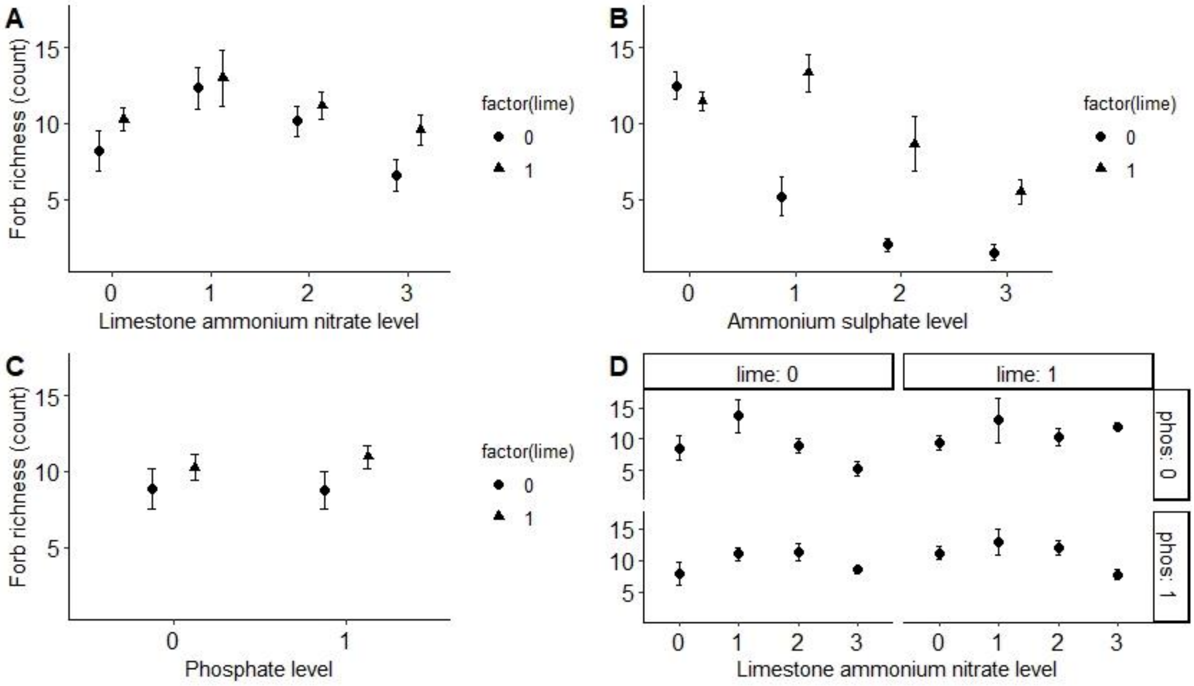


**Figure 2.5.** The interaction effect of limestone ammonium nitrate and lime (A), ammonium sulphate and lime (B), main effect of phosphorus (C) and three-way interaction effect of limestone ammonium nitrate, phosphorus, and lime (D) on mean (±SE) forb rich at the UGNE. Key: limestone ammonium nitrate (0 (control), 7.1, 14.1, and 21.2 g m $^{-2}$), ammonium sulphate (0 (control), 7.1, 14.1, and 21.2 gm $^{-2}$), phosphorus (0 (control), 33.6 g m $^{-2}$) and lime (0 (control), 225 g m$^{-2}$).

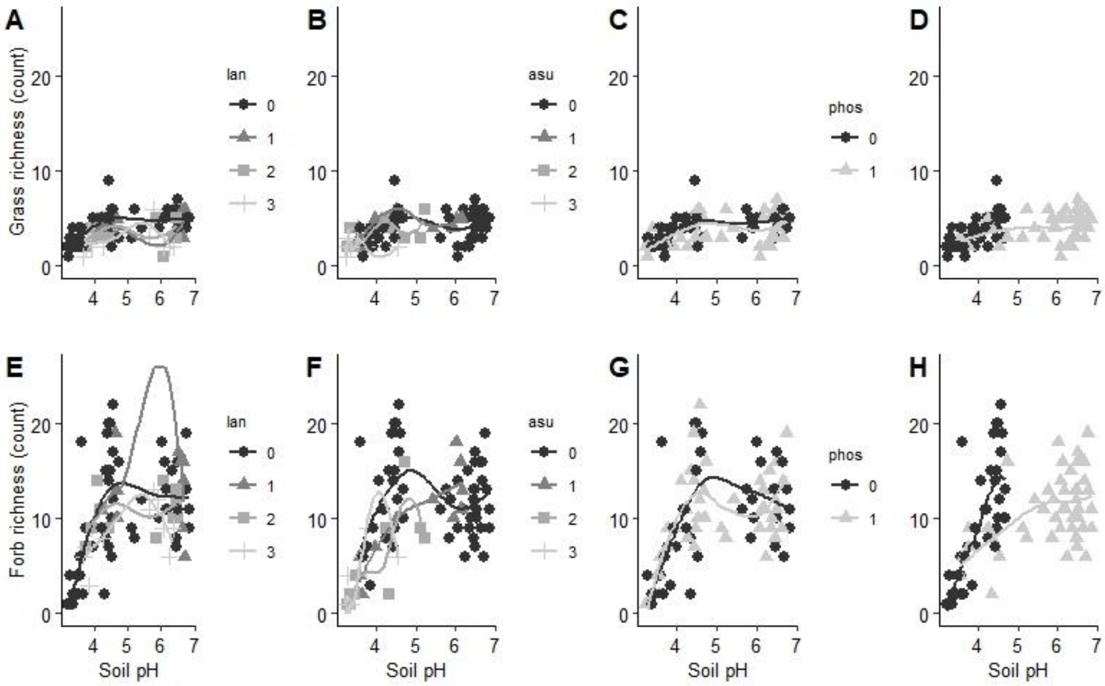


**Figure 2.6.** The relationship between soil pH (KCl) and grass richness (count) and soil pH and forb richness(count) at the UGNE. The same dataset was used to plot the effects of each nutrient and lime for visual purposes. A lowess line with a smoothing span of 2/3 was used here. Plots show the effects of each level for the following nutrients: A+E) limestone ammonium nitrate, B+F) ammonium sulphate, C+G) phosphorus and for D+H) lime. Key: limestone ammonium nitrate (0 (control), 7.1, 14.1, and 21.2 g m $^{-2}$), ammonium sulphate (0 (control), 7.1, 14.1, and 21.2 gm $^{-2}$), phosphorus (0 (control), 33.6 g m $^{-2}$) and lime (0 (control), 225 g m$^{-2}$).

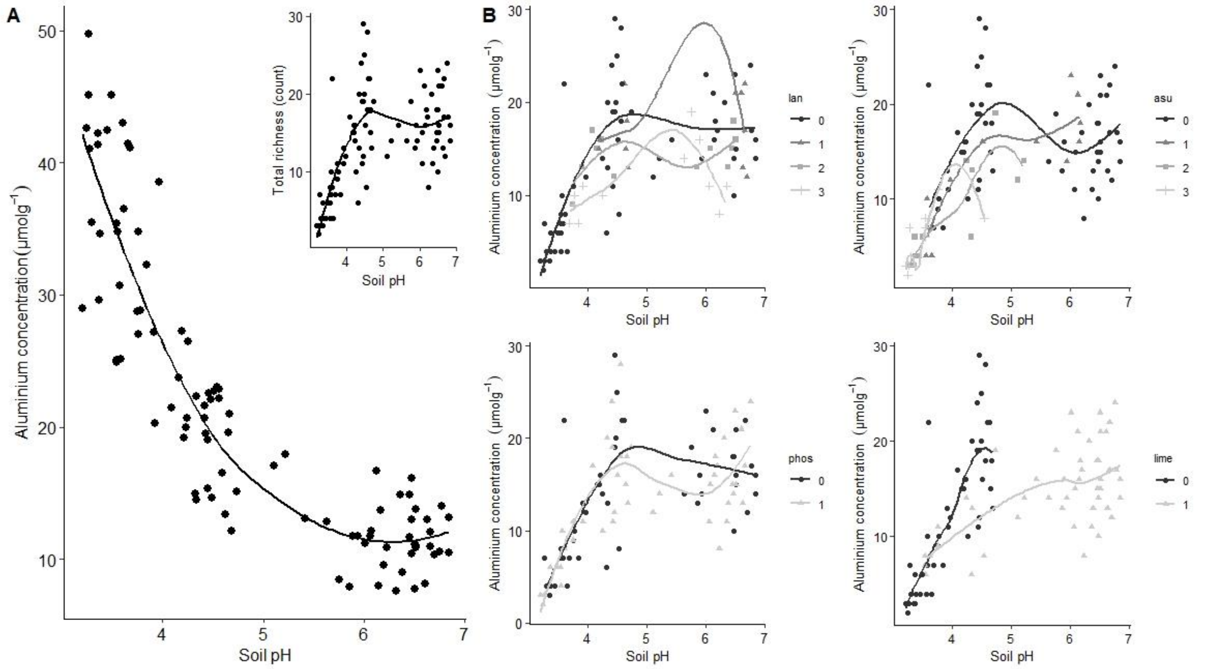


**Figure 2.7.** The relationship between soil pH (KCl) and aluminium concentration (µmolg$^{-1}$) and total species richness (A). The relationship between soil pH (KCl) and aluminium concentration (µmolg$^{-1}$) (B) at the UGNE. All lowess plots are shown with a smoothing span of 2/3. Plots in (B) represent show a visual for the effect of nutrients and lime. Key: limestone ammonium nitrate (0 (control), 7.1, 14.1, and 21.2 g m $^{-2}$), ammonium sulphate (0 (control), 7.1, 14.1, and 21.2 gm $^{-2}$), phosphorus (0 (control), 33.6 g m $^{-2}$) and lime (0 (control), 225 g m$^{-2}$).

## Discussion

Previously, the species occurring most frequently at the UGNE were *Setaria sphacelata*, *Eragrostis curvula*, *Tristachya leucothrix*, *Themeda triandra*, *Eragrostis plana* and *Megathyrsus maximus* (Fynn and O'Connor, 2005; Tsvuura and Kirkman, 2013). These species all accounted for the majority of the herbaceous ANPP (Fynn et al., 2005; Tsvuura and Kirkman, 2013). We assessed the species composition separately for grasses and forbs and found differences in how the two functional groups respond to nutrient and lime addition. The latest data reveals that N form affected the two functional groups differently. Grass species composition were influenced by both LAN and ASU. In contrast, forb species composition was mainly influenced by LAN. In both cases, plots with the highest LAN level application had more bare ground than the lower levels. Phosphate and lime enrichment explained less of the species composition for both functional groups. In this experiment, short species (approx. <1.5 m in height) such as *T. triandra*, *T. leucothrix*, *Brachiaria serrata* (Thunb.) Stapf (*B. serrata*) and *S. nigrirostris* declined in abundance with an increasing N level addition. Similar results were previously observed on the UGNE (Fynn et al., 2005). These short species were replaced by taller grass species such as *M. maximus*, *P. aequinerve* and *C. validus* that are known to respond positively to nutrient enrichment (Fynn et al., 2005). However, in this experiment not all tall grasses (i.e *H. hirta* and *E. curvula*) were competitive in nutrient rich plots. This could suggest that other traits (Fynn et al., 2005) or environmental factors are involved in determining species replacement. For example, grasses such as *M. maximus* and *H. hirta* have tall broad or narrow leaves, that increases their competitive abilities over shorter species like *T. triandra* in fertile soils (Fynn et al., 2005). In addition, a low specific leaf area is associated with slow growth rates and a reduction in competitive ability in fertile soils (MacDougall and Turkington, 2004). Furthermore, shade tolerance may explain success in fertilized plots, but other models emphasize that long-term dominance in a fertilized site requires shade tolerance and a tall growth form (Huston and Smith, 1987). This supports observations indicating that tall broad-leaved species are the most dominant in productive sites (Gaudet and Keddy, 1995; Lepš, 1999). The observed pattern of species replacement may also be related to accessibility to light. Nitrogen addition tends to decrease the amount of photosynthetically active radiation reaching the ground due to a high biomass thus reducing light accessibility to shorter species (Tsvuura and Kirkman, 2013; Ward et al., 2017).

In this experiment, nitrogen was the primary limiting nutrient as demonstrated in other studies (Fynn and O'Connor, 2005; Lebauer and Treseder, 2008; Tilman, 1987; van Dobben et al., 2017; Vitousek and Howarth, 1991). More specifically, limestone ammonium nitrate was the only nitrogen form to result in significant changes in ANPP. When LAN was applied at the lowest level, it resulted in an increase in ANPP relative to the control plots. However, when LAN was applied with lime it resulted in the highest ANPP, this indicates that N is a limiting nutrient unlike previous research on the site where N and P were both limiting nutrients. Previously, N and P were identified as limiting factors on the same experiment (Fynn et al., 2005).

Nutrients and lime had a significant effect on grass and forb richness. This negative effect of both N forms on richness has previously been reported on the site (Scott and Booysen, 1956; Ward et al., 2017). The decline in species richness with N addition is a common response recorded in other countries (Crawley et al., 2005; Inouye and Tilman, 1995; Jenkinson et al., 1994; Shaver et al., 2001; Silvertown et al., 2006; Socher et al., 2012; Tilman, 1987). Another study revealed that the effects of fertilization by acidic ammonium vs limestone ammonium nitrate differed (Ward et al., 2017). We had a similar result, however there was a slight difference whereby the lowest level of LAN increased forb richness. This increase was also prevalent when LAN was combined with lime. The addition of lime alone or with LAN, ASU or P prevented the decline in forb richness but not grass richness. This suggests that forb species may be more sensitive to nutrient and lime enrichment than grass species. This sensitivity can be linked to the effects of soil pH (Crawley et al., 2005; Silvertown et al., 2006; Ward et al., 2017). Liming ameliorated the negative effects of both forms of nitrogen on forb richness but less so for ammonium sulphate (Fynn and O'Connor, 2005). This is consistent with the current study.

Aboveground-net primary productivity was not a good predictor of total species richness. This contrasts a previous study at the UGNE where a negative relationship was observed between species richness and ANPP (Ward et al., 2017). A negative relationship was also reported in the Park Grass Experiment (Crawley et al., 2005). In an earlier study at a UGNE, a non-significant relationship was observed in one year and a significant relationship the following, suggesting that rainfall variability may be responsible for the differences (Tsvuura and Kirkman, 2013).

This non-significant relationship between ANPP and richness experimentally demonstrated here contrasts with the commonly recorded pattern of a positive/unimodal relationship (Cornwell and Grubb, 2003; Mittelbach et al., 2001; Waide et al., 1999). We expected a negative relationship based on previous studies assuming interspecific competition for light under high nutrients to be the main cause (Tsvuura and Kirkman, 2013). Our results are in support of findings of a review of experiments in 48 sites on five continents (Adler et al., 2011). Some studies indicate that short-term studies are not good predictors of the relationship between ANPP and species richness because they are normally affected by small term perturbations whereas the pattern of species richness in natural grasslands is influenced by long-term ecological and evolutionary processes such as dispersal and speciation respectively (Gough et al., 1994; Henry et al., 2004). Furthermore, the richness of a region reflects the level of plant biomass allowed by a local climate and soil conditions, rather than the cause of increased biomass (Huston, 1997). In addition, co-varying environmental factors can limit the positive effects of species richness (Ma et al., 2010). This could explain why species richness is weakly correlated with biomass in natural communities (Li et al., 2020) and potentially the UGNE.

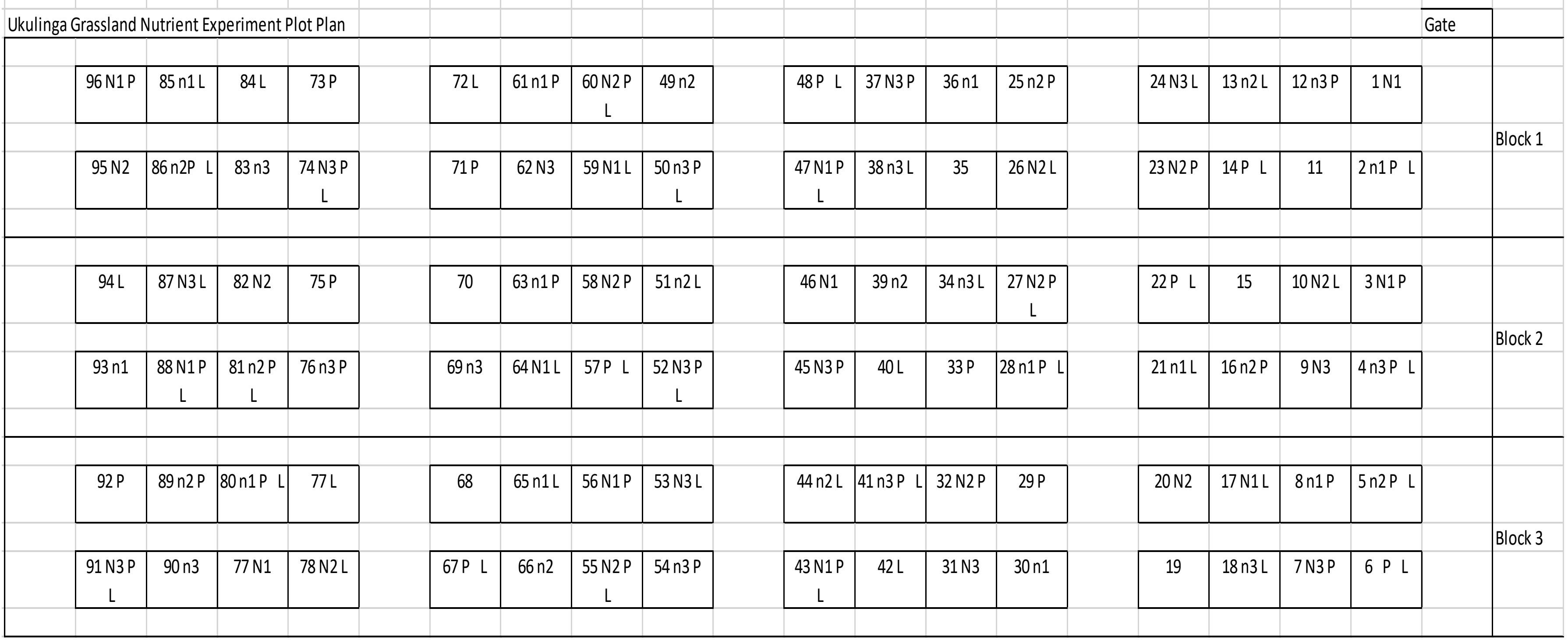
A very interesting result in this study was that the two N forms did not affect soil pH and both forb and grass species in a similar manner. Ward et al (2017) previously recorded a significant positive relationship between soil pH and total species richness at the UGNE. We too found a similar positive relationship at the site. In addition, a similar result was found at the Park Grass Experiment (Crawley et al., 2005; Silvertown et al., 2017). Unlike most studies we assessed the effect of pH on forb and grass species richness and found clear differences between the two functional groups. The most noteworthy effects on soil pH were associated with liming (Crawley et al., 2005; Ward et al., 2017) and extreme acidification by ammonium sulphate decreased species numbers (Storkey et al., 2015; Ward et al., 2017).

The most important causes of soil acidification on agricultural land are the application of ammonium-based fertilizers and urea, elemental sulphur fertilizer and the growth of legumes (Bolan and Hedley, 2003). Ammonium salts strongly acidify soils through the process of nitrification (Goulding, 2016). Another response of acidic soils is the dissolution of Al minerals that causes concentrations of soluble Al that are rhizotoxic to various plant species (Kopittke and Blamey, 2016). In this study, the highest ammonium sulphate application increased the soils acidity (pH < 4). This effect would strongly increase the solubility of Al. Kopittke and Blamey (2016) recorded Al toxicity occurring in soils with pH between 4.5 and 5.0. These acidic and high Al content soils are suitable for certain species and has implications for biodiversity. Aluminium-sensitive species can be replaced by more Al-tolerant species (Sade et al., 2016). For example, in this study, acidic soils were dominated by *M. maximus* and *E. curvula*. Both species are considered somewhat Al-tolerant (Almeida et al., 2000; Miles and De Villiers, 1989) and nitrophilous (Noukeu et al., 2019; Tsvuura and Kirkman, 2013). These advantages and morphological features such as tall broad leaves enhance their ability to capture light and outcompete neighbouring species in acidic soils (Fynn and O'Connor, 2005; Wilson and Tilman, 1991). *Cephalaria pungens* was the most dominant forb species however its dominance has not yet been linked to acidic soils or Al-tolerance, to our knowledge. A strategy to alleviate the negative effects of acidity is liming as it can provide a resistance against Al toxicity (Sade et al., 2016). However, liming may not be feasible because of costs and in situations where acidity occurs at deep soil layers (Sade et al., 2016). This study shows that plots that have not received nutrients in 70 years had the highest pH, lowest Al content and ultimately more species.

## Conclusions

Our findings reveal that changes in species composition, aboveground net-primary productivity, species richness and soil variables have been influenced by nutrients and lime enrichment over the past 70 years. High nutrient conditions may directly influence species compositional shifts from short species like *T. triandra* and *T. leucothrix* to more physiologically tolerant species such as *M. maximus*. However, there appears to be other important traits that enhance a plants ability to outcompete neighbouring species. Specifically, limestone ammonium nitrate and lime were considered most limiting in terms of aboveground net-primary productivity. Phosphorus and limestone ammonium nitrate did not influence species richness as strongly as ammonium sulphate. The latter nutrient increased soil acidity, creating a more suitable environment for high aluminium concentrations and reducing both grass and forb species richness. The soil pH threshold for the lower limit for species richness was approximately 4.6, like previous work (4.5) at UGNE. Aluminium toxicity poses a threat to agricultural production since it inhibits root growth and ultimately overall plant growth.

# Appendix A



| Ukulinga Grassland Nutrient Experiment Plot Plan | | | | | | | | | | | | | | | | Gate |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 96 N1 P | 85 n1 L | 84 L | 73 P | 72 L | 61 n1 P | 60 N2 P L | 49 n2 | 48 P L | 37 N3 P | 36 n1 | 25 n2 P | 24 N3 L | 13 n2 L | 12 n3 P | 1 N1 | |
| 95 N2 | 86 n2P L | 83 n3 | 74 N3 P L | 71 P | 62 N3 | 59 N1 L | 50 n3 P L | 47 N1 P L | 38 n3 L | 35 | 26 N2 L | 23 N2 P | 14 P L | 11 | 2 n1 P L | Block 1 |
| 94 L | 87 N3 L | 82 N2 | 75 P | 70 | 63 n1 P | 58 N2 P | 51 n2 L | 46 N1 | 39 n2 | 34 n3 L | 27 N2 P L | 22 P L | 15 | 10 N2 L | 3 N1 P | |
| 93 n1 | 88 N1 P L | 81 n2 P L | 76 n3 P | 69 n3 | 64 N1 L | 57 P L | 52 N3 P L | 45 N3 P | 40 L | 33 P | 28 n1 P L | 21 n1 L | 16 n2 P | 9 N3 | 4 n3 P L | Block 2 |
| 92 P | 89 n2 P | 80 n1 P L | 77 L | 68 | 65 n1 L | 56 N1 P | 53 N3 L | 44 n2 L | 41 n3 P L | 32 N2 P | 29 P | 20 N2 | 17 N1 L | 8 n1 P | 5 n2 P L | |
| 91 N3 P L | 90 n3 | 77 N1 | 78 N2 L | 67 P L | 66 n2 | 55 N2 P L | 54 n3 P | 43 N1 P L | 42 L | 31 N3 | 30 n1 | 19 | 18 n3 L | 7 N3 P | 6 P L | Block 3 |

**Figure 2. A1.** A plot plan of the Ukulinga Grassland Nutrient Experiment. The plot plan shows the 96 plots with the nutrient treatment applied (plot number alone = control plot; N1, N2, N3 = LAN level 1, 2 and 3 respectively; n1, n2 and n3 = ASU level 1, 2 and 3 respectively; P = phosphorus and L = lime. Nutrients applied in combination are shown per plot. The treatments are replicated into here block, totalling 32 plot per block.

**Table 2. A1.** Results of Canonical Correspondence Analysis (CCA) for grass and forb plant communities (Biplot scores for constraining variables). Limestone ammonium nitrate – LAN, ammonium sulphate – ASU, phosphorus – PHOS and Lime – Lime

| | **CCA1** | | **CCA2** | | **CCA3** | | **CCA4** | | **CCA5** | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Grass** | **Forb** | **Grass** | **Forb** | **Grass** | **Forb** | **Grass** | **Forb** | **Grass** | **Forb** |
| **Control** | 0.489 | -0.495 | 0.236 | -0.475 | -0.784 | -0.676 | -0.101 | 0.261 | -0.303 | -0.075 |
| **LAN** | -0.469 | 0.854 | -0.124 | 0.013 | -0.014 | -0.390 | -0.855 | -0.305 | -0.187 | 0.161 |
| **ASU** | -0.474 | 0.153 | -0.211 | -0.334 | -0.089 | 0.625 | 0.839 | 0.688 | -0.140 | -0.030 |
| **PHOS** | -0.439 | 0.321 | 0.724 | 0.479 | 0.067 | 0.114 | 0.003 | 0.102 | 0.528 | -0.803 |
| **LIME** | 0.014 | 0.152 | 0.363 | 0.671 | 0.715 | 0.103 | 0.089 | 0.485 | -0.591 | 0.529 |

**Table 2. A2.** Results of Canonical Correspondence Analysis (CCA) for grass and forb plant communities (Eigenvalues for constrained axes)

| | **CCA1** | | **CCA2** | | **CCA3** | | **CCA4** | | **CCA5** | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Grass** | **Forb** | **Grass** | **Forb** | **Grass** | **Forb** | **Grass** | **Forb** | **Grass** | **Forb** |
| **Eigenvalue** | 0.535 | 0.357 | 0.271 | 0.183 | 0.103 | 0.147 | 0.017 | 0.122 | 0.006 | 0.064 |
| **p value** | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.112 | 0.001 | 0.695 | 0.008 |

**Table 2. A3.** Grass species found at the UGNE in 2019

| Species name | Abbrev |
|---|---|
| *Aristida junciformis* Trin. & Rupr. subsp. *junciformis* | *A. junc* |
| *Brachiaria serrata* (Thunb.) Stapf | *B. serr* |
| *Cymbopogon caesius* (Hook. & Arn.) Stapf | *C. caes* |
| *Cymbopogon validus* (Stapf) Stapf ex Burrt Davy | *C. vali* |
| *Eragrostis curvula* (Schrad.) Nees | *E. curv* |
| *Eragrostis racemosa* (Thunb.) Steud. | *E. race* |
| *Hyparrhenia hirta* (L.) Stapf | *H. hirta* |
| *Panicum aequinerve* Nees | *P. aequ* |
| *Megathyrsus maximus* Jacq. | *P. maxi* |
| *Setaria nigrirostris* (Nees) T. Durand & Schinz | *S. nigr* |
| *Setaria sphacelata* (Schumach.) Stapf & C.E Hubb ex M.B Moss | *S. spha* |
| *Themeda triandra* Forssk. | *T. tria* |
| *Tristachya leucothrix* (Trin. ex Nees) | *T. leuc* |

**Table 2. A4.** forb species found at the UGNE in 2019

| Abbreviation | Family and species name |
|---|---|
| | **Asteraceae/Compositae** |
| *B.pilo* | *Bidens pilosa* L. |
| *A.hisp* | *Afroaster hispidus* (Thunb.) J.C.M |
| *B.seti* | *Berkheya setifera* DC. |
| *B.umbe* | *Berkheya umbellata* DC. |
| *C.flori* | *Conyza floribunda* Kunth |
| *D.argy* | *Dicoma argyrophylla* Oliv. |
| *G.ambi* | *Gerbera ambigua* (Cass.) Sch. Bip |
| *H.mico* | *Helichrysum miconiifolium* DC. |
| *H.nudi* | *Helichrysum nudifolium* (L.) Less. var *nudifolium* |
| *H.pilo* | *Helichrysum nudifolium var pilosellum* (L.) Less. var *pilosellum* |
| *H.tric* | *Helichrysum tricostatum* (Thunb.) Less. |

| | |
|---|---|
| *S.burp* | *Senecio bupleuroides* DC. |
| *S.coro* | *Senecio coronatus* (Thunb.) Harv. |
| *S.rhom* | *Senecio rhomboideus* Harv. |
| *T.minu* | *Tagetes minuta* L. |
| *V.nata* | *Vernonia natalensis* Sch. Bip. Ex. Walp |
| | **Fabaceae/Leguminosae** |
| *V.vexi* | *Vigna vexillata* (L.) A. Rich. |
| *E.cord* | *Eriosema cordatum* E. Mey |
| *R.mini* | *Rhynchosia minima* (L.) D.C. var *minima* |
| *R.totta* | *Rhynchosia totta* (Thunb.) DC. var. *totta* |
| *V.nilo* | *Vachellia nilotica* (L.) P.J.H. Hurter & Mabb |
| | **Acanthaceae** |
| *C.hirs* | *Crabbea hirsuta* Harv. |
| *D.burc* | *Dyschoriste burchellii* (Nees) Kuntze |
| *R.baur* | *Ruellia baurii* C.B. Clarke |
| *T.atri* | *Thunbergia atriplicifolia* E.Mey. ex. Nees |
| | **Commelinaceae** |
| *C.erec* | *Commelina erecta* L. |
| *C.afri* | *Commelina africana* L.var.africana |
| *C.spec* | *Cyanotis speciosa* (L.F.) Hassk. |
| | **Malvaceae** |
| *H.aeth* | *Hibiscus aethiopicus* L.var. *aethiopicus* |
| *H.pusi* | *Hibiscus pusillus* Thunb. |
| *S.dreg* | *Sida dregei* Burtt Davy. |
| | **Hypoxidaceae** |
| *H.arge* | *Hypoxis argentea* Harv. ex Baker var. argentea |
| *H.fili* | *Hypoxis filiformis* Baker |
| *H.rigi* | *Hypoxis rigidula* Baker var. *rigidula* |

| | |
|---|---|
| | **Euphorbiaceae** |
| *A.angu* | *Acalypha angustata* Sond. |
| *T.meye* | *Tragia meyeriana* Mull.Arg. |
| | **Asclepiadoideae** |
| *A.gibb* | *Asclepias gibba* (E.Mey.) Schltr |
| *P.scab* | *Pachycarpus scaber* (Harv.) N.E. Br |
| | **Dipsacaceae** |
| *C.pung* | *Cephalaria pungens* Szabó |
| *S.colu* | *Scabiosa columbaria* L. |
| | **Geraniaceae** |
| *P.alch* | *Pelargonium alchemilloides* (L.) L'Her |
| *P.luri* | *Pelargonium luridum* (Andrews) Sweet |
| | **Solanaceae** |
| *P.peru* | *Physalis peruviana* L. |
| *S.pand* | *Solanum panduriforme* E.Mey. |
| | **Amaranthaceae** |
| *A.aspe* | *Achyranthes aspera* L. |
| | **Boraginaceae** |
| *C.geom* | *Cynoglossum geometricum* Baker & C.H. Wright |
| | **Alliaceae/Allioideae** |
| *D.viri* | *Dipcadi viride* (L.) Moench |
| | **Orobanchaceae** |
| *G.scab* | *Graderia scabra* (L.f.) Benth. |

| | |
|---|---|
| | **Lamiaceae** |
| *O.obov* | *Ocimum obovatum* E.Mey.ex Benth |
| | **Apiaceae** |
| *L.kolb* | *Lichtensteinia kolbeana* Bolus |
| | **Oxalidaceae** |
| *O.comi* | *Oxalis corniculata* L. |
| | **Rubiaceae** |
| *S.nata* | *Spermacoce natalensis* Hochst. |
| | **Araceae** |
| *S.nati* | *Stylochiton natalensis* Schott |
| | **Cyperaceae** |
| *A.ovat* | *Abildgaardia ovata* (Burm.f.) Kral |
| | **Thymelaeaceae** |
| *G.krau* | *Gnidia kraussiana* Meisn. |
| | **Campanulaceae** |
| *W.undu* | *Wahlenbergia undulata* (L.f.) A. DC. |
| | **Cucurbitaceae** |
| *C.zeyh* | *Cucumis zeyheri* Sond. |
| | **Not classified** |
| *Unkown1* | Unknown 1 |
| *Unkown2* | Unknown 2 |

## Appendix B

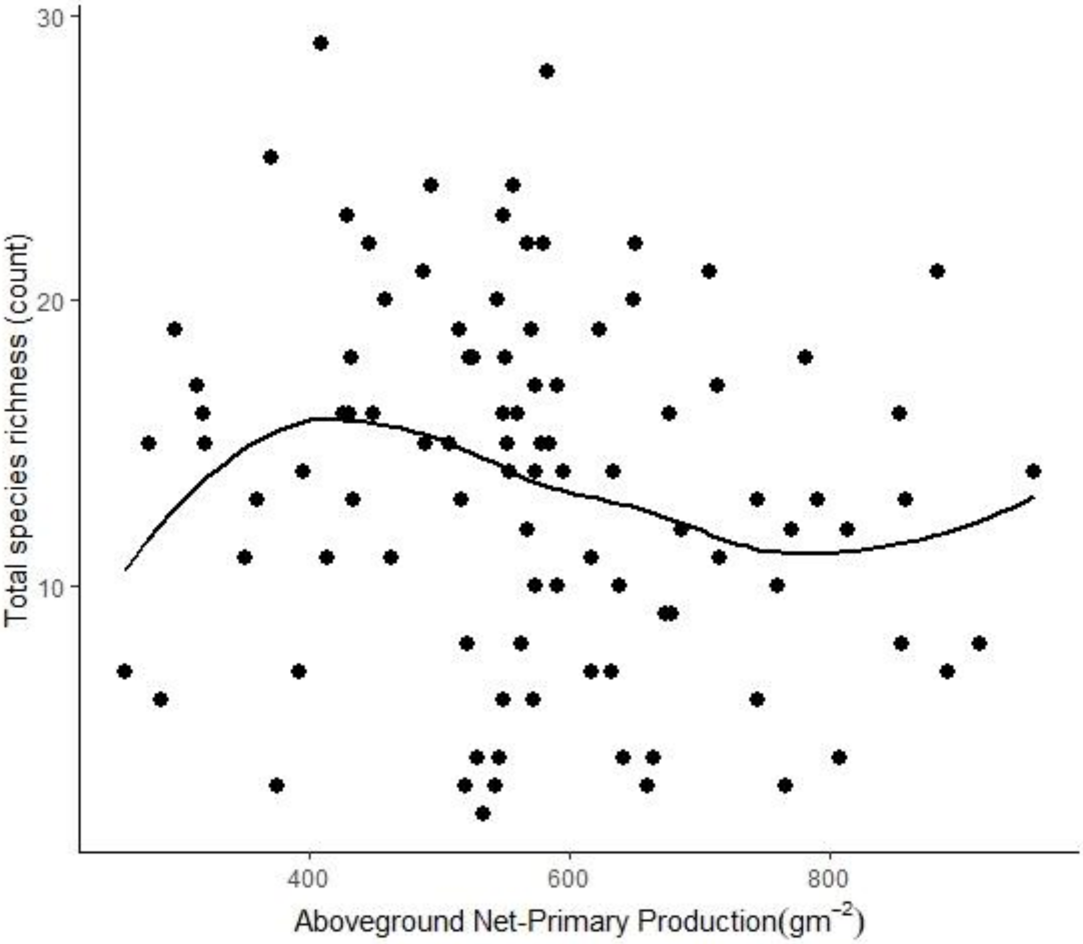


**Figure 2. B1.** The relationship between Aboveground Net-Primary Production (gm$^{-2}$) and total species richness (count) at the UGNE. A lowess plot with a smoothing span of 2/3 is shown here.

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# Chapter 3

# Short-term cessation of phosphorus enrichment does not alter plant species abundance, indicator species, productivity, or diversity in a mesic grassland

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## Abstract

Phosphorus (P) is considered a limiting nutrient in acidic and nutrient-poor South African grassland soils. It is expected that P enrichment would increase above-ground net primary productivity (ANPP), leading to competitive exclusion among plant species and ultimately reduce species diversity. The effect of short-term cessation of P enrichment on plant dynamics in a South African mesic grassland is unknown. Here, selected plots (size 4 x 2.7 m, N = 18) from the control, P enriched, and cessation of P (three years) treatment on the Ukulinga Grassland Nutrient Network (UGNE) were used to investigate responses to continued P enrichment and cessation of P enrichment. To evaluate the effects of the treatments on species, indicator species, ANPP and diversity we conducted species rank-abundance curves, indicator species analyses and multivariate analyses of variances. In the field, findings show that plant species abundance has drastically transformed since the inception of the experiment in 1951. This change is however not attributed to the cessation of P enrichment. All treatment plots showed a decline in cover abundance for previously dominant short species such as, *Themeda triandra* and *Tristachya leucothrix*. An increased cover abundance for *Setaria nigrirostris* was prevalent in all plots. Additionally, species identity was the most important factor influencing changes in cover abundance regardless of the plot treatment, suggesting individual species morphological and physiological traits to be important. Interestingly, treatments were associated with different individual and species combination indicator species. Notably, P enriched plots had the greatest number of indicator species, with *Achyranthes aspera* showing the greatest specificity. The use of functional diversity methods is recommended, to link diversity indices to ecological changes. The cessation of P enrichment applied here had not resulted in any significant changes over the period under investigation. This was linked to potentially negligible differences in the soil macronutrients among treatments. Phosphorus fertilizer residues can accumulate in the soil over time if continuously applied. Even after cessation of P enrichment, legacy effects may cause a delay in recovery.

**Key words:** biomass, fertilizer abandonment, Poaceae, forbs, species richness

## Introduction

Globally, the effects of elevated eutrophication of nitrogen (N) and phosphorus (P) levels are generally well known to decrease plant diversity in grasslands (Ceulemans et al., 2014, 2013;

Duprè et al., 2010; Humbert et al., 2016). Additionally, nutrient enrichment can alter shoot biomass of certain functional groups (Avolio et al., 2014; Suding et al., 2005). Functional groups can be defined as a collection of plants that have a similar response to environmental conditions and thus have similar effects on ecological processes within a plant community (Woodward and Cramer, 1996). For example, field experiments show that N enrichment alone favours the growth of grass species over forb species (Ceulemans et al., 2013; Tian et al., 2016), whereas P enrichment alone alleviates P limitation and ultimately favours the growth of N-fixing forb species (Benner and Vitousek, 2007). In terms of differences between life forms, annual species have a greater efficiency in using the supplied nutrients and therefore have a greater competitive advantage over perennial species (Avolio et al., 2014; Heger, 2016). Considering the abovementioned factors, differentiating between different growth forms and life forms may assist in better interpreting how nutrient enrichment alters plants communities (Yang et al., 2021).

Plant communities are impacted by altering resource availability through N deposition, elevated carbon dioxide ($CO_2$), precipitation fluctuations (Avolio et al., 2019) and increasing input of P because of agricultural activities (Peñuelas et al., 2013). In natural ecosystems, soil P is obtained from atmospheric P deposition through dust or from parent bedrock (Cui et al., 2022). However, P is predominantly added to the soil through the application of fertilizers, to support crop production and livestock grazing (Mahowald et al., 2008; Schoumans and Groenendijk, 2000). Continuously adding P disrupts and alters the internal balance of the soil and can significantly affect plant growth, carbon cycling, plant biomass and microbial biomass carbon (Bond-Lamberty and Thomson, 2010). Studies indicated that high P levels may have a negative effect on biodiversity in terrestrial ecosystems, regardless of N levels (Fujita et al., 2013; Wassen et al., 2005). Most soils in KwaZulu-Natal (KZN) ecosystems are nutrient-poor and acidic and nutrient availability is considered a limiting factor, receiving an annual precipitation of $\geq$ 600 mm (Magadlela et al., 2022; Mandiringana et al., 2005; Roberts et al., 2003). Therefore, we emphasize the importance of research focussing on the effects of P alone on KZN grasslands.

It is important for ecologists to utilise tools that assist in predicting and understanding community changes in response to environmental drivers (Avolio et al., 2019). This is imperative, given increasing concerns about the biodiversity crisis (Sala et al., 2000). Certain measures, such as species richness are commonly used to assess changes at a local scale (Dornelas et al., 2014; Vellend et al., 2013). However, assessing species richness solely, may not fully explain ecological dynamics within communities (Hillebrand et al., 2018; Jones et al., 2017; Wilsey et al., 2005) particularly because it does not consider either species identity nor abundance, potentially overlooking species turnover and species dominance re-ordering (Avolio et al., 2015; Collins et al., 2008; Hillebrand et al., 2018). Research suggests the use of rank abundance curves (RACs) to be used in combination with species richness to assess changes more clearly across time and different communities (Avolio et al., 2015, 2019).

Most research conducted on the long-term Ukulinga Grassland Nutrient Experiment (UGNE) has focussed on the responses of dominant grass species (Fynn and O'Connor, 2005; Tsvuura and Kirkman, 2013; Ward et al., 2017) thus ignoring the effects of nutrients on dominant forb species. Grassland forb richness and abundance have been neglected in studies or included under a combined non-grass category in veld assessments (Scott-Shaw and Morris, 2015). However more recently, research focus has shifted to forb species' responses to disturbances such as fire, grazing or mowing (Andrade et al., 2022; Chamane et al., 2017; Huang et al., 2018; Watson et al., 2021). Grassland forb species usually outnumber grass species (Bond and Parr, 2010) and this is particularly common in mesic grasslands (Drury, 2016; Uys et al., 2004). As a result, forbs provide various ecosystem services such as habitat and food for invertebrates and other taxa (Damhoureyeh and Hartnett, 1997), foraging opportunities for pollinators (Woodcock et al., 2014), enhanced pollinator diversity and wildflower reproduction (Orford et al., 2016), are used in cultural ceremonies (Klein et al., 2008; Quaempts et al., 2018) and have medicinal value (Dzerefos and Witkowski, 2001). Therefore, understanding how long-term nutrient enrichment and cessation of nutrient enrichment would affect both grass and forb abundance and diversity is important, especially at the UGNE which is a species-rich mesic grassland community, with a great diversity of plant families. In cases where the long-term application of nutrients has ceased, it is expected that the community structure will, over time, revert to its natural state. However, a study of this nature is yet to be conducted in a South African mesic grassland. To date, there is limited research on the effects of cessation of nutrient enrichment on community parameters, particularly in long-term studies (Heinsoo et al., 2020; Jan et al., 2007; Olff and Bakker, 1991; Semelová et al., 2008; Spiegelberger et al., 2006).

Studies conducted at the UGNE, support the notion that long-term P addition either "marginally" increases (Tsvuura and Kirkman, 2013) or significantly increases aboveground net-primary production (ANPP) (Ward et al., 2017). Additionally, P does not affect species richness or diversity (Tsvuura and Kirkman 2013; Ward et al. 2017; Zama et al. 2022). However, the possibility of species shifts, and replacements is difficult to isolate when solely assessing the abovementioned variables within a plant community. For example, if we assume that P enrichment does not affect plant species assemblages, then there should be no difference in the common species, rare species and indicator species identified among nutrient treatments. One useful method to approach this issue, is to use an Indicator Species Analysis (ISA) that uses indices to associate the abundance of a single or a combination of organisms to a specific group (De Cáceres et al., 2010; De Cáceres and Legendre, 2009). This should be useful in identifying species that are relatively more abundant and frequently occur among sites of interest. Another method used to assess the successful recovery of degraded grasslands is an increase in abundance of a target species after the cessation of nutrient enrichment (Mountford et al., 1996). The few studies conducted in European grasslands report fast or slow recovery (Mountford et al., 1996; Prach et al., 2021; Semelová et al., 2008; Walker et al., 2004). It was hypothesized that the grassland restoration after cessation of nutrient enrichment is more likely to rapidly occur in productive grasslands as opposed to unproductive grasslands (Kralovec et al., 2009). In this instance, productive grasslands are those with high aboveground net-primary productivity. Other factors that may influence recovery of grasslands after cessation of nutrient enrichment are linked to local conditions, metapopulation dynamics (Silvertown et al., 2009), species pools (Bakker and Berendse, 1999) and dispersal limitations (Lepš et al., 2007). The UGNE allows for ecological questions related to both long-term P enrichment and short-term cessation of P enrichment to be assessed for the first time in a South African mesic grassland.

To date, many studies have investigated the effects of N alone or N and P, while the effects of P alone have received less attention (Avolio et al., 2014), partially, because of the assumption that grasslands are more limited by N than P (Avolio et al., 2014). Acidic soils with low nutrient availability reduce agricultural productivity (Buresh et al., 1997; Haumaier and Zech, 1995; Onwuka et al., 2016). However, P enrichment of grasslands can replenish the required nutrients and improve soil fertility and productivity (Zhang et al., 2019). Additionally, an increase in productivity is associated with an increase in P availability and negatively with plant species richness (Ceulemans et al., 2013). Long-term P enrichment legacies are known to last for decades after cessation of P enrichment (Dupouey et al., 2002; Mclauchlan, 2006). Hence, it is possible that species that should begin to increase or decrease in relative abundance may not do so following cessation of P enrichment. With these considerations, we attempt to ask questions to find out how the cessation of P enrichment and long-term P enrichment affect plant species cover, Above-ground net-primary productivity and biodiversity. Thus, we aim to provide answers for the following questions: (1) what is the effect of long-term P enrichment on plant species relative cover abundance; (2) what is the effect of short-term (3 years) cessation of P enrichment on plant species relative cover abundance (3) what is the effect of short-term (3 years) cessation of P enrichment on dominant grass and forb species proportional cover abundance; (4) which indicator species are associated with the control treatment, P enrichment treatment and cessation of P enrichment treatment; and (5) what is the effect of short-term cessation of P enrichment on ANPP and biodiversity?

## Methods

### Site description

The Ukulinga Grassland Nutrient Experiment (UGNE) is located at Ukulinga Research Farm (29°39′58.42″ S, 30°24′04.6245″ E), University of KwaZulu-Natal, South Africa and was initiated in 1950. The experimental site is located on a south-east facing plateau with a mean annual precipitation and temperature of 838 mm and 18°C respectively (Ward et al., 2020) with majority of the rainfall occurring between October and April (Tsvuura and Kirkman, 2013). The soil is classified as Westleigh and is relatively infertile (Soil Classification Working Group, 1991).

The vegetation of the UGNE is classified as KwaZulu-Natal Hinterland Thornveld of Sub-Escarpment Savanna (Mucina and Rutherford, 2006). It consists of a dense tall grassland with scattered *Vachellia sieberiana* (D.C) Kyal & Boatwr and an understory of *Hyparrhenia hirta* (L.) Stapf and *Themeda triandra* (Forssk.) with other herbaceous species (Fynn and O’Connor, 2005). The most frequently occurring species in the UGNE plots according to previous studies include *Themeda triandra, Setaria sphacelata* (Schumach.) Stapf & C.E. Hubb. ex M.B*,* Moss, *Eragrostis curvula* (Schrad.) Nees, *Tristachya leucothrix*, *Eragrostis plana* Nees. and *Megathyrsus maximus* Jacq. (Fynn and O’Connor, 2005; Tsvuura and Kirkman, 2013). The most recent study on the UGNE shows that P enrichment has no influence on grass and forb species composition and biodiversity (Zama et al., 2022). All plots are mown on a yearly basis to prevent carry over plant growth from the previous season (Fynn and O’Connor, 2005). Grazing and fire have been excluded since 1951, and there are no trees on the study site.

### Experimental design and nutrient enrichment

Prior to 2019, the UGNE involved the manipulation of nitrogen (N), phosphorus (P) and lime in combination. There were 96 plots of 9.0 x 2.7 m in size with a minimum 1 m spacing between plots. The experiment was replicated in three blocks, each block containing 32 plots, resulting in a 4 x $2^3$ factorial design. The UGNE is located on a flat area, to minimize leaching of nutrients onto other plots. Further details of the abovementioned experimental design are available in previous studies (Fynn and O’Connor, 2005; Tsvuura and Kirkman, 2013; Ward et al., 2020, 2017; Zama et al., 2022). Since superimposing a withdrawal experiment in 2019, each plot has been split into subplots (4.0 x 2.7 m - with a 1 m corridor between subplots) with one subplot continuing to receive nutrients at the same application rate per unit area, while the other subplot does not. The number of plots has thus doubled to 192. In terms of phosphorus enrichment, the application was in the form of super-phosphate, once a year in October. Treatment summary is provided (Figure 3.1).

For this study we selected control plots (no nutrient enrichment) and plots enriched with P only to examine the cumulative (67 years) effect on species abundance, based on the abovementioned conditions.

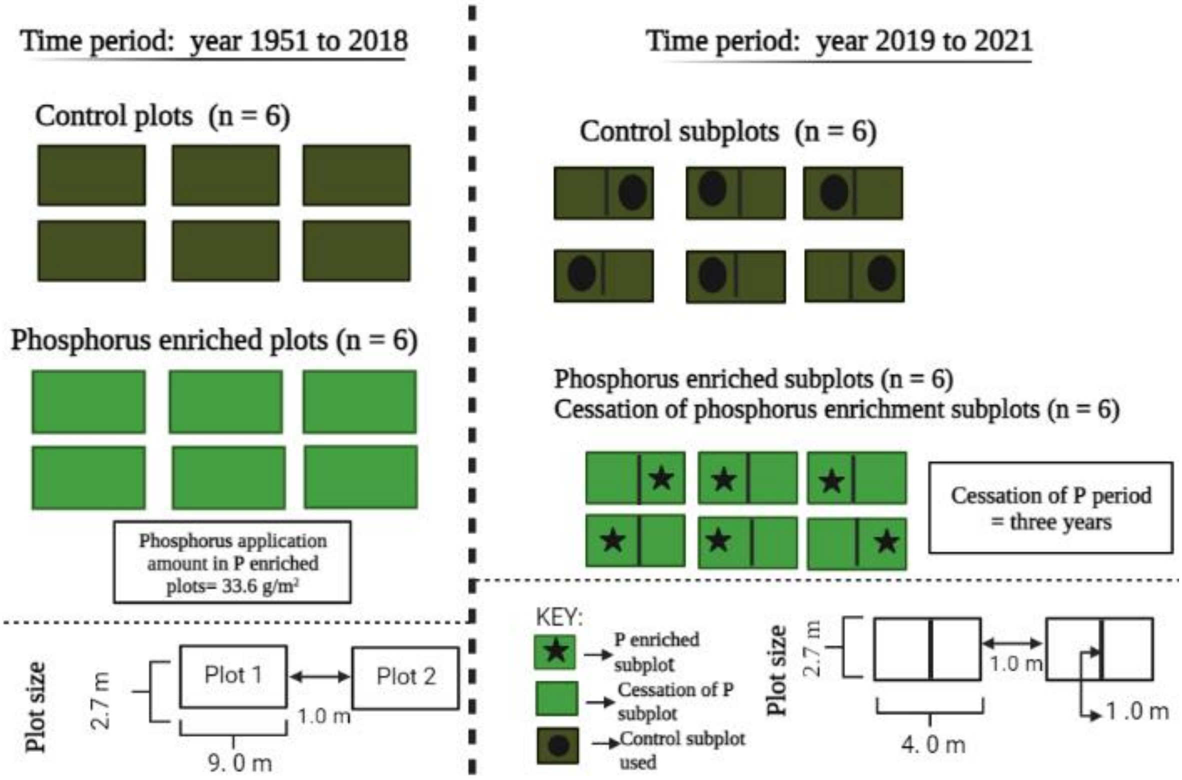

**Figure 3.1.** Illustration of the treatments (control, phosphorus enriched and cessation of phosphorus enrichment) and plot sizes (4.0 x 2.7 m) used for data collection in the study. The sample size per treatment was six. Both control and phosphorus plots were split to ensure correct scale of sampling.

### Sampling

Aboveground net primary productivity was measured in each plot by mowing a 2.13 m wide strip per plot at the end of the growing season. All plant material present (grasses and forbs) was mown to 5 cm above ground level. The fresh material in each plot was collected and weighed on site. The dry biomass was determined with the use of a grab sample that was weighed fresh, dried at 60 °C for 2 days and weighed again. To perform species-rank abundance, we used the species cover abundance data, for each plot. Plant cover abundance was physically measured per species present and measured as the cover percentage it utilized per quadrat. Plant cover abundance can be described as the cover proportion of each species occurring in a quadrat measured as a percentage. For both the 2019 and 2021 datasets, plant cover was sampled systematically using (1 m x 1 m) quadrats. The same dataset was used to determine species richness and further plant diversity measures. Species richness is one simple indicator and is described as the number of plant species present (Keylock, 2005). This is a widely used measure, previously used at the UGNE (Fynn and O’Connor, 2005; Ward et al., 2017; Zama et al., 2022) and similar study sites elsewhere (Fynn and O’Connor, 2005; Harpole and Tilman, 2007; Hejcman et al., 2010; Kidd et al., 2017; Yang et al., 2011). In addition, we calculated the Shannon-Weiner index and Pielou’s evenness. The Shannon-Weiner diversity index is described as a function of relative frequency of the varying plant species and Pielou’s evenness index refers to the similarity in the relative abundance of different species (Keylock, 2005; Mendes et al., 2008). To address the cumulative effects of P enrichment on species abundance, we used species abundance data from one growing season survey in 2019. To address the effect of short-term P withdrawal on dominant grass and forb species abundance, indicator species, ANPP and biodiversity we used data from one growing season survey in 2021.

### Data analyses

### Species rank-abundance

All statistical analyses were performed using RStudio (version 4.2.1) (R Core Team, 2022). Prior to performing the species-rank abundance curves and diversity analyses, we transformed and manipulated the cover plant cover abundance dataset. This was performed using packages 'dplyr' and 'tidyverse' packages to filter the replicates from the dataset and obtain mean values for each species occurring in each plot to avoid pseudo-replication (Wickham et al., 2019) . To address research question *(1) what is the effect of P enrichment and, (2) the effect of short-term (3 years) cessation of P enrichment on plant species proportional cover abundance?* Species rank-abundance curves (RACs) were constructed to assess the cumulative effect of P enrichment on plant species using the plant cover abundance data set for 2019. A separate species RAC was conducted to assess the effect on the plant cover abundance data set for 2021. The 'BiodiversityR' package was used to construct the RACs (Kindt, 2022). The *rankabundance* function in the 'BiodiversityR' package was used to calculate the rank-abundance curves from the plant cover abundance and treatment data sets. The abovementioned function allows for the RAC for a selected treatment to be selected. The resulting output produced information regarding the proportional abundance, accumulated proportional abundance, logarithmic abundance, whilst providing the confidence limits for each species (Kindt, 2022). The vertical axis scaling method used here was proportional abundance, calculated as,

$$Proportional\ cover\ abundance\ (\%) = \frac{total\ species\ abundance\ in\ treatment}{total\ abundance\ of\ all\ species} * 100$$

### Plant species cover abundance

*To assess what was the effect of short-term cessation of P enrichment on dominant grass and forb species relative cover abundance,* a two-way analysis of variance (ANOVA) was conducted. The ANOVA was conducted to determine whether mean (±SE) grass and forb cover abundance were significantly affected by nutrient treatment * species interaction. We selected the species with greatest cover abundance values across all treatments in line with the reported dominant species at the UGNE (Fynn and O'Connor, 2005; Tsvuura and Kirkman, 2013; Zama et al., 2022) and others identified from the present data. The 2021 growing seasons' species composition dataset was used to perform two separate ANOVAs for grass and forb species. For the grass two-way ANOVA, a log transformation was performed on the plant cover percentage data, to meet the assumptions of normality (W = 0.979, p = 0.171) and homogeneity of variance (p = 0.232). Forb plant cover data required removal of identified outliers and a log transformation to meet the assumptions of normality (W = 0.980, p = 0.124) and homogeneity of variance (p = 0.06). Normality was checked visually using Q-Q plots and further supported using the Shapiro-Wilk test performed on the ANOVA model residuals. Homogeneity of variance was assessed using a Levene's test from the *leveneTest* () function in the 'car' package. Pairwise comparison of the Estimated Marginal means (EMMeans) with a multiple comparisons' adjustment (Tukey's HSD) was performed as a post hoc test, with α = 0.05.

### Indicator species analysis (ISA)

*Additionally, an Indicator Species Analysis was conducted to address research question 4, which aimed to determine which indicator species were associated with the different treatments (control, P-enriched and cessation of P enrichment)*. For this analysis, the *multipatt* function with the IndVal.g association index from the 'indicspecies' package (De Cáceres et al., 2010; De Cáceres and Legendre, 2009) was used. This analysis was performed on single, species pairs and trio species combinations. The analysis was performed with 999 permutations. For each species, sensitivity (how well a species covered all plots for a species treatment group) and specificity (how strongly a species is restricted to a specific treatment group) was calculated (De Cáceres et al., 2012). Only species with a statistically significant association to the treatment groups (based on *p*-values < 0.05 and Indicator values > 0.750 from the permutation test) were considered as a strong indicator species here.

### Species richness and diversity

Plant species richness, Shannon-Weiner diversity index and Pielou's evenness were calculated for each treatment. The abovementioned measures of diversity are commonly used in vegetation studies, with richness expressing the number of individual forb and grass plants, Shannon-Weiner diversity index revealing abundance information besides richness and lastly, evenness extrapolating the equality of the species abundance within the vegetation community (Heip and Engels, 1974; Hutcheson, 1970). The 'vegan' package was used to calculate the descriptive community diversity values and indices (Oksanen, 2013), using the plant cover abundance data. Species richness was conducted using the *specnumber* function. Shannon-Weiner diversity indices (H') were calculated using the *diversity* function (Hill, 1973). The calculation for Shannon-Weiner diversity index is:

$$H' = -\sum_{i=1}^{S} p_i \ \ log_b\, p_i$$

where, pi is the proportion of species i, S is the number of species so that $\sum_{i=1}^{S} p_i$=1 and b refers to the base of the logarithm.

The ‘vegan’ package does not have an index for evenness, but the most common of these – Pielou’s evenness (J’), was calculated as:

$$J' = H'/\log\,(specnumber(S))$$

where, H’ = Shannon-Weiner diversity index *specnumber* (S) = is a simple vegan function to find the number of species in a species data S.

To address the question “*what are the effects of short-term P enrichment cessation on ANPP and biodiversity”*, a one-way multivariate analysis of variance (MANOVA) was performed. The MANOVA tested for differences in the two sets of response variables (ANPP and species richness) and diversity indices (Shannon-Weiner diversity index and Pielou’s evenness) among the treatments. Two separate ANOVAs were conducted to meet the assumption of no multicollinearity. The 2021 data was evaluated with treatment: control, P-enriched and cessation of P enrichment, as the grouping independent variable. The assumptions of MANOVA were tested; 1) sample size n > number of outcome variables, 2) no univariate outliers, 3) no multivariate outliers, 4) univariate normality, 4) multivariate normality, 5) no multicollinearity, 6) linearity, 7) homogeneity of covariances and 8) homogeneity of variances. All assumptions excluding assumption 7 were met. To address this, a log transformed was performed on all response variables to satisfy the assumptions of MANOVA. This was followed by Tukey’s HSD to determine the significant effects of treatment on the different levels of the response variables. The ‘rstatix’ package and the following functions: *identify_outliers, shapiro_test, cor_test, levenes_test* were used to evaluate four assumptions (Kassambara, 2021). To assess linearity, the ‘GGally’ package (Schloerke et al., 2021) was used. Lastly, the *boxM* function in the ‘heplots’ package was used to assess homogeneity of covariances (Fox et al., 2021).

## Results

### What is the effect of P enrichment and, (2) the effect of short-term (3 years) cessation of P enrichment on plant species cover abundance?

The most proportionally abundant grass and forb plant species were identified using the rank abundance curves (RACs) for each year. In 2019, prior to the cessation of P enrichment treatment, *T. leucothrix* was the most abundant species in both the control and P enriched plots (38.8 % and 24.9 % respectively). However, its proportional cover abundance was predominately greater in the control plots. The proportional cover abundance of *T. triandra* was slightly lower in P enriched plots (8.8 %) when compared to control plots (16.6 %). In addition to this, the proportional cover abundance of *Setaria nigrirostris* in P enriched plots (18.6 %) was similar to that of *T. triandra* in control plot (16.1 %) (Figure 3.2). On average, *S. nigrirostris*, *Rhynchosia cooperi* and *Vigna vexillata* had a greater cover abundance in P enriched plots compared to the control plots in 2019 (Table 3.1). *Eragrostis curvula* and a *Ledebouria* species were present in P enriched plot and not the control plots (Table 3.1). Notably, *Hibiscus aethiopicus* and *Cymbopogon caesius* were absent in P enriched plots (Table 3.1).

Following three years since imposing the cessation of P enrichment treatment on the UGNE, the plant species proportional cover abundance changed. For example, the proportional cover abundance of *T. triandra* in control plots decreased from 16.1% to 6.0 %. Within the same plots, *T. leucothrix* decreased from 38.8 % in 2019 to 18.9 % in 2021. *Setaria nigrirostris* had the greatest proportional abundance in all treatments (control = 18.5 %; P enriched = 21.2 %; cessation of P enrichment = 18.9 %) in 2021, contrary to 2019, whereby *T. leucothrix* was the most abundant species on all treatments (Figure 3.2). In terms of the P enriched and cessation of P enrichment plots, the proportional cover abundance of *T. triandra* decreased since 2019 to 0.5 % and 0.2 % (not shown in Figure 3.3 due to low value). Plant species that did not previously occur in the top ten highest proportional abundances in the P enriched plots in 2019 were abundant in plots where P enrichment had been ceased in 2021 include *Tagetes minuta, Achyranthes aspera, Megathyrsus maximus, Cephalaria pungens* and *Eriosema cordatum* (Figure 3.3). *Setaria nigrirostris*, *T. leucothrix*, *C. pungens* and *E. curvula* were present in all treatments and had the highest mean cover abundance (Figure 3.4). *Hibiscus aethiopicus*, *S. coronatus*, *C. africana*, *C.caesius* and *T. triandra* had the lowest mean cover abundance in P enriched plots and plots where P enrichment had been ceased (Figure 3.5). In contrast, the mean cover abundance was highest for *E. cordatum*, *M. maximus*, *A. aspera*, *R. cooperi* and *T. minuta* under the same treatment conditions (Figure 3.6).

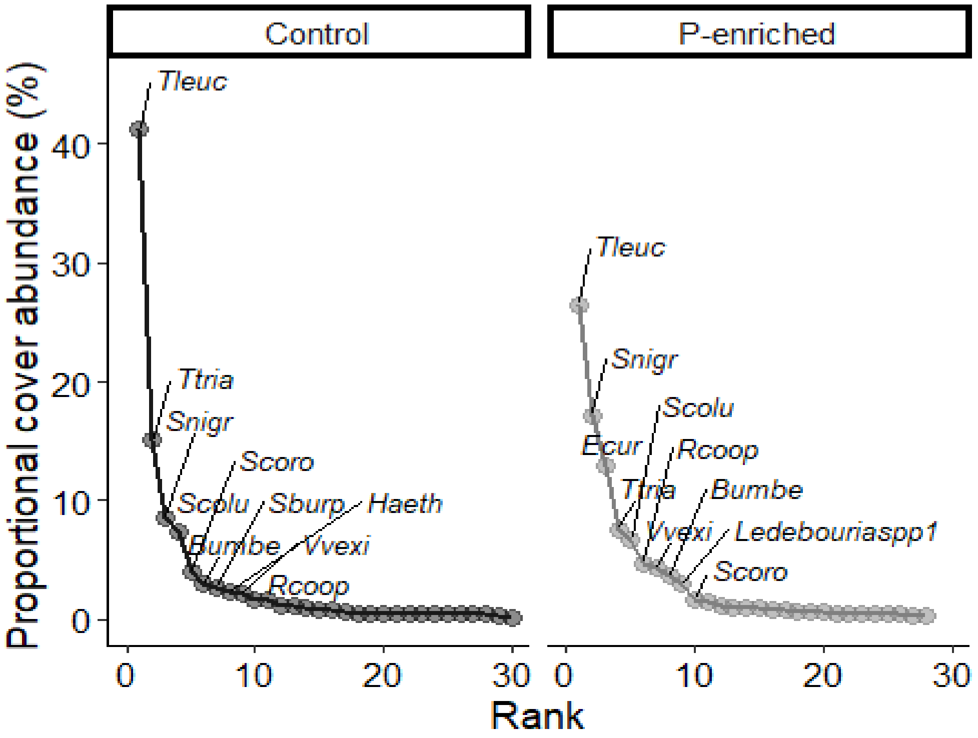

**Figure 3.2.** Species rank abundance curves for Control (no phosphorus added) and phosphorus enriched (P-enriched) plots at the Ukulinga Grassland Nutrient Experiment. Data used from 2019 showing the cumulative effect of the treatment over sixty-seven years. Data represents a plot size of 4 x 2.7 m. *Tleuc* = *Tristachya leucothrix*, *Ttria* = *Themeda triandra*, *Snigr* = *Setaria nigrirostris*, *Scolu* = *Scabiosa columbaria*, *Bumbe* = *Berkheya umbellata*, *Scoro* = *Senecio coronatus*, *Vvexi* = *Vigna vexillata*, *Rcoop* = *Rhynchosia cooperi*, *Ecurv* = *Eragrostis curvula*, *Haeth* = *Hibiscus aethiopicus*, *Ccaes* = *Cymbopogon caesius*, *Ledebouriaspp1* = *Ledebouriaspp1*.

**Table 3.1.** Cover abundance (%) (mean ± se) for the ten plant species (per treatment) with the greatest proportional abundance in 2019. Values calculated separately per treatment

| Treatment<br>Control | | Treatment<br>P-enriched | |
|---|---|---|---|
| Species | Mean ± se | Species | Mean ± se |
| *Tristachya leucothrix* | 56.67 ± 7.15 | *Tristachya leucothrix* | 30.83 ± 13.87 |
| *Setaria nigrirostris* | 11.67 ± 6.67 | *Setaria nigrirostris* | 20.00 ± 16.18 |
| *Themeda triandra* | 20.83 ± 4.73 | *Themeda triandra* | 8.83 ± 3.77 |
| *Berkheya umbellata* | 4.17 ± 2.99 | *Berkheya umbellata* | 4.17 ± 4.17 |
| *Senecio coronatus* | 5.50 ± 0.81 | *Senecio coronatus* | 2.00 ± 1.00 |

|  |  |  |  |
|---|---|---|---|
| *Rhynchosia cooperi* | 2.33 ± 1.50 | *Rhynchosia cooperi* | 5.50 ± 2.63 |
| *Vigna vexillata* | 2.83 ± 1.14 | *Vigna vexillata* | 5.00 ± 3.03 |
| Scabiosa *columbaria* | 10.17 ± 2.52 | Scabiosa *columbaria* | 7.83 ± 1.62 |
| *Hibiscus aethiopicus* | 3.17 ± 1.25 | *Hibiscus aethiopicus* | Absent |
| *Cymbopogon caesius* | 2.17 ± 1.64 | *Cymbopogon caesius* | Absent |
| *Eragrostis curvula* | absent | *Eragrostis curvula* | 15.00 ± 9.31 |
| *Ledebouriaspp1* | absent | *Ledebouriaspp1* | 3.33 ± 3.33 |

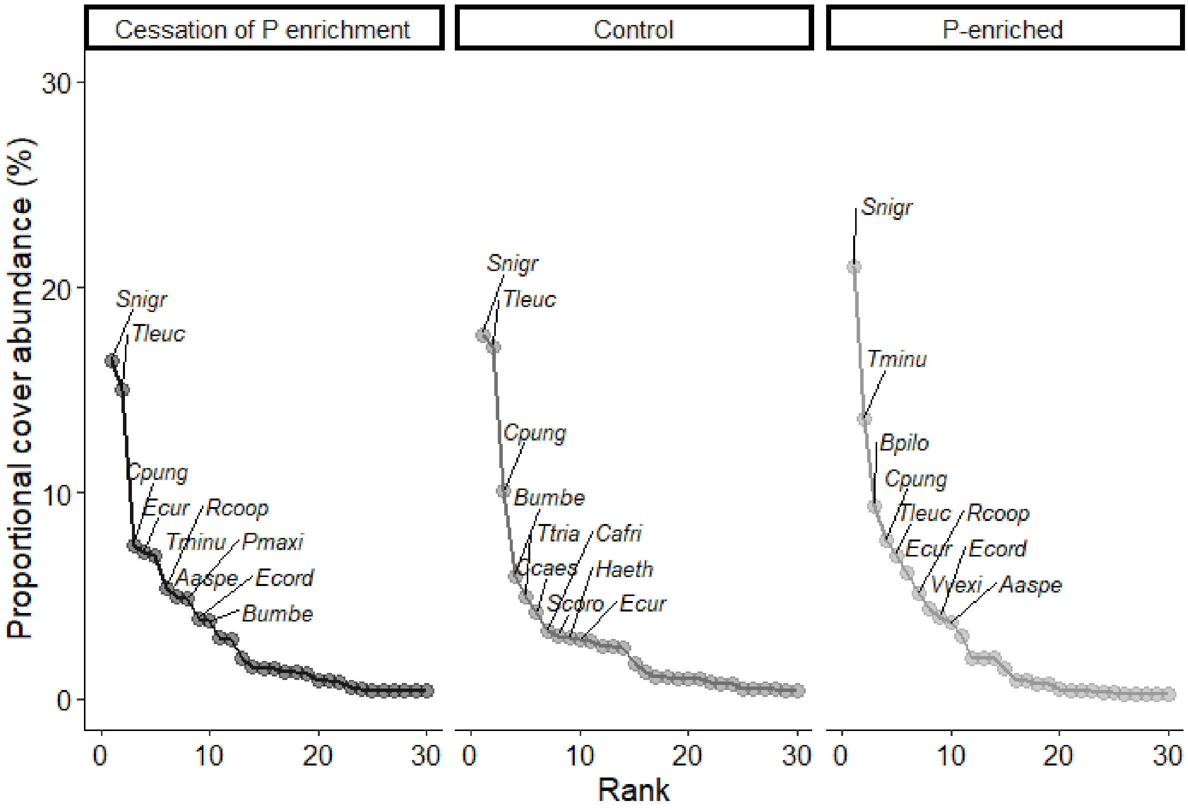

**Figure 3.3.** Species rank abundance curves for control (no phosphorus added), phosphorus enriched (P enriched) and cessation of phosphorus enrichment (cessation of P enrichment) plots at the Ukulinga Grassland Nutrient Experiment in 2021. Data represents a plot size of 4 x 2.7 m. *Tleuc* = *Tristachya leucothrix*, *Ttria* = *Themeda triandra*, *Snigr* = *Setaria nigrirostris*, *Bumbe* = *Berkheya umbellata*, *Scoro* = *Senecio coronatus*, *Vvexi* = *Vigna vexillata*, *Rcoop* = *Rhynchosia cooperi*, *Ecurv* = *Eragrostis curvula*, *Cpung* = *Cephalaria pungens*, *Ccaes* = *Cymbopogon caesius*, *Tminu* = *Tagetes minuta*, *Pmaxi* – *Megathyrsus maximus*, *Bpilo* – *Bidens pilosa*, *Haeth* = *Hibiscus aethiopicus*, *Aaspe* = *Achyranthes aspera*, *Cafri* = *Commelina africana*, *Ecord* = *Eriosema cordatum*.

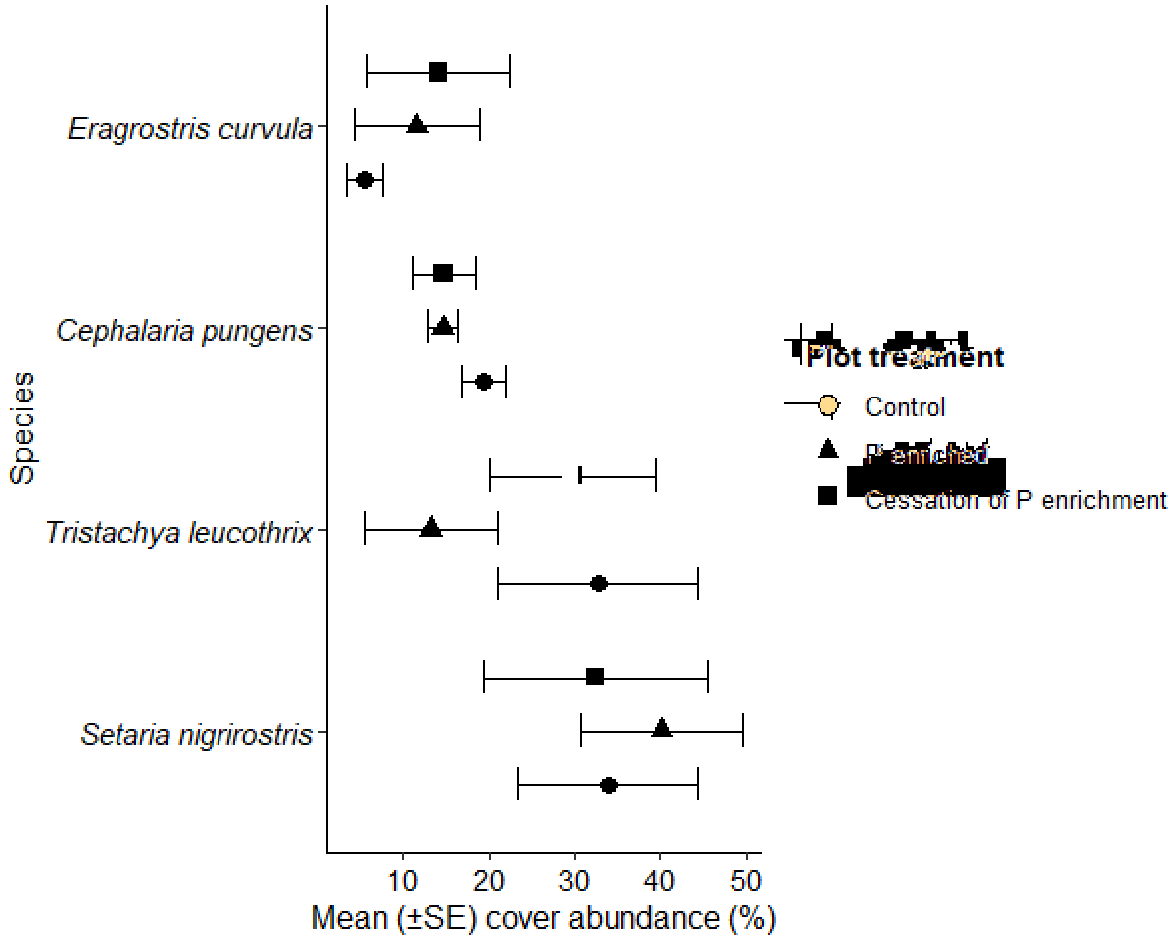

**Figure 3.4.** Plot showing plant species with the highest mean (± se) cover abundance (%) and that occurred within all three plot treatments in 2021 at the UGNE.

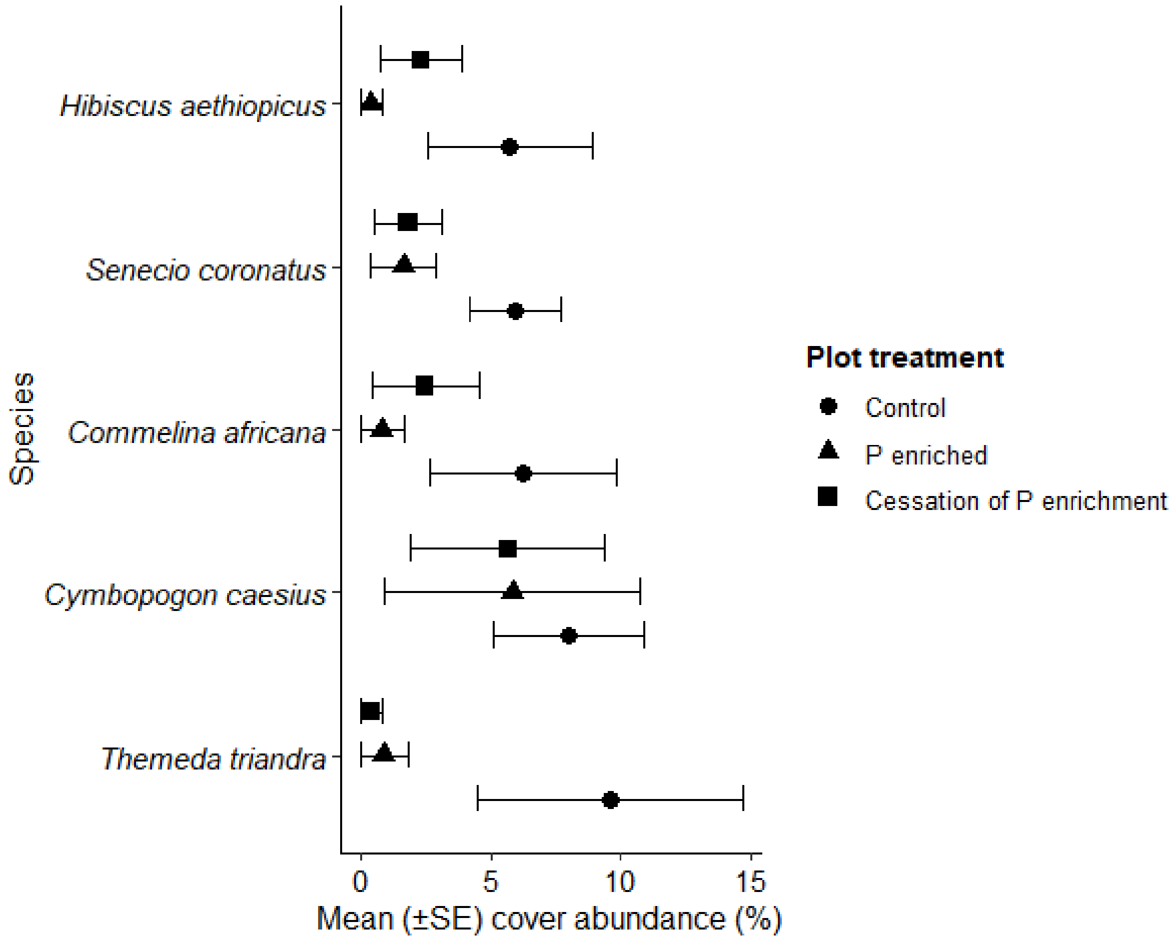

**Figure 3.5.** Plot showing plant species with a higher mean (± se) cover abundance (%) in the control plots than in P enriched plots and plots where P enrichment has been ceased at the UGNE.

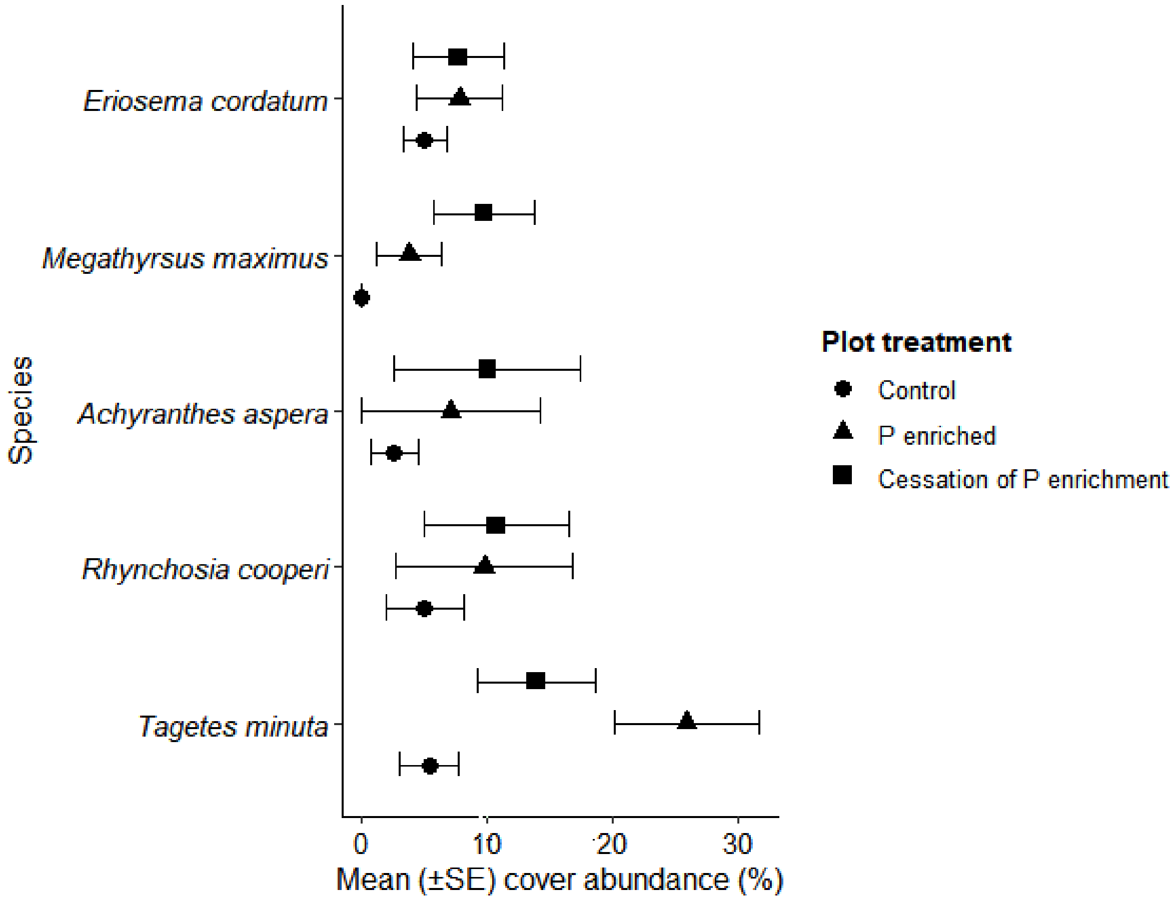

**Figure 3.6.** Plot showing plant species with a high mean (± se) cover abundance (%) in the P enriched plots and plots where P enrichment has been ceased than in the control plots at the UGNE.

### What is the effect of short-term (3 years) cessation of P enrichment on dominant grass and forb species proportional cover abundance?

For plant cover abundance for the most dominant grass and forb species, the main effect of species was significant, but the main effect of treatment (control, P enrichment, cessation of P enrichment) and interaction of treatment and species were not significant (Table 3.2) for both separate analyses. The cover abundance (mean ± standard error) for *Cymbopogon caesius* (5.528 ± 1.803), *E. curvula* (8.236 ± 3.003) and *T. triandra* (3.639 ± 1.919) were the lowest and not significantly different to each other (Figure 3.7A). The mean cover abundances for *S. nigrirostris* (35.513 ± 6.064) and *T. leucothrix* (25.264 ± 5.701) were significantly greater than those for *C. caesius*, *E. curvula* and *T. triandra* (Figure 3.7A). The cover abundance (mean ± standard error) for *Cephalaria pungens* was significantly higher (16.319 ± 1.594) than all other forbs except *T. minuta* (15.069 ± 3.189) (Figure 3.7B).

Regarding plant families, numerous species belonging to Asteraceae occurred in the control (16 species), P enriched (11 species) and cessation of P enrichment (9 species) (Figure 3.8). Furthermore, species belonging to the Poaceae, and Fabaceae were also more numerous than those belonging to Rubiaceae and Acanthaceae (Figure 3.8). A total of 23 plant families were described, with 20, 16 and 13 occurring in the control, P enriched and cessation of P enrichment plots, respectively (Figure 3. A1). There were 7 species that occurred in the control and/or P enriched plots and not in the plots where P enrichment had been ceased. For the control plots, these were *Graderia scabra* (L.f.) Benth. (Orobanchaceae), *Oxalis corniculata* L. (Oxalidaceae), *Pelargonium alchemilloides* (L.) L'Her (Geraniaceae), *Stylochiton natalensis* Schott (Alliaceae) and *Pelargonium luridum* (Andrews) Sweet (Geraniaceae). For the P enriched plots, these were *Oxalis corniculata* L. (Oxalidaceae), *Stylochiton natalensis* Schott (Alliaceae) and *Wahlenbergia undulata* (L.f.) A. DC (Campanulaceae). *Teucrium kraussii* Codd (Lamiaceae) and *Commelina erecta* L. (Commelinaceae) occurred in low cover abundance (0.83 ± 0.83) and (1.75 ± 1.65) respectively, only in plots where P enrichment had been ceased.

**Table 3.2.** Effects of treatment (control, phosphorus enrichment, cessation of P enrichment), plant species and the interaction of treatment * plant species on plant cover abundance in 2021 at the Ukulinga Grassland Nutrient Experiment. Significant differences (p-value < 0.05) are in bold

| Grass plant cover | | | | | |
|---|---|---|---|---|---|
| | df | Sum sq | Mean sq | F value | p-value |
| Treatment | 2 | 1.544 | 0.772 | 2.250 | 0.113 |
| Species | 4 | 12.830 | 3.207 | 9.346 | < 0.001 |
| Treatment * Species | 8 | 3.457 | 0.432 | 1.259 | 0.278 |
| Residuals | 75 | 25.70 | 0.343 | | |
| Forb plant cover | | | | | |
| | df | Sum sq | Mean sq | F value | p-value |
| Treatment | 2 | 0.721 | 0.360 | 1.469 | 0.236 |
| Species | 5 | 12.576 | 2.515 | 10.256 | < 0.001 |
| Treatment * Species | 10 | 2.090 | 0.209 | 0.852 | 0.580 |
| Residuals | 87 | 21.336 | 0.245 | | |

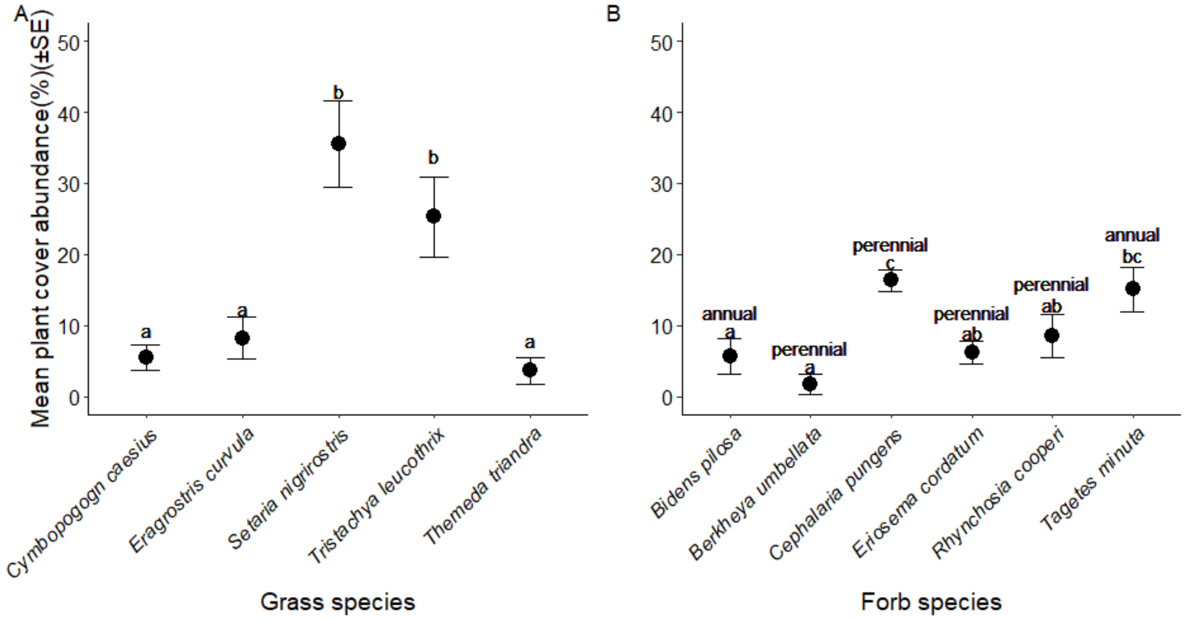

**Figure 3.7.** Patterns of the main effect of mean (± SE) cover abundance (%) for dominant grass (A) and (B) forb plant species at the Ukulinga Grassland Nutrient Experiment in 2021. Different letters indicate statistical significance between plant species.

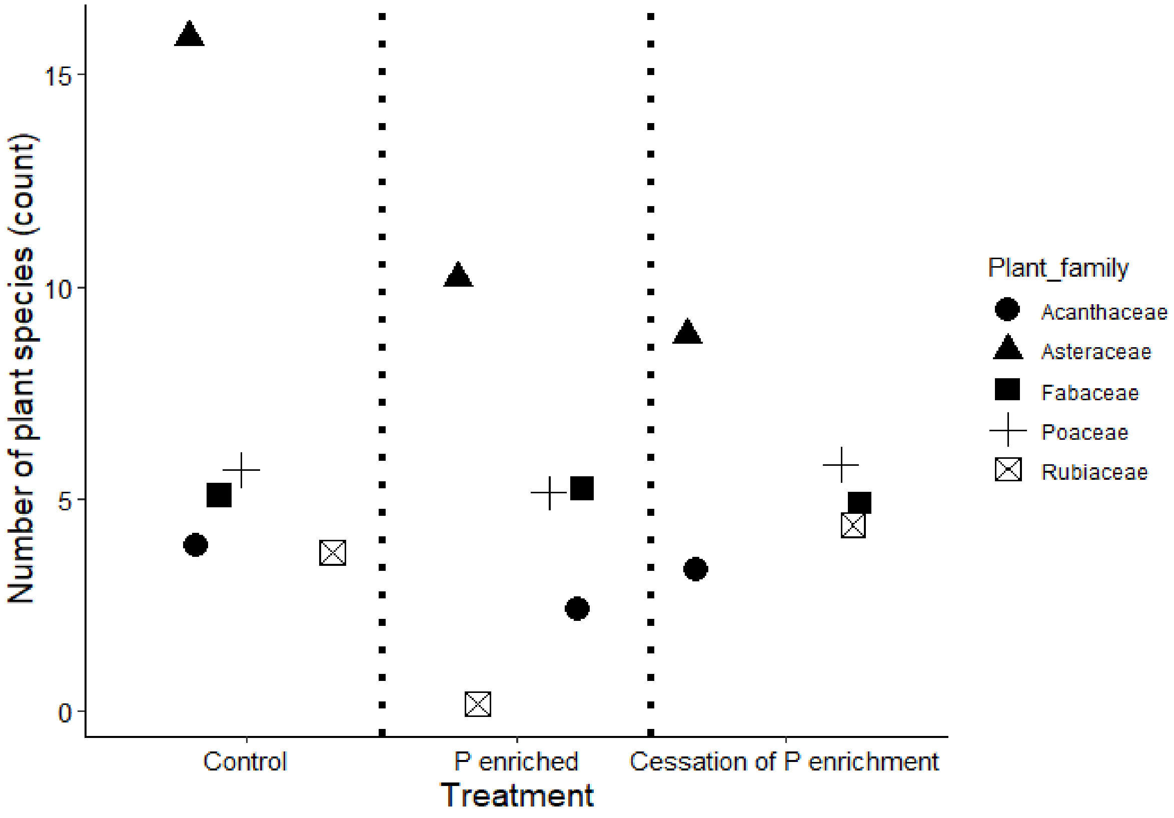

**Figure 3.8.** Number of plant species per plant family for control (no phosphorus added), phosphorus enriched (P enriched) and cessation of phosphorus enrichment (cessation of P enrichment) plots at the Ukulinga Grassland Nutrient Experiment in 2021. Shapes indicate, black circle – Acanthaceae, black triangle - Asteraceae, black square – Fabaceae, black cross – Poaceae and black square cross – Rubiaceae. Total plant families found across treatments are shown in Figure 3. A1.

### Indicator species

Across all treatments, species combination of *T. triandra* + *T. leucothrix* + *S. nigrirostris* had the highest specificity (specificity = 1.000, sensitivity = 0.833, p = 0.005) and it was strongly associated with control plots. This was followed by *T. triandra* + *T. leucothrix* (specificity = 0.924, sensitivity = 0.833, p = 0.005), and *T. triandra* + *T. leucothrix* + *C. pungens* (specificity = 0.924, sensitivity = 0.833, p = 0.005). Phosphorus enriched plots were strongly characterized by single species, *Achyranthes aspera* L. (specificity = 0.972, sensitivity = 0.667) and less strongly by *T. minuta* (specificity = 0.652, sensitivity = 1.000) (Table 3.3). Additionally, species combinations associated with P enriched plots included new species not identified in the control plots. In decreasing specificity, these were: *A. aspera* + *T. minuta* (specificity = 0.966), *T. leucothrix* + *A. aspera* (specificity = 0.962), *T. leucothrix* + *A. aspera* + *C. pungens* (specificity = 0.962) and *T. leucothrix* + *A. aspera* + *T. minuta* (specificity = 0.962), and *E. curvula* + *T. minuta* (specificity = 0.788) (Table 3.3). Species combinations of *S. nigrirostris* + *B. pilosa* (specificity = 0.735, sensitivity = 0.833, p = 0.005), was revealed to be the best indicator species combination for plots where P enrichment had been ceased (Table 3.3).

**Table 3.3.** Results of Indicator species analysis (ISA) for treatments (control, phosphorus enrichment, phosphorus withdrawal). Indicator values > 0.750 with p-value < 0.05 are shown here. The significance level was set at 0.05

| **Indicator species and combinations** | Control | Phosphorus enriched | Cessation of P enrichment | Specificity | Sensitivity | sqrtIV | p-value |
|---|---|---|---|---|---|---|---|
| 1. *Themeda triandra* Forssk. + *Tristachya leucothrix* Trin.ex Nees + *Setaria nigrirostris* (Nees) T. Durand | ✓ | | | 1.000 | 0.833 | 0.913 | 0.005 |
| 2. *Achyranthes aspera L.* | | ✓ | | 0.972 | 0.667 | 0.805 | 0.005 |
| 3. *Achyranthes aspera* L. + *Tagetes minuta* L. | | ✓ | | 0.966 | 0.667 | 0.802 | 0.005 |
| 4. *Tristachya leucothrix* Trin.ex Nees + *Achyranthes aspera L* | | ✓ | | 0.962 | 0.667 | 0.800 | 0.005 |
| 5. *Tristachya leucothrix* Trin.ex Nees + *Achyranthes aspera L + Cephalaria pungens* Szabo | | ✓ | | 0.962 | 0.667 | 0.800 | 0.005 |
| 6. *Tristachya leucothrix* Trin.ex Nees + *Achyranthes aspera L + Tagetes minuta* L. | | ✓ | | 0.962 | 0.667 | 0.800 | 0.005 |
| 7. *Themeda triandra* Forssk. + *Tristachya leucothrix* Trin.ex Nees + *Cephalaria pungens* Szabo | ✓ | | | 0.924 | 0.833 | 0.878 | 0.005 |
| 8. *Themeda triandra* Forssk. + *Tristachya leucothrix* Trin.ex. Nees | ✓ | | | 0.924 | 0.833 | 0.878 | 0.005 |
| 9. *Eragrostis curvula* (Schrad.) Nees + *Tagetes minuta* L. | | ✓ | | 0.788 | 0.833 | 0.810 | 0.005 |
| 10. *Setaria nigrirostris* (Nees)T. Durand + *Bidens pilosa* L. | | | ✓ | 0.735 | 1.000 | 0.808 | 0.005 |
| 11. *Tagetes minuta* L. | | ✓ | | 0.652 | 0.667 | 0.800 | 0.005 |
| 12. *Setaria nigrirostris* (Nees)T. Durand | | | ✓ | 0.619 | 1.000 | 0.787 | 0.005 |

### Aboveground Net-Primary Productivity and biodiversity metrics

Analyses using separate MANOVAs for firstly, Aboveground Net-Primary Productivity and species richness and secondly, Shannon-Weiner and Pielou's evenness indices as dependent variables were inconsistent with our prediction that short-term cessation of P enrichment would alleviate the negative impacts of long-term P enrichment. Neither P enrichment nor cessation of P enrichment had a significant effect on both ANPP and species richness (MANOVA $F_{4,30} = 1.093$, $p = 0.377$) (Table 3.4). Treatment had a significant effect on Shannon's diversity and evenness (MANOVA $F_{4,30} = 3.5357$, $p = 0.017$) (Figure 3.9A). This difference was manifested by a considerable increase in Shannon's diversity on the P enriched plots (mean ± standard error) (0.644 ± 0.042) and plots where P enrichment had been ceased (0.609 ± 0.018) compared to the control plots (0.494 ± 0.032) (Figure 3.9A). The same pattern was observed for evenness with the control plots showing a mean < 0.2 and P enriched plots and plots where P enrichment had been ceased showing a mean < 0.2 (Figure 3.9B). Overall results show that no significant effect between the two treatments (P enrichment vs cessation of P enrichment) on ANPP and biodiversity metrics. A difference is observed only when compared to the control plots and this associated with Shannon's diversity and Pielou's evenness indices.

**Table 3.4.** Mean ± standard error values for dependent variables on untransformed data

| | ANPP (gm $^{-2}$) | Richness |
|---|---|---|
| Treatment | | |
| Control | 496.604 ± 38.499 | 17.777 ± 1.202 |
| P enriched | 506.548 ± 94.406 | 14.500 ± 0.620 |
| Cessation of P enrichment | 416.928 ± 52.491 | 16.333 ± 1.520 |

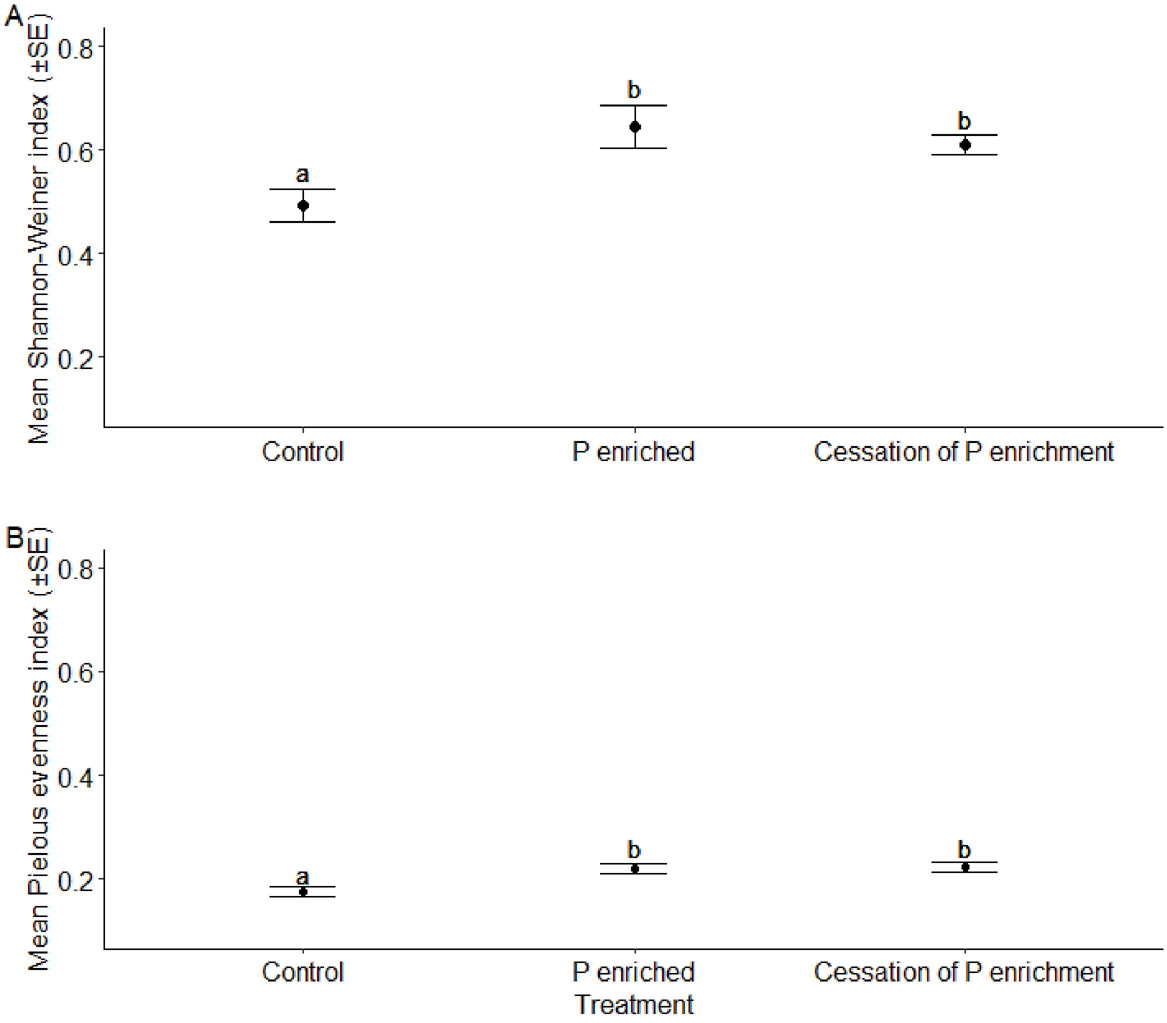

**Figure 3.9.** Biodiversity metrics for control (no phosphorus added), phosphorus enriched (P enriched) and cessation of phosphorus enrichment (cessation of P enrichment) plots at the Ukulinga Grassland Nutrient Experiment in 2021. Plots showing A) mean (±SE) Shannon-Weiner's index and B) mean (±SE) Pielou's evenness index. Different letters indicate statistical significance between treatment groups. The significance level was set at 0.05.

## Discussion

Since imposing the cessation of P enrichment on the UGNE, considerable changes have occurred on the plant cover abundance. However, these changes are not attributed to the short-term cessation of P enrichment period applied here. This is because the observed changes occurred across all treatments, thus are not unique to P enrichment cessation. Findings indicated that *T. leucothrix* dominated in 2019 but its dominance was replaced by *S. nigrirostris* in 2021 in all three treatments. Therefore, the observed changes can potentially be attributed to plant species traits considering these are likely to affect plant colonization, competitive ability, and tolerance to resource availability in different environmental conditions (Diaz et al., 1998; Fynn et al., 2009; Goldberg, 1996; Lavorel and Garnier, 2002; Suding et al., 2003; Wright et al., 2004), in accordance with the habitat template theory (Southwood, 1988). Previous research on the UGNE indicated that *T. leucothrix* and *T. triandra* were the two most stable dominant species in control plots for over fifty years (Fynn and O'Connor, 2005). However, the findings in this study, suggest otherwise. Most recently there has been a switch in species dominance in the control and P enriched plots by *S. nigrirostris* that has a greater leaf height (mean ± standard error) (603.64 ± 32.98 mm), leaf width (8.29 ± 0.30 mm) and stem width (1.12 ± 0.40mm) than both *T. triandra* (leaf height: 517. 00 ± 17.83, leaf width: 4.05 ± 0.19, stem width: 0.18 ± 0.18) and *T. leucothrix* (leaf height: 504.00 ± 13.43, leaf width: 4.55 ± 0.23, stem width: 0.00 ± 0.00) (Fynn and O'Connor, 2005). The ability to tolerate shade increases competitive ability in light-limited and productive sites (Ghebrehiwot et al., 2006). In productive sites that are infrequently disturbed, such as the UGNE, seedlings are likely exposed to competition for asymmetrical light and survival is dependent on an individuals' ability to access this light in the shade of other neighbouring plants (Goldberg, 1996).

Additionally, P enrichment alleviates P limitation and increases the growth of N-fixing forbs (Benner and Vitousek, 2007). Therefore, it was expected that P enriched plots would have a greater cover abundance of forbs, including legumes. Legumes, belonging to the Fabaceae plant family are the second most important following Poaceae (Graham and Vance, 2003), further warranting the need to assess how they respond to P level changes within grasslands. Soil acidification is a concern is some parts of the South Africa, specially in areas receiving high amounts of rainfall (Barnard and du Preez, 2004). In this study, P enrichment increased the cover abundance of *V. vexillata* and *R. cooperi* which is important because legumes provide essential services and contribute towards soil fertility through N-fixation (Cleveland et al., 1999; Peoples and Craswell, 1992). *Vigna vexillata* occurred in low cover in all treatments, suggesting that this legume species may be adapted low soil P, partially explaining its wide distribution in southern Africa. Relevant literature on forb morphological trait-environment relations in South African grasslands is lacking.

This study confirmed that different individual species and species combinations were associated with the plot treatments. The main outcome for conducting the ISA, was to determine how strongly species are restricted to a particular treatment (specificity). The presence of *T. leucothrix*, *T. triandra* and *S. nigrirostris* occurring together was strongly associated with control plots. Although these species differed in their cover, they still occupied the control plots. *Themeda triandra* and *T. leucothrix* are considered perennial bunchgrasses (Chamane et al., 2017, p. 36). *Setaria nigrirostris* was also considered a bunchgrass (personal observation). A study conducted at Ukulinga found that bunchgrasses differed in their ability to recruit secondary tillers (Tomlinson and O'Connor, 2005). For example, *T. triandra* had a greater secondary tiller recruitment rate than *Eragrostis curvula*, allowing it to efficiently colonize sites that are moderately defoliated (Fynn et al., 2005). Even though *T. triandra* is short grass with a slow growth rate (Tomlinson and O'Connor, 2005), its ability to recruit tillers may explain its presence in most plots. However, its decline may occur in swards that have many species from different plant families with different growth forms. Further studies on tiller recruitment of other grass species are recommended. Species combination of *Bidens pilosa* and *S. nigrirostris* was significantly associated with plots where P enrichment had been ceased. This is particularly interesting because *B. pilosa* was identified as having a strong tolerance to heavy metals and it can also be used a potential cadmium hyperaccumulator (Sun et al., 2009). Future studies should focus on how growth form, morphological and physiological plant traits enhance survival in soils of varying nutrient content.

Short-term cessation of P enrichment and P enrichment did not affect ANPP and species richness. However, P enrichment not having an effect of species richness supports previous research at the experimental site (Ward et al., 2017; Zama et al., 2022). Interestingly, the result of this study further suggests that applying P or a three-year cessation of P enrichment has the same effect on productivity and species richness as not adding nutrients altogether. Therefore, P enrichment at the UGNE may not be necessary if the aim is to increase yield or species richness. These findings are not in accordance with the niche dimension hypothesis (Harpole and Tilman, 2007; Hutchinson, 1957), which suggest that adding nutrients should reduce the number of limiting resources or niche dimensions, creating an environment where fewer species co-exist. However, it is acknowledged that this hypothesis was not tested here, considering changing soil nutrient content alongside changes in biomass was not assessed (Kralovec et al., 2009). The cessation of P enrichment period applied here may also not have been long enough to observe drastic changes, possibly because lowering soil P content is not simple as it is the least mobile soil nutrient compared to most macronutrients (Sharma et al., 2011). Moreover, P enrichment legacies can potentially last for decades (Dupouey et al., 2002; Mclauchlan, 2006), perhaps an extended cessation of P enrichment is required to observe ecological impacts. Research assessing P leaching rates is required on the UGNE, considering that leaching was determined to b strongly correlated with soil texture (Leinweber et al., 1999). Previous research indicates that mowing efficiently removes N due to high mobility of nitrate (Storkey et al., 2015) but it does not lower soil P, especially in intensely fertilized environments (Smits et al., 2008). Considering the UGNE is infrequently disturbed during the rowing season, additional decrease of soil P coupled with cessation of P enrichment is likely not to affect soil P content.

Diversity was assessed using the Shannon-Wiener and Pielou's evenness indices. No significant difference was identified between P enriched plots and plots where P enrichment had been ceased, suggesting that the conditions influencing these biodiversity metrics are most likely similar. Phosphorus enrichment and cessation of P enrichment increased diversity and evenness relative to the control. The results from this study were unexpected because previous studies conducted at the UGNE show no effect of P enrichment on diversity (Ward et al., 2017; Zama et al., 2022). This is further supported by studies conducted in China (Zhao et al., 2019) and Sweden (Sundqvist et al., 2014). Other studies indicate the P enrichment decreases diversity (Avolio et al., 2014; Ceulemans et al., 2013; Dorrough et al., 2006). Experiments show that N, P, and potassium (K) are important and limiting soil nutrients for plant diversity (Bakelaar and Odum, 1978; During and Willems, 1984). Therefore, it was expected that the addition of P would increase ANPP, leading to an increase in competition among plants for light (competitive exclusion) and reduce species richness (Huston, 1979) and diversity. Here, however, P enrichment did not result in such changes. There is a possibility that there is a relatively small difference in soil N, P and K content among the sampling plots. This is speculative, considering we didn't statistically test for any soil macronutrients. The P enrichment and cessation of P enrichment plots had similar species proportional cover abundances, and this could have influenced the results.

Lastly, it is acknowledged that many variables influence species composition and species turnover in terrestrial ecosystems. For example, ecologists are now able to quantify trait variation differences within communities, also known as functional diversity (FD) (Cadotte et al., 2009). The use of FD measures the diversity of species' traits and links this to the diversity of species' niches within a community (McGill et al., 2006; Petchey and Gaston, 2006; Villéger et al., 2008). It is suggested that assessing traditional methods such as species richness solely, cannot accurately quantify how diversity can respond to ecosystem disturbances and stress (Cadotte et al., 2009; Flynn et al., 2011; Norberg et al., 2001; Suding et al., 2008)

## Conclusions

The current study reveals that short-term cessation of P enrichment on the UGNE, has not modified plant species cover abundance. Rather, the observed changes have occurred in all treatment plots, manifested by the replacement of short species such as *T. triandra* and *T. leucothrix* by taller species such as *S. nigrirostris*. This suggests the importance of assessing plant species traits, using functional diversity methods.

Cessation of P enrichment was uniquely and strongly associated with the presence of both *S. nigrirostris* and *B. pilosa*, With the latter species being characterized as tolerant to soils containing heavy metals. It is suggested that the use of ISA strengthens the understanding of species importance in response to changes in the environment.

Unexpectedly, the applied three-year cessation period did not influence changes in ANPP, richness and diversity indices. Although not tested here, this could be associated with slight differences in crucial macronutrient among the soil plot treatments. Particularly between P enriched and cessation of P enrichment plots since P can have legacy effects. The study of the impacts over a longer period may reveal ecological impacts.

## Appendix A

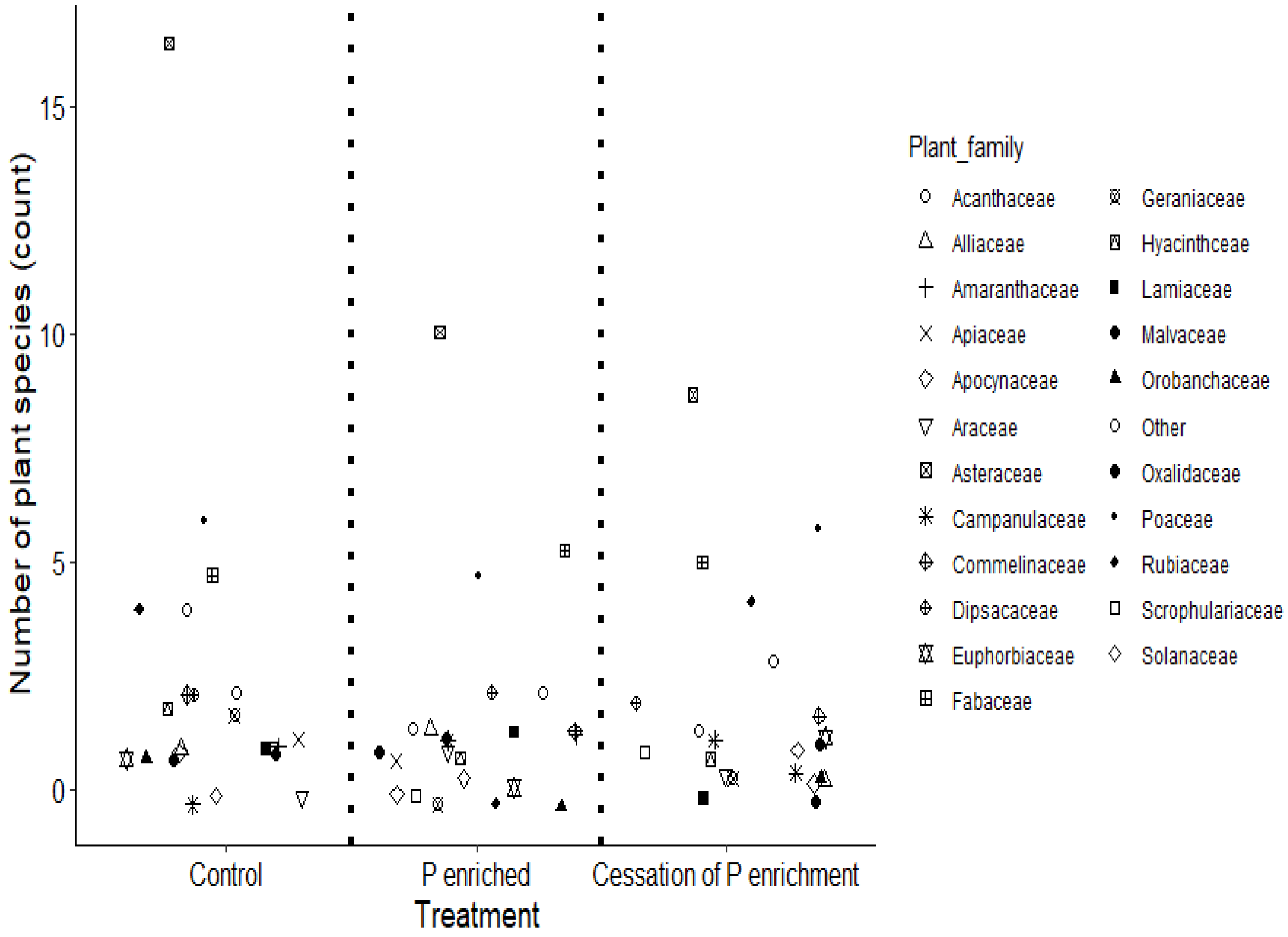

**Figure 3. A1.** The full set of plant species per plant family for control (no phosphorus added), phosphorus enriched (P enriched) and cessation of phosphorus enrichment (cessation of P enrichment) plots at the Ukulinga Grassland Nutrient Experiment in 2021.

## Appendix B

**Table 3. B1.** Species names, abbreviation, and family

| Species full name | Abbreviation | Family |
|---|---|---|
| *Brachiaria serrata* (Thunb.) Stapf | Bserr | Poaceae |
| *Cymbopogon caesius* (Hook. & Arn.) Stapf | Ccaes | Poaceae |
| *Eragrostis curvula* (Schrad.) Nees | Ecur | Poaceae |
| *Hyparrhenia hirta* (L.) Stapf | Hhirt | Poaceae |
| *Panicum aequinerve* Nees | Paequ | Poaceae |
| *Paspalum dilatatum* Poir. | Pdila | Poaceae |
| *Megathyrsus maximus* Jacq. | Pmax | Poaceae |
| *Sporobolus africanus* (Poir.) Robyns & Tournay | Safri | Poaceae |
| *Setaria nigrirostris* (Nees) T. Durand & Schinz | Snigr | Poaceae |
| *Setaria sphacelata* (Schumach.) Stapf & C.E Hubb ex M.B Moss | Sspha | Poaceae |
| *Themeda triandra* Forssk | Ttria | Poaceae |
| *Tristachya leucothrix* (Trin. ex Nees) | Tleuc | Poaceae |
| *Achyranthes aspera* L. | Aaspe | Amaranthaceae |
| *Agathisanthemum chlorophyllum* (Hochst.) Bremek | Achlo | Rubiaceae |
| *Afroaster hispidus* (Thunb.) J.C. Manning & Goldblatt | Ahisp | Asteraceae |
| *Asclepias gibba* (E.Mey) Schltr | Agibb | Apocynaceae |
| *Albuca setosa* Jacq. | Aseto | Hyacinthaceae |
| *Berkheya sertifera* DC. | Bsert | Asteraceae |
| *Albuca setosa* DC. | Bumbe | Asteraceae |
| *Bidens pilosa* L. | Bpilo | Asteraceae |
| *Cephalaria pungens* Szabo | Cpung | Dipsacaceae |
| *Commelina erecta* L. | Cerec | Commelinaceae |
| *Commelina africana* L. | Cafri | Commelinaceae |
| *Conyza floribunda* Kunth | Cflori | Asteraceae |
| *Crabbea hirsuta* Harv. | Chirs | Acanthaceae |
| *Cyanotis speciosa* (L.f.) Hassk. | Cspec | Commelinaceae |
| *Chaetacanthus burchellii* Nees | Dburc | Acanthaceae |
| *Dicoma argyrophylla* Oliv. | Dargy | Asteraceae |
| *Diclis reptans* Benth. | Drept | Scrophulariaceae |
| *Dipcadi viride* (L.) Moench | Dviri | Hyacinthaceae |
| *Eriosema cordatum* E. Mey. | Ecord | Fabaceae |
| *Gerbera ambigua* (Cass.) Sch. Bip. | Gambi | Asteraceae |
| *Graderia scabra* (L.f.) Benth. | Gscab | Orobanchaceae |
| *Helichrysum miconiifolium* DC. | Hmico | Asteraceae |
| *Helichrysum nudifolium* (L.) Less | Hnudi | Asteraceae |
| *Helichrysum nudifolium* (L.) Less | Hnudi2 | Asteraceae |
| *Hibiscus aethiopicus* L. | Haeth | Malvaceae |
| *Kohautia amatymbica* Eckl. & Zeyh. | Koama | Rubiaceae |

| | | |
|---|---|---|
| *Lichtensteinia kolbeana* Bolus | Lkoli | Apiaceae |
| *Oxalis corniculata* L. | Ocorn | Oxalidaceae |
| *Ocimum obovatum* E. Mey. Ex Benth | Oobov | Lamiaceae |
| *Pelargonium alchemilloides* (L.) L'Her | Palch | Geraniaceae |
| *Pentanisia angustifolia* (Hochst.) Hochst. | Pangu | Rubiaceae |
| *Pelargonium luridum* (Andrews) Sweet | Pluri | Geraniaceae |
| *Physalis peruviana* L. | Pperv | Solanaceae |
| *Rhynchosia cooperi* (Harv. ex Baker F.) Burtt Davy | Rcoop | Fabaceae |
| *Rhynchosia minima* (L.) DC. | Rmini | Fabaceae |
| *Rhynchosia totta* (Thunb.) DC. | Rtott | Fabaceae |
| *Ruellia baurii* C.B.Clarke | Rbaur | Acanthaceae |
| *Scabiosa columbaria* L. | Scolu | Dipsacaceae |
| *Senecio bupleuroides* DC. | Sburp | Asteraceae |
| *Senecio coronatus* (Thunb.) Harv. | Scoro | Asteraceae |
| *Solanum panduriforme* E. Mey. | Spand | Solanaceae |
| *Spermacoce natalensis* Hochst. | Spnat | Rubiaceae |
| *Stylochiton natalensis* Schott | Snata | Araceae |
| *Tagetes minuta* L. | Tminu | Asteraceae |
| *Thunbergia atriplicifolia* E. Mey. Ex Nees | Tatri | Acanthaceae |
| *Tragia meyeriana* Mull. Arg | Tmeye | Euphorbiaceae |
| *Tulbaghia acutiloba* Harv. | Tacut | Alliaceae |
| *Teucrium kraussii* Codd. | Tkrau | Lamiaceae |
| *Vernonia* spp | Vbraz | Asteraceae |
| *Vigna vexillata* (L.) A. Rich. | Vvexi | Fabaceae |
| *Wahlenbergia undulata* (L.f.) A. DC. | Waund | Campanulaceae |
| *Spermacoce* spp | Spermacospp | Rubiaceae |
| *Scabiosa* spp | Scabiosassp | Dipsacaceae |
| *Ledebouria* spp | Ledebouriaspp | Hyacinthaceae |
| *Plectranthus* spp | Plectranthusspp | Lamiaceae |
| Unknown clustered – leaf spp | Clusteredleafspp | Not classified |
| An exotic weed spp | Exoticweed | Not classified |

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# Chapter 4

## Does the effect of nutrient enrichment on above-ground net primary productivity, and light reduce species richness in a mesic grassland?

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## Abstract

A common pattern in vegetation studies is that nutrient addition reduces plant species richness. However, the mechanisms used to explain this phenomenon are highly controversial. Popular hypotheses include the niche dimension hypothesis, nitrogen detriment hypothesis and the biomass-driven competition hypothesis. It was predicted that high levels of nutrient enrichment would increase above-ground net primary productivity (ANPP), enhancing competition for light and causing species loss. This was tested on the Ukulinga Grassland Nutrient Experiment, a mesic grassland located in KwaZulu-Natal, South Africa. Plots enriched with nitrogen (N) in the form of limestone ammonium nitrate (LAN), ammonium sulphate (ASU) and phosphorus (P) and the interactions of both N nutrient sources with P were selected. Results show that when LAN was added with P, this effect modified species composition causing the dominance of a fast-growing and grass species such as *Megathyrsus maximus* at the expense of nitrophobic species such as *Themeda triandra* and *Tristachya leucothrix*. Forb species composition was significantly predicted by the number of nutrients added and ANPP with *Bidens pilosa* dominating in the high fertility plots. The model revealed that the best predictor of species loss in LAN and LAN + P enriched plots was ANPP. This was associated with increased species over-abundance and potential biomass production of the grass and forb species in these plots, leading to a high litter accumulation and decreased light reaching the smaller plants. This result best supported the biomass-driven hypothesis. In contrast, declines in species richness in the ASU and ASU + P enriched plots were linked to the increasing number of nutrients added. However, a significant decline in species richness was prevalent when no nutrient addition was compared to either one nutrient or two nutrient additions, not between one or two nutrient additions. This suggested that the acidifying effects of ASU may be firstly, an important factor and secondly, P addition may not be influencing changes in species richness on the UGNE. Thus, it was concluded that the nitrogen-detriment hypothesis may partially explain the mechanisms of species loss, particularly in ASU enriched soils. The findings from this study provide a baseline for future research to consider investigating alternative ways to assess the interaction of light, ANPP and specific nutrient identity additions in mesic grasslands.

**Key words:** niche axes, species diversity, fertilization, species -turnover, nutrient availability, light asymmetry

## Introduction

Declining plant species richness with an increase in nutrient availability is a common phenomenon occurring globally (Midolo et al., 2019; Stevens et al., 2004). Universal examples of this trend are documented in several experiments (DeMalach et al., 2017a; Gough et al., 2000; Harpole et al., 2016; Midolo et al., 2019; Schrijver et al., 2011; Soons et al., 2017; Suding et al., 2005). Eutrophication is major threat to diversity (Sala et al., 2000; Tilman et al., 2001) and has therefore stimulated research on understanding the implications of increased nutrient addition in terrestrial environments (Bobbink et al., 2010; Stevens et al., 2010). However, the mechanisms by which increased nutrient availability causes a reduction in the number of species present within a community are not yet fully understood (DeMalach and Kadmon, 2017; Grace et al., 2016; Harpole et al., 2017; Rajaniemi, 2003). Explaining the mechanisms that influence diversity is an important objective of ecology (Chesson, 2000). It is therefore important to understand how species can stably coexist with increasing anthropogenic environmental changes that cause a loss in diversity (Newbold et al., 2016; Turnbull et al., 2016). Grasslands occupy more than one third of the vegetation in terrestrial environments and their importance is linked to food production (O'Mara, 2012). Nutrient enrichment in grasslands is a common management tool used to increase production (Humbert et al., 2016; Socher et al., 2012). Additionally, high levels of atmospheric nitrogen (N) deposition also decreased grassland diversity (Bobbink et al., 2010; Stevens et al., 2010, 2004). Various sources of nutrient enrichment (nutrient fertilizers or atmospheric N depositions) have the same outcome of reducing species richness and diversity, suggesting they share common underlying mechanisms (DeMalach and Kadmon, 2017).

One explanation for a decrease in species richness with increasing nutrient addition is associated with higher above-ground productivity and this is believed to cause competition among species (Grime, 1973; Newman, 1973). This hypothesis is referred to as the "biomass-driven competition hypothesis". This hypothesis suggests that high soil resources create an environment where large and fast-growing species outcompete smaller and slow-growing species (Aerts, 1999; Grime, 1973; Newman, 1973; Rajaniemi, 2003). Furthermore, this competition is proposed as being primarily related to light (Hautier et al., 2009; Newman, 1973). Root traits also play an important role in determining competition for belowground resources and influencing changes in plant species composition, when nutrients are supplied (Zheng et al., 2019). However, it is noted that the relationship between species diversity and above-ground productivity in communities when nutrients are added is not direct and can differ in strength and direction across different sites and systems (Harpole et al., 2016).

Another hypothesis that has gained support is known as the “niche dimension hypothesis” (Harpole and Tilman, 2007). This hypothesis is based on the idea that high levels of resources will reduce the number of species that can coexist within an environment, by reducing the number of limiting resources or niche axes (Band et al., 2022; Harpole and Tilman, 2007). The niche dimension hypothesis is strongly supported by a global-scale experiment incorporating data obtained from five continents (Harpole et al., 2016). The results from the abovementioned study, revealed that a decline in species diversity was strongly associated with an increase in the number of nutrients added. However, the evidence used to support the niche dimension hypothesis is questioned by some authors (DeMalach et al., 2017b; DeMalach and Kadmon, 2017). These authors indicate that the decline in species diversity following an increase in the number of added resources shows a “pattern” and does not explain the mechanism behind this observation.

There is also a hypothesis indicating a decline in species richness under an increase in soil nutrients, specifically focussing on N. This hypothesis is known as the “nitrogen detriment hypothesis” and it suggests that high levels of N will negatively affect plant performance in several ways including acidification (Crawley et al., 2005), aluminium toxicity (Britto and Kronzucker, 2002) and causes changes to the soil microbiome (Farrer and Suding, 2016). Support for this hypothesis is reported in several studies (Ceulemans et al., 2013; Gough et al., 2000; Soons et al., 2017).

The type of nutrient in question is important when attempting to assess the patterns and mechanisms affecting plant species loss. Evidence shows that greater losses in species richness were observed with increasing ammonium and nitrate deposition owing to increased soil acidification (Kleijn et al., 2008; Paulissen et al., 2004). However, soil acidification tends to be more severe when ammonium fertilizer is applied instead of nitrate fertilizer (van den Berg et al., 2008). Thus, distinguishing between the nitrogen sources added may provide greater insights regarding the factors influencing species losses in nutrient- enriched grassland soils.

Nitrogen and P enrichment have different effects on ecosystem functioning. For example, N enrichment results in a loss of species (Soons et al., 2017) and changes species dominance (Song et al., 2019). On the other hand, P enrichment was identified as having a slightly increasing effect on grassland biomass (Tsvuura and Kirkman, 2013) and no pronounced effect on species composition (Song et al., 2019). The combined effects of N and P may be stronger than the individual effects of N and P therefore assessing multiple effects is crucial. In this sense, the following questions were asked: 1) What is the influence of nutrient number, light and ANPP on grass and forb species composition? and 2) Are the number of nutrients added, ANPP and light good predictors of overall species richness? The following predictions were made: 1) The addition of nutrients (N or P) will increase ANPP and the grass canopy height of fast-growing species over slow-growing species. 2) This change will lead to a reduction in the light that reaches the understory and result in a loss of species. 3) Species richness will be more strongly and negatively affected by ASU than both LAN and P owing to the increased soil acidification. 4) Phosphorus addition has previously shown to not significantly change ANPP or species composition on the current study site and other experimental sites, therefore it is assumed that the effect on species richness will be minimal.

## Methods

### Study area

The Ukulinga Grassland Nutrient Experiment is located at the Ukulinga Research Farm (29° 24'E, 30° 24'S) of the University of KwaZulu-Natal, South Africa. The growing season of the grass and forb species is regulated by rainfall and temperature, with the optimal growth period for the vegetation between October and April (Fynn and O'Connor, 2005). The UGNE is located on top of a plateau with soils having a low pH, and are classified as Westleigh form (orthic A over soft plinthic B horizons) (Soil Classification Working Group, 1991), with a soil depth ranging from 500 to > 1000 mm (Fynn and O'Connor, 2005; Tsvuura and Kirkman, 2013). The vegetation is classified as KwaZulu-Natal Hinterland Thornveld of Sub-Escarpment Savanna (Mucina and Rutherford, 2006). The site is dominated by C4 grasses and has a high diversity of forbs. The dominant grass species include *Setaria sphacelata* var. *sericea*, *Eragrostis curvula* (Schrad.) Nees, *Themeda triandra* Forssk., *Megathyrsus maximus* Jacq., *Tristachya leucothrix* Nees and *Hyparrhenia hirta* (L.) Stapf (Fynn and O'Connor, 2005; Tsvuura and Kirkman, 2013). Fire has been excluded on the site for >70 years.

### Experimental design

The UGNE was established in 1951 to assess how a combination of nutrients (N and P) can transform biomass production and species composition. The nutrients applied were as follows: limestone ammonium nitrate (LAN, 28 % N), ammonium sulphate (ASU, 21 % N), superphosphate (10.5 % P), lime and control treatments. The plot treatments were placed randomly, totalling 96 plots (9 m by 2.7 m) in three blocks from year 1951 to year 2018. Both nitrogen and phosphorus nutrients were applied annually, and lime was applied once every five years. Nitrogen application each year was the same for LAN and ASU at 0, 7.1, 14.1 and 21.2 g $m^{-2}$. Phosphorus was applied at two levels of 0 and 33.6 g $m^{-2}$. Lime was also applied at two levels (0 and 225 g $m^{-2}$). The N amount was split and applied twice a year, at the start of rainy season in October and during the rainy season (between December and January). The N forms were not applied together, only in combination with P and L. In 2019, a cessation of nutrient enrichment was imposed on the UGNE, resulting in each plot being split into subplots (4.0 x 2.7 m - with a 1 m corridor between subplots) with one subplot continuing to receive nutrients, while the other subplot does not. The number of plots has thus doubled to 192. The amount of nutrients applied has been modified to accommodate the decreased plot sizes.

## Sampling

We collected all the data for this study in 2021. Above-ground net primary productivity (ANPP) was estimated in each plot by mowing a 2.13 m wide strip per plot at the end of the growing season at 5 cm above ground level. The fresh material in each plot was collected and weighed on site and a grab sample was collected, weighed, and dried at 60 °C for 2 days before weighing again to determine dry matter percentage. We measured photosynthetically active radiation to determine the incoming solar radiation in all plots during the growing season. These measurements were conducted on sunny days between 10h00 and 14h00 hours (Tsvuura and Kirkman, 2013) using an AccuPAR model LP-80 ceptometer (Decagon Devices, Inc., Pullman, Washington). In each plot, 2 ground level (beneath the grass layer) and 2 above canopy level measurements were taken. These measurements were used to calculate the percentage of light intercepted by the grass canopy. Species composition in each plot was sampled using two systematically placed quadrats (1m x 1m). Plant cover abundance was physically measured per species present and measured as the cover percentage it utilized per quadrat and it can be described as the cover proportion of each species occurring in a quadrat measured as a percentage.  Species composition was determined using cover abundance, for each subplot and species richness was determined as a count from that data. Species richness is a simple indicator of species diversity (Keylock, 2005). For this study, the data from the following treatment plots were selected; control (0 nutrients), LAN (21.2 g $m^{-2}$) only enriched, LAN (21.2 g $m^{-2}$) * P enriched, ASU (21.2 g $m^{-2}$) only enriched and ASU (21.2 g $m^{-2}$) * P enriched. The highest N application level was selected to assess the strongest effects.

## Calculations

### *Light:*

$$\text{Light intercepted by the grass canopy }\% = \left(\frac{\text{ground level light reading}}{\text{above grass canopy light reading}}\right) * 100$$

## Statistical analyses

### Canonical correspondence analysis (CCA)

This study aimed to firstly determine the influence of nutrients on grass and forb species composition. To address this, a canonical correspondence analysis (CCA) was conducted using the relative contribution of the environmental variables to plant cover abundances and the association between plant species. A CCA is a form of eigenvalue ordination that allows for an easy assessment of relationships between a species distribution matrix and its associated environmental explanatory matrix. This direct gradient analysis allows for a species composition matrix to be directly related to a single or a combination of environmental variables (Palmer, 1993). Permutation tests were conducted to test the model and the environmental terms used in the full model were significant ($p < 0.05$). A separate CCA was conducted for grass and forb species composition. Furthermore, the CCAs were separated by nutrient identity whereby LAN, ASU and P were analysed separately to elucidate the differences better, totalling six independent CCAs.

### Generalized linear models (GLM)

The second research question aimed to determine if the number of nutrients, ANPP and light were good predictors of overall species richness. To address this, separate generalized linear models (GLM) were fitted for the nutrient sources (i.e., LAN, ASU, and P). Generalized linear models are a useful tool used in ecology to fit non-normal data using link functions (Bolker et al., 2008). The response variable for all GLMs was overall species richness and predictor variables were either, number of nutrients, ANPP, light or an interaction of ANPP and number of nutrients or an interaction of all three variables. An AIC model selection procedure was used to distinguish and compare possible models using the abovementioned predictor variables (Akaike, 1974). The most parsimonious model was selected and used as the final model for interpretation for each nutrient identity (Table 4.1). For the P nutrient identity GLM, two results are presented due to similarities in AIC scores. Generalized linear models with light were only included as a full model. Reduced models with light interacting with the other variables or alone as predictive variables are not shown due to poor model performance scores. This could be linked to the similarities in mean light measurements (Appendix B, Figure 4. B3).

Poisson distributions in the GLM were used for LAN and P nutrient identity models. To account for the over dispersed species richness in the ASU dataset, we selected a negative binomial distribution for its superiority over the Poisson (White and Bennetts, 1996). In this case, a GLM with a negative binomial distribution. All analyses were analysed using R software on R Studio version 4.2.2 (R Core Team, 2022).

**Table 4.1.** The AIC values for the different GLM models. The selected model is listed first and in bold. AICc = Akaike information criterion test that can account for small sample sizes, AICc.Wt = proportion of total predictive power and Cum.Wt = sum of the AICc weights

| Plot enrichment | Model | AICc | AICc.Wt | Cum.Wt |
|---|---|---|---|---|
| **LAN and LAN + P** | **ANPP** | **50.62** | **0.81** | **0.81** |
| | Nutrient number | 53.25 | 0.18 | 0.99 |
| | Nutrient number * ANPP | 60.03 | 0.01 | 1.00 |
| | Nutrient number * ANPP * light | 61.27 | 0.00 | 1.00 |
| **ASU and ASU + P** | **Nutrient number** | **58.87** | **0.85** | **0.85** |
| | ANPP | 62.30 | 0.15 | 1.00 |
| | Nutrient number * ANPP | 70.59 | 0.00 | 1.00 |
| | Nutrient number * ANPP * light | 74.79 | 0.00 | 1.00 |
| **P** | **Nutrient number** | **36.12** | **0.55** | **0.55** |
| | **ANPP** | **36.55** | **0.44** | **0.99** |
| | Nutrient number * ANPP * light | 45.44 | 0.01 | 1.00 |
| | Nutrient number * ANPP | 46.17 | 0.00 | 1.00 |

## Results

### Relationship between environmental variables and species composition

Canonical correspondence analyses were conducted to statistically test if the environmental variables were significantly affecting the grass and forb species cover abundance. The results of the CCA models showed that the grass species cover abundance for LAN and LAN + P was significant ($\chi^2 = 1.277$, F = 1.640, df = 5, p = 0.048) (Figure 4.1A) and were not significant for ASU and ASU + P ($\chi^2 = 1.134$, F = 1.559, df = 3, p = 0.125) (Figure 4.1B) and P enrichment treatment ($\chi^2 = 0.707$, F = 1.469, df = 3, p = 0.157) (Figure 4.2). Considering the latter mentioned CCA models were not significant, results related to the CCA will be limited to the LAN and LAN + P enrichment treatment. According to this CCA, the most important environmental term was the number of nutrients added ($\chi^2 = 0.849$, F = 3.274, df = 1, p = 0.001) (Figure 4.1A). Plots that had not been enriched with any nutrients were significantly associated with a high cover abundance of *Tristachya leucothrix* (*T. leucothrix*), whereas plots enriched with only one nutrient (LAN) were associated with a significantly high cover abundance of *Aristida junciformis* (*A. junciformis*). Lastly, when two nutrients were added in the form of LAN + P, a significantly high abundance of *Megathyrsus maximus (M. maximus)* was observed (Appendix A, Table 4. A3).

Similarly to the grass CCA analyses, the biplots for CCA conducted for forb species cover abundance produced a significant model for LAN and LAN + P enrichment treatment ($\chi^2$ = 1.414, F = 1.438, df = 5, p = 0.015) (Figure 4.3A) and not for ASU and ASU + P ($\chi^2$ = 1.471, F = 1.271, df = 3, p = 0.217) (Figure 4.3B) and P enrichment treatment ($\chi^2$ = 1.170, F = 1.102, df = 3, p = 0.342) (Figure 4.4). Considering the latter mentioned CCA models were not significant, results related to the CCA will be limited to the LAN and LAN + P enrichment treatment. Accordingly, the most important environmental terms were the number of nutrients added ($\chi^2$ = 0.486, F = 1.485, df = 1, p = 0.035) and ANPP ($\chi^2$ = 0.544, F = 1.659, df = 1, p = 0.038). Plots that had not received any nutrients were significantly associated a high cover abundance of *Cephalaria pungens*. In contrast, no forb species were significantly associated with the enrichment of one nutrient, in the form of LAN (Appendix A, Table 4. A4). The results however show that a significant high cover abundance of *Bidens pilosa* when two nutrients (LAN + P) are applied, and this is also closely related to increased ANPP (Figure 4.3B).

### Relationship between environmental variables and species richness

For LAN, the reduced model for overall species richness showed ANPP as a significantly and negatively related variable (z = −0.002, P = 0.045). Therefore, ANPP reduced species richness in LAN and LAN + P enriched plots, regardless of the nutrient number or light entering the canopy (Figure 5A). According to the negative binomial reduced model conducted on the ASU dataset, the number of nutrients added had significant and decreasing effect on species richness. When applying a single nutrient (ASU) (z = - 3.647, p < 0.001) or 2 nutrients (ASU * P) (z = -4.155, p < 0.001), richness declines compared to no nutrient application (Figure 4.5B). Lastly, for P, none of the tested models significantly predicted species richness (Table 4.1). However, two models were similar in predictive strength (Table 4.1). These were nutrient number (z = -1. 144, p = 0.235) (Figure 4.6A) and ANPP (z = -0.931, p = 0.352) (Figure 4.6B).

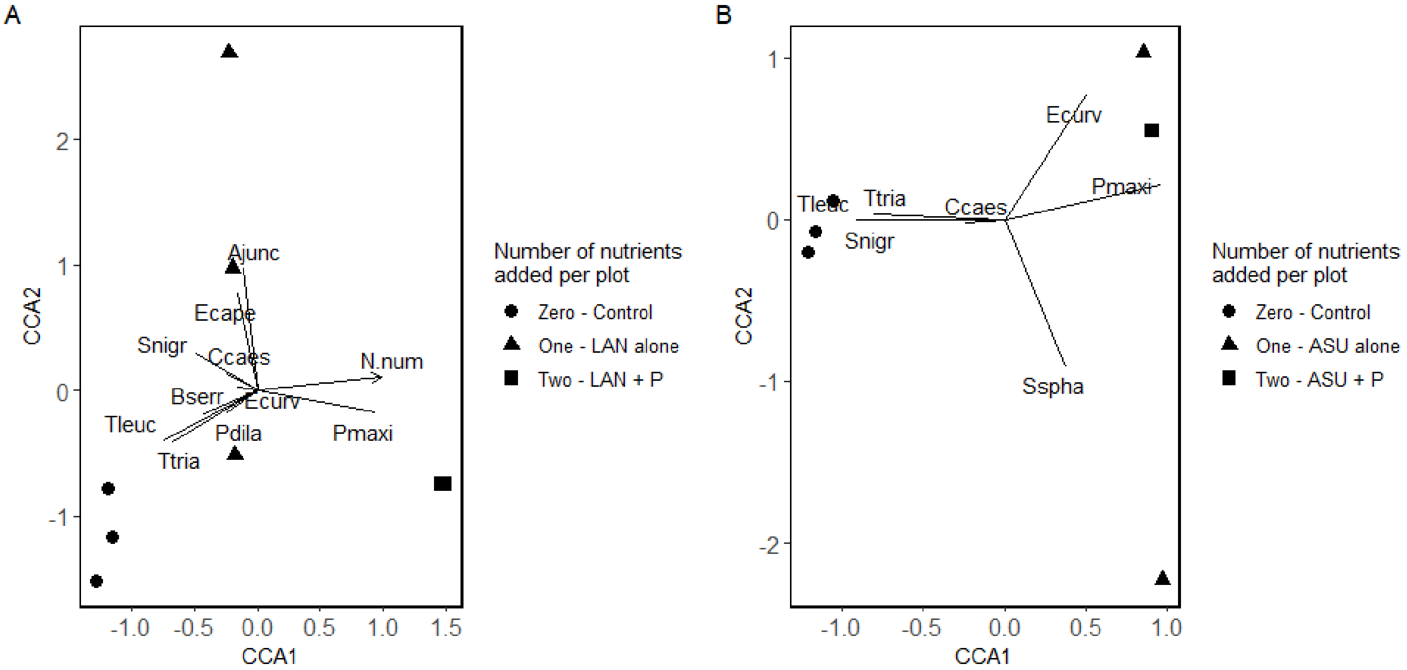

**Figure 4.1.** A Canonical Correspondence Analysis ordination of grass species community composition in 2021 from the Ukulinga Grassland Nutrient Experiment (UGNE), South Africa. For (A) limestone ammonium nitrate, axes one and two account for 67.05 % and 27.89 % of the total variability of the data set and for (B) ammonium sulphate axes one and two account for 80.74 % and 17.29 % of the total variability of the data set. Total and constrained inertia for were (A) 2.575 and 1.277, (B) 1.620 and 1.134 respectively. The plots shown here are separated into the two nitrogen sources. The nutrients numbers are as follows; limestone ammonium nitrate (0(no nutrient added); 1= only LAN added; 3 = LAN + P and ammonium sulphate (0 (no nutrient added); 1 = only ASU added; 2 = ASU + P. For ammonium sulphate, we show only the grass species with the greatest cover abundance due to overlap. Significant predictor variables only are shown by the arrow/s, N.num = number of nutrients added. Full species names are shown in Appendix A, Table 4. A1. Further model information is shown in Appendix A, Table 4. A3.

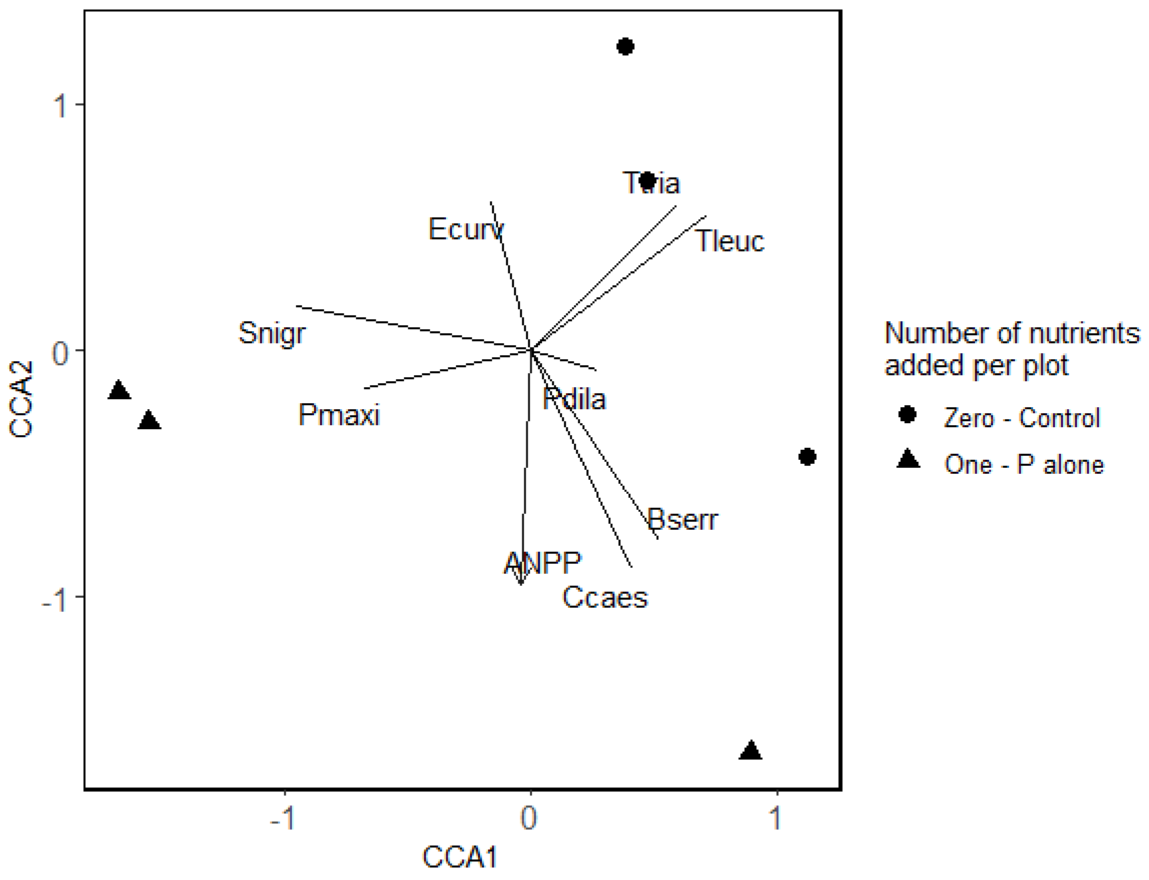

**Figure 4.2.** A Canonical Correspondence Analysis ordination of grass species community composition in 2021 from the Ukulinga Grassland Nutrient Experiment (UGNE), South Africa. For phosphorus, axes one and two account for 44.47 % and 19.65 % of the total variability of the data set of the total variability of the data set. Total inertia = 1.027 and constrained inertia = 0.707. The nutrients numbers are as follows; (0(no nutrient added); 1= only P added. Only the grass species with the greatest cover abundance due to overlap. Significant predictor variables only are shown by the arrow/s, ANPP = above-ground net primary productivity. Full species names are shown in Appendix A, Table 4. A1. Further model information is shown in Appendix A, Table 4. A3.

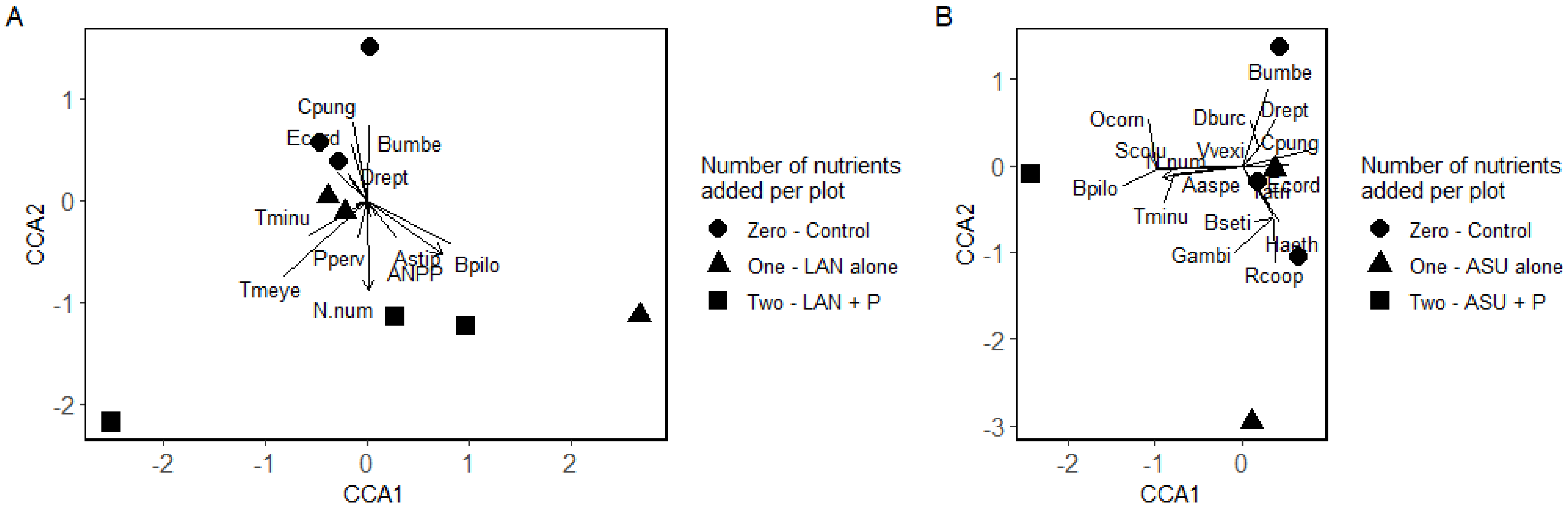

**Figure 4.3.** A Canonical Correspondence Analysis ordination of forb species community composition in 2021 from the Ukulinga Grassland Nutrient Experiment (UGNE), South Africa. For limestone ammonium nitrate, axes one and two account for 18.29 % and 16.23 % of the total variability of the data set for ammonium sulphate axes one and two account for 35.84 % and 20.29 % of the total variability of the data set. Total and constrained inertia for were (A) 3.054 and 1.415, (B) 2.243 and 1.471 respectively. The plots shown here are separated into the two nitrogen sources. The ordihull polygons and number show the number of nutrients applied. The nutrients numbers are as follows; limestone ammonium nitrate (0(no nutrient added); 1= only LAN added; 3 = LAN + P and ammonium sulphate (0 (no nutrient added); 1 = only ASU added; 2 = ASU + P. Significant predictor variables only are shown by the arrow/s, N.num = number of nutrients added and ANPP = above-ground net primary productivity. Full species names are shown in Appendix A, Table 4. A2. Further model information is shown in Appendix A, Table 4. A4.

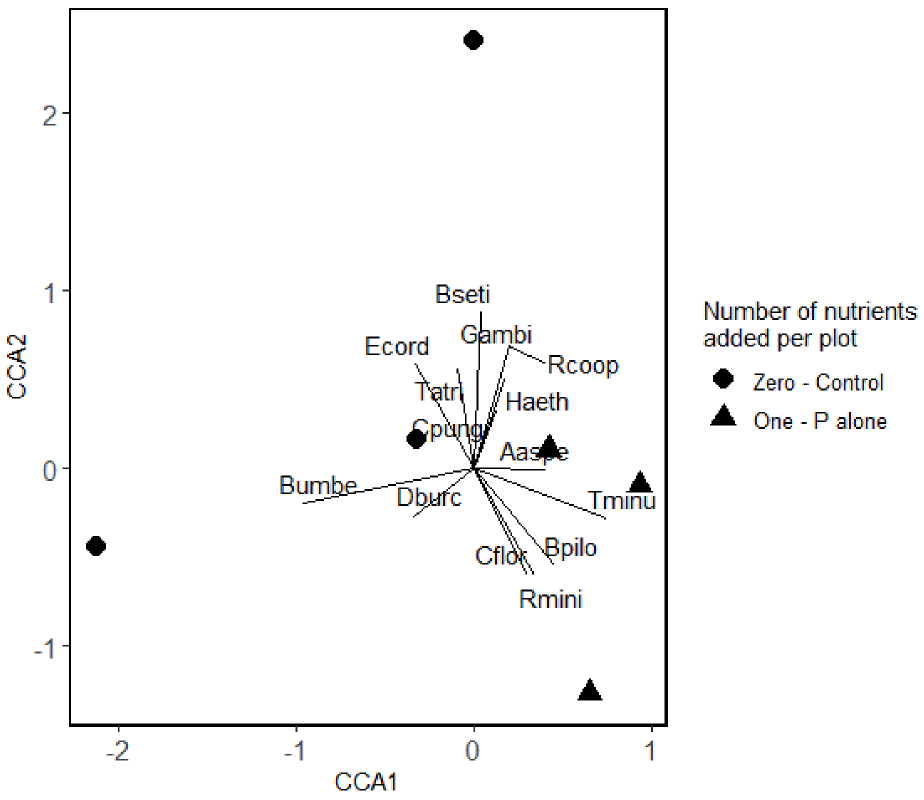

**Figure 4.4.** A Canonical Correspondence Analysis ordination of forb species community composition in 2021 from the Ukulinga Grassland Nutrient Experiment (UGNE), South Africa. Axes one and two account for 32.30 % and 27.79 % of the total variability of the data set. Total inertia = 1.879 and constrained inertia = 0.708. The plots shown here are separated into the two nitrogen sources. The ordihull polygons and number show the number of nutrients applied. The nutrients numbers are as follows; (0(no nutrient added); 1= only P added. Full species names are shown in Appendix A, Table 4. A2. Further model information is shown in Appendix A, Table 4. A4.

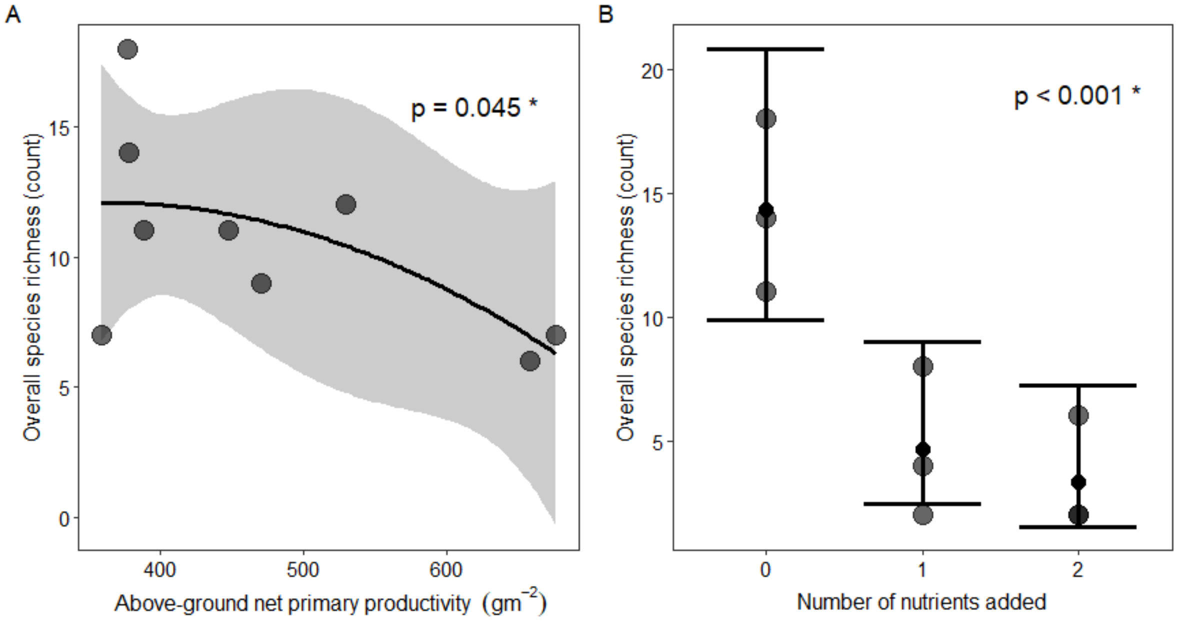

**Figure 4.5.** Plots showing (A) when ANPP (continuous variable) significantly predicts overall species richness in LAN and LAN+P enriched plots and (B) when the number of nutrients (categorical variable) added significantly predicts overall species richness in ASU and ASU+P enriched plots on the UGNE. The grey area in plot A represents the 95 % confidence interval with a poly curve fitted. The bars in plot B represents the 95 % confidence interval/prediction around the plot points.

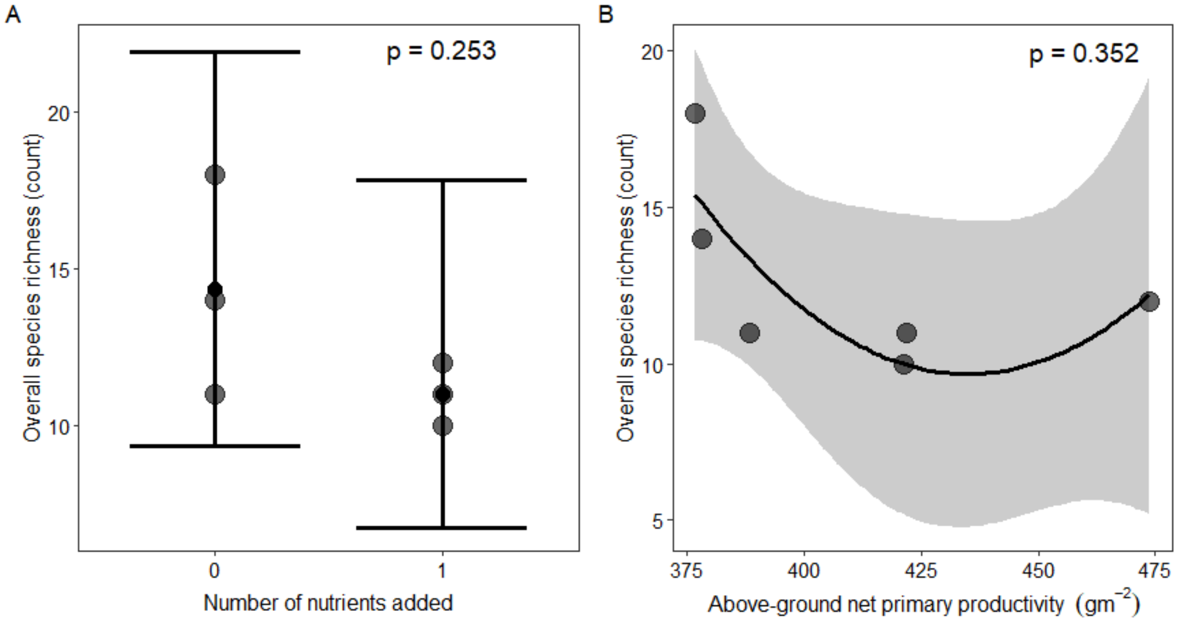

**Figure 4.6.** Plots showing (A) that adding P alone does not influence overall species richness and (B) ANPP is not a good predictor of overall species richness in P enriched plots on the UGNE. The bars in plot A represents the 95 % confidence interval/prediction around the plot points. The grey area in plot B represent the 95 % confidence interval with a poly curve fitted.

## Discussion

This study shows that in general, the UGNE primarily consisted of more non-grass species over grass species. However, the grass species had proportionally more cover abundance than all the other plant forms. In addition, the species cover abundance and composition differed among the three nutrient sources and forms described here. This observation further supported the notion of distinguishing among nutrient sources and forms since they uniquely modified species compositional shifts on the UGNE.

The number of nutrients added was significantly important in determining grass species distribution in the UGNE. Plots enriched with LAN in combination with P were distinctly dominated by *M. maximus*. This grass species is tolerant of changes in the environment linked to drought, soil conditions, intense grazing, and shade (Benabderrahim and Elfalleh, 2021; Malaviya et al., 2020). A study conducted elsewhere, identified *M. maximus* as an invasive species that is tolerant to soil salinity, a range of soil moisture conditions and pH, aiding its dominance as an invasive plant species (Holland et al., 2022). It was also considered to be found abundantly in high N and high soil moisture habitats (Holland et al., 2022). The findings from this study justify the identification of *M. maximus* as a nitrophilic species (Ward et al., 2017), present in mesic grasslands, such as the UGNE. The addition of LAN alone was also significantly associated with *Aristida junciformis* in this study. This finding is interesting and considered problematic because if *A. junciformis* expands with LAN application, it could invade these fertilized plots very quickly. However, another study showed that *A. junciformis* was a slow-growing species that was not competitive when fertility levels were highest (Fynn et al., 2011). Previous reports indicate that *A. junciformis* and *Tristachya leucothrix* are dominant grasses in soils of low nutrient quality (Fynn and O’Connor, 2005; Morris, 2016). This partially explains the significant abundance of *T. leucothrix* in the control plots, in this study. In addition, *Aristida junciformis* and *T. leucothrix* had significant cover abundances in the low nutrient plots, owing to their short height and slow growth forms in comparison to taller species such as *M. maximus,* dominant in highly fertile soils (Fynn et al., 2011).

Unlike for grass species composition, both the number of nutrients added and ANPP significantly influenced forb species composition. In control plots, *Cephalaria pungens* was the most abundant. This finding concurs with a previous study, showing *C. pungens* dominance in control plots associated with low soil pH (Zama et al., 2022). The presence of *C. pungens* in low nutrient soils is an important finding because it suggests that this species is present and dominant in South African nutrient-poor soils and thus can be utilised as a medicinal plant. The known medicinal uses include treatment for infertility and colic (Kose et al., 2015). In this study, none of the forb species were significantly associated with the addition of LAN only. In contrast, *Bidens pilosa* had a significantly higher abundance in LAN + P enriched plots. *Bidens pilosa* is an alien annual weed in South Africa and this may have ramifications for biodiversity. The presence of alien invasive plants reduces plant biodiversity (Gallien et al., 2017; Vilà et al., 2011), and poses a threat (Van Kleunen et al., 2018) to the functioning of the ecosystem. In this study, the occurrence and abundance of *B. pilosa* was associated with an increase in ANPP, further emphasizing its dominance in high fertility soils on the UGNE. In this study, light was not identified as an important environmental predictor but previous studies indicate that light is crucial for the seed germination of *B. pilosa* and that if the seeds are buried too deep in the soil, they do not emerge (Chauhan et al., 2019; Cristaudo et al., 2007). Further experimental support is needed to assess the importance of light for alien invasive weeds such as *B. pilosa* on the UGNE.

Ecosystems that are N limited tend to be high in plant diversity (Bobbink et al., 2010) and can better resist invasion (Holland et al., 2022). The results from this study partially support the abovementioned statement, considering a higher species richness was observed in the control plots compared to the nutrient enriched plots (Appendix B Figure 4. B1). A clustering of grass species with similar cover abundance was observed in the control plots, suggesting that the conditions present in the control (low nutrients) plots were better suited to accommodate a wider number of grass species. This trend was not so obvious for the forb species on the UGNE. This may be partially explained by the greater number of forbs over grass species generally occurring at Ukulinga. Forb species occurring on site belonged to various plant families that differ in their phenology and physiology, possibly accommodating more microhabitats within the plots.

The findings reveal that the different nutrient sources influence changes in species richness in different ways. Although, it is commonly assumed that an increase in nutrient supply is linked with declines in the number of species in terrestrial plant communities (Borer et al., 2014; Hautier et al., 2009; Midolo et al., 2019), this idea may be deemed too general. This is because the findings from this study suggest that the observed declines in species richness are best explained by different predictors and this is different across the three nutrient sources. Increasing ANPP on LAN and LAN + P enriched plots significantly reduced species richness. This trend is not surprising and was reported in the most recent studies conducted on the UGNE (Ward et al., 2017; Zama et al., 2022). Limestone ammonium nitrate enriched plots had a greater ANPP relative to the control plots when compared to ASU enriched plots and this could partially explain ANPP as a strong predictor in the model (see Figure 4. B2). However, the fact that ASU decreased species richness more than LAN but had lower ANPP than LAN enrichedaccplots suggests that ANPP had a weaker effect than acidification. Previously, it was noted that a shift in species dominance was observed when LAN or LAN + P was added, and this could be associated with differences in species growth, N-use efficiency, and N utilisation strategies (Standish et al., 2012; Xia and Wan, 2008). It is believed that the differential growth responses of *M. maximus* and *B. pilosa* aided in increasing the ANPP, causing competitive exclusion of slower growing species. The results from this study support the biomass-driven competition hypothesis (Grime, 1973; Rajaniemi, 2003). It is also suspected that LAN and LAN + P enrichment may have suppressed litter decomposition, causing litter above-ground biomass accumulation, ultimately decreasing light intensities within plots. Light was not a good predictor of species richness in this study. This is thought to be caused by the method employed to measure light on site. For example, it is suggested that experiments should rather focus on manipulating the asymmetry of light to provide more meaningful findings (DeMalach and Kadmon, 2017). Therefore, it is assumed that lower light intensities reaching the ground surface may have suppressed the seed germination and establishment of small plants and increased their mortality rates (Fang et al., 2012). In addition, increased shading may reduce tillering in shorter grass species such as *T. triandra* (Everson et al., 1988), while taller species such as *M. maximus* and *Eragrostis curvula* continue producing tillers (Ghebrehiwot et al., 2006).

Ideally, it was predicted that for all nutrient sources, increasing the number of nutrients added would increase ANPP and decrease light transmitted to the lower grass canopy (Harpole et al., 2016). Additionally, it was expected that the only difference would be related to the intensity or severity of the decline in species for the different nutrient sources. The results here, differed to the abovementioned predictions because the model best explaining species loss in plots enriched with ASU and ASU + P was significantly determined by only the number of nutrients added and not ANPP or light. Interestingly, species loss in this case was only observed between zero and one (ASU) nutrient additions and between zero and two (ASU + P) nutrients additions, suggesting that P addition alone may not be a nutrient that is enhancing species loss on the UGNE. This may be associated with the application amount of 33.6 gm $^{-2}$ year $^{-1}$ applied on the UGNE. A study on an alpine grassland in China revealed that the enrichment of N and P suppressed legume growth more than the N enrichment alone, indicating that P did not alleviate the negative impact of N enrichment. However, the nutrients were applied at a lower amount of 10 gm $^{-2}$ year $^{-1}$ and 5 gm $^{-2}$ per $^{-1}$ for N and P respectively (Ren et al., 2017). Therefore, it may be useful to investigate a range of P applications to determine at what point changes in species richness occur, when N is applied with P within grasslands.

The addition of inorganic fertilizers like ASU over organic fertilizers like manure over a long period can result in soil acidification and negatively affect crop production (Kidd et al., 2017). Increasing soil acidification reduces nitrification rates, base cation availability and the solubility of P (Chen et al., 2015; Haynes and Mokolobate, 2001; Stevens et al., 2011). Ammonium sulphate and other N-containing fertilizers are known to significantly reduce species richness and diversity and this also reduces the variety of invertebrate and vertebrate species (Kidd et al., 2017; Manning et al., 2015).

At the Park Grass Experiment, acidic ASU application had a greater negative effect on plant species richness than sodium nitrate application (Crawley et al., 2005). A similar result was found in this study where ASU had a greater reducing effect on species richness over LAN. It is clear that the UGNE has undergone significant changes since 1951, because an earlier study indicated that ASU and LAN had a similar effect (Le Roux and Mentis, 1986). However, a more recent study showed that nitrophobic species like *T. triandra*, *T. leucothrix* and *Setaria sphacelata* are negatively correlated with N addition and are more abundant in more alkaline soils (Ward et al., 2017). This may explain their decreased abundance in the ASU and ASU + P enriched plots. Perhaps, the addition of lime would increase the soil pH and eventually modify the species composition to accommodate species that are sensitive to low pH levels (Fynn and O’Connor, 2005; Ward et al., 2017).

The reduced species richness with increasing nutrient number addition on the ASU and ASU + P plots, provide some support for the nitrogen detriment hypothesis. Similar species richness declines caused by N are described elsewhere (Band et al., 2022). It is believed here that species loss is caused by ASU and not necessarily the number of nutrients added because species richness did not significantly change from one to two nutrients. Therefore, the result here does not provide evidence for the niche dimension hypothesis. Furthermore, the addition of ASU may also increase the supply of other nutrients such a sulphur (Powlson and Dawson, 2022). This then made it complicated to characterise the discrete number of limiting resources described by the niche dimension hypothesis (Grime, 1973; Harpole et al., 2016; Kaspari and Powers, 2016), alongside other concerns outlined in other studies (Band et al., 2022; DeMalach et al., 2017b). Thus, the observed decline caused by ASU is believed to have been driven by the toxicity of ammonia (Bobbink et al., 2010; Chen et al., 2019; Tian and Niu, 2015). Ammonia toxicity leads to soil acidification and results in reduced nutrient availability for plants (Bobbink et al., 2010), possibly reducing overall plant growth. This may explain the slightly lower increase in ANPP in ASU and ASU + P plots relative to the control plots.

Irrespective of the abovementioned predictors of species loss in soils enriched with various nitrogen sources and combinations, other mechanisms may provide greater insights. For example, extremely high levels of soil N availability and increases in N uptake can cause delays in plant maturation and can alter vulnerability to drought (Bobbink et al., 2010; Britto and Kronzucker, 2002; Güsewell and Güsewell, 2004). The results presented here support the conclusion that the drivers and predictors of species declines are closely associated with nutrient identity. Further experiments are required to understand the exact mechanisms underlying species richness declines following long-term nutrient enrichment, particularly for N on the UGNE.

## Conclusions

In cases where grassland management aims to increase plant species richness or diversity, the addition of high levels of N (either LAN or ASU), alone or in combination with P, is not recommended. Management of forage species could also incorporate regularly testing N and P soil availability since certain species such as *M. maximus* are known to dominate in nutrient-rich and moist soils.  The potential of increased invasions from weeds such as *B. pilosa* are also associated with high nutrient availability and further studies are necessary to assess environmental factors that enhance distribution. This has implications for more nitrophobic and desirable species with a high palatability score such as *T. triandra* and *T. leucothrix* that may be less adapted to deal with high N levels. Studies investigating nutrient availability need to potentially consider other factors that limit plant growth such as soil salinity, moisture, and pH (Bui, 2013; Chen et al., 2010; Holland et al., 2022) to provide further insights. An increase in nutrient availability is also associated with soil microbes including ammonia-oxidizing archaea and various bacteria (Erguder et al., 2009). This warrants further study on the UGNE. Lastly, the addition of acidifying ASU over long periods is highly discouraged considering that grassland recovery is a fairly slow process (Storkey et al., 2015).

## Appendix A

**Table 4. A1.** Grass species found at the UGNE in 2021 in the control, ammonium sulphate, limestone ammonium nitrate and phosphorus enriched plots

| **Species name** | **Abbrev** |
|---|---|
| *Aristida junciformis* Trin. & Rupr. subsp. J*unciformis* | *A. junc* |
| *Brachiaria serrata* (Thunb.) Stapf | *B. serr* |
| *Cymbopogon caesius* (Hook. & Arn.) Stapf | *C. caes* |
| *Eragrostis curvula* (Schrad.) Nees | *E. curv* |
| *Eragrostis capensis* (Thunb.) Trin. | *E. cape* |
| *Megathyrsus maximus* Jacq. | *P. maxi* |
| *Paspalum dilatatum* Poir. | *P. dila* |
| *Setaria nigrirostris* (Nees) T. Durand & Schinz | *S. nigr* |
| *Setaria sphacelata* (Schumach.) Stapf & C.E Hubb ex M.B Moss | *S. spha* |
| *Themeda triandra* Forssk. | *T. tria* |
| *Tristachya leucothrix* (Trin. ex Nees) | *T. leuc* |

**Table 4. A2.** Forb species found at the UGNE in 2021 in the control, ammonium sulphate, limestone ammonium nitrate and phosphorus enriched plots

| **Species name** | **Abbrev** |
|---|---|
| *Achyranthes aspera* L. | *A. aspe* |
| *Bidens pilosa L.* | *B. pilo* |
| *Berkheya umbellata* DC. | *B. umbe* |
| *Berkheya setifera* DC. | *B. seti* |
| *Conyza floribunda* Kunth | *C. flor* |
| *Cephalaria pungens* Szabó | *C. pung* |
| *Dyschoriste burchellii* (Nees) Kuntze | *D. burc* |
| *Diclis reptans* Benth. | *D. rept* |
| *Eriosema cordatum* E. Mey | *E. cord* |
| *Gerbera ambigua* (Cass.) Sch. Bip | *G. ambi* |
| *Hibiscus aethiopicus* L.var. *aethiopicus* | *H. aeth* |
| *Lichtensteinia kolbeana* Bolus | *L. kolb* |
| *Oxalis corniculata* L. | *O. corn* |
| *Physalis peruviana* L. | *P. perv* |
| *Rhodohypoxis baurii* (baker) Nel | *R. baur* |
| *Rhynchosia cooperi* (Harv. ex Baker f.) Burtt Davy | *R. coop* |
| *Rhynchosia totta* (Thunb.) DC. var. *totta* | *R. tott* |
| *Scabiosa columbaria* L. | *S. colu* |
| *Senecio coronatus* (Thunb.) Harv. | *S. coro* |
| *Thunbergia atriplicifolia* E. Mey. ex. Nees | *T. atri* |
| *Tagetes minuta* L. | *T. minu* |
| *Tragia meyeriana* Mull. Arg | *T. meye* |
| *Vigna vexillata* (L.) A. Rich | *V. vexi* |

**Table 4. A3**. Grass species list with p-value (indicating if a species was significant ($p < 0.05$*) or not ($p > 0.05$) per canonical correspondence analysis

| Species name | Abbrev | p-value |
|---|---|---|
| LAN and LAN + P enriched (model significant) | | |
| *Aristida junciformis* Trin. & Rupr. subsp. *junciformis* | *A. junc* | 0.017* |
| *Brachiaria serrata* (Thunb.) Stapf | *B. serr* | 0.559 |
| *Cymbopogon caesius* (Hook. & Arn.) Stapf | *C. caes* | 0.941 |
| *Eragrostis curvula* (Schrad.) Nees | *E. curv* | 0.981 |
| *Eragrostis capensis* (Thunb.) Trin. | *E. cape* | 0.057 |
| *Megathyrsus maximus* Jacq. | *P. maxi* | 0.001* |
| *Paspalum dilatatum* Poir. | *P.dila* | 0.891 |
| *Setaria nigrirostris* (Nees) T. Durand & Schinz | *S. nigr* | 0.357 |
| *Themeda triandra* Forssk. | *T. tria* | 0.057 |
| *Tristachya leucothrix* (Trin. ex Nees) | *T. leuc* | 0.042* |
| ASU and ASU + P enriched (model not significant) | | |
| *Cymbopogon caesius* (Hook. & Arn.) Stapf | *C. caes* | 1.000 |
| *Eragrostis curvula* (Schrad.) Nees | *E. curv* | 0.091 |
| *Megathyrsus maximus* Jacq. | *P. maxi* | 0.017* |
| *Setaria nigrirostris* (Nees) T. Durand & Schinz | *S. nigr* | 0.133 |
| *Setaria sphacelata* (Schumach.) Stapf & C.E Hubb ex M.B Moss | *S. spha* | 0.167 |
| *Themeda triandra* Forssk. | *T. tria* | 0.267 |
| *Tristachya leucothrix* (Trin. ex Nees) | *T. leuc* | 0.083 |
| P enriched (model not significant) | | |
| *Brachiaria serrata* (Thunb.) Stapf | *B. serr* | 0.067 |
| *Cymbopogon caesius* (Hook. & Arn.) Stapf | *C. caes* | 0.017* |
| *Eragrostis curvula* (Schrad.) Nees | *E. curv* | 0.553 |
| *Paspalum dilatatum* Poir. | *P.dila* | 1.000 |
| *Megathyrsus maximus* Jacq. | *P. maxi* | 0.500 |
| *Setaria nigrirostris* (Nees) T. Durand & Schinz | *S. nigr* | 0.021* |
| *Themeda triandra* Forssk. | *T. tria* | 0.194 |
| *Tristachya leucothrix* (Trin. ex Nees) | *T. leuc* | 0.153 |

**Table 4. A4.** Forb species list with p-value (indicating if a species was significant ($p < 0.05$*) or not ($p > 0.05$) per canonical correspondence analysis

| Species name | Abbrev | p-value |
|---|---|---|
| LAN and LAN + P enriched (model significant) | | |
| *Bidens pilosa L.* | *B. pilo* | 0.013* |
| *Conyza floribunda* Kunth | *C. flor* | 0.989 |
| *Cephalaria pungens* Szabó | *C. pung* | 0.037* |
| *Dyschoriste burchellii* (Nees) Kuntze | *D. burc* | 0.558 |
| *Diclis reptans* Benth | *D.rept* | 0.450 |
| *Eriosema cordatum* E. Mey | *E. cord* | 0.275 |

| | | |
|---|---|---|
| *Hibiscus aethiopicus* L.var. *aethiopicus* | *H. aeth* | 0.927 |
| *Lichtensteinia kolbeana* Bolus | *L. kolb* | 0.900 |
| *Physalis peruviana* L. | *P. perv* | 0.464 |
| *Rhynchosia cooperi* (Harv. ex Baker f.) Burtt Davy | *R. coop* | 0.538 |
| *Rhynchosia totta* (Thunb.) DC. var. *totta* | *R. tott* | 0.832 |
| *Thunbergia atriplicifolia* E. Mey. ex. Nees | *T. atri* | 0.610 |
| *Tagetes minuta* L. | *T. minu* | 0.153 |
| *Tragia meyeriana* Mull. Arg | *T. meye* | 0.116 |
| ASU and ASU + P enriched (model not significant) | | |
| *Achyranthes aspera* L. | *A. aspe* | 0.967 |
| *Bidens pilosa L.* | *B. pilo* | 0.167 |
| *Berkheya setifera* DC. | *B. seti* | 0.500 |
| *Berkheya umbellata* DC. | *B. umbe* | 0.167 |
| *Cephalaria pungens* Szabó | *C. pung* | 0.122 |
| *Dyschoriste burchellii* (Nees) Kuntze | *D. burc* | 0.833 |
| *Diclis reptans* Benth | *D.rept* | 0.833 |
| *Eriosema cordatum* E. Mey | *E. cord* | 0.767 |
| *Gerbera ambigua (*Cass.) Sch. Bip | *G. ambi* | 0.500 |
| *Hibiscus aethiopicus* L.var. *aethiopicus* | *H. aeth* | 0.400 |
| *Oxalis corniculata* L. | *O. corn* | 0.167 |
| *Rhynchosia cooperi* (Harv. ex Baker f.) Burtt Davy | *R. coop* | 0.500 |
| *Scabiosa columbaria* L. | *S. colu* | 0.167 |
| *Thunbergia atriplicifolia* E. Mey. ex. Nees | *T. atri* | 0.633 |
| *Tagetes minuta* L. | *T. minu* | 0.333 |
| *Vigna vexillata* (L.) A. Rich | *V. vexi* | 1.000 |
| P enriched (model not significant) | | |
| *Achyranthes aspera* L. | *A. aspe* | 0.633 |
| *Bidens pilosa L.* | *B. pilo* | 0.400 |
| *Berkheya setifera* DC. | *B. seti* | 0.333 |
| *Berkheya umbellata* DC. | *B. umbe* | 0.033* |
| *Conyza floribunda* Kunth | *C. flor* | 0.433 |
| *Cephalaria pungens* Szabó | *C. pung* | 0.861 |
| *Dyschoriste burchellii* (Nees) Kuntze | *D. burc* | 0.667 |
| *Eriosema cordatum* E. Mey | *E. cord* | 0.417 |
| *Gerbera ambigua (*Cass.) Sch. Bip | *G. ambi* | 0.333 |
| *Hibiscus aethiopicus* L.var. *aethiopicus* | *H. aeth* | 0.583 |
| *Rhynchosia cooperi* (Harv. ex Baker f.) Burtt Davy | *R. coop* | 0.367 |
| *Rhynchosia minima* (L.) D.C. var *minima* | *R. mini* | 0.500 |

| | | |
|---|---|---|
| *Thunbergia atriplicifolia* E. Mey. ex. Nees | *T. atri* | 0.567 |
| *Tagetes minuta* L. | *T. minu* | 0.223 |

## Appendix B

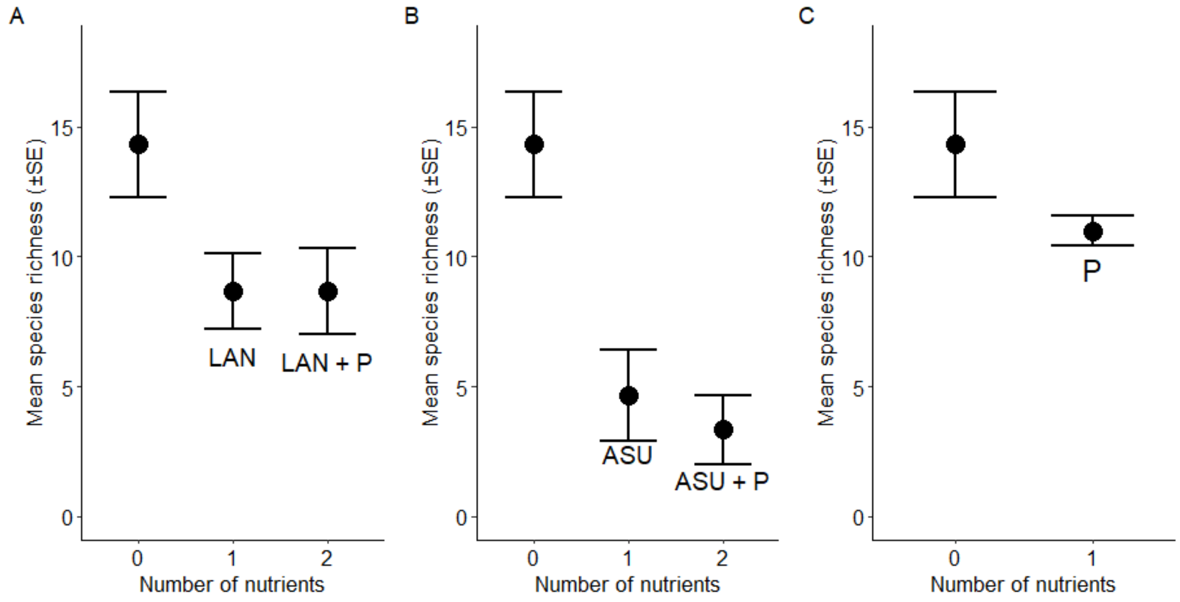

**Figure 4. B1.** Mean species richness ± se for A) when the single nutrient added is LAN and when LAN + P are added, B) when the single nutrient added is ASU and when ASU+P are added and C) when P is added alone on the UGNE.

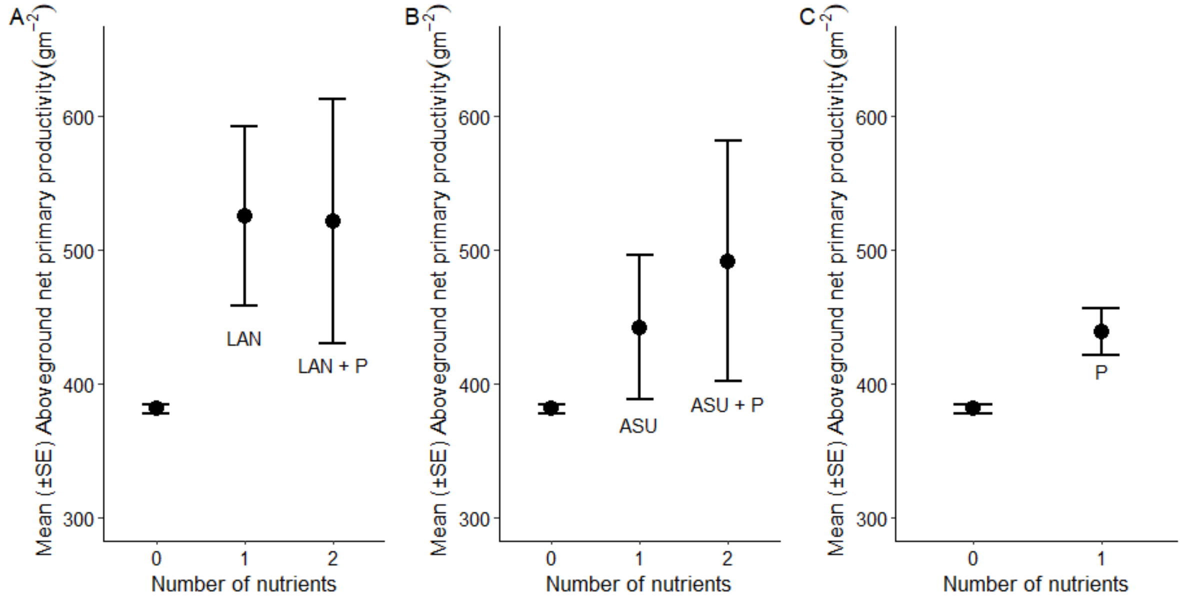

**Figure 4. B2.** Mean above-ground net primary productivity ± se for A) shows when the single nutrient added is LAN and when LAN+P are added, B) when the single nutrient added in ASU and when ASU + P are added and C) when P is added alone on the UGNE.

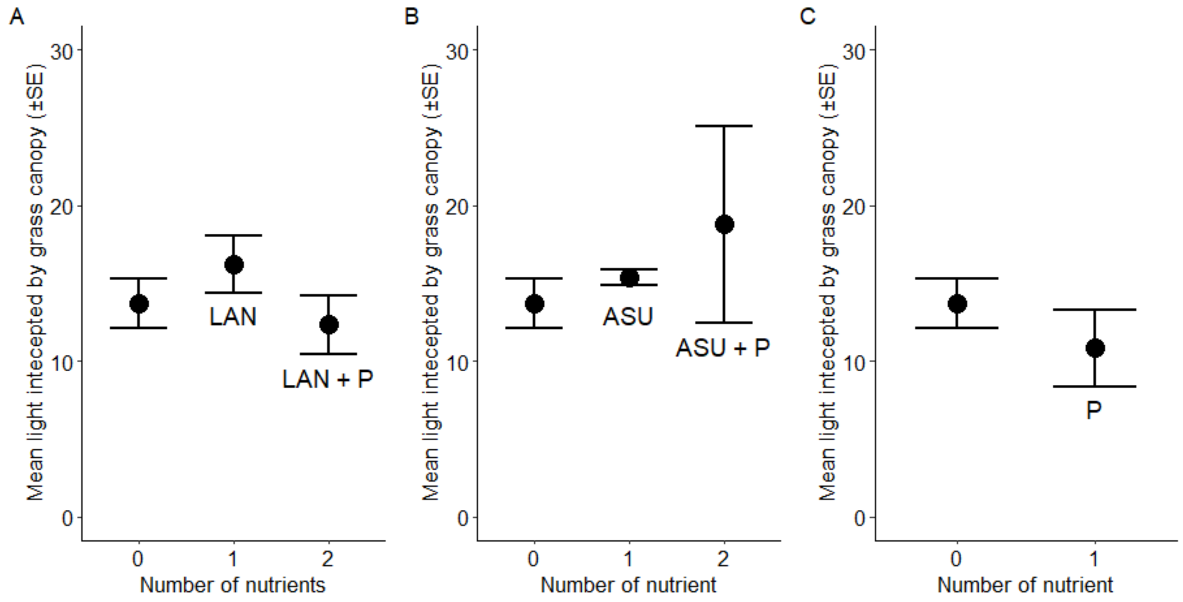

**Figure 4. B3.** Mean light intercepted by the grass canopy ± se for A) when the single nutrient added is LAN and when LAN + P are added, B) when the single nutrient added in ASU and when ASU + P are added and C) when P is added alone on the UGNE.

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# Chapter 5

# Soil Acidification in Nutrient-Enriched Soils Reduces the Growth, Nutrient Concentrations, and Nitrogen-Use Efficiencies of *Vachellia sieberiana* (DC.) Kyal. & Boatwr Saplings

(Published journal article*)

Zama, N*., Kirkman, K., Mkhize, N., Tedder, M., Magadlela, A., 2022. Soil acidification in nutrient enriched soils reduces the growth, nutrient concentrations, and nitrogen-use efficiencies of *Vachellia sieberiana* (DC.) Kyal. & Boatwr saplings. Plants. doi: 10.3390/plants11243564.

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## Abstract

Nitrogen (N) and phosphorus (P) nutrient enrichment is important for grasslands. This study aimed to determine how soils enriched with N and P influenced soil concentration correlations and affected the growth kinetics, mineral nutrition, and nitrogen-use efficiencies of *Vachellia sieberiana* grown in a greenhouse experiment. The soils used as the growth substrate were analysed and showed extreme acidity (low soil pH, 3.9). Nitrogen-enriched soils were more acidic than P-enriched soils. Exchangeable acidity was strongly negatively correlated with an increase in soil pH, with soil pH between 3.9 and 4.1 units showing the strongest decline. Plant saplings showed increased root biomass, shoot biomass, total biomass, and plant N and P concentrations when grown in soils with high soil P concentrations. Extreme soil acidification in N-enriched soil was one of the main factors causing P unavailability, decreasing sapling growth. Extreme soil acidification increased concentrations of toxic heavy metals, such as Al which may be alleviated by adding lime to the extremely acidic soils. Research implications suggest that soil pH is an important chemical property of the soil and plays a significant role in legume plant growth. Legume species that are unable to tolerate acidic soils may acquire different strategies for growth and functioning.

**Keywords:** mesic grassland; nutrient addition; P deficiency; biological nitrogen fixation; liming

## Introduction

In Africa, sustainable plant growth and agricultural practices are threatened by the poor nutrient status of soils (Goldman, 1995; Henao and Baanante, 1999). Increasing populations worldwide coupled with increased demand for agricultural products are cause for concern, and the need to increase soil fertility in agricultural regions is heightened (Zungu et al., 2020). South African soils are characterised as having nutrient-poor and acidic conditions (Mafongoya et al., 2006; Nandwa, 2001). The acidic soil pH and low cation-exchange capacity negatively affect the availability of nutrients such as potassium ($K^+$) and calcium ($Ca^{2+}$) (Aprile and Lorandi, 2012). In addition, acidic conditions result in the sequestration of certain nutrients like phosphorus (P), causing it to be insoluble through binding with cations (Sharma et al., 2013). Furthermore, soils with high concentrations of cations including aluminium (Al), iron (Fe) and manganese (Mn) have also been associated with P sequestration and making P unavailable for plant use (Afonso et al., 2020; Mitra et al., 2020). In sub-Saharan Africa, legumes are important in smallholder farming systems, as they are a source of income and food security (Vanlauwe et al., 2019). Thus, understanding the impact of soil quality on legume growth and persistence in grasslands is important. Nitrogen (N) and P are important for plant growth and occur in both an organic and inorganic form. Phosphate also occurs in the energy molecule adenosine triphosphate (ATP), involved in the metabolic activities in plants, and even more in legume plants during biological nitrogen fixation (BNF) (Mitran et al., 2018; Rotaru and Sinclair, 2009). A symbiotic relationship may occur between certain legumes and N-fixing bacteria, resulting in BNF (Ndabankulu et al., 2022). Large amounts of ATP are required as the energy driver for BNF (Rotaru and Sinclair, 2009). This is used by microbes in legume plant nodules for metabolic pathways during dinitrogen ($N_2$) reduction (Vance et al., 2003). Although N is abundantly present in the atmosphere (ca. 78%), soils remain N deficient (Ferguson et al., 2010; Zahran, 1999). Legumes can use N in the atmosphere and convert it into usable forms such as nitrate and ammonia through N-fixing microbes housed in the nodules (Herridge et al., 2005). This emphasizes the importance of BNF in farming and agricultural practices as it reduces the need to apply expensive chemical fertilizers that can be harmful to the environment (Herridge et al., 2005; Zahran, 1999). However, the efficiency of BNF is influenced by certain factors, including P deficiency (Sulieman and Tran, 2015; Vance et al., 2003), fluctuating pH conditions, temperature and water status (Werner and Newton, 2005). Because P is fixed by cations in acidic soils, thereby making it unavailable to plants (Balemi and Negisho, 2012; Chen and Liao, 2016), acidic soil conditions are likely to affect nodulation and N fixation (Bekere, 2013; Mohammadi et al., 2012). In contrast, large amounts of P support productivity, nodulation, growth, and N fixation in legume plants (Zaidi et al., 2009).

*Vachellia sieberiana* (DC.) Kyal. & Boatwr (formerly known as *Acacia sieberiana*), commonly known as the paperbark thorn, occurs in nutrient-poor environments and can survive due to its ability to fix atmospheric N (Otuba and Weih, 2015). This species encroaches on savannas and mountainous grasslands in the mesic environments of southern Africa (Grellier et al., 2013). Water availability, water uptake and soil properties are strong determinants of successful tree establishment and encroachment in grasslands (Grellier et al., 2021; Schleicher et al., 2011). Furthermore, growth of *V. sieberiana* saplings is limited by nutrient availability within grassland soils (Wakeling et al., 2010). Research suggests that nutrient-rich soils promote *Vachellia* sapling growth rates when compared to growth in soils of low nutrients (Wakeling et al., 2010). Therefore, it is expected that, soil nutrient status has an extreme effect on legume growth and survival (Mohammadi et al., 2012; Reverchon et al., 2012). When N was applied to topsoil (0–20 cm), an exponentially negative relationship was observed between N addition rate and N fixation (Mohammadi et al., 2012; Salvagiotti et al., 2008; Santachiara et al., 2019). It should be noted that nutrient stress can be indirectly caused by changes in soil pH, such as acidity, that suppress nutrient bioavailability, rather than the lack of nutrients themselves (Mohammadi et al., 2012). However, when assessing nutrient conditions affecting N fixation, the factors influencing legume growth and those influencing microbe/symbiotic interactions must be distinguished (Mohammadi et al., 2012). For instance, water stress and acidity may alter legume root growth, indirectly affecting nodule formation and N fixation (Mohammadi et al., 2012; Shah et al., 2021).

Most concerns regarding nutrient addition focus on N and its impact on biodiversity and overall productivity (Fowler et al., 2013; Steffen et al., 2015). The changing levels of P and K cycles and other nutrients potentially influence the abundance and diversity of legume species in line with resource competition theory (Bauer et al., 2012; Peñuelas et al., 2013; Steffen et al., 2015; Suding et al., 2005; Tilman, 1980). Soil nutrient status is important for N-fixing legumes. Owing to their physiological demands, legumes tend to require greater amounts of K, P and other micro-nutrients, including Fe and Ca, than plants that do not fix N. However, altering the natural soil nutrient status by adding nutrients may have synergistic effects (Fay et al., 2015; Harpole et al., 2011). Furthermore, limited information is available regarding the long-term nutrient enrichment effects of N, P, and their interaction on soil nutrient status and the growth response and N-use efficiency of *V. sieberiana*. *Vachellia sieberiana* saplings found growing in such ecosystems enriched by nutrients for over 67 years are predicted to show varying growth patterns and mineral nutrition. The Ukulinga Grassland Nutrient Experiment (UGNE) in South Africa is the longest-running nutrient enrichment experiment in Africa (Morris and Fynn, 2001) and provides a unique opportunity for assessing important ecological and agricultural questions.

The aim of this study was to determine how nutrient-poor soils enriched with N and P influenced soil concentration correlations and affected the growth kinetics, mineral nutrition, and nitrogen-use efficiencies of *Vachellia sieberiana* grown in a greenhouse experiment. We set out to test the following hypotheses:

1. A low soil pH will result in a high exchangeable acidity (EA); thus, there will be negative correlation between the two variables.
2. Soil Al concentration will increase with increasing soil EA and Mn.
3. Soil P concentration will increase with soil Ca concentration, and this phenomenon will be stronger in the P-enriched soils.
4. *Vachellia sieberiana* saplings grown in soils with extremely low pH (below 4) will possess significantly reduced root, stem, leaf, and overall biomass compared to saplings grown in conditions with slightly higher pH (above 4).
5. Sixty-seven years of N and P enrichment will significantly increase both plant N and P concentrations in *V. sieberiana* saplings.
6. *Vachellia sieberiana* saplings grown in N-enriched soils will have significantly reduced nitrogen-use efficiencies compared to those grown in soils not enriched with N.

## Methods

### Study Species and Soil Collection Site

The soil samples used to plant the *V. sieberiana* seedlings were collected from the UGNE located at the Ukulinga research farm, University of KwaZulu-Natal, Pietermaritzburg, South Africa (29°24′ E, 30°24′ S). The UGNE is located at an altitude ranging from approximately 838 to 847 m above sea level (Ward et al., 2017). The climatic conditions of the site are as follows; mean annual temperature of 18 °C and a mean annual precipitation of approximately 838 mm (Ward et al., 2020). The soils are relatively infertile (Soil Classification Working Group, 1991). More specifically, the soil pH differs depending on the source of N in the nutrient application in the plots. A high-level application of ammonium sulphate (ASU) was linked to generally low pH (below 4), whereas high-level limestone ammonium nitrate (LAN) supplemented soils demonstrated slightly higher pH (above 5) (Zama et al., 2022). The vegetation is described as a grassland with a few *V. sieberiana* trees accompanied by an understory layer of common grass and forb species (Fynn and O’Connor, 2005; Morris and Fynn, 2001; Zama et al., 2022).

### Experimental Site and Soil Treatment Selection

The UGNE was initiated in 1951 and all plots are mown annually to prevent carry-over plant growth from the previous season (Fynn and O'Connor, 2005). Grazing has been excluded from the study site since its establishment. Originally the experiment was replicated in three blocks, with 96 plots (each 9.0 × 2.7 m) receiving various combinations of N and P. There were two forms of N applied, namely limestone ammonium nitrate (28% N) and ammonium sulphate (21% N) (henceforth LAN and ASU, respectively). For both LAN and ASU, there were four application rates applied annually (0(control/no nutrients), 7.1, 14.1 and 21.2 gm$^{-2}$). Both N applications were performed twice per year during the months of October and December and were either applied alone or in combination with P ((0 (control/no nutrients) and 33.6 gm$^{-2}$) applied once in October and lime (0 (control/no nutrients) and 225 gm$^{-2}$) applied at five-year intervals (Tsvuura and Kirkman, 2013). Since superimposing a nutrient cessation experiment on the UGNE in 2018/19, each plot was split into two subplots (4.0 × 2.7 m) separated by a 1 m buffer corridor, with one subplot continuing to receive nutrients, while the other subplot did not. This was then used as the growth substrate for the seedlings to grow in a greenhouse pot trial experiment. We selected soils in the buffer zone from (1) the control plots, (2) LAN-enriched level-2 plots (14.1 g m$^{-2}$), (3) P- enriched plots (33.6 gm$^{-2}$), and (4) plots enriched with both LAN level-2 and P plots, for an interaction effect. This resulted in the four treatments used in this study. We selected LAN over ASU as our N source because it is often used alongside urea in agriculture in South Africa (Coetzee et al., 2017), for example, in maize production, and is subject to a range of conversion processes within the soil (Coetzee et al., 2017).

In preparation for soil analyses, soil samples were collected from a depth ranging from the surface (0 cm) to 15 cm below the surface. This topsoil region is considered the most active site for fine roots to absorb nutrients (Du et al., 2020; Xia and Wan, 2013). For the purposes of this study, we selected only certain plots for soil collection and pooled these together for uniformity. There were three plots per treatment and two soil samples were collected per plot per treatment. We selected Ca, Fe, Mg, Mn, P, N, Zn for our study, since these nutrients are considered important for plant growth and development (Fageria and Moreira, 2011). Among these, N, P, Ca, and Mg are required in large amounts by plants and thus considered to be macronutrients (Fageria and Moreira, 2011). Additionally, we included soil pH because it is defined as an important predictor of plant occupancy (Paal et al., 2013). Although soil pH is not a primary resource, it does influence nutrient availability and the presence of toxic $Al^{3+}$

cations in soils (Li et al., 2022). Comprehensive soil analyses were performed on the soil samples at the Institute for Commercial Forestry Research located in the Pietermaritzburg Campus of the University of KwaZulu-Natal.

### Seed Germination and Growth Conditions

The pot trial experiment was conducted under ambient conditions in a greenhouse at the Neil Tainton Arboretum, University of KwaZulu-Natal, Pietermaritzburg (30°40‘ E, 30°24′ S). Prior to planting, the *V. sieberiana* seeds were soaked overnight in warm water to soften the hard seed coat. Thereafter, the seeds were planted in at a depth of approximately 1–2 cm below the soil surface in pots containing the soils collected from the UGNE. The pot trial experiment was arranged in a randomized complete block design with the four-soil nutrient status treatments (Figure 5.1). We blocked to account for microenvironmental differences within the greenhouse. Each treatment had a minimum of five replicates, and plants were checked and watered every two days in the morning.

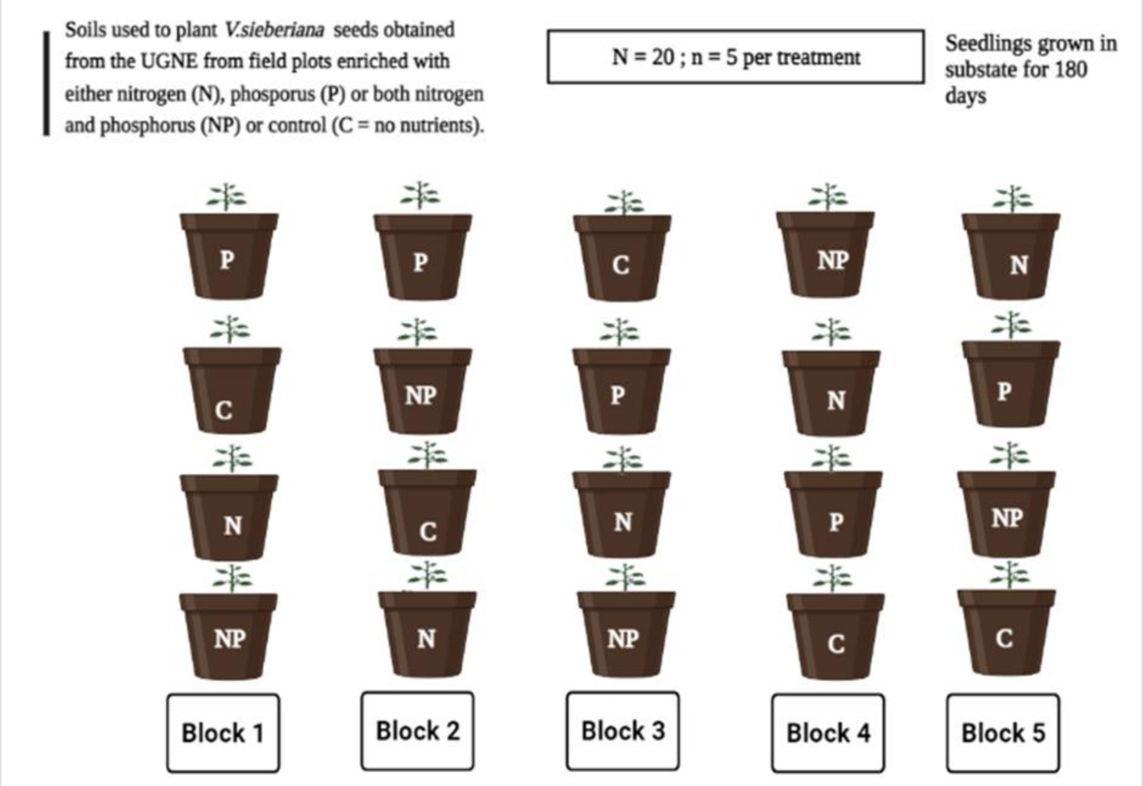

**Figure 5.1.** A simple diagram of the layout of treatments in a randomized complete block design in the greenhouse. Key: N = nitrogen, P = phosphorus, NP = nitrogen and phosphorus, and C = control. Sample size n = 5.

### Plant Preparation and Nutrient Analysis

Five plants per treatment were harvested 30 days following seed germination for initial dry weights and N concentrations. Thereafter, final harvesting took place 180 days after the seedlings emerged. During each harvest, the plant material was rinsed with distilled water and separated into roots, leaves, and stems and thereafter oven-dried at 65 °C for four days. Their dry weights were measured and recorded; thereafter plant material was pulverized in a pestle and mortar under laboratory conditions. The ground material was transferred into 2 mL Eppendorf tubes and sent to the Archaeometry Department at the University of Cape Town for C and N isotope analysis and to the Central Analytical Facilities of Stellenbosch University for P analysis.

### Calculation of the Specific N Absorption Rate

The initial and final values for total plant N were incorporated in the calculation of the specific N absorption rate (SNAR). This is described as the net N absorption rate by the plant through the roots (mg $Ng^{-1}$ root dw $day^{-1}$):

$$\mathrm{SNAR} = ((N\_2 - N\_(1)))/((t\_2 - t\_(1))) \ast ([(\llbracket log \rrbracket\_(e)\, R\_2 - \llbracket log \rrbracket\_(e)\, R\_1))/((R\_2 - R\_(1))]) \qquad (1)$$

where *N* represents the total N content present in the plant, *t* represents the period it took the specific plant to grow, and *R* represents the dry root weight (Nielsen et al., 2001).

The specific N use rate (SNUR) is described as the dry weight gained or acquired by the specific plant during the time of N uptake (g dw $mg^{-1}$ N $Day\text{-}^{1}$):

$$\mathrm{SNUR} = ((W\_2 - W\_(1)))/((t\_2 - t\_(1))) \ast ([(\llbracket log \rrbracket\_(e)\, N - \llbracket log \rrbracket\_(e)\, N\_1))/((N\_2 - N\_(1))]) \qquad (2)$$

where *N* represents the total N content present in the plant, *t* represents the period it took the specific plant to grow, and W represents the plant dry weight.

### Statistical Analyses

All statistical analyses were performed in R software (version 4.2.1) (R Core Team, 2022). To address hypotheses 1, 2 and 3, we conducted separate Principal Components Analyses (PCA) for the soil chemical variables (pH, exchangeable acidity, P, Al, Mn, Zn, Fe, N, Mg, and Ca) and the plant mineral nutrition and growth kinetics traits. We used the “FactoMineR” package in R software (Le et al., 2008); this was supported by the “FactoExtra” package, which is suited for visualization (Kassambara and Mundt, 2017). The benefit of using “FactoMineR” is its ability to transform data to fit a standardized normal distribution. Additionally, we used the “Hmisc” package to assess the correlations between the soil chemical variables by selecting both non-parametric Spearman correlation and parametric Pearson’s correlation as options (Harrell, 2022) (Appendix A , Table 5. A1). To visualize and assess the correlations between the selected soil variables we used Spearman’s rank correlation coefficient as it is a distribution-free statistic (Spearman, 2010) that suited the limited soil data samples.

To address hypothesis 3, we carried out our analyses separately for plant traits (N = 20; *n* = five plant seedlings/samples per treatment), plant mineral nutrition and standard corrected $^{15}N/^{14}N$ (N = 20; five seedlings/sample per treatment). This was done to meet the assumptions of no multicollinearity. We evaluated the original data with treatment: control (no nutrients), nitrogen, phosphorus, and nitrogen*phosphorus as the grouping independent variable. We tested for the following assumptions of MANOVA: (1) sample size n > number of outcome variables, (2) no univariate outliers, (3) no multivariate outliers, (4) univariate normality, (5) multivariate normality, (6) no multicollinearity, (7) linearity, (8) homogeneity of covariances, and (9) homogeneity of variances. All assumptions of MANOVA were met in both cases. In our assessments, we employed Pillai’s trace as the multivariate test statistic. Additionally, we accepted a statistic significance of $p < 0.05$ in our study. This was followed by Tukey’s HSD to determine the significant difference effects of treatment on the response variables (followed by a Tukey HSD pairwise post hoc comparison test for each response variable). We used the ‘rstatix’ package and the following functions: *identify_outliers*, *shapiro_test*, *cor_test*, *levenes_test* to evaluate four assumptions (Kassambara, 2021). To assess linearity, we used the ‘GGally’ package (Schloerke et al., 2021). Lastly, to assess homogeneity of covariances we used the *boxM* function in the ‘heplots’ package (Fox et al., 2021)**.**

Lastly, to further address hypothesis 3, we performed a separate analysis of variance (ANOVA) for SNAR and SNUR response variables (N = 20, *n* = five individual samples per treatment) to detect significant differences among the independent variables of control (no nutrients), nitrogen, phosphorus, and nitrogen*phosphorus. The assumptions for one-way ANOVA of independence of observations, normal distribution of data for each factor and homogeneity of variances were satisfied. Normality of residuals and homogeneity of variances were assessed using the Shapiro–Wilk test and Levene's test, respectively. When significant differences were observed ($p < 0.05$), we proceeded with multiple comparisons using the function: *pairwise.wilcox.test* to calculate pairwise comparisons between treatment levels with corrections for multiple testing ($p < 0.05$).

## Results

### Soil Chemical Properties

The soil chemical concentrations (μmol $g^{-1}$/Cmol $L^{-1}$) that where highly positively correlated were P and Ca (Figures 5.2 and 5.3B), Al and Mn (Figures 5.2 and 5.4A), and Al and EA (Figures 5.2A and B). There was a high negative correlation between pH and exchangeable acidity (Ea) (Figures 5.2 and 2A). High concentrations of Mg (mean ± se, 17.188 ± 0.336) and less acidic conditions (pH = 4.347 ± 0.147) were more associated with control soils (Figure 5.2 and Table 5.1). Phosphorus-enriched soils were also associated with slightly higher pH units (when compared to the other treatments) and higher concentrations of Ca, Zn and P (Figure 5.2 and Table 5.1). Nitrogen-enriched soils were associated with more acidic soils (low pH units) and high concentrations of Mn, N and EA (Figure 5.2 and Table 5.1). Interestingly, soils enriched with both N and P had a more similar soil profile to soils enriched with P only than N only.

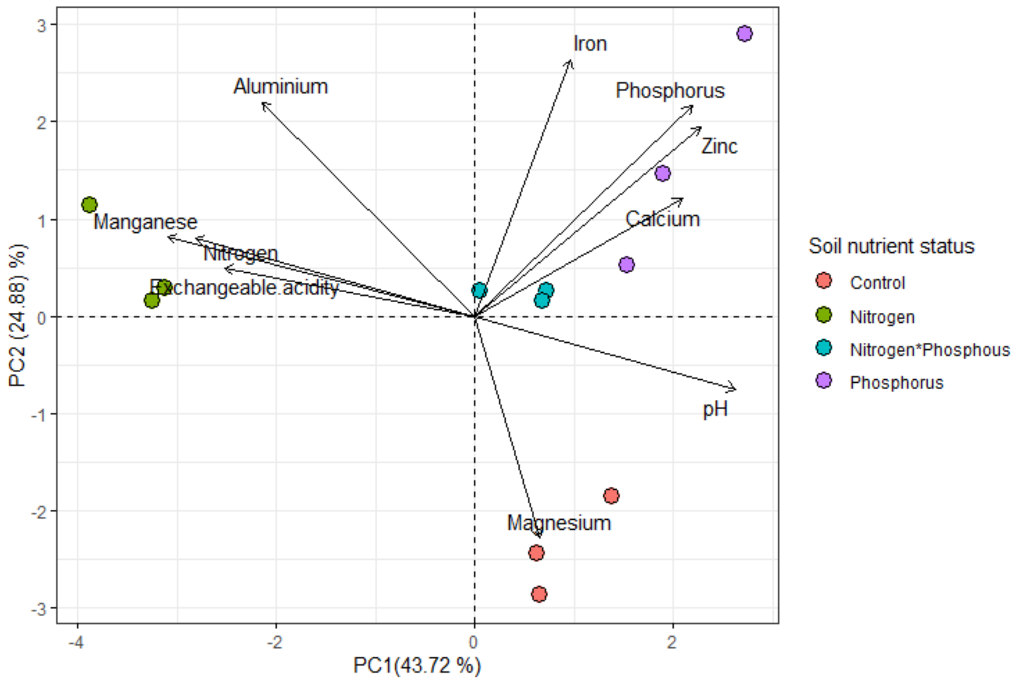

**Figure 5.2.** Principal component analysis (PCA) for all standardized soil chemical concentrations under four different soil nutrient status treatments: control (no nutrients), nitrogen (limestone ammonium nitrate), phosphorus only and both nitrogen (limestone ammonium nitrate) and phosphorus. The plot shows the relationship among the soil chemical variables. Positively correlated variables are grouped together, and negatively correlated variables are positioned on the opposite ends from the plot origin. The quality of the variables is assessed by the distance between the variable and the origin. The longer the length of the variables, the greater its level of contribution. Key: control (pink solid circle), N only (green solid circle), N and P (blue solid circle, P only (purple solid circle).

**Table 5.1.** The chemical parameters of control soil (no nutrients) and soils enriched with nitrogen (limestone ammonium nitrate), phosphorus and both nitrogen (limestone ammonium nitrate) and phosphorus obtained from the Ukulinga Grassland Nutrient Experiment, South Africa

Each value is a mean ± standard error of three replicates.

| **Soil Parameter** | **Control** | **Nitrogen (N)** | **Phosphorus (P)** | **N*P** |
|---|---|---|---|---|
| **pH (KCl)** | 4.347 ± 0.147 | 3.945 ± 0.119 | 4.455 ± 0.089 | 4.261 ± 0.107 |
| **Exchangeable acidity** (C mol L $^{-1}$) | 0.998 ± 0.809 | 1.799 ± 0.659 | 0.218 ± 0.031 | 0.471 ±0.112 |
| **Aluminium** (µmol Al $g^{-1}$) | 17.071 ± 0.954 | 26.409 ± 409 | 21.914 ± 0.207 | 22.854 ± 1.883 |
| **Calcium** (µmol Ca $g^{-1}$) | 27.224 ± 2.327 | 24.156 ± 6.071 | 36.519 ± 2.101 | 32.003 ± 3.445 |
| **Iron** (µmol Fe $g^{-1}$) | 1.328 ± 0.059 | 1.586 ± 0.104 | 2.064 ± 0.322 | 1.444 ± 0.148 |
| **Magnesium** (µmol Mg $g^{-1}$) | 17.188 ± 0.336 | 11.838 ± 2.824 | 12.025 ± 0.999 | 10.446 ± 1.361 |
| **Manganese** (µmol Mn $g^{-1}$) | 0.892 ± 0.084 | 2.051 ± 0.105 | 0.988 ± 0.062 | 0.863 ± 0.074 |
| **Phosphorus** (µmol P $g^{-1}$) | 0.084 ± 0.012 | 0.090 ± 0.020 | 0.451 ± 0.042 | 0.364 ± 0.054 |
| **Nitrogen** (µmol N $g^{-1}$) | 0.002 ± 0.522 | 0.029 ± 0.002 | 0.024 ± 0.001 | 0.023 ± 0.001 |
| **Zinc** (µmol Zn $g^{-1}$) | 0.017 ± 0.006 | 0.013 ± 0.001 | 0.048 ± 0.003 | 0.017 ± 0.005 |

We further separated the correlations into their soil nutrient status to assess relationships more closely (Figures 5.3 and 5.5). Here, we show only relationships with a correlation coefficient >0.700 and <−0.700 (Appendix A, Table 5. A1 for addition correlation coefficients). The correlation between pH and EA was negative, but a strikingly steep decline in EA occurred between a soil pH of 3.8 and 4.1. This occurrence was associated with only N-enriched soils (Figure 5.3A). The correlation between P and Ca revealed that an increase in P concentration was associated with an increase in Ca concentrations, especially in soils enriched either with P only or with both N and P (Figure 5.3B). However, this was more prominent in the P-enriched soils. The trend for soil Al concentration for the different soil nutrient statuses were as follows: control < NP < P < N. Manganese was slightly increased when Al was higher, this was noticeable in N-enriched soils (Figure 5.4A). Exchangeable acidity was strongly negatively correlated with increased concentrations of Al in N-enriched soils (Figure 5.4B).

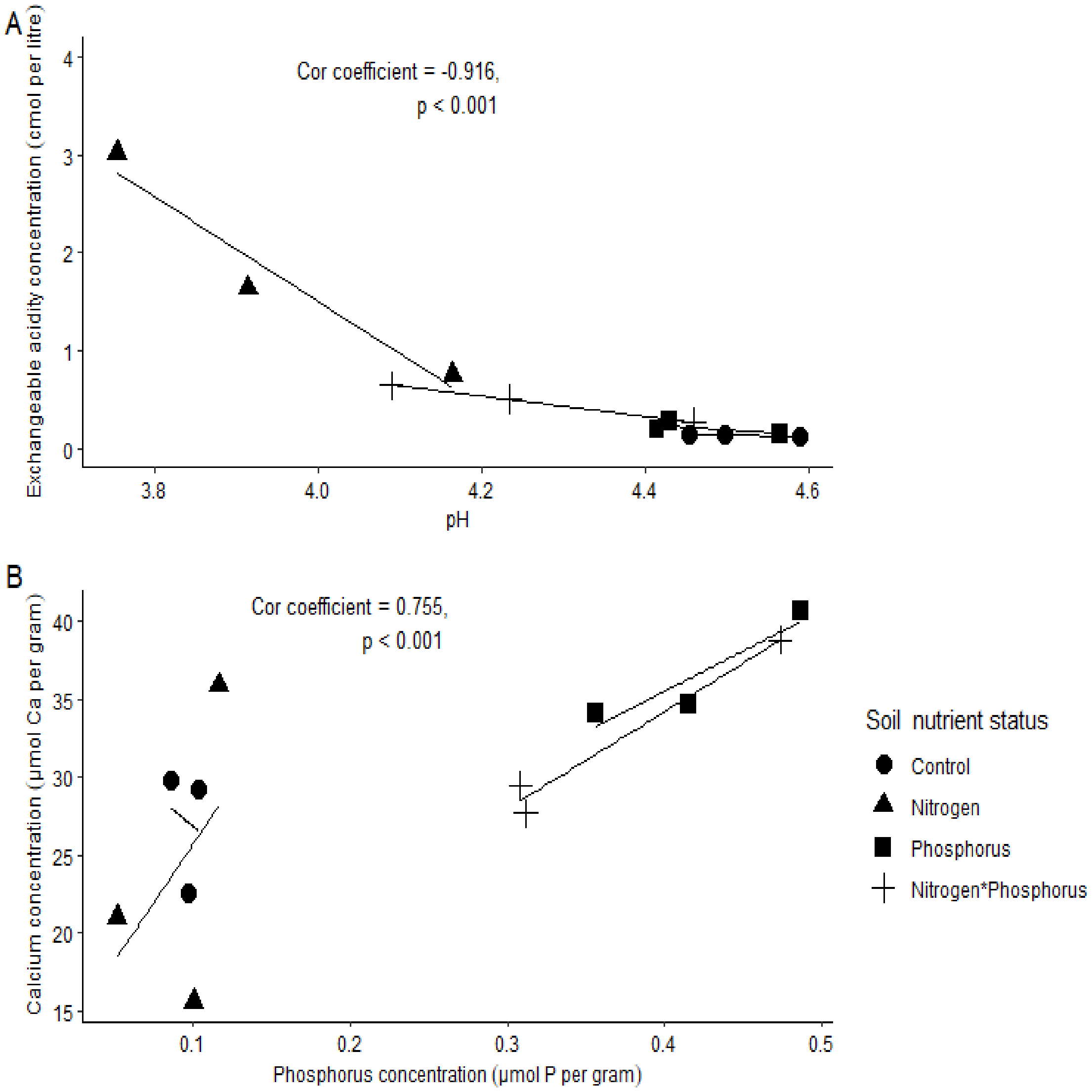

**Figure 5.3.** Correlations for soil chemical parameters between (A) pH and exchangeable acidity and (B) phosphorus and calcium concentrations. Spearman's correlation coefficient > 0.700 and $p < 0.05$ is shown here. Key: ● = control (no nutrients added); ■ = phosphorus-enriched soils; ▲ = nitrogen-enriched soils and + = nitrogen and phosphorus-enriched soils.

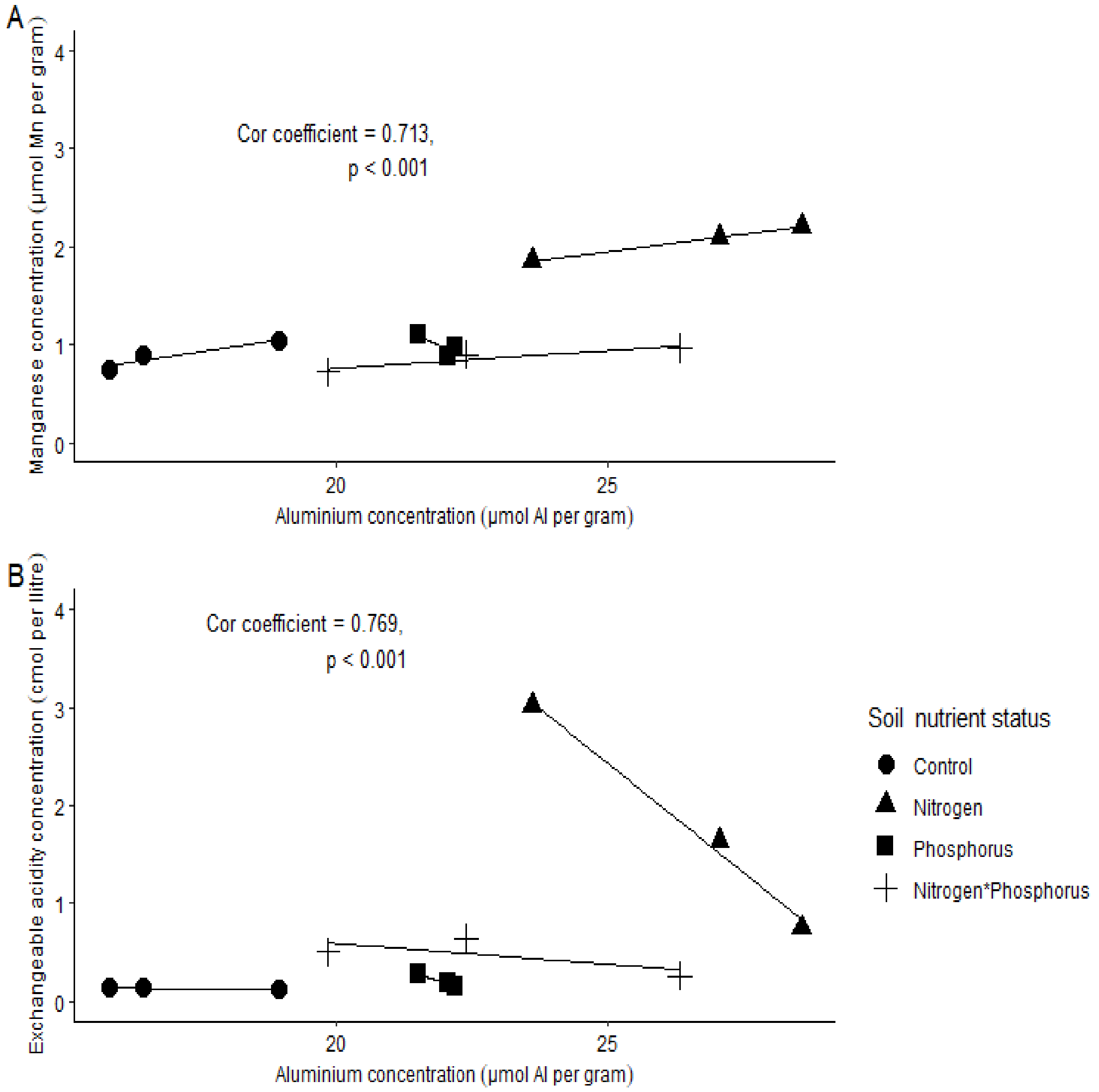

**Figure 5.4.** Correlations for soil chemical parameters between (A) aluminium and manganese concentrations and (B) aluminium and exchangeable acidity. Spearman's correlation coefficient > 0.700 and p < 0.05 is shown here. Key: ● = control (no nutrients added); ■ = phosphorus-enriched soils; ▲ = nitrogen-enriched soils and + = nitrogen and phosphorus-enriched soils.

**Growth dynamics**

*Vachellia sieberiana* saplings grown in the different soil nutrient statuses, showed distinct differences in their growth and mineral nutrition (Figure 5.5). Saplings grown in N only-enriched soils showed significantly less root biomass compared to saplings grown in the other soil conditions (Table 5.2). Additionally, these saplings also had significantly less leaf biomass and overall biomass compared to saplings grown in both P only enriched and N*P-enriched soils (Table 5.2). Saplings grown in P-enriched soils had significantly more stem biomass than those grown in N-enriched soils (Table 5.2). The observation for plant P concentration in saplings grown in the different treatments was as follows; control = N < P = N*P. In contrast, for plant N concentration, it was N < control = P = N*P (Table 5.2). *Vachellia sieberiana* saplings grown in N-enriched soils had significantly higher SNUR compared to the rest, while saplings grown in N*P-enriched soils had significantly less (Table 5.2 and Figure 5.6B).

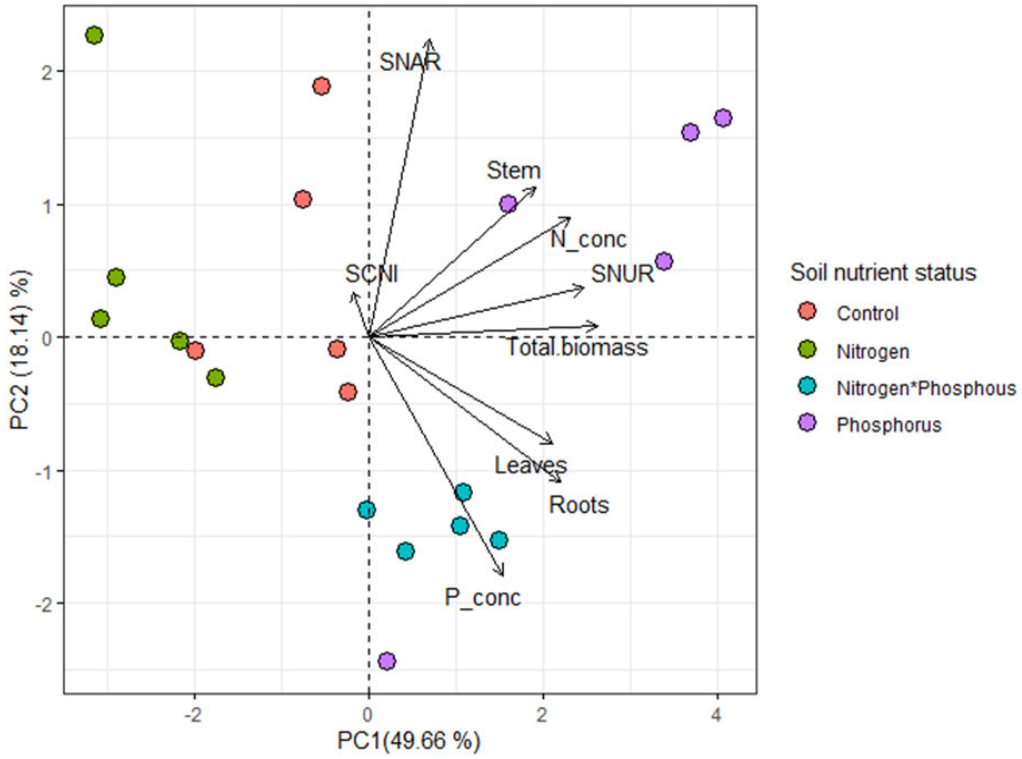

**Figure 5.5.** Principal component analysis (PCA) for standardized plant mineral nutrition and growth kinetics trait variables under four different soil nutrient status treatments control (no nutrients), nitrogen (limestone ammonium nitrate), phosphorus only and both nitrogen (limestone ammonium nitrate) and phosphorus. The plot shows the relationship among the plant growth, mineral and nitrogen-use efficiency variables. Positively correlated variables are grouped together, and negatively correlated variables are positioned on the opposite ends from the plot origin. The quality of the variables is assessed by the distance between the variable and the origin. The longer the length of the variables, the greater its level of contribution. Labels: Leaves—leaves biomass (g); Roots—roots biomass (g); stems—stem biomass (g); Total.biomass—overall plant biomass (g); P_conc—plant phosphorus concentration (µmol P.

g$^{-1}$); N_conc—plant nitrogen concentration (mmol Ng$^{-1}$); SCNI—standard corrected $^{15}$N/$^{14}$N; SNAR—specific nitrogen absorption rate (mg Ng$^{-1}$ root dw day$^{-1}$) and SNUR—specific nitrogen use rate (g dw mg$^{-1}$ N day$^{-1}$). Key: control (pink solid circle); N only (green solid circle); N and P (blue solid circle); P only (purple solid circle).

**Table 5.2**. *Vachellia sieberiana* biomass and mineral nutrition grown in control soils (no nutrients) and soils enriched with nitrogen (limestone ammonium nitrate), phosphorus and both nitrogen (limestone ammonium nitrate) and phosphorus obtained from the Ukulinga Grassland Nutrient Experiment, South Africa. Different letters indicate significant differences among treatments per parameter, at $p \leq 0.05$

**Plant Traits**

| **Parameter** | **Control** | **Nitrogen** | **Phosphorus** | **Nitrogen * Phosphorus** |
|---|---|---|---|---|
| **Roots (g)** | 4.379 ± 0.623 $^{b}$ | 2.269 ± 0.464 $^{a}$ | 5.828 ± 0.587 $^{b}$ | 5.815 ± 0.368 $^{b}$ |
| **Leaves (g)** | 0.769 ± 0.070 $^{ab}$ | 0.696 ± 0.079 $^{a}$ | 1.211 ± 0.125 $^{bc}$ | 1.282 ± 0.176 $^{c}$ |
| **Stems (g)** | 2.972 ± 0.866 $^{ab}$ | 2.367 ± 0.578 $^{a}$ | 5.909 ±1.400 $^{b}$ | 2.954 ± 0.240 $^{ab}$ |
| **Total biomass (g)** | 8.119 ± 0.604 $^{ab}$ | 5.331 ± 1.034 $^{a}$ | 12.949 ± 1.482 $^{b}$ | 10.051 ± 0.553 $^{b}$ |

**Plant mineral Nutrition**

| **Parameter** | **Control** | **Nitrogen** | **Phosphorus** | **Nitrogen * Phosphorus** |
|---|---|---|---|---|
| **Total plant P (μmol P. g$^{-1}$)** | 25.319 ± 2.779 $^{a}$ | 26.699 ± 1.139 $^{a}$ | 37.193 ± 2.433 $^{b}$ | 41.306 ± 1.963 $^{b}$ |
| **Total plant N (mmol Ng$^{-1}$)** | 48.017 ± 3.046 $^{b}$ | 31.137 ± 2.697 $^{a}$ | 56.851 ± 6.259 $^{b}$ | 48.171 ± 0.972 $^{b}$ |
| **Standard corrected $^{15}$N/$^{14}$N** | 6.021 ± 0.886 $^{a}$ | 4.562 ± 0.505 $^{a}$ | 3.896 ± 1.142 $^{a}$ | 5.405 ± 0.283 $^{a}$ |

Each value is a mean ± standard error of five replicates. Values with the same letter superscript within a row under the same parameters are not significantly different at $p \leq 0.05$.

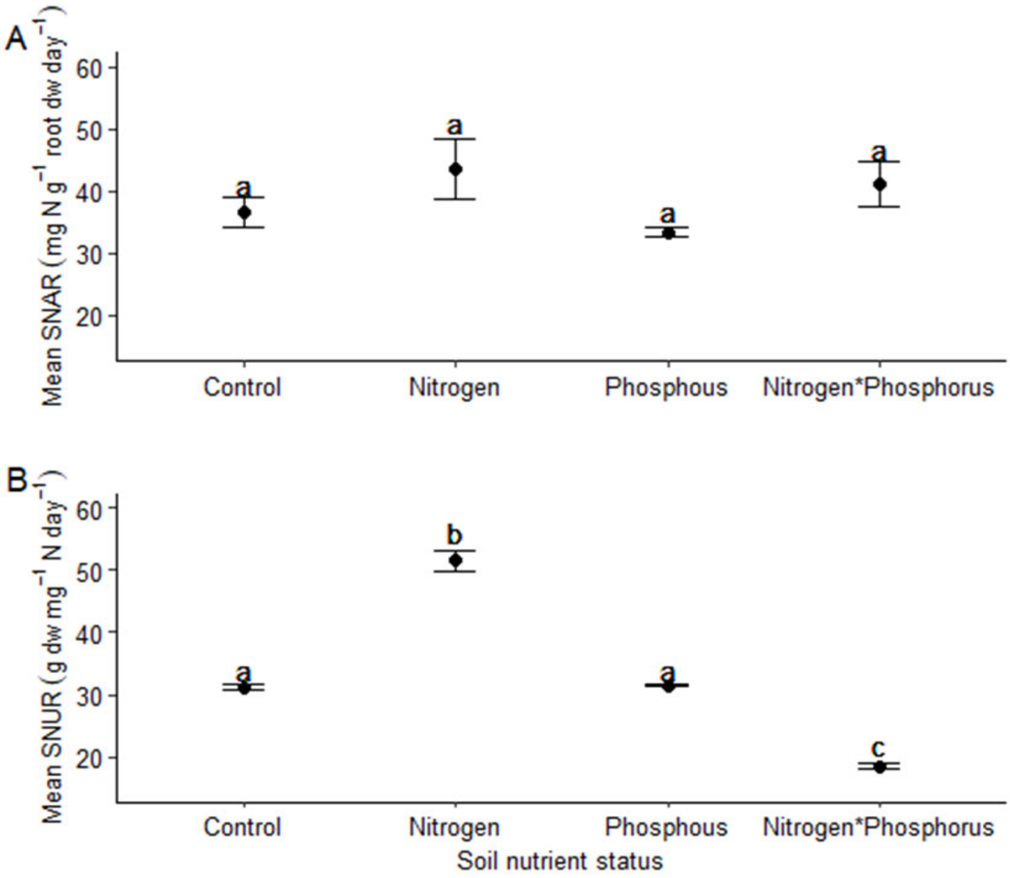

**Figure 5.6.** The mean ± standard error (A) specific N absorption rate (mg $Ng^{-1}$ root dw $day^{-1}$) and (B) specific N use rates (g dw $mg^{-1}$ N $day^{-1}$) of *V. sieberiana* saplings grown in soils with various nutritional statuses: control (no nutrients); nitrogen (limestone ammonium nitrate); phosphorus and both nitrogen (limestone ammonium nitrate) and phosphorus obtained from the Ukulinga Grassland Nutrient Experiment; South Africa. Means ± se are shown here, with different letters indicating significant differences ($p \leq 0.05$) among treatments, n = 5 per treatment.

## Discussion

The long-term effect of nutrient enrichment on the UGNE has resulted in soil chemical variables differentiating based on the single nutrient or nutrient combination treatments applied. We believe that this is one of the main drivers for the differences observed in the growth kinetics, N and P mineral nutrition and N-use demand for *V. sieberiana* plants.

Soils that had been enriched with N, P, and both N and P exhibited a decrease in soil pH by approximately 0.402, 0.108 and 0.084 units on average when compared to soils that had not been enriched with nutrients since 1951. This extreme decrease in soil pH caused by N enrichment is consistent with other long-term studies (Cai et al., 2015; Schroder et al., 2011).

Estimations indicate that soils are considered to be acidic when top soil pH is below 5.5 and that most of the agricultural land is made up of acidic soils (von Uexkull and Mutert, 1995). In this study, soils for all nutrient treatments (including the control) were found to be acidic. However, it is noteworthy to specify that nutrient enrichment of N and P further lowered the soil pH, with N having the greatest decreasing effect. This intensified soil acidification effect is generally associated with numerous conditions that restrict plant growth (Liu et al., 2020). Furthermore, soil acidification and leaching reduces the availability of base cations including Ca, Mg, sodium (Na) and K, leading to lower soil fertility and production yield decline (Zhang et al., 2016). In particular, in this study, N-enriched soils were associated with reduced Ca and Mg concentrations, supporting the notion that leaching of these minerals occurred and could have activated high Al concentrations that would be detrimental to plant growth (Aber et al., 1998). When soil pH was below 4.5, the release of high and toxic Al and Mn concentration levels resulted in root damage and decreased overall plant production (Hue et al., 2001). Considering the above-mentioned statement, we assume that all the soils used are acidic and potentially have toxic levels of Al and Mn. Surprisingly, this seems to be strongly supported when soils are enriched with N, since the concentrations of Al and Mn were markedly higher. This effect was observed through the significantly lower root biomass in *V. sieberiana* plants grown in N-enriched soils. Our results support hypothesis 1, with a strong relationship being observed between soil pH and exchangeable acidity, where exchangeable acidity decreased with increasing soil pH (Cai et al., 2015; Moir and Moot, 2014). Exchangeable acidity is defined as the measure of $Al^{3+}$ and $H^{+}$ ions that is retained on the most active constituent of the soil (colloids) (Dai and Richter, 2000). When this is high, and with a resultant low soil pH, the soil conditions and the processes that occur within the soil are affected (Onwuka et al., 2016). We identified a threshold at soil pH = 4.2 following which there appears to be no significant change in exchangeable acidity. The critical changes in decreasing exchangeable acidity occurred between soil pH values of 3.9 and 4.1. However, we observed an increase in EA and Mn with increasing soil Al, which supports hypothesis 2. We associate this with increased soil acidification, especially because under N enrichment, toxic elements such as Al and Mn are released (Cai et al., 2017; Mao et al., 2017).

The macronutrients most limiting to photosynthetic production in both aquatic and terrestrial environments are N and P (Elser et al., 2007; Fay et al., 2015). Research on KwaZulu-Natal grasslands has revealed that the soils are acidic, contain cations, and are deficient in P (Magadlela et al., 2023). We further support this notion and maintain that this is true even for soil that has not been enriched with additional nutrients. Consequently, we expected that the measured growth parameters of *V. sieberiana* would be directly influenced by the amount of P available in the soil (Somavilla et al., 2021). The factors that are attributed to low available P include low soil pH (acidic soils) and highly weathered soils, whereby P is absorbed by the presence of soil minerals and P precipitation through Al and Fe (Tate and Salcedo, 1988; Zhan et al., 2015). In ecosystems with low available P, application of mineral P fertilizer can increase the P content (Kisinyo et al., 2014). This is in agreement with our results, in which P concentration in soils enriched with P only and with both N and P were approximately 5.3 and 4.3 times greater than in soils without any nutrient enrichment. Additionally, an increase in soil P concentration was strongly positively associated with an increase in soil Ca concentration, favouring hypothesis 3. We suspect that the increased soil Ca concentration observed here is linked to the calcium-containing superphosphate [$Ca(H_2PO_4)_2$] P fertilizer (Keller et al., 2022; Tian et al., 2016) used in this study. However, chemical fertilizers are used to increase productivity but are also considered to have negative impacts coupled with high costs and further strengthens the motivation for use of organic fertilizer usage instead (Bekele et al., 2018). Higher soil P availability favours the development and growth of leguminous plants, allowing them to thrive and become dominant within the ecosystems in which they occur (Ceulemans et al., 2013; Harpole et al., 2016). In our study, N-enriched soils were associated with extremely low pH and soil P, as well as significantly reduced sapling root, stem, leaf and overall biomass. As a result, we accept hypothesis 4, stating that *Vachellia sieberiana* saplings grown in soils with a pH below 4 will have significantly reduced growth dynamics. Additionally, high soil P concentration coupled with high plant biomass of *V. sieberiana* plants occurred in P only- and N and P-enriched soils.

The nutritional status of the soils had a strong effect on both belowground and aboveground biomass of *V. sieberiana* plants. This is supported by soil conditions with higher soil P concentration resulting in greater leaf and root biomass. Ultimately, the same pattern was noticeable for plant P mineral concentration. This was not the case for plant N mineral concentration. Due to these findings, we reject hypothesis 5, indicating that long-term N and P enrichment would increase mineral concentration in *V. sieberiana* saplings. We therefore assume that soil P concentration more strongly positively affects plant P concentration than plant N concentration for *V. sieberiana*. The P requirements for legumes are high, and P deficiencies can impair nodulation and symbiotic N fixation, affecting the growth and respiration of the host [73–75]. In this study, plants failed to produce nodules in all soil conditions. A previous study on *Vachellia nilotica* saplings grown in similar N-enriched soil conditions indicated that nodulation did not occur (Sithole et al., 2021). This occurrence was explained by the low P levels in the soils, considering that P is necessary for regulating energy required for nodule formation and BNF (Taylor et al., 1991). When nodules do not form, legume species are able to rely on actinomycetes and other Gram-positive bacteria for BNF (Bhatti et al., 2017; Boonkerd, 1998). Additionally, it is cheaper to assimilate N from the soil than to fix N (Valentine et al., 2017). We also note that the extreme soil acidity in all soil treatments could have further enhanced the processes that make P unavailable for plant use (Khan et al., 2009). We also further emphasize that the P application amount or rate applied at the UGNE over the lifetime of the experiment may not be suitable to support the conditions required for nodule formation in legumes. For example, a 35 percent reduction in nodule numbers for soybean plants occurred when P was oversupplied, and when P was deficient, smaller nodules were present (Tsvetkova and Georgiev, 2003). Hence, we believe that P concentration in the soil is important, and high and low levels can retard structural development and reduce efficient BNF (Batterman et al., 2013; Weisany et al., 2013). Additionally, N fixation decreases when P is deficient, through an adaptation to low N demand, caused by feedback mechanisms within plants (Almeida et al., 2000). Therefore, we agree that optimum P concentration is important (Afzal et al., 2015), and thus this should be considered when performing such studies.

*Vachellia sieberiana* saplings grown in N-enriched soils did not have significantly lower nitrogen-use efficiencies when compared to those not enriched with N. Thus, we reject hypothesis 6. Specifically, soil nutrient status did not appear to affect SNAR. This is dissimilar to previous findings on *Vigna unguiculata* saplings grown in soils obtained from soils of varying nutritional status (Sithole et al., 2019). In this case, a high soil potassium (K) concentration was related to increased SNAR to support increased biomass (Sithole et al., 2019). We did not consider soil K concentration here, but previous results from the same soils show that K concentrations were 2.531 ± 0.335 (mean ± se) and 4.234 ± 0.681 for the control and P-enriched soils, respectively (Ndabankulu et al., 2022). However, we believe the main cause for our findings is linked to soil acidification. The soil pH units reported in (Ndabankulu et al., 2022; Sithole et al., 2021) are higher than those reported here, suggesting that more acidic conditions could affect SNAR. In contrast, *V. sieberiana* saplings grown in N-enriched soils under extreme soil acidity and low P concentration showed an increased N use rate (SNUR).

## Conclusions

Our findings show that the soils at the UGNE are acidic, regardless of nutrient enrichment. Therefore, we conclude that applying N at the current rate decreases the soil pH and increases the availability of potential toxic heavy metals. We recommend the addition of lime as a solution to this. It is estimated that to neutralize the acidity from applying 1 kg of N as urea and ammonium sulphate, approximately 1.72 and 5.2 kg of lime would be required, respectively (Bolan and Hedley, 2003).

We acknowledge that under natural conditions, *V. sieberiana* would grow in competition with other plant species and thus recommend competition to be incorporated in future studies. Competition between neighbouring plants can result in trade-offs between defence, production and reproduction.

*Vachellia sieberiana* is a common tree in the humid grasslands of KwaZulu-Natal, and we concede that further studies incorporating more samples and variables occurring under natural conditions would reinforce our findings (Figure 5.7). We also recommend that the growth responses of common legumes species with commercial or cultural significance in nutrient-poor soils be investigated.

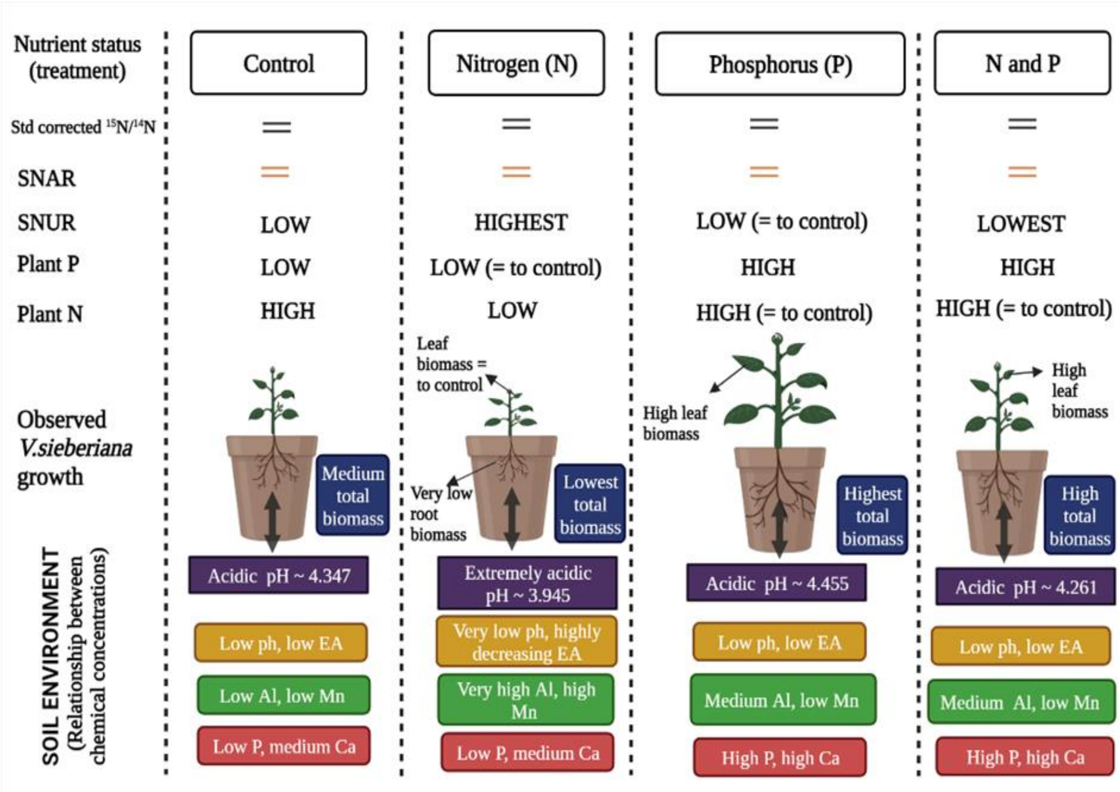

**Figure 5.7.** A visual representation of the effects of nitrogen and phosphorus enrichment on soil chemical properties and growth dynamics of Vachellia sieberiana. "=" means that the effect of the treatment was non-significant.

## Appendix A

**Table 5. A1.** Pearson's and Spearman's correlation for the soil chemical parameters. Distribution of all soil variables were not performed; thus, we show both parametric and non-parametric statistical procedures

| **Soil variable 1** | **Soil variable 2** | **Pearson's correlation** | **Spearman's correlation** |
|---|---|---|---|
| Aluminium | Calcium | -0.228 | -0.070 |
| Aluminium | Magnesium | -0.676 | -0.545 |
| Calcium | Magnesium | 0.271 | 0.350 |
| Aluminium | Manganese | 0.738 | 0.713 |
| Calcium | Manganese | -0.489 | -0.126 |
| Magnesium | Manganese | -0.223 | -0.049 |
| Aluminium | Zinc | -0.116 | -0.182 |
| Calcium | Zinc | 0.596 | 0.594 |
| Magnesium | Zinc | -0.090 | 0.077 |
| Manganese | Zinc | -0.324 | -0.021 |
| Aluminium | pH | -0.506 | -0.573 |
| Calcium | pH | 0.095 | 0.021 |
| Magnesium | pH | 0.102 | 0.196 |
| Manganese | pH | -0.684 | -0.329 |
| Zinc | pH | 0.402 | 0.133 |
| Aluminium | Exchangeable acidity | 0.420 | 0.769 |
| Calcium | Exchangeable acidity | -0.006 | -0.056 |
| Magnesium | Exchangeable acidity | 0.119 | -0.357 |
| Manganese | Exchangeable acidity | 0.687 | 0.524 |
| Zinc | Exchangeable acidity | -0.346 | -0.133 |
| pH | Exchangeable acidity | -0.909 | -0.916 |
| Aluminium | Nitrogen | 0.586 | 0.622 |
| Calcium | Nitrogen | -0.456 | -0.378 |
| Magnesium | Nitrogen | -0.288 | -0.266 |
| Manganese | Nitrogen | 0.802 | 0.483 |

| | | | |
|---|---|---|---|
| Zinc | Nitrogen | -0.417 | -0.545 |
| pH | Nitrogen | -0.559 | -0.448 |
| Exchangeable acidity | Nitrogen | 0.547 | 0.587 |
| Aluminium | Phosphorus | 0.036 | 0.014 |
| Calcium | Phosphorus | 0.730 | 0.755 |
| Magnesium | Phosphorus | -0.354 | -0.231 |
| Manganese | Phosphorus | -0.523 | -0.329 |
| Zinc | Phosphorus | 0.658 | 0.538 |
| pH | Phosphorus | 0.230 | -0.028 |
| Exchangeable acidity | Phosphorus | -0.350 | 0.049 |
| Nitrogen | Phosphorus | -0.387 | -0.301 |
| Aluminium | Iron | 0.188 | 0.280 |
| Calcium | Iron | 0.386 | 0.203 |
| Magnesium | Iron | -0.324 | -0.462 |
| Manganese | Iron | -0.022 | 0.196 |
| Zinc | Iron | 0.708 | 0.476 |
| pH | Iron | 0.117 | -0.007 |
| Exchangeable acidity | Iron | -0.060 | 0.147 |
| Nitrogen | Iron | 0.040 | 0.000 |
| Phosphorus | Iron | 0.478 | 0.406 |

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# Chapter 6

# Synthesis and recommendations

The main aim in this chapter is to summarise and integrate the key findings obtained in the preceding chapters. It is also an opportunity to discuss the new findings and ideas that I have contributed within this thesis to the current understanding of the long-term effects of nutrient enrichment and cessation of nutrient enrichment in a mesic South African grassland. In this chapter I will draw conclusions from my main results and provide recommendations where applicable. In addition, I will assess the limitations encountered throughout and provide suggestions for further research. My main purpose here is to reinforce the notion that long-term enrichment of nutrients (in the form of nitrogen and phosphorus) has greatly modified the soil chemistry and the plant community structure on the Ukulinga Grassland Nutrient Experiment (UGNE). Considering that these nutrients have been applied since the year 1951, short-term cessation of the nutrient application (three-year period) does not appear to have been a reasonable period to 'reverse' the long-term effects, given the persistent nature and thus slow turnover of the species occurring on the site.

## Soil acidification

One of the key findings that stood out in this thesis is the acidifying effects of nitrogenous fertilisers. In chapter 2, both limestone ammonium nitrate (LAN) and ammonium sulphate (ASU) decreased soil pH, but ASU had a greater acidifying effect. In fact, ASU and ammonium nitrate are both known to have a negative effect on forb species because these fertilizers promote soil acidification (Carbutt and Kirkman, 2022). This observation was further supported in chapter 2. To minimise the loss of forb species in mesic grasslands, I recommend the use of non-acidifying fertilisers such as potassium nitrate, sodium nitrate and inorganic fertilizers such as manure, in situations where nutrient addition is important for agricultural production.

South African grasslands are considered nutrient-poor and acidic. Thus, continued excessive use of ammonium-based fertilisers in these grasslands and the agricultural lands located within the grassland biome is bound to only intensify soil acidification. This has implications for leguminous plants that occur within natural grasslands as well as grass and forb species that occur within the grassland biome. In chapter 5, I showed that root and shoot biomass of a legume species grown in nitrogen (N) fertilized soils was significantly reduced. Such conditions were correlated with a low soil phosphorus (P) concentration. The implication for this is enhanced P deficiency in the soil which can stunt nodulation and nitrogen fixation of legumes (Graham and Vance, 2003). Therefore, alternate methods to improve soil fertility in an agricultural context are necessary. Such methods should incorporate socio-economic and biophysical methodologies. Existing acidic conditions in South African grasslands provides an explanation for low occurrence of legumes in South African grasslands, and nitrogen deposition and associated soil acidification is likely to further reduce legume occurrence.

### Aluminium toxicity

Plant growth is limited on acidic soils ($pH < 5.5$). As a consequence of the soil acidity, the availability of important elements such as molybdenum (Mo) and P is compromised in soils with a low pH (Hafner et al., 1992; Mitran et al., 2018). On the other hand, highly acidic soils exacerbate the levels of aluminium (Al), a non-essential yet abundant metallic element (Kidd and Proctor, 2001; Sade et al., 2016). This finding was supported in chapter 2, where I described the increased soil Al levels in soils with low pH. I consider this to be an important finding within this thesis because emphasis on heavy metal accumulation in fertilized soils has not been previously highlighted on the UGNE. This may have been overlooked because the symptoms of Al toxicity are not easy to identify. However, reports show that Al toxicity is linked to reduced root growth (Gupta et al., 2013) and yellowing of the leaf tips (Rahman and Upadhyaya, 2021). In chapter 5, I determined high soil Al concentrations in N fertilized soils, and these were associated with significantly lower root biomass in *Vachellia sieberiana*. This could be associated with the high soil Al levels, but I acknowledge that this was not directly tested, and there could have been other factors contributing to the reduced root biomass. I recommend that further studies to directly assess changes in heavy metal levels and morphological traits with various leguminous plants. This is because plants may show symptoms of Al toxicity in unique ways (Rahman and Upadhyaya, 2021), including reduced root growth, yellowing of leaf tips, leaf veins and stems turning purple and calcium deficiency in young leaves (Clark et al., 1981; Foy and Fleming, 1982; Frantzios et al., 2001) . Additionally, some plants are known to mitigate high Al concentrations via internal detoxification and externally excluding Al by minimizing its’ entry to the roots (Chen and Liao, 2016; Ma et al., 2001; Wang et al., 2020; Zhang et al., 2019). The findings within this thesis warrant further studies to investigate plant adaptations to metal toxicity on the UGNE and other fertilized/acidic South African grasslands.

### Phosphorus and fertility in acidic soils

Phosphorus use efficiency is between 10 to 15 % in acidic soils owing to high Al and iron oxide concentrations (Scherer and Sharma, 2002; Thomas Sims and Pierzynski, 2005). However, there are plants that have evolved strategies to deal with P limitation. For example, such plants increase the length of root hairs (Haling et al., 2018; Richardson et al., 2011) and some legumes associate with mycorrhizae to strengthen the utilization of P (Beltayef et al., 2021; Hou et al., 2021; Richardson et al., 2009). I did not investigate mycorrhizal association in chapter 5 but encourage such studies considering limited work has been published regarding this on the UGNE. However, a study conducted on three sites occurring in KwaZulu-Natal (KZN) South African soils revealed the presence of fungal species, that can potentially improve soil fertility (Zungu et al., 2020). The authors further state that soils obtained from the Bergville site (approximately ± 155 km away from the UGNE) had low dehydrogenase activity. Measuring soil enzymes activity is important because they provide an index for the assessing changes in soil quality (Wolinska and Stepniewsk, 2012). Soil dehydrogenase enzymatic activity is conducted in soils and commonly used for determining enzyme activity (Visser and Parkinson, 1992). I suggest further assessments of soil microflora and enzyme activity on the fertilized soils of the UGNE, since these may also influence soil fertility and plant growth. To date, I am aware of one study conducted on the UGNE that showed a decline in soil respiration with increasing N application (Ward et al., 2017). The authors' findings contrasted the general idea of soil respiration serving as a predictor of soil fertility that suggests that soil respiration will increase when more fertilizers are applied (Haney et al., 2008). In chapters' 2 and 5, I focused more on heavy metals, macro- and micro-nutrients present in the soil whereas a combination of factors still need further investigating.

### Liming

In this chapter, I have already highlighted the negative impacts of soil acidification. A simple and effective management practice to alleviate soil acidity and maintain crop production is liming (Li et al., 2019). However, clear instructions on the application of lime may be complicated because managers and farmers may have different objectives when it comes to their land. Dolomitic limestone, as applied on the UGNE, is one of the more commonly used liming materials alongside calcitic limestone. However, the latter material is known to be more soluble (Conyers et al., 1995). In chapter 2, I show that when LAN, ASU or P are applied with lime, forb species richness was significantly higher. I associate this pattern with lime application. Another study conducted on KZN soils revealed that soil pH increased with an increase in lime rate (Mkhonza et al., 2020). I believe that, the addition of lime alleviates the acidifying effects of fertilizer application, potentially supports microbial activity and increases the rate of organic matter decomposition (Biasi et al., 2008; Grover et al., 2017).

In terms of restoration, lime has been used as a strategy to counteract acidification (De Graaf et al., 1998). In tropical grasslands where soils have been fertilized or limed for agricultural purposes, methods to restore the soil back to its low nutrient status must be employed (Sampaio et al., 2019). This should allow for slow-growing species to recover (Sampaio et al., 2019), however, that recovery may be extremely slow or impossible (Andrade et al., 2015). In chapter 3, I observed that species composition dominance shifted from smaller and slower growing grasses to taller species with broader leaves and the short period applied for restoration had a minimal impact.

### Multiple nutrients, above-ground net primary productivity and light

In chapter 4, I assessed which predictor variables best explain species richness patterns in fertilized soils on the UGNE. I attempted to link my results to known and documented hypotheses used to explain species losses in grasslands. My observations from the other chapters within thesis led me to believe that the different types of fertilizers could affect species losses at different intensities or not all. This was indeed the case. Above-ground net primary productivity strongly reduced species richness in LAN and LAN + P enriched plots. Plots enriched with ASU and ASU + P showed declines in species richness, and I associated this with the acidifying effect of ASU and not necessarily the nutrient numbers. I failed to associate my results with the most popular hypothesis (niche dimension hypothesis) used to explain species losses after fertilization because of technical flaws in methods and insufficient number of nutrients to test. Overall, there are numerous factors that explain changes in species diversity in grassland and the use of only certain hypotheses like the light asymmetry or niche dimension hypotheses may only provide limited insights (Grace et al., 2016). An approach to test the direct and indirect effects of various environmental variables on species richness/diversity would be via structural equation modelling, provided the relationships are linear (Malaeb et al., 2000).

### Cessation of nutrient (fertilizer) enrichment

The minimal impact of the three-year cessation of phosphorus enrichment highlighted that ecological changes may take longer to manifest within the UGNE. Theoretically, the addition of a formerly limiting nutrient should result in its accumulation and the cessation of this nutrient and its decreasing levels should cause for it to be limiting again and for diversity to recover (Tilman and Isbell, 2015). In this thesis, I did not assess the effects of nitrogen cessation enrichment due the vast research already published on the topic. These research papers highlighted that plant diversity recovery following cessation of N addition to be low (Isbell et al., 2013) and difficult because of persistence of soil acidification (Bakker and Berendse, 1999) and required a longer period of time (Carpenter et al., 1999). The nutrient addition reversal component of the UGNE provides an opportunity to study N and P reversal over longer time periods in future, as this experiment will continue indefinitely.

In chapter 3, I presented findings on the effects of short-term cessation of phosphorus addition. The observed changes in species composition were not associated with cessation of P. In fact, there was no significant effect of this treatment on dominant plant species or diversity indices, suggesting that other factors need to be considered when investigating the effect of cessation of phosphorus. I associate this finding with the ability of P to strongly bind with the soil, which means when P is applied, it may take a long time for it to decline in concentration (Dodd et al., 2012). Additionally, the persistence of P in the soil even after cessation, raises concerns about other soil nutrients on the UGNE. For example, topsoil cadmium (Cd) concentration decreased in two New Zealand sites after cessation of P addition for 21 and 26 years. In these cases, P was applied at 24 kg ha$^{-1}$ y$^{-1}$ and the decline in Cd was linked to moderate rainfall amounts of 1630 mm and 740 mm that may have enhanced leaching (McDowell and Gray, 2022). I assume that the same scenario could occur at the UGNE over a longer period because the site receives an annual rainfall of approximately 790 mm and may also experience leaching. However, it could take even longer than the 26 years because P was applied at a greater amount of 336 kg ha$^{-1}$ yr$^{-1}$ on the UGNE.

The combined results from this long-term study were conducted within a natural grassland. Fertilization of natural grasslands is no longer recommended or performed but a study of this kind allows for scientific testing for the effects of chronic increases in nutrient availability caused by anthropogenic activities. This increase in nutrient availability could occur through atmospheric depositions or fertilizer use in agricultural lands with associated nutrient pollution. For example, fertilizers are used in South Africa extensively, thus the long-term impact of fertilizer use on soil properties and quality in crucial for sustainable use. In addition, considerable increases in the use of fertilizers has been reported between 1961 and 2018 (Suri and Udry, 2022). This substantial increase in fertilizer use is bound to affect soil quality therefore I further emphasize the need to investigate the presence of heavy metals such as Cd, especially because it is not an important element for plant growth and an impurity in P fertilizers. Although I did not assess the effect of fertilizers on other systems, excessive or the incorrect use of fertilizers is expected negatively affect the soil and reliant plants. A study conducted in the western Cape province of South Africa found that various vegetable crops exceeded the limits of Cd, lead and zinc concentrations as stipulated by the South African legislation and regulations, according to the Foodstuffs, Cosmetics and Disinfectant Act 54 of 1972 (Government Gazette, 1972; Malan et al., 2015). Thus, fertilized soils used for agricultural purposes need to be monitored carefully.

## Concluding remarks

In line with expectations, high nutrient enrichment has reduced plant species richness on the UGNE, but I highlight that this reducing effect is stronger for ammonium sulphate than limestone ammonium nitrate or phosphorus. This trend may be exacerbated by the already acidic and nutrient-poor soils from this mesic grassland. The addition of nutrients on these soils increased soil acidification. In addition, the soils on the UGNE were also identified as having high soil aluminium concentration levels. I conclude that the key drivers of change are 1) nutrient-poor soils, 2) type of nitrogen used for enrichment, 3) soil acidification and 4) high soil aluminium concentrations and these factors explained how the soil environment affected the observed belowground and aboveground growth dynamics of grasses and forbs. A noticeable reduction in root growth affected aboveground growth under extreme soil acidification in a leguminous species. To emphasize the negative impact of specifically ammonium sulphate enrichment and soil acidification, I observed that forb species richness declined drastically in comparison to grass species. This effect presents consequences for biodiversity, considering that more diverse plant communities can assist in providing greater ecosystem services for other organisms. To add onto to the existing literature regarding hypotheses used to explain the mechanisms by which species decline following nutrient enrichment occur, the UGNE provided partial evidence for the nitrogen detriment hypothesis and biomass-driven hypothesis over the niche dimension hypothesis. This suggests that high levels of nitrogen enrichment and changes in aboveground biomass are also important factors that alter species compositional shifts. From the results here, I inferred that certain known (not measured here) morphological features and growth dynamics (such as tillering) have allowed certain species to persist or dominate within the UGNE. The UGNE has provided the opportunity to assess the key drivers of species composition, species diversity, species richness and the soil chemical properties within a mesic grassland. Furthermore, and for the first time in Africa, I was able to determine that a three-year cessation of phosphorus enrichment following consistent phosphorus enrichment, has not provided noticeable changes. One would expect that cessation of nutrient enrichment should restore the condition of an ecosystem, as a form of rehabilitation. Perhaps in environments exposed to large nutrient inputs though fertilizer addition or atmospheric nutrient deposition, other forms of rehabilitation should be employed. Research suggests that using fire, defoliation regimes and the introduction of native herbivores are possible management techniques to overcome the effects of high nutrient levels (Isbell et al., 2019). In natural grasslands, the focus should be on defoliation (Mentis, 2006), since it is a cheap strategy (Carbutt and Kirkman, 2022) over pH ameliorates.

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