The impact of nitrogen deposition on semi-natural ecosystems

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Declaration

This thesis has not been submitted in support of an application for another degree at this or any other university. It is the result of my work and includes nothing that is the outcome of work done in collaboration except where specifically indicated. Many of the ideas in this thesis were the product of discussion with my supervisor Prof. Carly Stevens.

Mahmoud Alharbi

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Abstract

In the context of increasing anthropogenic impacts, understanding the effects of nutrient addition, particularly N deposition, on plant communities and ecosystems has become increasingly important. This thesis explores the implications of N deposition and nutrient enrichment on different ecosystems, including grasslands and heathlands, through multiple long-term and short-term experimental approaches. The main questions addressed were: 1) how does N deposition affect plant traits such as biomass, flowering, and seed production in grassland species? 2) how does N deposition influence seed banks and species composition in heathland ecosystems? 3) how does nutrient addition alter soil characteristics, and interact with disturbance to impact species diversity and biomass in grassland habitats?

The first part of thesis was focused on the study of the impact of N addition on traits associated with flowering in four plant species: *Achillea millefolium* L., *Lotus corniculatus* L., *Plantago lanceolata* L., and *Prunella vulgaris* L. Despite the well-documented effects of N deposition on plant growth, this study revealed no significant differences in the biomass, plant height and phenology across all four species but *Lotus corniculatus* L. and *Achillea millefolium* L. showed significant differences in the total number of seeds and flowers, respectively, indicating a potential reproductive response to N addition. Furthermore, carbon content and C:N ratios exhibited no change across all species, except *Plantago lanceolata* L., which exhibited significant changes in nitrogen content and C:N ratios.

The second chapter, conducted in Ruabon and Budworth long-term N addition experiments, investigated seed banks. The study showed that *Calluna vulgaris* dominated the seed banks at both Ruabon and Budworth sites, correlating well with the vegetation present. While *C. vulgaris* exhibited strong seed bank presence, other species such as *Deschampsia flexuosa* and *Rumex acetosella* were less abundant, suggesting limited contributions to species diversity and potential ecosystem degradation in the long term. Reductions in some of these species were significantly impacted by N additions.

The thesis further examined the impact of nutrient addition, disturbance, and their combined effects on species richness, diversity, light availability, as well as the biomass. Nutrient addition had limited effects on species richness and diversity in a nutrient-rich, species-poor grassland. Disturbance initially increased diversity by favoring less competitive species, but dominant species recovered quickly, reducing diversity in the following year. Grass biomass decreased in the second year, and forb benefits from disturbance declined, possibly due to seed bank depletion. The combination of disturbance and fertilization ultimately led to reduced diversity due to nutrient-fueled dominance by certain species.

Lastly, a long-term study in Norway assessed the impact of recovery from N deposition on soil chemistry from 2015 to 2020. No significant changes were observed in soil chemistry due to N deposition, indicating recovery over time. Mowing had no significant impact on recovery. This finding is particularly relevant to the current understanding of ecosystem management, as it suggests that soil chemistry may recover from N deposition. However, literature suggests vegetation recovery might not follow as readily.

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1. Introduction

As a result of global emissions reactive nitrogen (Nr) deposition is being enhanced (Galloway et al., 2021). While there are various different forms of reactive nitrogen in the environment, oxidized (NO_y) and reduced (NH_x) nitrogen components are two generalized forms. Primarily, oxidized nitrogen is released through the combustion of fossil fuels, whereas reduced nitrogen primarily makes its way into the atmosphere via agricultural practices, including the creation and application of mineral fertilizers and animal waste (Sutton et al., 2011).

N deposition has increased significantly since around 1850 due to a rapid increase in nitrogen emissions (NEGTAP, 2001, Galloway et al., 2004). The global deposition of inorganic nitrogen saw an upsurge of 8% from 1984-2016, going from 86.6 teragrams of nitrogen (TgN) to 93.6 TgN (Ackerman et al., 2019).

Within terrestrial ecosystems, the nitrogen cycle involves the conversion of both organic and inorganic nitrogen forms (such as NH_4^+ and NO_3^-), alongside the immobilization and absorption by plants and microorganisms (Sutton et al., 2011). This cycle, depicted in (Figure 1), encompasses several phases:

(i) Decomposition and mineralization are key to the turnover of nitrogen in terrestrial environments, where soil becomes enriched with nitrogen as microorganisms transform dissolved organic nitrogen (DON) into ammonium through a process known as mineralization or ammonification (Sutton et al., 2011). The cycle of litter production and breakdown is integral to the movement of nitrogen within soil organic matter, primarily through plant contributions. The breakdown of organic materials is facilitated by fungi and bacteria, with ammonification being conducted by heterotrophic microbes.

(ii) Nitrification takes place, a step where ammonium (NH_4^+) or ammonia (NH_3) undergo biological oxidation through hydroxylamine into nitrite (NO_2^-) or nitrate (NO_3^-). This stage is crucial for the potential nutrient loss within ecosystems, affecting atmospheric composition, soil acidity, and the quality of stream water. Nitrification increases the risk of N escaping the ecosystem through the leaching of nitrates and nitrites, influenced by several factors such as the availability of soil oxygen, soil pH, temperature, and the consumption of ammonium by plants, which acts as the base for nitrification.

(iii) Microbial immobilization of N in the soil occurs when soil microorganisms take up organic and inorganic (ammonium, nitrate) N species. This stage is largely regulated by gross ammonification and available C. Sutton et al. (2011) note that research has shown that undisturbed soils have higher immobilization compared to physically disturbed soils.

(iv) Plants take up inorganic N forms such as NH_4^+ in preference to NO_3^- that is utilized for growth (Sutton et al., 2011). In low temperature and N-poor ecosystems, plants may also uptake amino acids, peptides and proteins from the soil. However, in N deposition affected ecosystems or agricultural locations where there can be reduced limitation of N, inorganic N is utilized.

Finally, (v) Dissimilatory nitrate reduction to ammonium is catalyzed by microorganisms that reduce NO_3^- via (NO_2^-) to (NH_4^+). This process allows NH_4 to be available for plant uptake and microbial immobilization stages. This reduces N loss from the cycle through denitrification and nitrate leaching.

The addition of N to terrestrial ecosystems leads to increased soil NO_3 concentration, nitrification, soil NH_4^+, N leaching and denitrification. N levels below ground and aboveground increase with increased plant pools, litter and dissolved organic N along with reduced microbial biomass N (Lu et al., 2011).

The introduction of N into litter and plant reservoirs results in increased N sequestration. This increment in plant-based nitrogen storage, stemming from N enrichment, correlates with an upsurge in both the plant's biomass as well as its internal nitrogen concentrations. Such an increase in the nitrogen reserves of litter aligns with the nitrogen accumulation noted in the plant’s above and below ground biomass. It is crucial, however, to acknowledge that a rise in nitrogen levels often results in a reduction of nitrogen within the microbial biomass. The surge in the soil's inorganic nitrogen, a direct result of N addition, may interact with the soil's organic constituents, fostering the formation of resistant compounds. Furthermore, the addition of N causes a drop in soil pH, which triggers the leaching of carbon and N. This process modifies the physical as well as chemical characteristics of the soil and creates conditions that limit microbial biomass growth. Although N enrichment does facilitate N capture in the mineral soil and organic layer, the level of sequestration in these areas is significantly less than what is seen in plant and litter reservoirs (Lu et al., 2011).

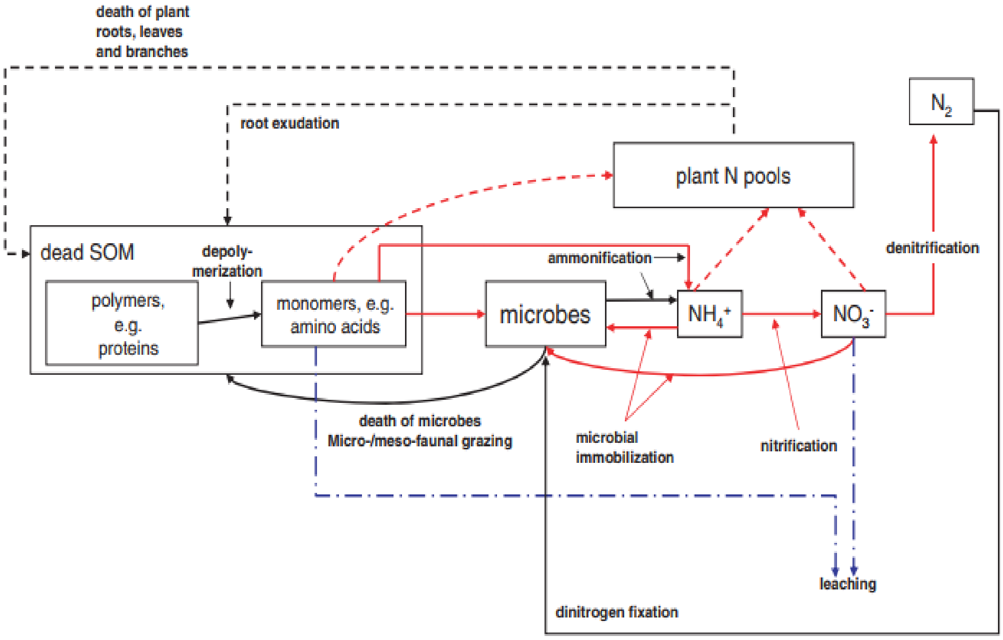

Figure 1 Ecosystem process of N cycling including the internal N retention pathways. Soil Organic Matter is represented by SOM. Taken from Rennenberg et al. (2009).

The use of industrial goods, agricultural products, and fossil fuel has resulted in a dramatic alteration in the cycling of Nr at various scales. Nr can contribute to many environmental problems such as air pollution, greenhouse gas production, acidification, and eutrophication (Sutton et al., 2011). Over last 30 years, the patterns of Nr have found to be altered as Southern Brazil and Asia showed an increased N deposition, whereas Europe and North America has shown a decline in N deposition (Ackerman et al., 2019). Ackerman et al. employed the GEOS-Chem Chemical Transport Model to calculate the worldwide wet and dry deposition of inorganic nitrogen. This estimation was conducted at a spatial resolution of 2° × 2.5° over a span of 12 years, from 1984 to 2016. During this period, their research uncovered an 8% rise in worldwide deposition of inorganic nitrogen, escalating from 86.6 reaching up to 93.6 Tg of nitrogen per year. This pattern included a mix of different regional trends that collectively accounted for the noted growth. For example, areas like East Asia and the southern part of Brazil saw a rise in the deposition of inorganic nitrogen, while regions such as Europe noted a decline**.** Additionally, the share of deposition attributed to non-organic, reduced nitrogen forms saw a global increase from 30% to 35%, predominantly across the United State of America, due to a surge in chemically reduced N deposition (Ackerman et al., 2019).

After being released into soil, groundwater, rivers, and the atmosphere, the forms of Nr in the environment can change as a result of environmental processes (Galloway, 1998). Figure 2 illustrates nitrogen cascade, and how the non-reactive N is produced from Nr after denitrification during the movement of Nr in the aquatic ecosystem, terrestrial ecosystems as well as in the atmosphere. The figure also shows how environment is impacted by Nr (Sutton et al., 2011). The results of nitrogen emissions encompass air contamination, the nutrient enrichment of soils and water bodies, the drinking water pollution, soil acidification, and the release of greenhouse gases (Sutton et al., 2011). While N_2O is a notable greenhouse gas, its contribution to atmospheric N deposition is minimal, and thus, it is not featured in Figure 2.

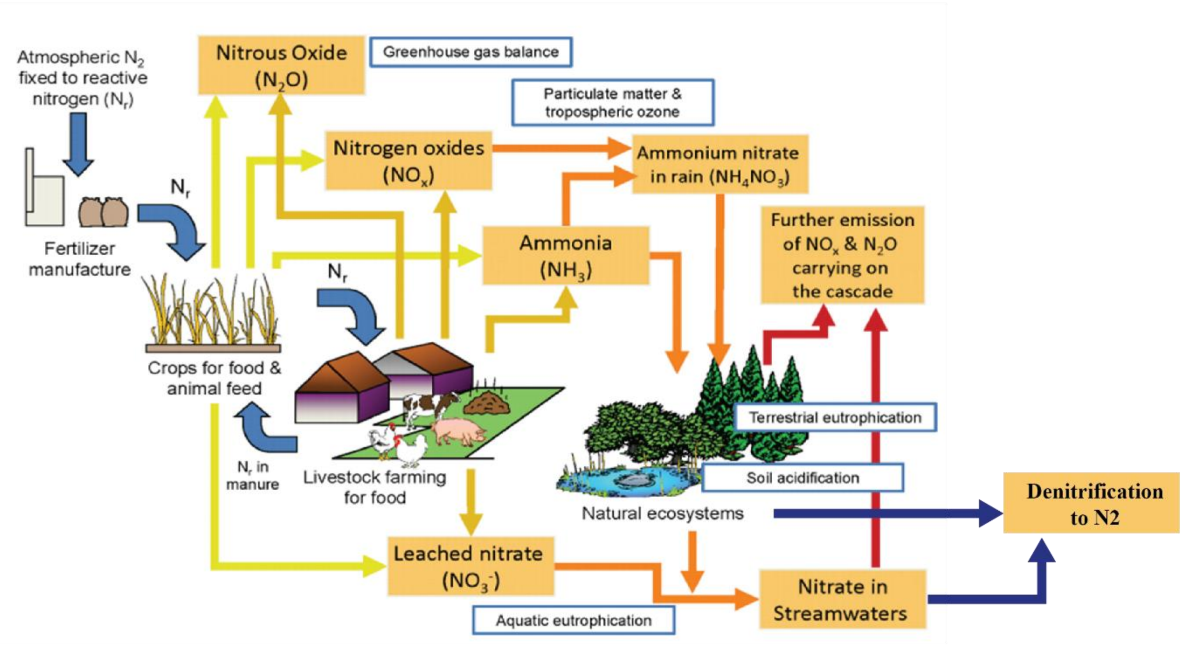

Figure 2 The nitrogen cascade in nature (Sutton et al., 2011).

Nitrogen is deposited from the atmosphere to the biosphere in the form of precipitated gases. These gases are either in the form of dry deposition as gas or dust particles, or dissolved in precipitation (Air Pollution Information System, 2020). Nitrogen transitions to the biosphere from the atmosphere as gases that precipitate. These gases either dissolve in precipitation or exist as dry deposition in the form of gas or dust particles (Air Pollution Information System, 2020). Additionally, nitrogen is able to enter soil through plant litter, such as leaves, decomposed within it.

In the UK, most of the nitrogen is deposited from the atmosphere as wet deposition and enters ecosystems in precipitation. The nitrogen loading in precipitation depends not only on the amounts of nitrogen present but also on the amount of precipitation. In contrast, dry deposition involves direct disposal of gases such as ammonia onto the soil and plant surfaces via wind and gravitational processes (NEGTAP, 2001). Dry N deposition of nitrogen oxides is abundant near highways and in densely populated settlements (Air Pollution Information System, 2020) but in the UK, 380 kilotons yearly total N deposition is split between 43% oxidized nitrogen and 57% reduced nitrogen, even though reduced nitrogen emissions are far lower (287 kilotons) than oxidized nitrogen emissions (560 kilotons) (NEGTAP, 2001). Particularly noteworthy are the proportional contributions of oxidized and reduced nitrogen to semi-natural vegetation, which functions as an exceptionally effective sink for NH_3 (Fowler et al., 1998).

Oxidized nitrogen is composed of NO_2, NO_3, HNO_2, HNO_3 (Sutton et al., 2011). These elements remain in the atmosphere for a duration of 4 to 15 days, and contribute to remote ecosystem by their removal via precipitation following extensive transport (Asman et al., 1998). The primary source of nitrogen dioxide emissions is fossil fuel combustion, which includes vehicles and heating systems. The process of atmospheric oxidation results in the formation of nitric acid and NO_3^- in both particulate and aqueous forms (Air Pollution Information System, 2020). In the UK, N deposition studies since 1986 indicate that the deposition levels of both reduced and oxidized forms of nitrogen have consistently decreased by up to 20% or more in southern and eastern England (Crittenden et al., 2023). Annual emission of oxidized nitrogen has reduced from 2.89 million tons in 1970 to 0.61 million tons in 2022 (Annual Emissions Of Nitrogen Oxides, 2022). The trend of this drop has been a gradual reduction of emissions every year. The energy industries, manufacturing industries, and road transport (vehicles) had emissions of 861 kilotons, 436 kilotons, and 1238 kilotons respectively in 1990. By 2022, nitrogen oxide emissions have reduced in all sectors with energy industries contributing 0.122 kilotons, manufacturing industries 0.09 kilotons, road transport 0.19 kilotons and 0.13 kilotons from other sources (Annual Emissions Of Nitrogen Oxides, 2022).

In the UK, the application and storage of fertilizers, slurry, manure as well as other agricultural activities are the primary contributors to ammonia emissions, which constitute the reduced form of nitrogen (Annual Emissions Of Nitrogen Oxides, 2022). Additionally, in the UK, the waste and various non-point sources also add to the reduced nitrogen levels (Annual Emissions Of Nitrogen Oxides, 2022). This category of nitrogen encompasses ammonia (NH_3), ammonium aerosol (NH_4^+), and ammonium that is deposited via precipitation. Between 1986 and 2005, there was an average decrease of 35% in ammonium levels found in precipitation. The peak of NH_3 emissions in the UK occurred during the 1980s, with a subsequent reduction of 15% noted (Air Pollution Information System, 2020).

Figure 3 shows regions of deposition of both oxidized and reduced nitrogen in the UK. NH_x deposition in the UK is lowest in the north of Scotland. These regions have a low deposition of less than 5 kg ha^{-1} yr^{-1}. NH_x deposition is typically between 5 and 10 kg ha^{-1} $yr^{-}1$ across much of the UK. The highest levels of NH_x deposition, occur in high altitude areas such as Cumbria and the Pennines as well as low altitude areas with intensive farms such as the England-Wales border and East Anglia. On the other hand, the NO_x map shows the lowest N depositions of 0 to 2 kg ha^{-1} yr^{-1} in the northern Scotland.

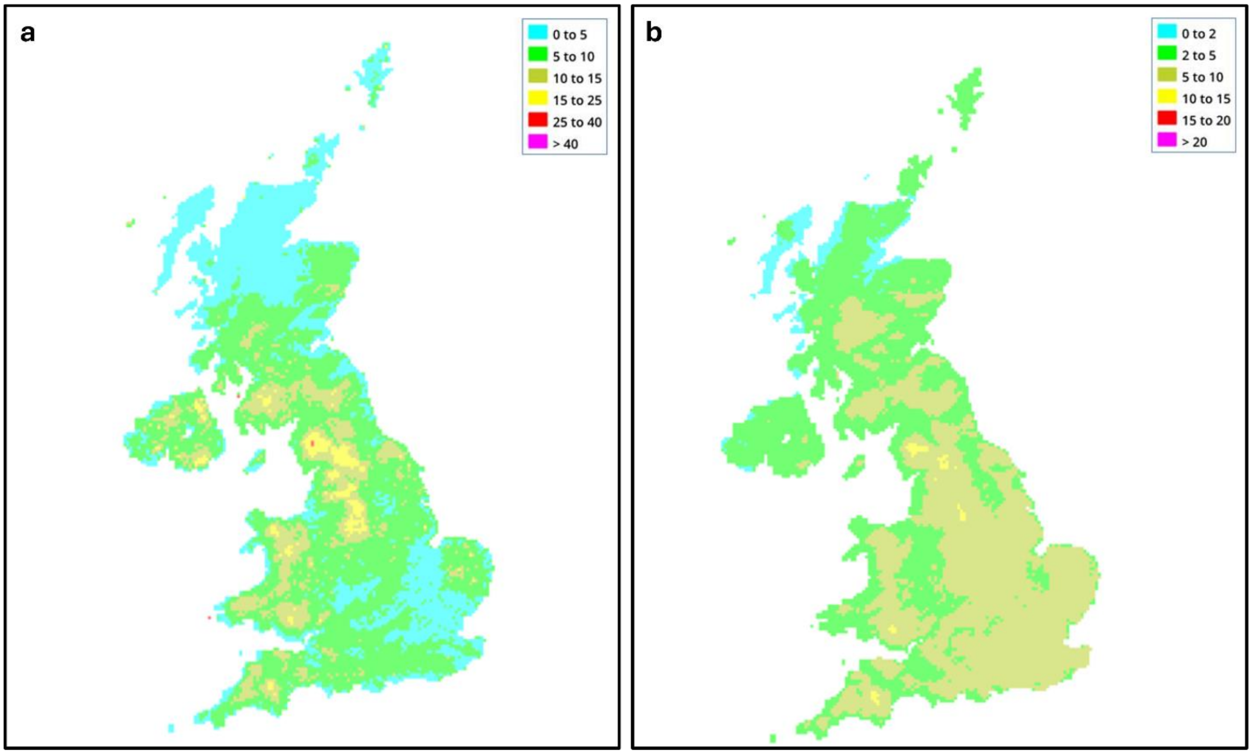

Figure 3 The map of the range of N deposition in the UK (Critical Load maps - Defra, UK). (a) shows NHx and (b) shows NOx.

Historically, in 1990, research indicates that the total N deposition reached its highest value. Fowler et al. (2004) calculated that N deposition in UK was around 430 kilotons N in 1990, with reduced nitrogen (mainly ammonia) making up 54% of this. RoTAP (2012) revised the 1990 total deposition of nitrogen estimate for the United Kingdom to about 380 kilotons N, while Smith et al. (2018) later provided an estimate of 410 kilotons N deposited. There has been a decrease in deposition since early 1990s as policies aimed at reducing nitrogenous emissions, primarily NH_3 and NOx, have been implemented. However, the deposition rate has plateaued at roughly 300 kilotons N per year since around 2010. A balance has to be established to achieve linear decrease in acidification by reduction in emission and other methods of deposition.

Biodiversity faces a major threat in the form of N deposition along with climate change and habitat loss. Modern agricultural and fossil fuel combustion add more nitrogen to aquatic and terrestrial ecosystems than all natural processes combined (Sutton et al., 2011).

1.1 Impacts on Biodiversity

Atmospheric deposition of nitrogen is the key driver of the loss of biodiversity in plants (Clark et al., 2019). N deposition impacts in terrestrial settings occur through several mechanisms such as direct toxicity, eutrophication, an increase in susceptibility to secondary stressors and acidification in soil (Carter et al., 2017).

1.1.1 Direct toxicity

The process of direct toxicity on plants occurs most commonly when plants have no way of filtering excessive intake of nitrogen. Additionally, toxicity in plants can take place due to ammonia and ammonium when the amount of nitrogen in the soil rises to excessively high levels leading to poor shoot and root development (Stevens et al., 2018). Exposure to elevated ammonium levels typically results in diminished growth, smaller foliage, and compromised root systems in most plant species, with extreme cases causing chlorosis. Chlorosis is thought to occur through the disruption of the electron means of transport that takes place in the chloroplasts, leading to the yellowing of leaves (Pearson and Stewart, 1993, Van der Eerden, 1982). Other potential causes include mineral deficiency, halted secondary growth, internal pH shifts, disrupted energy production, and altered metabolism of polyamines and hormones (Gerendás et al., 1997). Although the precise cause of ammonia toxicity is still unknown, it may result from physiological disruption as opposed to the ion's toxicity. The release of protons during ammonia absorption by leaves has significant effects on the regulation of acid-base balance in cells and has the potential to induce cellular acidosis. The fundamental characteristics of the plant's metabolism, which are reliant on the ecology of nitrogen nutrition through root vs leaf uptake under typical circumstances, govern this regulation (Pearson and Stewart, 1993). Lichens are especially sensitive to direct toxicity. The intake of nitrogen in lichens occurs because they have no protective surface or roots, making them absorb the air that is available in the surroundings (Askham, 2020).

1.1.2 Acidification

The resultant change in the pH of soil or surface water as a result of deposition of acidifying gases is called acidification. It is a process that is characterized by increasing concentrations of hydrogen ions (H^+) in the soil (NEGTAP, 2001). In the United Kingdom, sources of acidity in the atmosphere include oxidation of SO_2 and (NO_x), leading to the formation of acids such as sulfuric acid (H_2SO_4) and nitric acid (HNO_3). Nitric acid is formed when nitrogen oxides (NO_x), primarily nitrogen dioxide (NO_2), react with water in the atmosphere. Similarly, sulphur dioxide contributes to acidity through its oxidation to sulphur trioxide which further reacts with water to produce sulphuric acid. These processes release hydrogen ions (H^+), increasing the acidity of rainwater and contributing to acid rain. Nitric acid primarily adds one hydrogen ion per molecule, while sulfuric acid contributes two hydrogen ions, making it a more significant contributor to acidification. These substances provide the majority of the acid deposited to precipitation or “acid rain”. Dry deposition of sulphur dioxide and nitric oxide from terrestrial surfaces also contribute to acid deposition. The acidic substances are oxidized within the soil and vegetation surfaces. Gaseous ammonia can also be oxidized and leached from the soil contributing to the acidification process. Gaseous ammonia is taken up due to its high solubility and is readily absorbed as NH_3 producing NH_4^+ in vegetation or soil. Metabolism of NH_4^+ ions by plants converts it to R-NH_2 producing one hydrogen (H^+) ion which further contributes to soil acidification. Another method of soil acidification is through oxidation of NH_4^+ to NO_3^- which is leached from the soil when it is leached it takes a cation with it. This is called the mobile anion concept (NEGTAP, 2001). The exact contribution of each acidifying input to the soil or landscape is difficult to calculate.

Over the last 20 years, emission of nitrogen and sulphur compounds in the atmosphere has resulted in the acidification of freshwaters and soils in the UK. Many grassland and arable soils have a pH that is below the optimum. A publication on the analysis of more than 200,000 samples revealed that around 40% of UK arable soils have a pH of less than 6.5 and nearly 57% of the grassland soils have less than the recommended pH level of 6 (Goulding, 2016). Addition of N reduces soil pH by 0.26 on average worldwide (Kirk et al., 2010).

The impact of soil acidification on the levels of exchangeable metals in the soil is profound. Stevens et al. (2009) conducted an extensive evaluation of the chemical properties of the surface and deeper layers of soil across 68 acid grassland sites throughout the UK. These sites were chosen to represent a range of acid deposition levels. Through the use of statistical analysis, the study uncovered associations between the concentrations of exchangeable soil metals and various environmental variables, with acid deposition being identified as a key influencer on the concentration of 8 out of 13 exchangeable metals in the upper soil layer and 5 out of 14 metals in the lower soil layer. The study particularly noted significant increases in the levels of exchangeable aluminum and lead when the soil's pH level fell below roughly 4.5, indicating a strong connection to acid deposition (Stevens, et. al., 2009). Plant growth is inhibited in the presence of high aluminum in acidic soil through a mechanism of function inhibition and root growth (Kochian et al., 2005). Blake and Goulding (2002) studied the plant samples originally taken from the Geescroft and Broadbalk Wildernesses (a natural woodland regeneration) and Park Grass (a hay meadow) to show how acidification influences the soil chemistry and the accessibility of metals including Cu, Mn, Pb, Cd, Zn, and Ni to plants. They analyzed both new and archived samples of soil and plants collected from more than 100-year-old experiments. The amount of metals increased over time due to deposition, but soil pH was key to the long-term buildup of metals in the soil. For example, Mn and Cd were mobilised in the soil at pH 6.0 to 5.5 as they were sensitive to soil acidity, whereas Cu, Ni and Zn showed mobilisation at pH 5.5–5.0. Pb however did not mobilise until pH below 4.5 (Blake and Goulding, 2002).

Soil pH can directly and indirectly affect the microbial environment. The high concentration of hydrogen ions in soil with low pH can directly disrupt cell membranes of microorganisms, alter their processes of enzyme production and reduces their reproduction (Birgander et al., 2014). A low soil pH is favored by fungi. This means soil pH produce a shift in the balance of microorganisms in the soil, leading to an environment where fungi predominate (Rousk et al., 2010).

There is widespread acidification of soil in the UK that has affected the soils, freshwater and vegetation (NEGTAP, 2001). This has been subject of different studies and legislations. Since the 1850s, there have reports of widespread acidification of freshwaters particularly in the northern portions of UK. Changes in species and ecosystem in freshwaters have been observed. Although decreases in sulphur dioxide concentrations have improved the state of vegetation and expanded the lichen species, N deposition significantly contributes to acidification and causes a change in the composition of species in vegetation (NEGTAP, 2001).

Stevens et al. (2006) studied how N addition affects the diversity of plants within acidic grasslands throughout the UK. This investigation was conducted across grasslands experiencing N deposition rates ranging from 6 kg N ha^{-1} yr^{-1} as the lowest and 36 kg N ha^{-1} yr^{-1} as highest N level. Samples were collected from both the surface and subsoil layers, species diversity was observed to declined corresponding with rising N deposition levels, attributing this trend to the resultant soil acidification (Stevens et al., 2006). These data were collected from 68 different grasslands across the UK, where the 2 x 2 m quadrat covered 6-27 species. Identification of competitive responses of plant species was through mean C-S-R signatures and soil acidity preference index. This research tested two hypotheses. One hypothesis stated that soil acidification increases the number of species capable of tolerating low pH conditions; the second stated that soil eutrophication increases the number of highly competitive and nitrophilic species (Stevens et al., 2010). In calcifuge grasslands, the relationship between pH and soil nitrogen enrichment, both of which are influenced by N deposition, exhibit a strong correlation within the ecosystem. To assess the impact on plant communities, this study examined whether shifts in pH, nutrient enrichment, or a combination of both are driving changes in plant community characteristics (Stevens et al., 2010). The results show that the alteration in diversity as well as the composition of species in these grasslands, due to N deposition, are mainly impacted by soil acidification. This acidification fosters an environment favorable to species that thrive in acidic conditions, rather than being a result of eutrophication and the subsequent competitive pressures (Stevens et al., 2010).

Sulphur deposition has reduced considerably leading to reduced acid inputs. In recent decades there are signs of recovery from acidification in UK soils. Kirk et al. (2010) analysed the data gathered between 1978-2003 by National Soil Inventory (NSI) and reported the change measurements in soil pH to compare the results with acid deposition change during the period of survey. Soil specimens were collected, with approximately 40% of the initial sites being re-sampled between 12 and 25 years after the first collection. Kirk et al. (2010) found that the soils became less acidic in both England and Wales during the period. They found no definite regional trends of the pattern of pH change, except in south of Wales and North of England where evidence showed little or no pH change. Overall, there was an increase in soil pH up to 0.04 pH unit annually and 0.5 pH units over a period of 12.5 years, making the soil less acidic. Furthermore, in a countrywide survey measuring soil change in the UK (mainly Great Britain, Scotland and Wales) from 1978 to 2007, found that the mean pH in organic rich soils rose significantly during this period (Reynolds et al., 2013). Among the three constituent countries comprising Great Britain, the topsoils of England exhibited the highest mean pH values, attributed to the substantial presence of mineral soils and the extensive cultivation practices prevalent in the region. Conversely, Scottish topsoils consistently demonstrated the highest acidity levels, indicative of the prevalence of organic-rich soils across Scotland. Meanwhile, the mean pH values of Welsh topsoils fell intermediate between those of England and Scotland. Over the period from 1978 to 2007, there was a significant overall increase in mean topsoil pH across Great Britain and its individual constituent countries. However, it is noteworthy that Wales exhibited no significant alteration in topsoil acidity during this period.

1.1.3 Eutrophication

The term eutrophication comes from the Greek word *eutrophos,* meaning ‘well-nourished’. In terrestrial habitats or soil, eutrophication refers to the accumulation of excess minerals or nutrients. In soil, nitrogen and phosphorous are the elements most commonly responsible for eutrophication (Broadmeadow et al., 2004).

One notable N deposition impact on land-based ecosystems is eutrophication, which involves the enrichment of an ecosystem with nutrients that are otherwise in limited supply (Clark et al., 2017). In terrestrial ecosystems including fresh water ecosystems, phosphorous and nitrogen are considered limiting nutrients as plants require large quantities of these nutrients daily. A soil nutrient in leads to reduced plant life in terrestrial ecosystem and fresh water ecosystems (Clark et al., 2017).

Human-induced eutrophication occurs mainly via two principal mechanisms: a) N deposition from the atmosphere and b) the application of fertilizers in agriculture. The former affects nutrient concentrations over broad areas, whereas the latter, including agricultural runoff, differs regionally and is shaped by elements like the closeness to sources of pollution and the geographical features of watersheds.

The diversity of bog, heathland, dune, and grassland ecosystems of UK has been affected by soil eutrophication (Phoenix et al., 2012). They found out that alterations in soil chemistry, resulting from acidification and eutrophication, can significantly influence ecosystem reactions to N deposition. The processes that lead to shifts in community composition can occur in stages, initially involving the disappearance of sensitive or particularly rare species due to eutrophication or toxicity. Subsequently, changes in species driven by acidification may emerge at a later stage.

The structural changes resulting from long-term fertilization can persist for decades or even become permanent (Semelová et al., 2008). Since many grasslands are naturally limited by nitrogen, their native plant species have evolved to survive in low-nitrogen conditions (Chapin, 1980). When nitrogen is no longer a limiting factor, faster-growing species with higher nitrogen needs outcompete slower-growing species that are adapted to nutrient-poor soils (Bobbink, 1991).

Increased atmospheric N has led to changes in UK grasslands by affecting the N-cycling process. Phoenix et al. (2003) notes that in the UK, increased reactive atmospheric N deposition led to changes in grasslands located in the White Peak area of Derbyshire. High inputs of pollutant nitrogen deposition in the grassland soil led to increased N loss from leaching of soluble organic N (mostly nitrate), ammonia volatilization and through denitrification during winter. Overall, N deposition did not affect the grassland plant cover however N-cycling processes were affected (Phoenix et al., 2003). N deposition even in remote and 'unpolluted' regions lead to an increase in N cycling rates, soil %N, 'plant available' KCl extractable N, and acid–base status resulting in significant effects such as loss of sensitive species in terrestrial ecosystems in the UK (Phoenix et al., 2012).

In natural grasslands, human activities like climate change and eutrophication are altering biodiversity and potentially affecting ecosystem stability (Ives and Carpenter, 2007, Reich, 2009). Nutrient inputs, such as fertilization and atmospheric deposition, generally increase productivity but reduce plant diversity (Jie-Qi et al., 2010, Stevens et al., 2004). While reduced diversity is expected to lower the stability of productivity due to decreased species asynchrony, the overall impact of eutrophication on stability remains unclear. Eutrophication might stabilize ecosystems by increasing average productivity, but it could also cause instability through changes in temporal variability (Hautier et al., 2014).

Furthermore, nitrogen deposition i.e. nutrient enrichment also intensifies competition for light, a key mechanism contributing to biodiversity loss. Increased nitrogen availability leads to rapid growth of taller species, which reduces light availability to lower-growing plants. This imbalance in light availability drives the decline in biodiversity, as shorter species are unable to compete with faster-growing or taller species for light (Tilman, 1988). The study by Hautier et al. (2009) highlights that the loss of plant diversity in grasslands following eutrophication is primarily due to competition for light, rather than competition for soil nutrients. In nitrogen-enriched environments, taller species preemptively capture light, shading understory species and causing a reduction in species richness. This competition for light exacerbates the impacts of eutrophication, leading to further loss of diversity, particularly of smaller or slower-growing plants.

The study by Suding et al. (2005) states the theory of selection based on functional traits posits that specific environments preferentially support species possessing traits that are optimally adapted to those settings, thereby enabling them to surpass species with traits that are less beneficial under those conditions. Within this context, the nutrient enriched environment marks the reduction in biodiversity by indicating that environments with enhanced nutrients tend to support species characterized by rapid growth and greater height, at the expense of species that are adapted to environments with lower nutrient availability (Keddy, 1992, Diaz et al., 1998).

To study the effect of eutrophication on grasslands, Chen et al. (2023b) investigated the effects of eutrophication on five distinct stability metrics (temporal consistency, resilience in the dry and wet conditions and the ability to recover after both dry and wet conditions) in 55 different grasslands, concerning three aspects of community structure: biomass, diversity of species, and the species composition of community. Their findings indicated that while the addition of nutrients diminished the stability of species diversity and community structure, it had no significant effect on the stability of biomass. The study also revealed that these various measures of stability were largely independent of one another, highlighting the complexity and multi-dimensional nature of stability in grassland ecosystems.

1.1.4 Secondary stress

N deposition induces secondary stress in plants by modifying the concentrations of phenolic compounds and soluble nitrogenous substances, including free amino acids. (Sutton et al., 2011).

N deposition also increases available nitrogen to plants leading to increased organic N levels, which increase palatability to pests and insects (Bobbink & Hicks, 2014).

Sun et al. (2020) found that the deposition of N can alter plant's phenolic metabolism negatively. This reduction is associated with the inhibition of the phenylalanine ammonia-lyase enzyme, which is crucial for producing phenolic compounds. Additionally, the expression of genes related to this enzyme is also suppressed by the simulated N deposition. This interference is partly due to a lowered carbon to nitrogen (C/N) ratio as a result of diminished efficiency in photosynthetic nitrogen use, leading to reduced sugar levels. Consequently, there are fewer substrates available for the creation of phenolics. Regarding plant development, simulated N deposition is likely to enhance growth and primary metabolic activities, which could negatively affect phenolic levels through a "dilution effect" or according to the Growth-Differentiation Balance Hypothesis. Additionally, the buildup of nitrogen may suppress the MYB genes transcription factor, thereby hindering the regulatory mechanisms of genes and enzymes involved in phenolic synthesis. Collectively, these factors contribute to the global-scale reduction in phenolic metabolism triggered by nitrogen deposition.

These changes contribute to an increase in secondary stress sensitivity in plants, as phenolic compounds play a significant role in plant defense mechanisms (Kumar et al., 2020). The reduction in phenolics due to nitrogen deposition weakens the plants' ability to resist pests and environmental stressors, thereby heightening their overall stress sensitivity.

Increased susceptibility to frost desiccation is one of the main secondary stresses (Liu et al., 2015, Liu et al., 2017, Schaberg et al., 2002). Frost desiccation is the gradual dehydration of plant organs above-ground during winter. Liu et al. (2016) found increased plant N levels reduced plants tolerance to water stress and the ability to recover. Elevated N levels in moss affected C and N assimilation activities, leading to serious oxidative or osmotic stress in the mosses after water deficiency. This reduced the tolerance of N-sensitive plant species tolerance and also their ability to recover after environmental fluctuations such as changes in water levels reducing the biodiversity due to species decline.

An example of the impact of increased susceptibility to disease and pests is heathland decline and biodiversity losses that occurred in the Netherlands (Van der Eerden et al., 1991, Heil and Diemont, 1983). Here, N deposition was associated with decline of heather and increase in grass cover. N deposition increased the quality of *Calluna vulgaris* as a food plant to heather beetles, and hence *C. vulgaris* declined as a result of heather beetle infestation allowing grasses such as *Molinea caerulea* and *Deschampsia flexusosa* to become dominant in the damaged heathlands (Heil and Diemont, 1983, Van der Eerden et al., 1991).

1.2 Impacts on Biodiversity in Heathlands

N deposition has various effects on terrestrial ecosystems such as increased or decreased plant growth, changes in species distributions or changes in natural cycling processes resulting in biodiversity decline (Taboada et al., 2018). In the UK, several studies have shown that N deposition has negatively impacted the biodiversity of heathlands. One study noted that N deposition reduced plant diversity and altered ecosystem functioning in UK heathlands (Southon et al., 2013). The study was conducted on 52 heathlands across a N deposition gradient of 5.9 to 32.4 kg ha^{-1} yr^{-1}. The diversity and species composition were recorded against climate and N deposition levels. The researchers noted that higher temperature and N deposition levels were associated with reduced species richness. Also, in areas with higher N levels, the nitrophilous species abundance increased.

Addition of N in areas with low N deposition was associated with more significant species diversity decline. Litter C:N ratios and enzyme (phenol-oxidase and phosphomonoesterase) activities were directly linked to reduced species richness.

Edmondson et al. (2010) found a decrease in species richness following a N deposition gradient ranging from 7 to 31 kg N ha^{-1} yr^{-1} in heather moorland. They studied four moorland sites from Scotland, five Welsh sites, and nine moorlands from English southern Pennines. N deposition negatively affected litter phenol oxidase activity and bryophyte species richness (See Figure 4). The regional survey recorded lower levels of litter extractable NH_4^+ in Scottish and Welsh sites with low N deposition, however higher levels were recorded in Peak District with high N deposition rates. Bryophyte species richness reduced with N deposition, with the Welsh site recording 19 species, however the Peak District sites had only 12 species. The *C. vulgaris* foliar N:P ratio was positively affected by N deposition (Edmondson et al., 2010).

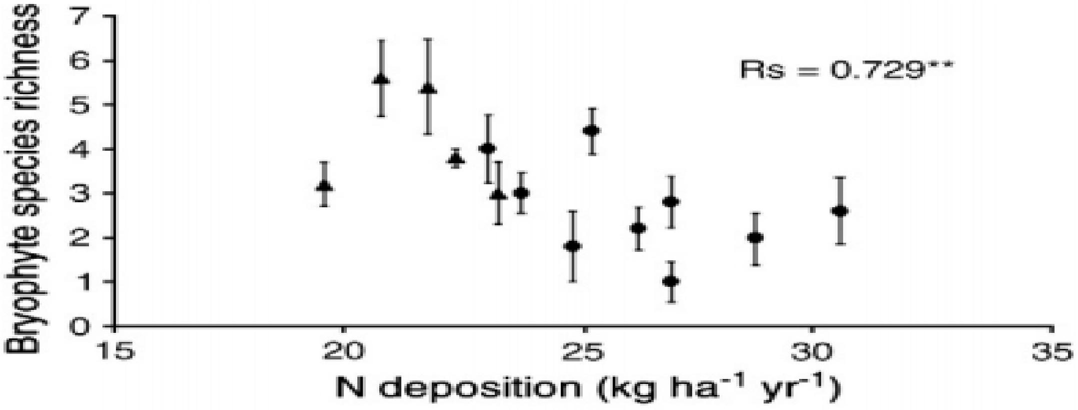

Figure 4 Increased N deposition is associated with reduced bryophytes species richness (Edmondson et al., 2010).

Similar to Edmondson et al. (2010), the study by Taboada et al. (2018) also observed a decrease in the presence of lichens and bryophytes across two stages of heathland life cycles, while noting no notable changes in the populations of annual graminoids and annual/perennial forbs throughout Europe. This research focused on evaluating the impact of N fertilizers across various nitrogen deposition gradients on *Calluna* plants, examining plant species richness, species composition and functional groups during you and mature life cycles. The results highlighted that N supplementation influenced both the shoot length and flower quantity in *Calluna* plants at mature as well as young stages. Ultimately, the study concluded that while young heathlands were more affected by N deposition, mature heathlands experienced lesser impacts. Nonetheless, lichens and bryophytes saw a significant reduction with increase in N addition.

In a study exploring the effects of N pollution on the microfauna, mesofauna, and plant populations within heathland ecosystems, Payne et al. (2012) observed a notable reduction in bryophyte populations following increased N applications at the Ruabon heathland while exploring how mesofauna, microfauna, and plant populations are affected by N addition within heathland ecosystems. This investigation aimed at finding the impact of ammonium nitrate treatments on various ecological groups within the context of a long-term experiment in the Ruabon heathland. The results demonstrated distinct differences in plant communities across treatment groups, with a significant decrease in bryophytes in areas where N was added. In contrast, enchytraeid worms showed a slight, though not statistically significant, increase in their populations following nitrogen application. Testate amoebae populations remained stable in terms of abundance and inferred biomass; however, there were significant shifts in their community composition, including a decrease in *Corythion dubium* numbers, likely as a direct consequence of bryophyte loss. The study concluded that N addition adversely affects the biodiversity of heathland plant communities, particularly manifesting as a decline in species richness and alterations in the composition of plant species, especially among non-dominant species. Ruabbon and Budworth are discussed in more detail in chapter 3.

1.3 Impacts on Biodiversity in Grasslands

The impact of N deposition on the UK grasslands has been recorded by multiple studies that have been carried out over the past three decades. Stevens et al. (2004) investigated 68 grasslands across the UK with N deposition rates of between 5 to 35 kg N ha^{-1} $year^{-1}$, recorded reduced plant species richness. One species was eliminated per 4 m^2 quadrat for every 2.5 kg N ha^{-1} $year^{-1}$ of N deposition. Regions that received at least 17 kg N ha^{-1} yr^{-1} N deposition had a 23% reduction in species. Furthermore, Stevens et al. (2006) also studied the impact of N addition plant diversity within acidic grasslands throughout the UK with N deposition rates ranging from 5 to 35 N kg ha^{-1} yr^{-1}. Studying the Atlantic region of Europe, Duprè et al. (2010) also noted N deposition was associated with vascular plant and bryophytes species richness decline. In particular, the number and proportion of dicots species reduced significantly. However, they also noted that the proportion covered by grass species increased despite the decrease in biodiversity. Tipping et al. (2013) also noted that 7.9 kg N ha^{-1} yr^{-1} for acid grassland and 23.6 kg N ha^{-1} yr^{-1} for calcareous grassland was the threshold for species retention with any N addition above that associated with species loss.

Confirming this, Phoenix et al. (2012), who used two grassland sites that involved wet and dry N deposition at rates of 0, 35, 70, and 140 kg ha^{-1} yr^{-1}, noted that addition of N promoted competition from nitrophilous species towards the dominant plant species. They noted that across different sites possessing various levels of N deposition, sites with low N deposition that received around 7.7–10 kg ha^{-1} yr^{-1} were the most sensitive to N addition compared to higher N deposition sites.

In grasslands, the addition of nitrogen causes a alterations in the natural ecosystem resulting in reduced biodiversity. Crawley et al. (2005) showed that addition of either ammonium sulfate or sodium nitrate led to changes leading to negative effects on biodiversity. The study involved the addition of 96 kg ha^{-1} yr^{-1} N as either ammonium sulfate, sodium nitrate or organic manures while another plot was left untouched. This level is higher than the N deposition in the UK. The findings of the study showed that plots with no addition of nutrients (N or P) from the experiment had the highest species richness while only N addition had a significant effect on species richness. P addition had no effect but N and P addition resulted in the largest species richness reduction (33.5 to 24.9 and 25.2 to 15.2). N addition led to a drop in pH with species richness declining linearly with pH until it fell below 4.5, where richness rapidly dropped (Figure 5). Soil pH plays a crucial role in shaping plant species richness by influencing nutrient availability, microbial activity, and soil chemistry. At extreme pH levels, either highly acidic or highly alkaline, essential nutrients such as nitrogen, phosphorus, and potassium become less accessible to plants, limiting species diversity. Additionally, acidic soils can increase the solubility of toxic metals like aluminum, which negatively impacts plant health and further reduces richness. Microbial activity, essential for nutrient cycling and decomposition, is most effective in neutral pH ranges but slows in extreme pH conditions, affecting ecosystem productivity. While some plant species are specialists adapted to specific pH conditions, such as calcicole species in alkaline soils and calcifuge species in acidic soils, extreme pH often limits the community to fewer specialized species. Overall, soils with moderate pH levels near neutrality support the highest species richness by offering balanced nutrient availability, minimal toxicity, and optimal microbial function.

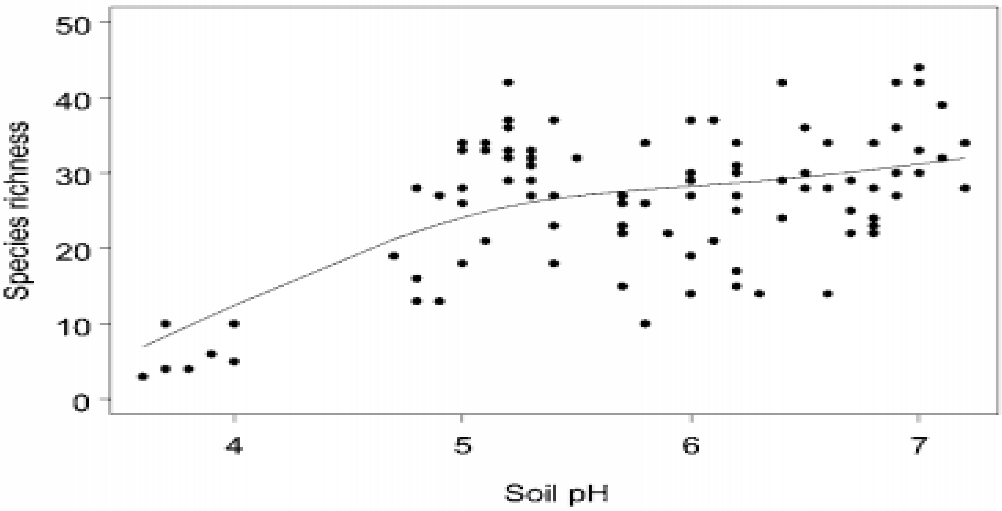

Figure 5 Correlation between species richness and pH. Species richness declines with drop in pH. (Crawley et al., 2005).

Increasing biomass in the study was also associated with a decline in species richness. An increase in biomass from 4 t ha^{-1} to 9 t ha^{-1} was linked to a reduction of species numbers from 32 to around 26 during the experiment (Crawley et al., 2005). In the study of Crawley et al. (2005), they found that the addition of 50 Kg N ha^{-1} led to loss of two species, and ammonium N lead to the loss of three more species compared to sodium nitrate whereas addition of organic manures in place of mineral fertilizers resulted in addition of two more species instead of losing.

1.4 Impacts on Biodiversity in Woodlands

N deposition effects on woodland in the UK have been documented in experimental and gradient studies. A modelling study conducted by Stevens et al. (2016) noted that N deposition caused several changes in deciduous woodlands. Of the twelve species that were examined in the study most showed a negative response to N deposition with only one species, *Juncus effusus,* showing a slight positive response. Changes in vegetation composition occurred with an increase in nitrophilous species and in grass cover. Canopy cover of the woodlands affected the response to N deposition. Nitrophilous species such as *Deschampsia flexuosa*, *Holcus lanatus, Rubus idaeus* and *Urtica dioica* were found in higher levels of cover while in low levels of cover N-sensitive species such as *Oxalis acetosella, Galium odoratum*, and ferns reduced. The most affected species was *Calluna vulgaris*. This species was affected negatively by cumulative N rates above 1817.6 kg N ha^{-1}. Different types of epiphytic lichen and bryophytes were found on different types of N stemflow. In the regions of high N stemflow, *Hypnun andoi, Hypogymnia physodes* and *Parmelia saxatilis* were able to survive and in regions with low N stemflow, *Isothecium myosuroides* and *Frullania tamarisci* were able to thrive (Stevens et al., 2016).

N accumulation is associated with competitive interaction between species and changes in composition favouring or against some species in the UK and around the world. In Scottish regions with a wide range of N deposition rates of 10, 20 and 50 kg N ha^{-1} yr^{-1}, there was a reduction in cover of *Racomitrium lanuginosum* while cover of *Carex bigelowii* increased. Similarly, there was decreased lichens cover (Bobbink et al., 2010).

A re-survey of 107 plots in Bedfordshire, England indicated that agricultural activities around terrestrial ecosystems such as woodlands affected their biodiversity (Walker et al., 2009). In woodlands around farms that typically used ammonia and other N rich products there was an increase of invasive species. Woodland species were resilient to N addition, however, invasive species, especially grass were increasing in woodlands. Keith et al. (2009) analysed 70 years of changes in the English county of Dorset and noted that eutrophication was a leading cause of taxonomic homogenization. In the 1930s, the Ellenberg N mean score was 5.02 but in 2008 it was

5.44, accounting for the loss of 117 species and gain of 47 species. The loss of biodiversity in the UK woodlands was attributed to eutrophication and more shaded conditions.

Mitchell et al. (2005) investigated the composition of Atlantic oak wood epiphytic communities with N deposition, which was associated with variations in epiphytic communities. The seven Atlantic oak woods receiving 10–53 kg N ha^{-1} yr^{-1} of N showed changes in bark pH and NH_4^+ concentration in the stemflow.

In an experimental study conducted in Sweden to understand the effects of N addition to the understory vegetation in boreal coniferous forest, the shrubs showed little response to N-addition (Palmroth et al., 2014). The study involved addition of either 0, 12.5, or 50 kg N ha^{-1} yr^{-1} of N. There was an increase in the foliar N and chlorophyl concentrations of *Vaccinium myrtillus* and *Vaccinium vitis-idaea* however there were no other changes due to increased shading under the *Pinus sylvestris* canopy. They concluded that despite increased N in the understory vegetation, the shrubs show little changes due to the increased shading as the canopies above increase their cover in response to the N addition.

Vanguelova et al. (2024) studied the impact of N deposition on biodiversity of woodland. They reported that the changes in N deposition (both increases and decreases) have been shown to affect ground flora. Different species respond variably, with some showing increases in growth and others declining, which can lead to shifts in species composition and dominance. These changes, however, are also influenced by other factors such as forest age, canopy management, and previous land use. Excessive N accumulates under conifers in UK forests, which can alter soil nutrient dynamics.

1.5 Impacts on Biodiversity in bogs

Multiple studies have shown how bogs are impacted by N deposition. Sheppard et al. (2014) noted that a slow decline in key species occurred in an ombrotrophic bog in Whim Scotland. In their experimental study, they added NH_4Cl or $NaNO_3$ at 8, 24 or 56 kg N ha^{-1} yr^{-1} and noted that sensitive non vascular plant species exhibited the most negative effects to N addition. They noted that *Calluna vulgaris* and *Hypnum jutlandicum* increased while *Pleurozium schreberi, Sphagnum capillifolium, Eriophorum vaginatum* and *Cladonia portentosa* declined. Ammonium had more negative effects than nitrate as it caused the most N accumulation. In another study recording the early effects of atmospheric N deposition, they noted that a 150–200 kg N ha^{-1} y^{-1} cumulative deposition over one year was associated with increased growth of *Calluna vulgaris* in an ombrotrophic bog (Carfrae et al., 2004). Cumulative ammonia deposition up to 400 kg N ha^{-1} y^{-1} was associated with a rise in foliar N content and a marked decrease in chlorophyll concentration. The researchers concluded that N deposition had significant impacts on plant stress measures.

A study conducted at Whim bog in Scotland, which explored the prolonged impact of nitrogen supplementation alongside phosphorus or potassium on the nitrogen status of Sphagnum mosses, found that long-term application of potassium and phosphorus does not counteract nitrogen saturation in Sphagnum (Chiwa et al., 2018). This research, spanning 11 years, involved administering ammonium or nitrate at 56 kg N ha^{-1} yr^{-1} to a location that naturally receives an ambient N deposition of 8 kg N ha^{-1} yr^{-1}, both with K and P as well as the absence of K and P. The findings indicated that nitrogen supplementation influenced the growth and coverage of Sphagnum by increasing nitrogen accumulation in the plant's tissue, which in turn enhanced growth and coverage. In the initial phase (under 5 years), the inclusion of phosphorus and potassium appeared to mitigate the effects of N supplementation. However, during the long-term experiment (beyond 5 years), these effects of nitrogen supplementation were not diminished. The study also noted no significant differences in plant nitrogen content between the use of ammonium and nitrate in the supplementation process (Chiwa et al., 2018).

Another study examining the effect of N addition on *Drosera rotundifolia* (carnivorous plant), which was conducted across 16 ombrotrophic bogs throughout Europe, found adverse effects (Millett et al., 2015). This investigation revealed that N deposition correlated with a decrease in interactions between carnivorous plants and their prey, thereby diminishing the nutrient exchange across different trophic levels. This was a result of the plants obtaining large amounts of N through root uptake instead of prey N that contributed between 20-60% of tissue N (Millett et al., 2015). (Sutton et al., 2004) studied ammonia emissions and deposition and its effects on semi-natural ecosystems of Scottish and noted that bogs were vulnerable to changes in available N. In 45% of the bogs, nutrient N deposition exceeded the estimated critical loads of N. Furthermore, *D. rotundifolia* were also found to be susceptible to negative effects of N deposition at 10 kg N ha^{-1} year^{-1}.

1.6 Impacts of Biodiversity on flowering

N deposition can significantly impact plant biodiversity, with one key mechanism being its effects on flowers and flowering. Flowering is crucial for plant reproduction, pollinator interactions, and genetic diversity maintenance within ecosystems (Cleland et al., 2007). The deposition of atmospheric nitrogen can modify the timing of flowering events, which in turn could change the quantity and accessibility of pollen and nectar rewards. This may lead to a mismatch in phenological timing (Stevens et al., 2018). The change in flowering time can result a change in biomass of the plants as Casler (2020) proved that the delay in flowering can cause an increase in biomass.

N deposition affects flowering differently across ecosystems. Britton and Fisher (2008) found that nitrogen increased shoot growth for *Calluna vulgaris* compared to burning and clipping. Over five years, there were no significant differences for different N additions ranging from 0 to 50 N ha^{-1} y^{-1}. In the third year, 50 kg N ha^{-1} y^{-1} plots had the lowest flowering. By the fourth year, flowering matched the control in these plots, while 10 as well as 20 kg N ha^{-1} y^{-1} plots saw increases. As fifth year approached, all nitrogen-treated plots showed increased flowering (Figure 6).

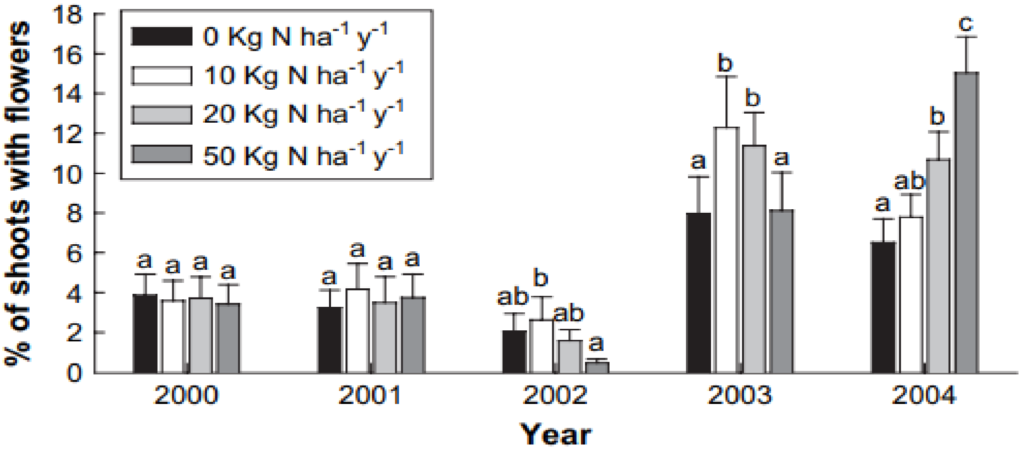

Figure 6 The addition of nitrogen affects *C. vulgaris* average percentage of flowering shoots, as indicated by the error bars showing the standard error of the mean. When comparing means within the same year, those that do not have identical letters differ significantly, with a significance level set at P that is less than 0.05 (Britton and Fisher, 2008).

Taboada et al. (2018) showed that N addition increased the annual flowering of *Calluna* plants. However, when N loading was increased to higher levels, the annual flowering of young plants was observed to decline. Their study involved N addition at rates of 0, 10, 20, 50 and 56 kg N ha^{-1} yr^{-1} and showed that flowering increase corresponded to 20, 50 and 56 kg N ha^{-1} yr^{-1}. However, at the end of nine years, the 56 kg N ha^{-1} yr^{-1} was associated with reduced flowering due to N accumulation. N addition had different effects in both young and mature life cycle stages with increase N associated with reduced flowering in young plants. Taboada et al. (2018) concluded that increased flowering observed with N addition may have no effect at increasing the reproduction of plants as well as the regeneration of heathlands due to other factors such as competition and absence of light.

Phoenix et al. (2012) showed that flowering in dominant shrub and forb species showed high sensitivity to N deposition with increases and declines observed at even the lowest N additions of 7.7–10 kg ha^{-1} yr^{-1}. They looked at a range of long-term experiments and noted that the minimum deposition rate (kg N ha^{-1} yr^{-1}) required to observe a significant effect in flowering. Increase in flowering was observed at the deposition rate of (7.7 kg N ha^{-1} yr^{-1} in Thursley), (10 kg N ha^{-1} yr^{-1} in Culardoch), 20 kg N ha^{-1} yr^{-1} in Ruabon, 60 kg N ha^{-1} yr^{-1} in Budworth, and 20 kg in Pwllperan while Wardlow acidic and calcareous grasslands showed a decrease in flowering at at 140 kg N ha^{-1} yr^{-1} and 35 kg N ha^{-1} yr^{-1} respectively. These changes in flowering were attributed to different species that are sensitive to N addition with grasslands showing a significantly reduced flowering with increased N deposition (Phoenix et al., 2012).

Vergeer et al. (2008) conducted an investigation to observe the response of *Arabidopsis lyrata petraea* to N deposition, explaining how variations in available N influenced the species' growth, leaf turnover rates, and flowering patterns. The experimental design involved the addition of N at rates of 3, 9, or 27 kg ha^{-1} yr^{-1}. The experimental works, categorized according to low and high deposition rate zones, observed that lower N deposition levels correlated with accelerated growth, elevated leaf turnover rates, and shorter time to flowering compared to sites experiencing higher N deposition. Consequently, it was concluded that the species acclimating to elevated N deposition rates exhibited diminished plasticity.

Ma et al. (2023) shows the effect of N deposition on flowering phenology of *Medicago sativa* in an experiment lasting one year. An experiment was conducted with N concentration of 0 and 6 g N m^{-2} $year^{-1}$. The results revealed that N addition had significant effects on the flowering phenology of *M. sativa*, although the effects varied across different phases of the flowering process. For *M. sativa*, N addition caused a delay of approximately 1.9 ± 0.39 days in the onset of first flowering day while plant species richness was observed to have no effect on it. Conversely, the last flowering day happened sooner by 1.2 ± 0.21 days in response to N addition. Additionally, plant species richness was associated with an earlier occurrence of the last flowering day, with reductions of 0.51 and 0.77 days observed in control and N addition plots, respectively. Overall, the duration of flowering shortened by approximately 3.2 ± 0.31 days. Furthermore, the study also found a significant decrease in flower numbers after N addition, although an increase was observed with higher levels of plant species richness.

Liu et al. (2017) studied the flowering response of three species *Anemone trullifolia var. linearis*, *Caltha scaposa*, and *Trollius farreri* to N addition in the form of urea 5 g N m^{-2} yr^{-1} for 2 years from April 2008 to 2010. The addition of nitrogen did not change the first flowering day in the three species. However, it caused a delay of 4.5 days in *A. trullifolia var. linearis* and 3.9 days in *C. scaposa*. Additionally, the N addition resulted in a delay of last flowering day for *A. trullifolia var. linearis* equal to 3.6 days and in *C. scapose* delay was observed to be equal to 6.0 days. Conversely, it extended the last flowering day of *T. farreri* by 3.4 days.

1.7 Impacts of Nitrogen on seed banks

One way that nitrogen can influence biodiversity is by affecting the seed bank (Wang et al., 2021). The seed bank is vital for sustaining plant communities, especially in times of environmental stress or change. It acts as a buffer, allowing for the regeneration of species that might otherwise be outcompeted or lost (Bobbink and Hicks, 2014). However, changes in the seedbank due to increased N deposition can have far-reaching consequences. N deposition promotes alteration in soil seed bank composition mainly through changes in seed germination (Ochoa-Hueso and Manrique, 2010, Plassmann et al., 2008, Zhong et al., 2019). In terms of ecological periods, seed banks serve as 'biodiversity reserves' within a locality, aiding in the continuation of local populations and the preservation of biodiversity via the effects of temporal storage (Faist et al., 2013, Plue and Cousins, 2013). Furthermore, seed banks play a crucial role in preserving genetic diversity and various traits within local communities, thereby offering a potential foundation for resilience against disruptions or shifts in the environment (Vandvik et al., 2016). Hence a change in seed bank as a result of N deposition can cause alterations in biodiversity.

Seed germination depletes the population of seeds stored in soil seed banks (Van Mourik et al., 2011). It can be postulated that any factor that increases the rate of seed germination negatively affects the population of seed stocks contained in respective seed banks (Gallandt, 2006). Plassmann et al. (2008) studied the impacts of N deposition on seed germination. Forty-five species collected from the southwest coast of the Isle of Anglesey and grown in greenhouse trays received either water or N in the form of ammonium nitrate at a dose of 15 kg ha^{-1} yr^{-1} once a week from September 2004 until February 2005. This level of N addition, 15 kg ha^{-1} yr^{-1}, approximates the actual N addition that is experienced in the area the seeds were collected from

(Mohd-Said, 1999). Plassmann et al. (2008) observed that trays that received N had significantly more seedlings than the control.

Basto et al. (2015) examined the effects of nitrogen deposition on the dynamics of the soil seed bank through a simulation study conducted in an acidic grassland at the Wardlow site in the UK. Initiated in 1995 and spanning 13 years, the study applied nitrogen at rates of 0, 35, and 140 kg of N per hectare per year to fixed vegetation plots. The findings indicated a significant reduction in the overall seed count within the seed banks due to nitrogen enrichment. Specifically, seed counts in plots treated at 140 kg N per hectare per year saw a 61% reduction by the study's conclusion, while those receiving 35 kg N per hectare per year experienced a 34% decrease. Furthermore, the seed banks that were adversely impacted by N addition showed no signs of recovery even four years after the cessation of N deposition.

The number of seeds in respective plots was negatively correlated with the amount of N deposited (Figure 7). Plots that received 140 kg N ha^{-1} yr^{-1} had significantly lower numbers of seeds in the seed bank than plots that received 0 or 35 kg N ha^{-1} yr^{-1}. It was observed that at depths of >14 cm, the number of seeds in respective plots was almost the same for all treatments. This seemed to contradict the conclusion that N negatively affected the quantity of seeds in soil seed banks (Basto et al. 2015). However, poor seed germination at depth, due to lack of oxygen Seiwa et al. (2002) is a possible explanation. The mechanisms behind the differences in seed population in plots that received different N concentrations were not explained. However, Basto et al. (2015), suggested that since N deposition stimulates seed germination, N pollution can increase the number of seeds that germinate, depleting the population of seeds within a seed bank.

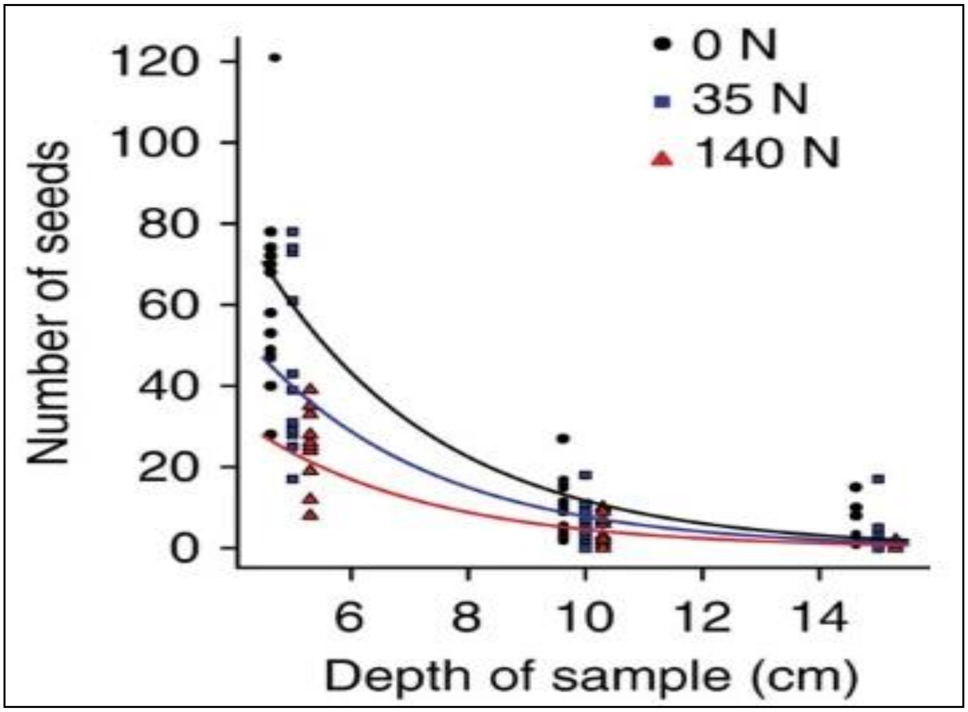

Figure 7 Impacts on N deposition on number seeds in soil seed banks at different depth (Basto et al., 2015).

Wang et al. (2021) conducted a practical experiment in a subtropical evergreen broadleaved forest to explore the impact of N addition on the seed bank's structure and diversity. Nitrogen was applied at two different rates, 50 kg N ha^{-1} yr^{-1} and 25 kg N ha^{-1} yr^{-1} to the understory (designated as UAN50 and UAN25) and forest canopy (designated as CAN50 and CAN25). The study observed the emergence of 1521 seedlings, encompassing 37 different species, from soil samples extracted from a depth of up to 10 cm. The results indicated that short-term simulated N deposition, particularly via understory N application, exerted minimal influence on the species diversity as well as the abundance of the seed in the soil seed bank. However, N addition to the canopy was found to affect both the quantity of seeds stored and the overall seed bank composition. Seed densities in the soil layer from 0 to 10 cm varied between 758.3 and 1634.4 seeds m^{-2}. In the top 0–5 cm soil layer, seed densities were generally increased in the control, CAN25, and CAN50 plots, with lower densities in the UAN50 as well as UAN25 plots, though these disparities did not reach statistical significance. Notably, the CAN50 plots exhibited the highest seed bank densities within the 0–5 cm layer compared to other nitrogen treatment plots. For the entire 0 to 10 cm soil layer, the CAN50 plots showed greater seed bank density than the control plots. The study also found differences in soil seed bank composition between the CAN50 as well as CAN25 plots versus the control plots, which indicates that the composition of species might be altered by canopy N additions within soil seed banks. In contrast, the soil seed bank communities in control, CAN25, and CAN50 plots showed more similarity to each other than to those in the canopy N-addition plots. Additionally, species richness or diversity indices showed no significant effect such as Simpson, Shannon-Wiener, or Pielou in either 0 to 5 cm or 5 to 10 cm soil layers. Niu et al. (2023) conducted research on how seed bank in the grassland of Loess Plateau in China is affected by N addition. The study utilized two forms of N supplementation: ammonium-nitrogen (NH_4^+-N) and nitrate-nitrogen (NO_3^--N), across six varying levels of concentration: (0, 0.05, 0.1, 0.15, 0.20 and 0.25 g N kg^{-1}). Additionally, two different soil moisture conditions were tested: a high watering (HW) condition, where soil was watered daily to maintain field capacity, and a low watering (LW) condition, where watering occurred every two days to reach field capacity. The study revealed that the LW moisture condition significantly inhibited the soil seed bank germination process as compared to the HW condition, and also led to a notable difference in the species composition of the seedlings that emerged. In conditions of high watering, the addition of both NH_4^+-N, and NO_3^--N significantly promoted the germination within the soil seed bank. Increasing levels of N addition impacts the seed germination in various ways, being negative for NO_3^--N and positive for NH_4^+-N. Remarkably, even under the LW condition, the addition of N still somewhat encouraged germination from the seed bank. The research also highlighted that composition of species that emerged from the seed bank was not altered with a single external application of N. Yet, under the LW condition, the variation in composition of species between N added plots and N absence plots grew progressively with higher levels of N. This study indicates the critical role of soil water conditions in the germination of seed banks and the determination of the species composition of emerging seedlings. N supplementation was found to foster seed bank germination, an effect that was modulated by the soil's moisture content. Over time, elevated N inputs could reduce the soil seed bank's viability if the influx of new seeds does not surpass the rate of seed germination and mortality, suggesting a nuanced interaction between N addition, soil moisture, and seed bank dynamics (Niu et al., 2023).

1.8 Impacts of Nitrogen on phenology

Phenology refers to the timing and length of biological occurrences, playing a very important role in the survival and cohabitation of species within plant communities (Forrest and Miller-Rushing, 2010). The phenological patterns of plants are highly responsive to changes in the surrounding environment, with adjustments in phenology due to species adapting to environmental conditions potentially leading to significant impacts on plant reproduction, the structure of plant communities, and overall ecosystem functionalities (Meineri et al., 2014, Parmesan and Yohe, 2003, Sparks and Carey, 1995). The response of functional groups' phenology to nitrogen enrichment varies, potentially influenced by their life history, physiological and morphological characteristics (Kliber and Eckert, 2004). Changes in phenology due to N deposition can significantly impact the biodiversity of plant ecosystems. Accelerated plant growth and earlier flowering times, often induced by increased N levels, can create competitive imbalances. Species that flower earlier may gain an advantage in resource acquisition, potentially outcompeting and suppressing later-flowering species (Hautier et al., 2009). This competitive exclusion can cause a decrease in richness of species along with the alteration in composition of community (Bobbink et al., 2010).

Seed germination is considered to be an important phenological phenomenon that defines the structure of an ecosystem (Lunt et al., 2012). Wang et al. (2020) conducted a study to determine the impacts of N deposition on seed germination in wheat seeds. The doses of N were set at 0, 5, and 10 $gm^{-2}/year^{-1}$ representing no N deposition (CK), low N deposition (NL), and high N deposition (NH) respectively. The NH and NL treatments germinated more quickly than the controls, indicating that N pollution negatively affects the phenological process of germination in wheat (Wang et al., 2020).

Power et al. (1998b) explored the impacts of N pollution on the phenological phenomenon of budding in *Calluna vulgaris* grown on acidic greensands within the Thursley Common National Nature Reserve, England. Different levels of N pollution were simulated by the addition of an equivalent of 7 ± 7 kg N $ha^{-1}year^{-1}$), 15 ± 4 kg N ha^{-1} $year^{-1}$, an artificial rain solution which was the control, or an alternation of the two N treatments. The experiment, which ran from 1994 to 1996, revealed that high N levels accelerated spring bud burst. On any given day in spring, the proportion of *C. vulgaris* shoots that began growing in high-N plots was significantly more than that observed in all other plots. Over the three years, high N plots had twice as many shoots bursting as the control plots (Power et al., 1998b). This is an indication that N has an impact on physiological activities that take place in the species at the onset of spring (Power et al., 1998b).

An experiment conducted by Xi et al. (2015) on Tibetan plateau in an alpine meadow investigated how plants' phenology responds to N addition. The findings highlight the significant influence of N addition on regulating plant phenology, particularly for dominant species. The study utilized a full block layout, incorporating four distinct nitrogen treatment intensities: 0N, 2.5N, 5N, and 10N (representing the amounts of N added per square meter per year, using urea as the source).

Phenological phases of seven species were carefully monitored, including five forbs (*Saussurea stolickai*, *P. saundersiana*, *P. bifurca*, *P. cuneata*, and *Y. simulatrix*), one sedge (*K. pygmaea*) and one graminoid species (*Festuca coelestis*). The results indicated that N addition significantly influenced the phenology of the dominant species. It led to a notable advancement in budding time and an extension in the duration of the flowering and reproductive phases for these dominant species. However, N addition did not show any obvious effects on the accompanying species. Additionally, different levels of N addition did not result in distinct phenological responses for each individual species, and there were no noticeable differences in phenology among the various N addition treatments.

Wang and Tang (2019) carried out an extensive examination of 117 plant species to explore the reaction of plant phenology to the addition of N across different terrestrial ecosystems. They also examined how these responses vary depending on functional groups, ecosystem types, and environmental conditions. In this study, eight key stages of plant phenology were considered, including the initiation and duration of different phases. Across all studied biomes, the initiation of phenological events was significantly delayed following N addition. This delay was observed to be 0.77 ± 0.02 (budding), 0.91 ± 0.01 (flowering), 1.0 ± 0.01 (fruiting), and 6.30 ± 0.01 (maturity) days. Conversely, the date of senescence advanced significantly (-0.43 ± 0.01 days).

Furthermore, the analysis revealed a significant reduction in -0.21 ± 0.01 (flowering duration), and 4.01 ± 0.02 days (reproductive duration) following N deposition. Conversely, there was a notable extension of -0.52 ± 0.01 days in fruiting duration.

Thesis structure

The objectives of this thesis are:

1. To understand the extent of the effect of N deposition on biomass, flowering, and phenology on sensitive or less species at different levels of N addition.
2. To further our knowledge of how the seed bank responds to long-term N addition and to compare this to the above-ground plant community in heathland habitat. This will help understand what the implications are for ecosystem function.
3. To understand the impact of nutrient addition and the disturbance and the interaction between them affect the species composition.
4. To understand how the soil of grassland recovers from N deposition and the role of mowing on the recovery process.

In chapter 2, I investigate the impact of N deposition on biomass, flowering, and phenology in a pot experiment with various levels of NH_4NO_3 addition. Four different grassland species selected because of their sensitivity to N deposition and two were less sensitive were analysed to identify changes in above-ground biomass, below-ground biomass, total biomass, plant height, flowering and seeds, plant tissue chemistry, and phenology.

In chapter 3, I study the influence of long-term N deposition on the seed bank of two long-term heathland experiments, Budworth and Ruabon. I examined the potential relationship between above-ground vegetation and seed bank responses following over twenty years of N addition.

In chapter 4, I look at the effect of nutrient additions and the disturbance and the interaction between them on the species composition as part of the global experimental collective

DragNet. I analyse two years pre-treatment data and two years post-treatment data, investigating seed banks, biomass, plant species composition and light levels.

In my final experimental chapter, chapter 5, I investigate the impact of N deposition on the recovery of soils from long-term N addition. To do this I use a long-term N addition experiment.

Chapter 6 discusses general conclusions and potential future research directions.

2 The effects of N addition on flowering and growth traits

2.1 Introduction

Nitrogen deposition has a significant effect on plants as shown in many studies (Stevens and Gowing, 2013, Liu et al., 2017, Lu et al., 2021, Allison, 2002). N deposition not only leads to change in above-ground and below-ground biomass (Stevens and Gowing, 2013) but also the phenology (Liu et al., 2017), seed number (Allison, 2002) and C:N ratio (Lu et al., 2021).

Flowering is defined as a shift from a plant's vegetative state to its reproductive phase (Zhang et al., 2022). The specific timing of occurrence of flowering is governed by many factors, including temperature variations, photoperiod, and stress factors (Zhang et al., 2022). A timely initiation of flowering is essential for plants to have a growth period optimized for the efficient utilization of light, water, nutrients, and other resources (Andrés and Coupland, 2012, Kazan and Lyons, 2016, Robledo et al., 2020). Furthermore, the availability of nutrients in the soil exerts a significant influence on flowering time (Cho et al., 2017). For instance, a decrease in nutrient availability to plants alters the flowering time, forcing the plants to have early flowering (Kolář and Seňková, 2008).

Many studies have demonstrated that N supplementation in grasslands leads to the augmentation of plant growth and biomass (Bai et al., 2010, Chen et al., 2011, Gong et al., 2011). As the quantity of supplemented N is increased, there is often an initial surge due to the alleviation of ecosystem N limitation and enhancement of soil nutrient status, followed by a subsequent decline in above-ground biomass (Fang et al., 2012, Li et al., 2015) as excessive N fertilization can induce soil acidification (Yao et al., 2014) and impede plant root growth (Liu et al., 2013). Consequently, when the level of N supplementation surpasses a certain threshold, above-ground biomass tends to decline. This initial boost in biomass can be attributed to due to N supplementation (Ladwig et al., 2012, Zhang et al., 2004).

Several studies have found that the increase in nitrogen deposition increases the number of flowers (Viik et al., 2012, Burkle and Irwin, 2009, Hoover et al., 2012, Munoz et al., 2005). For instance, Nams et al. (1993) *Achillea millefolium* L. produced an increased number of flowering individuals with fertilizer treatment containing N. The timing and duration of flowering, constitute crucial developmental stages in plant phenology, are influenced by a variety of abiotic factors, including temperature, photoperiod, and resource availability (Zhang et al., 2022). The modification of flowering timing due to either an excess or deficiency of specific mineral nutrients has been documented across various plant species (Cho et al., 2017, Kolář and Seňková, 2008, Kazan and Lyons, 2016, Wada and Takeno, 2010). N addition has been observed to cause a delay in first flowering day and advancement in last flowering day leading to a shortened flowering duration, for example *Medicago sativa* L. flowering duration was found to be reduced by 3.2 ± 0.31 days as observed by (Ma et al., 2023). Furthermore, nitrogen deposition also alters the seed production as discussed in chapter 3. For instance, Phoenix et al. (2012) found that N deposition negatively affects flowering leading to a decrease in seed production.

Some plants are more sensitive to N deposition while others are less sensitive. Four species were selected for this study, *Achillea millefolium* L., *Lotus corniculatus* L., *Plantago lanceolata* L. and *Prunella vulgaris* L. N deposition affects the plants in various ways, for instance, Stevens and Gowing (2013) show that N addition significantly increases above-ground biomass of *Plantago* *lanceolata* L and *Prunella vulgaris* L. Furthermore, N deposition has also been shown to significantly decrease the biomass of *Lotus corniculatus* L. (Zhao et al., 2022) whereas some other studies have reported no significant effect on it (Briggs, 1991). No significant effect of addition of N on biomass of *Achillea millefolium* L. has been reported (Plassmann et al., 2009), while N depositon has been shown to increase the yield of flowers in case of N addition to an *Achillea millefolium* L. cultivar (Tatar et al., 2013). Furthermore, N addition can increase the seed number of *Lotus corniculatus* L. (Briggs, 1991). N addition was observed to increase N concentration of the plant, leading to a decreased C:N (Ordoñez et al., 2009). Similarly, Shen et al. (2019) showed that the addition of N increases the N content of the tissues leading to a decrease in C:N in forbs. On the other hand, Schuster et al. (2016) observed no effect of N deposition on N concentration as well as C:N in tissue of forbs.

Only a few studies have shown the effect of N deposition on date of seed production. Peng et al. (2018) found that the addition of N had no effect on seed setting date of *Solidago canadensis*. Furthermore, Nogueira et al. (2017) also showed that N deposition has no effect on delaying or advancing plant phenology i.e. flowering and development of seeds.

Hypothesis

The effect of N deposition on plant traits shows a knowledge gap and few studies look at phenology. This experiment applied N additions at different levels of NH_4NO_3 in a pot experiment to *Achillea millefolium* L., *Lotus corniculatus* L., *Plantago lanceolata* L. and *Prunella vulgaris* L. to investigate the impact of N deposition on biomass, flowering and phenology. This experiment focused on plant traits in response to N under the same conditions across the treatments for four selected species.

The following are the study's hypotheses after adding N treatments:

1- N addition will increase the biomass of all plant species.

2- N addition will increase the number of flowers of in all species and increase the number of seeds.

3- There will be observable differences in plant tissue chemistry between control and N addition plots in all species in terms of increased N concentration and decreased C:N ratios.

4- N addition would change the phenology of all species resulting in delayed flowering.

2.2 Methods

2.2.1 Experimental design

Four species were selected for this study, *Achillea millefolium* L. (Forb)*, Lotus corniculatus* L. (Legume)*, Plantago lanceolata* L. (Forb) and *Prunella vulgaris* L. (Forb). The seeds were purchased from Emorsgate seeds (Emorsgate seeds, Kings Lynn, UK). They have been selected because they were sensitive to N deposition and others were less sensitive, The more sensitive species were: (1)- *P. lanceolata,* (2)- *L. corniculatus*, (3)- *A. millefolium* and (4)- *P. vulgaris* (Stevens and Gowing, 2013).

Four different species of plant seeds were sewn in seed trays in early February 2023, sewing a minimum of 75 seeds each. These were kept in an indoor greenhouse at Lancaster Environment Centre of Lancaster University, United Kingdom with a mean temperature 20°C, 61% relative humidity and light value 109.78 Wm^{-2}. Once seedlings germinated, they were transferred into plug trays for growth. At the end of April, 15 healthy plants were selected from each species, and were transplanted into pots (1 plant per pot). Two-litre pots (13.2 cm height, 16.7 cm diameter) were filled with soil-sand mix. Soil was sieved to 9 mm and mixed with horticultural sand with a ratio of 70 % soil and 30% sand. The soil had a pH of 8.1, total N concentration of 0.12 %, and total C concentration of 1.47 % (Davis, 2024). Pots were kept in an unheated greenhouse with a mean temperature of 19°C. The pots were watered 3 - 4 times per week from above with tap water to maintain soil moisture. The experiment was set up with 3 pots of each treatment for each species. N additions were applied in mid-May 2023 at rates of 10, 20, 50, and 70 kg N ha^{-1} $year^{-1}$ of NH_4NO_3 or tap water only (control plots).

During the first two months, the plants were checked 2 days a week in terms of flowering time and growth traits were measured. In addition, phenological measurements were recorded including the date of the first buds, the date of the first flower opened, and the date of flowers that produced seeds. In addition, the number of flowers for each species was counted. At the end of the experiment mesh bags were put over seed heads when they formed to ensure seeds were not lost. After flowering, the seeds of *Lotus corniculatus* L. and *Plantago lanceolata* L. were counted. Unfortunately, *Prunella vulgaris* L. did not flower. Seeds of *Achillea millefolium* L. were too small to count. During the experiment there was very hot weather for several days that affected the growth and very wet weather at other times led to some fungal infections. The experiment was terminated at the end of September 2023. At the end of the experiment, plant height was recorded. The plant height was measured in centimeters (cm) from the soil surface to the highest point of the plant. Above-ground vegetation was cut at the soil level of each pot and the heads of mature seeds were separated. Above-ground biomass was determined after drying at 60ºC for 72 hours. Root biomass was determined by washing soil from roots with tap water. Samples were dried at 60°C, the biomass was recorded.

A ball mill was used to grind the dehydrated materials into a fine powder. Then, C and N concentrations in above-ground biomass were determined by using an auto analyser (elementar, vario EL III).

2.2.2 Data analysis

The data was analyzed using SPSS version 27 (SPSS 27, IBM, released 15.0.1., 2020) (SPSS 27, 2020) with N treatment as a fixed factor by One-Way ANOVA. Before analysis, all variables were examined, and the normality was checked. For the data that were not normally distributed a logarithmic transformation (log 10) was used. Tukey's technique (SPSS 27, Microsoft Corporation, released 15.0.1., 2020) (SPSS 27, 2020) was used for post-hoc pair-wise comparisons of all treatments. 0.05 was set as the significance value.

2.3 Results

2.3.1 Above-ground biomass

There were no significant differences in above-ground biomass of *L. corniculatus*, *P. vulgaris*, *A. millefolium* and *P. lanceolata* between the treatments (ANOVA; F= 0.354 ; df= 4 ; P = 0.832, F= 1.557 ; df= 4 ; P = 0.299, F= 2.504 ; df= 4 ; P = 0.109 and F= 1.505 ; df= 4 ; P = 0.280 respectively). Although *A. millefolium* had the highest average biomass in the 70 N treatment compared to the control plot, this difference was not significant. The above-ground biomass of

P. lanceolata in the 50 N treatment was higher than in the control plot, but this difference was also not significant (Figure 8).

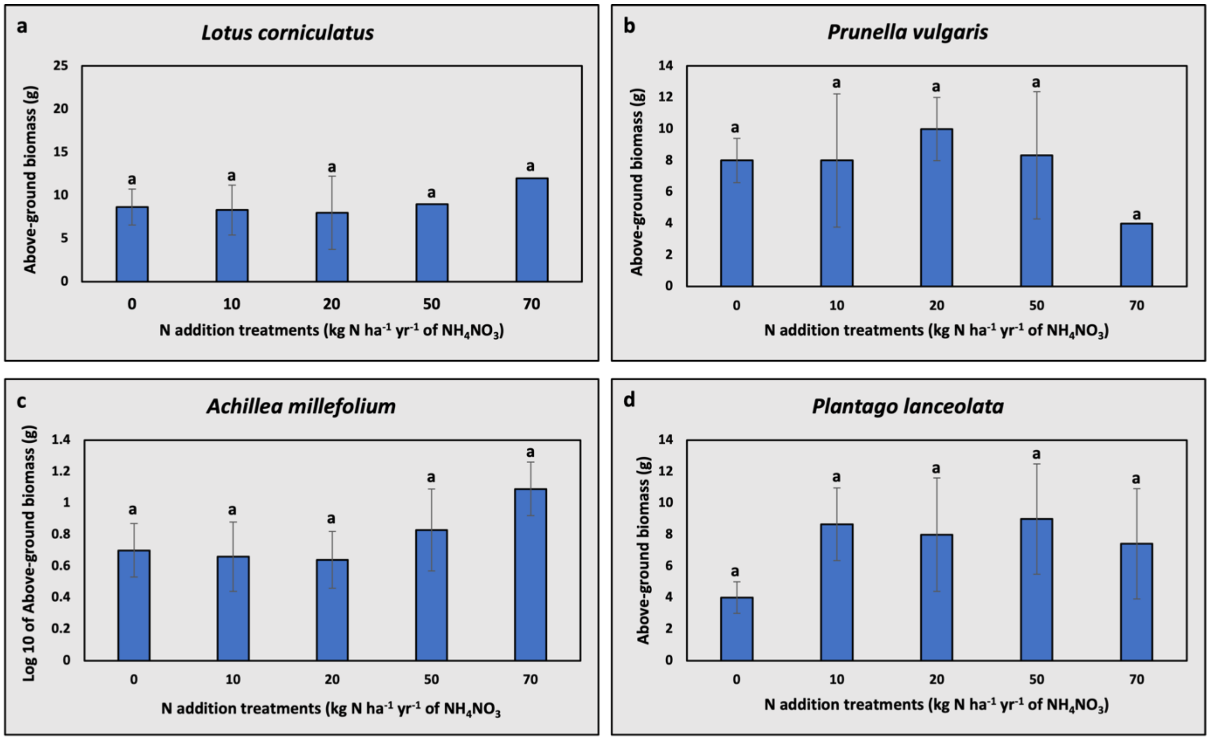

Figure 8 (a) The average of above-ground biomass for *L. corniculatus*, (b) *P. vulgaris*, (c) *A. millefolium* and (d) *P. lanceolata* N0, N10, N20, N50 and 70 indicate the previous N additions (control, 10 kg N ha^{-1}yr^{-1}, 20 kg N ha^{-1}yr^{-1}, 50 kg N ha^{-1}yr^{-1}, or 70 kg N ha^{-1}yr^{-1}). Values are Mean ± SE, those sharing the same letters denoted no significant differences between the groups based on Tukey's HSD post-hoc test.

2.3.2 Below-ground biomass

Below-ground biomass of *L. corniculatus*, (b) *P. vulgaris*, (c) *A. millefolium* and (d) *P. lanceolata* did not show any significant differences between the treatments (ANOVA; F= 3.512 ; df= 4 ; P = 0.100, F= 1.259 ; df= 4 ; P = 0.381, F= 1.875 ; df= 4 ; P = 0.191 and F= 1.154 ; df= 4 ; P = 0.392 respectively). While biomass of *L. corniculatus* increased with N treatment there were no significant difference between the treatments. *P. vulgaris* biomass showed a steady increase with N addition treatments but the loss of some plants resulted in low replication and meant that differences were not significant. The average of below-ground biomass of *P. lanceolata* in plots under all N addition treatments was higher than in the control plots, but data were very variable (Figure 9).

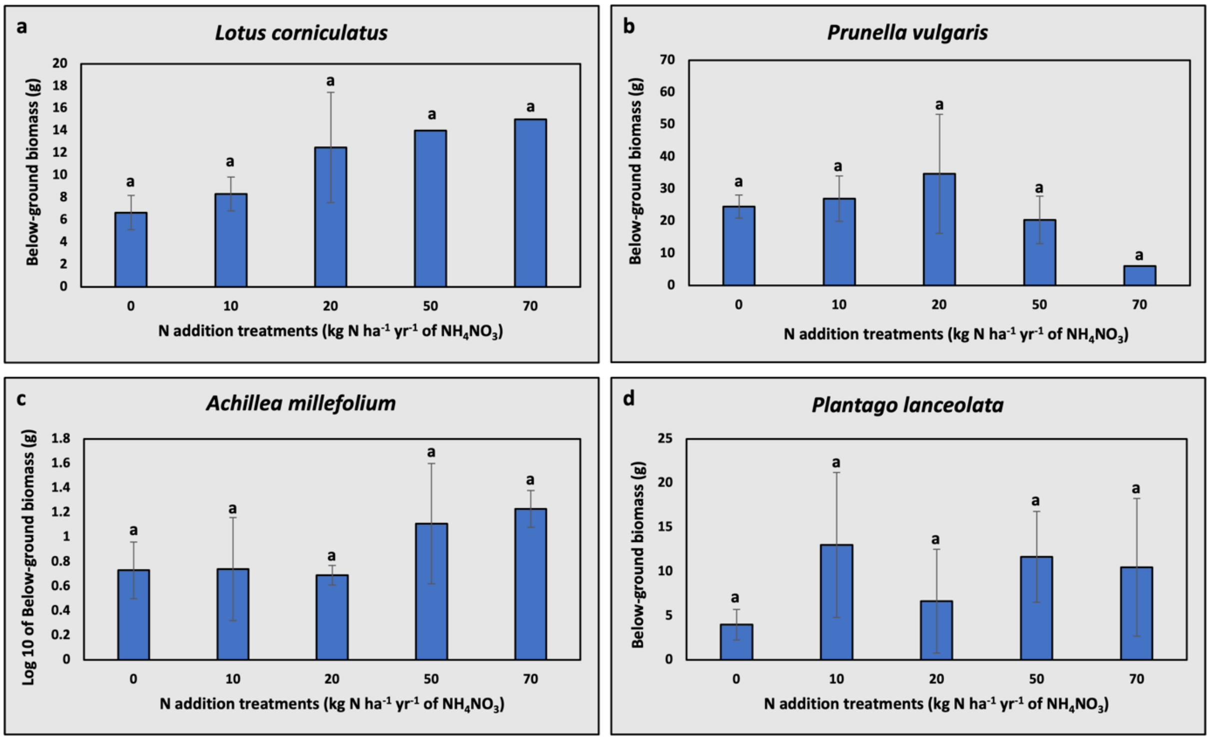

Figure 9 (a) The average of below-ground biomass for *L. corniculatus*, (b) *P. vulgaris*, (c) *A. millefolium* and (d) *P. lanceolata* for each treatment. Values are Mean ± SE, those sharing the same letters denoted no significant differences between the groups based on Tukey's HSD post-hoc test.

2.3.3 Total biomass

Total biomass did not differ significantly across treatments (ANOVA; F= 1.272 ; df= 4 ; *P* = 0.391, F= 0.1.683 ; df= 4 ; *P* = 0.271, F= 1.800 ; df= 4 ; *P* = 0.205 and F= 1.378 ; df= 4 ; *P* = 0.316 respectively), while a minor trend for increasing total biomass N was noted. The highest total biomass of *L. corniculatus* was found in the 70 N treatment, while the lowest was found in the control plot. However, no significant difference in the treatments was noted. For total biomass of

P. vulgaris the 70 N treatment was found the lower than other treatments, but not significantly so (Figure 10).

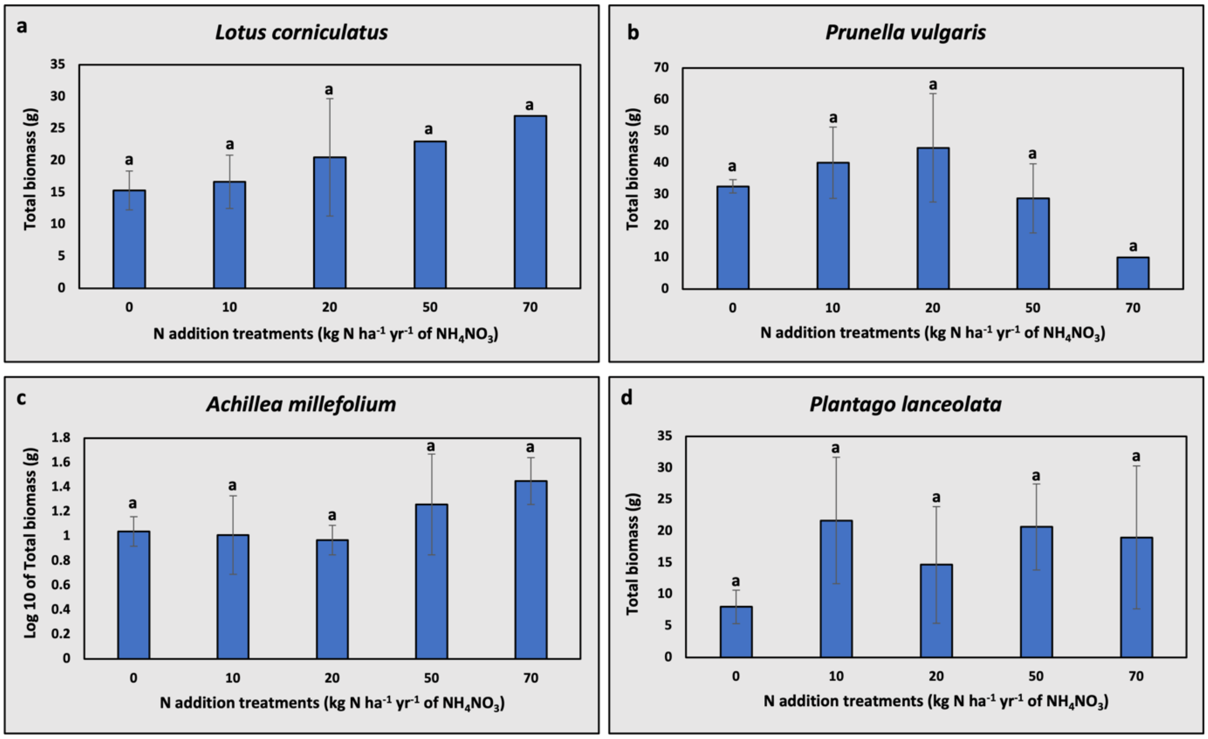

Figure 10 (a) The average of total biomass for *L. corniculatus*, (b) *P. vulgaris*, (c) *A. millefolium* and (d) *P. lanceolata* for each treatment. Values are Mean ± SE, those sharing the same letters denoted no significant differences between the groups based on Tukey's HSD post-hoc test.

2.3.4 Plant height

The plant height of *L. corniculatus*, *P. vulgaris*, *A. millefolium* and *P. lanceolata* were not significantly different across the treatments (ANOVA; F= 1.424; df= 4 ; *P* = 0.348, F= 2.164; df= 4 ; *P* = 0.190, F= 1.085 ; df= 4 ; *P* = 0.414 and F= 1.642 ; df= 4 ; *P* = 0.246 respectively). The average of *P. lanceolata* is very consistent throughout the treatments (Figure 11).

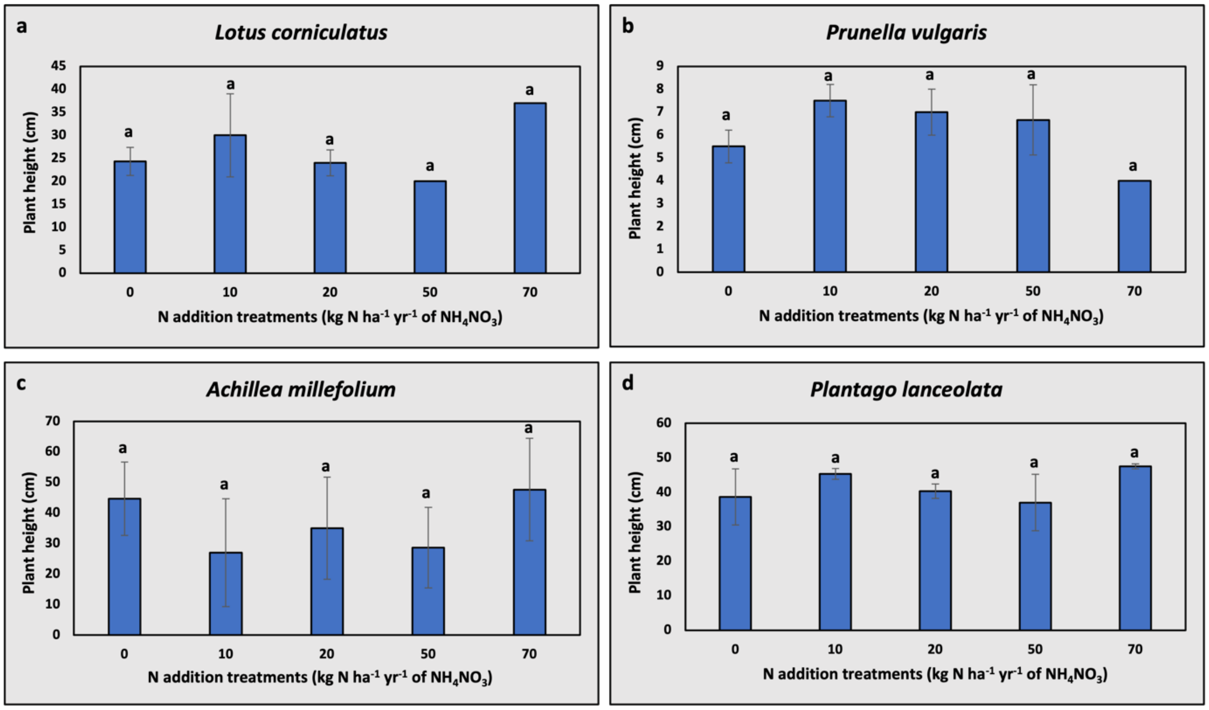

Figure 11 (a) The average of plant height for *L. corniculatus*, (b) *P. vulgaris*, (c) *A. millefolium* and (d) *P. lanceolata* for every treatment. Values are Mean ± SE, those sharing the same letters denoted no significant differences between the groups based on Tukey's HSD post-hoc test.

2.3.5 Flowering and Seeds

2.3.5.1 Number of flowers

The total number of *L. corniculatus* flowers in all N addition treatments was 139, that of *A. millefolium* was 1777 and that of *P. lanceolata* was 187 flowers. Unfortunately, *P. vulgaris* did not flower. The mean number of flowers of *L. corniculatus* and *P. lanceolata* was not significantly affected by N treatments (ANOVA; F= 1.768; df= 4 ; $P = 0.297$ and F= 1.973 ; df= 4 ; $P = 0.182$ respectively). The mean number of flowers of *L. corniculatus* was much higher in the control plot, while the lowest was in the 50 N and 70 N treatments but the data were very variable. The mean number of flowers of *A. millefolium* was found higher in plots under all N addition treatments than in the control plot, and there was significant difference between 70 N treatment and the control (F= 4.301; df= 4 ; $P < 0.045$) (Figure 12).

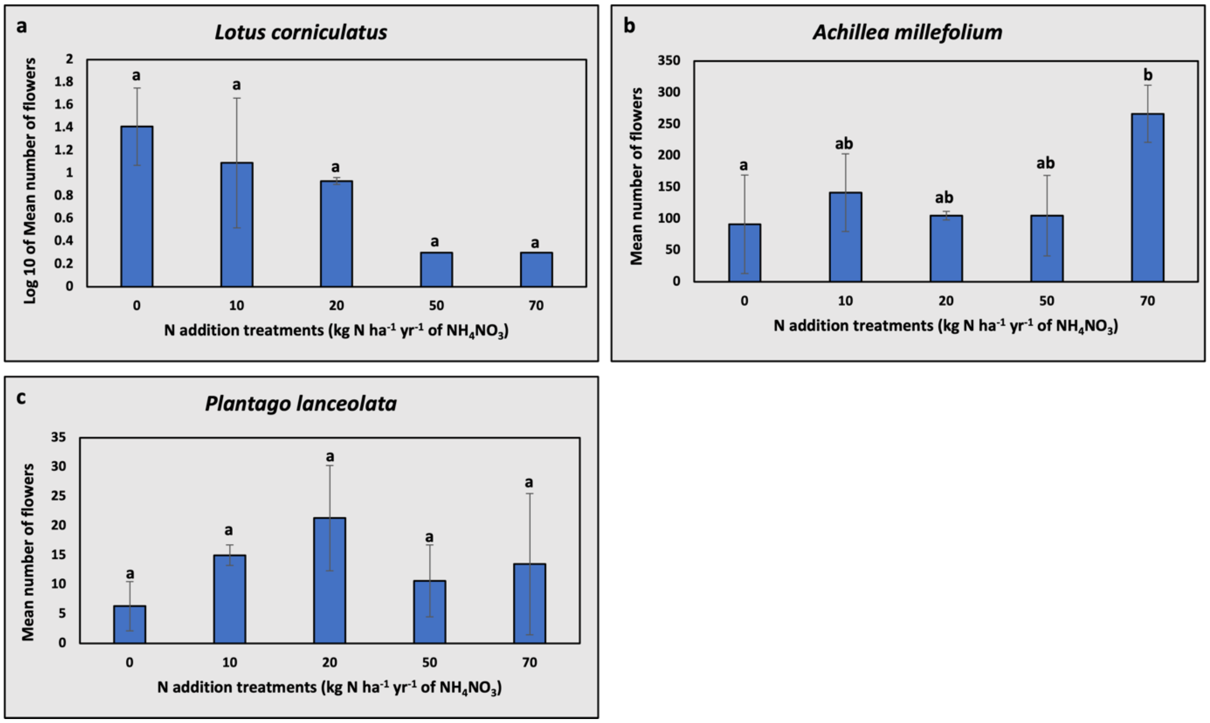

Figure 12 (a) The mean number of flowers for *L. corniculatus*, (b) *A. millefolium* and (c) *P. lanceolata* in each treatment. The error bars show the standard error of the mean, those do not have identical letters denoted significant differences between the groups based on Tukey's HSD post-hoc test, with a significant level set P that less than 0.05.

2.3.5.2 Number of seeds

The total number of seed that produced from *L. corniculatus* was 554 and *P. lanceolata* was 5478 seeds. The mean number of seeds of *P. lanceolata* was not considerably impacted by N treatments (ANOVA; F= 2.353; df= 4 ; *P* = 0.132). However, the mean number of seeds for *L. corniculatus* was found significantly higher in the 10 N treatment than other treatments. In addition, the 50 N treatment was significantly differences compared to the control and N addition treatments, and there was significant difference between the treatments (F= 5.857; df= 4 ; *P* < 0.04) (Figure 13).

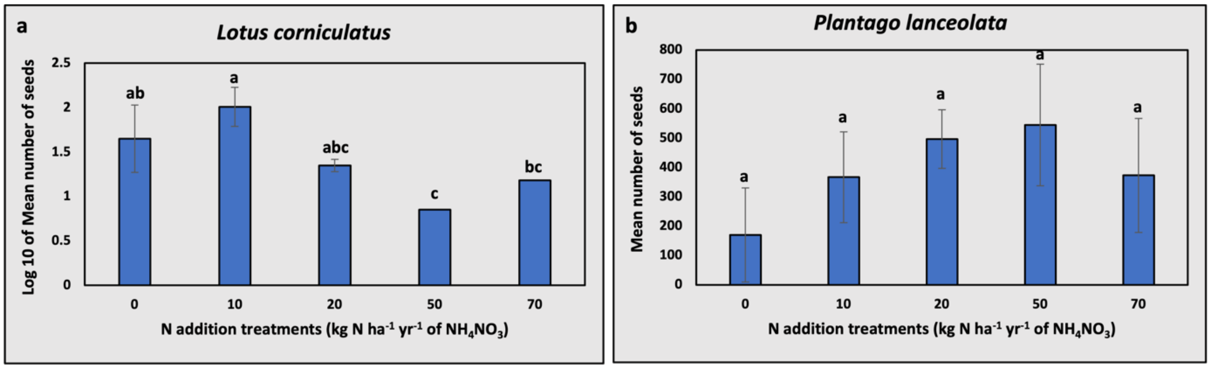

Figure 13 (a) The mean number of seeds for *L. corniculatus* and (b) *P. lanceolata* for each treatment. The error bars show the standard error of the mean, those do not have identical letters denoted significant differences between the groups based on Tukey's HSD post-hoc test, with a significant level set P that less than 0.05.

2.3.6 Plant tissue chemistry

2.3.6.1 Nitrogen concentration

The total percentage of nitrogen concentration of *L. corniculatus*, *P. vulgaris* and *A. millefolium* were not significantly influenced by N treatments (ANOVA; F= 0.335; df= 4 ; *P* = 0.844, F= 2.324; df= 4 ; *P* = 0.170 and F= 1.892; df= 4 ; *P* = 0.188 respectively). However, the total percentage of nitrogen concentration of *P. lanceolata* was significantly different. N content of *P. lanceolata* was significantly higher in the 50 N treatment than the control plot. In contrast, the control plot was significantly lowest among the treatments. Furthermore, the 70 N, 20 N and 10 N treatments were not significantly different compared to the control and the 50 N treatment and this treatment had high variability and the difference is significant (F= 5.098; df= 4 ; *P* < 0.02) (Figure 14).

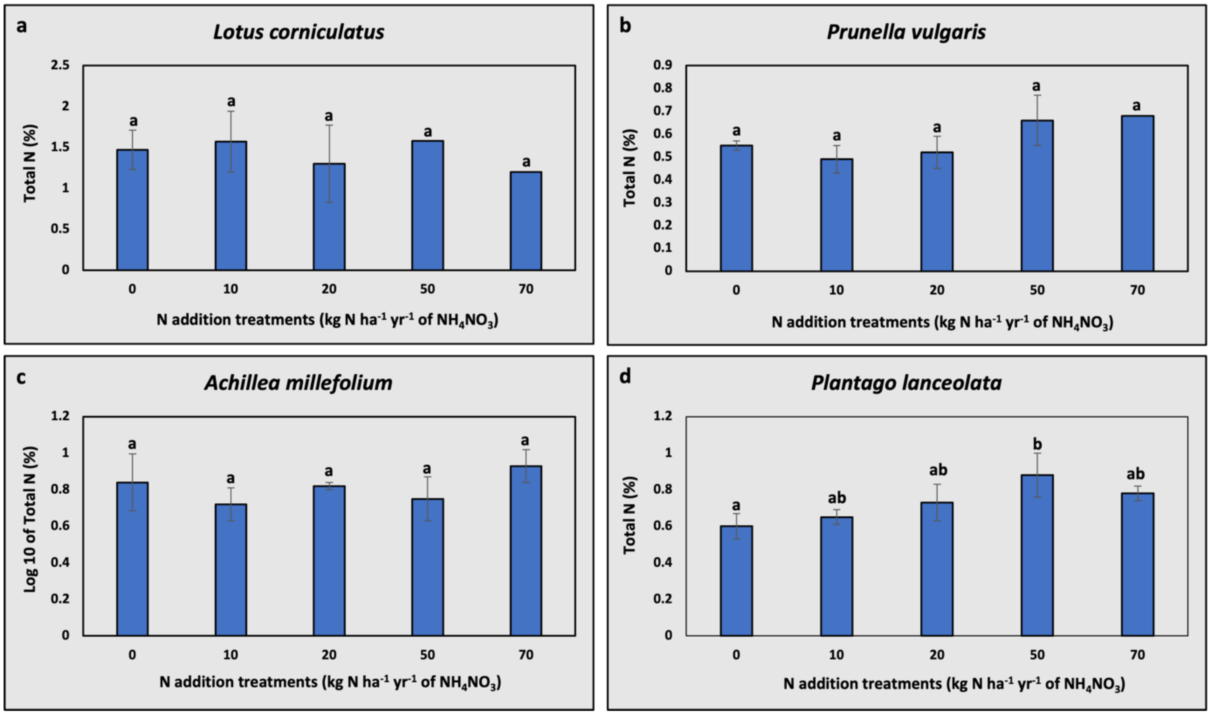

Figure 14 (a) The content of the total plant N in each treatment presented by percentage for *L. corniculatus*, (b) *P. vulgaris*, (c) *A. millefolium* and (d) *P. lanceolata* in each treatment. The error bars show the standard error of the mean, those do not have identical letters denoted significant differences between the groups based on Tukey's HSD post-hoc test, with a significant level set P that less than 0.05.

2.3.6.2 Carbon concentration

The total percentage of carbon concentration of *L. corniculatus*, (b) *P. vulgaris*, (c) *A. millefolium* and (d) *P. lanceolata* did not differ between the treatments (ANOVA; F= 1.097; df= 4 ; *P* = 0.449, F= 1.433; df= 4 ; *P* = 0.330, F= 1.706; df= 4 ; *P* = 0.225 and F= 1.537 ; df= 4 ; *P* = 0.271 respectively). The data for the four species is highly consistent across the treatments (Figure 15).

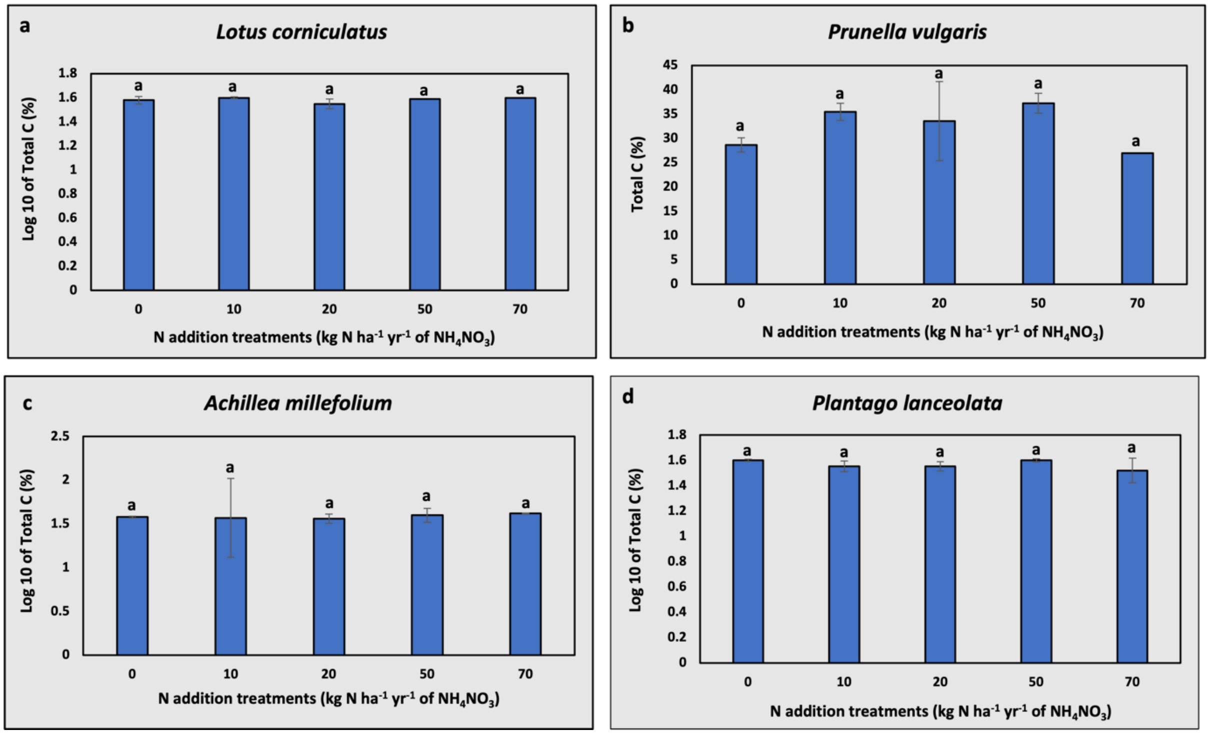

Figure 15 (a) The content of the total plant C in each treatment presented by percentage for *L. corniculatus*, (b) *P. vulgaris*, (c) *A. millefolium* and (d) *P. lanceolata* for every treatment. Values are Mean ± SE, those sharing the same letters denoted no significant differences between the groups based on Tukey's HSD post-hoc test.

2.3.6.3 C:N ratios

C:N ratios for *L. corniculatus, P. vulgaris* and *A. millefolium* were not significantly different among the treatments (ANOVA; F= 0.267; df= 4 ; P = 0.888, F= 2.671; df= 4 ; P = 0. 136 and F= 0.738; df= 4 ; P = 0.587 respectively). However, C:N ratios of *P. lanceolata* was significantly lower (F= 3.812; df= 4; P < 0.044) in the 10 N, 20 N, 50 N, 70 N treatments than they were in the control plot and there was significant difference between the treatments (Figure 16).

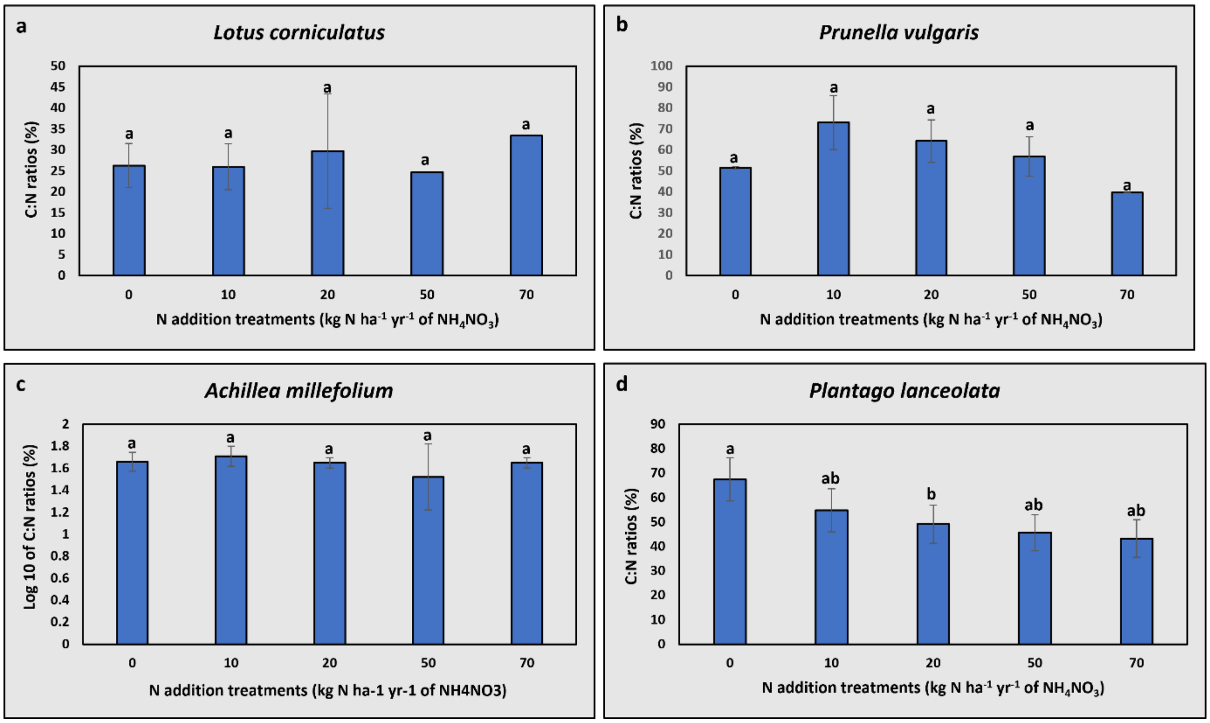

Figure 16 (a) The total ratios of C:N for each treatment presented by percentage for *L. corniculatus*, (b) *P. vulgaris*, (c) *A. millefolium* and (d) *P. lanceolata* for each treatment. The error bars show the standard error of the mean, those do not have identical letters denoted significant differences between the groups based on Tukey's HSD post-hoc test, with a significant level set P that less than 0.05.

2.3.7 Phenology

2.3.7.1 Date of first bud

For *L. corniculatus*, *A. millefolium* and *P. lanceolata* there were no significant differences between the treatments (ANOVA; F= 1.660; df= 4 ; P = 0.293, F= 0.525; df= 4 ; P = 0.720 and F= 1.884; df= 4 ; P = 0.190 respectively) for the date of first bud. There is very little variation in this data (Figure 17).

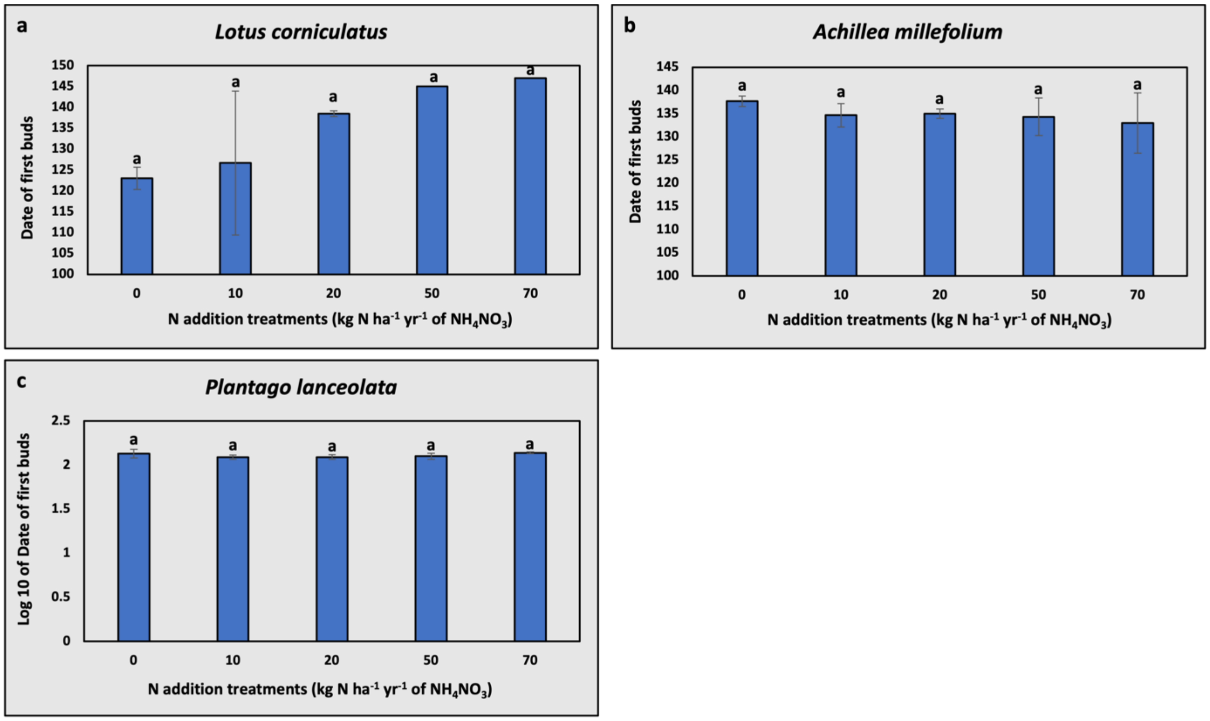

Figure 17 (a) The average of the date of the first bud emerged for *L. corniculatus*, (b) *A. millefolium* and (c) *P. lanceolata* for each treatment. Values are Mean ± SE, those sharing the same letters denoted no significant differences between the groups based on Tukey's HSD post-hoc test.

2.3.7.2 Date of first flowering

There were no significant differences in the date of the first flowering for *L. corniculatus*, *A. millefolium* and *P. lanceolata* between the treatments (ANOVA; F= 1.648; df= 4 ; P = 0.296, F= 0.436; df= 4 ; P = 0.780 and F= 2.090 ; df= 4 ; P = 0.157 respectively). This data contains relatively little variation (Figure 18).

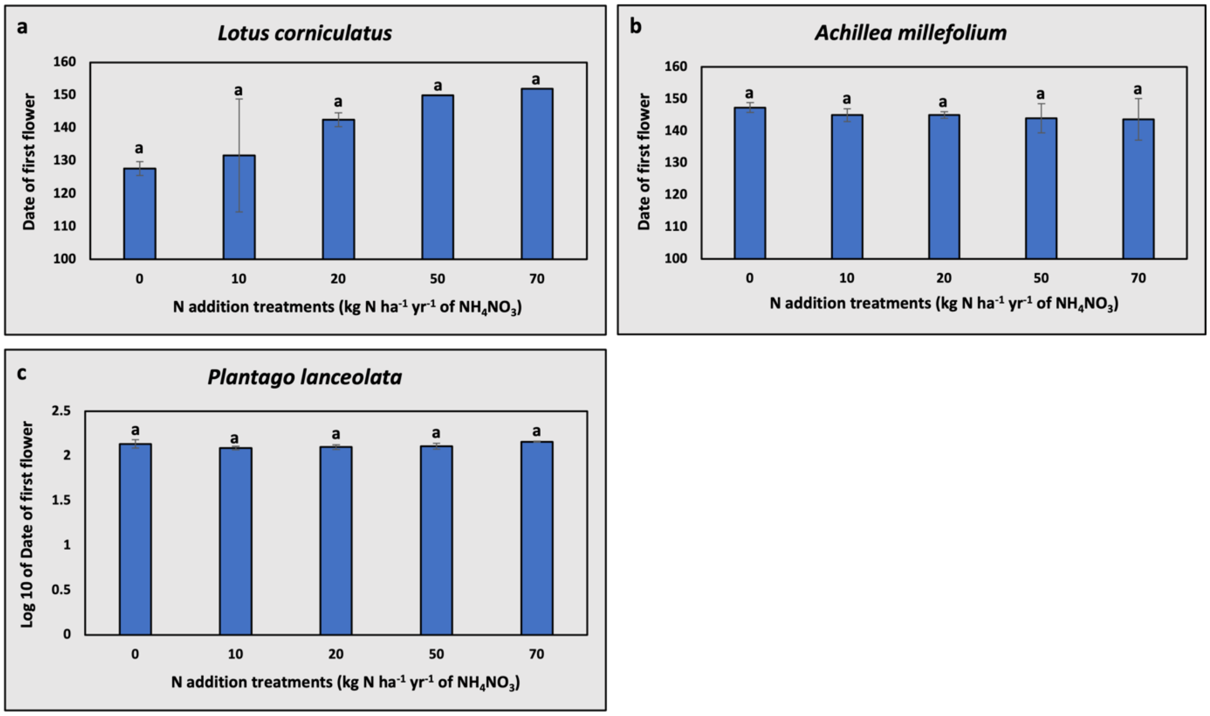

Figure 18 (a) The average of the date of the first flowering appeared for *L. corniculatus*, (b) *A. millefolium* and (c) *P. lanceolata* for each treatment. Values are Mean ± SE, those sharing the same letters denoted no significant differences between the groups based on Tukey's HSD post-hoc test.

2.3.7.3 Date of first setting seed

For *L. corniculatus* there was no significant difference between the treatments (ANOVA; F= 1.726; df= 4 ; $P = 0.280$) for the date of first seed set. The date of the first seed set of *P. lanceolata* was significantly higher in the 70 N treatment than 10 N treatment and all N addition treatments (ANOVA; F= 4.260; df= 4 ; $P < 0.029$) (Figure 19).

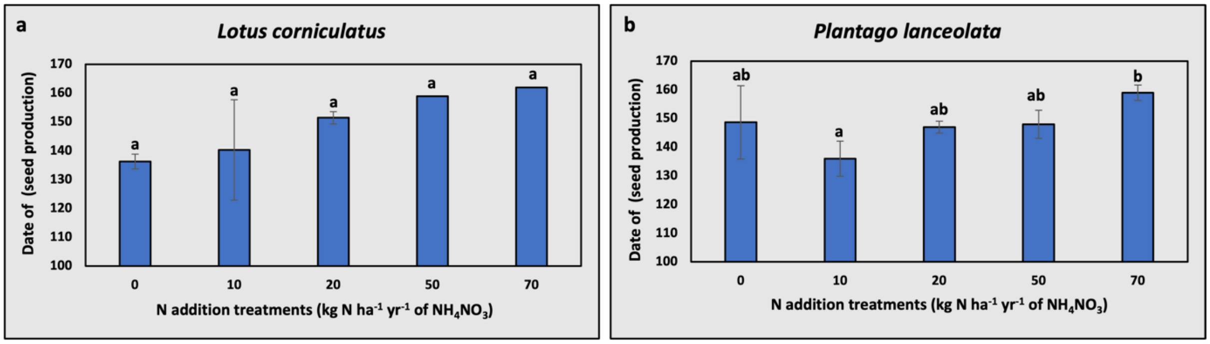

Figure 19 (a) The average of the date of the first setting seed for *L. corniculatus*, (b) *P. lanceolata* in each treatment. The error bars show the standard error of the mean, those do not have identical letters denoted significant differences between the groups based on Tukey's HSD post-hoc test, with a significant level set P that less than 0.05.

2.3.8 The relationship between the response variables

Table 1 The table shows Pearson correlation coefficients between the response variables (Tissue N, C:N ratio, Above-ground biomass, Below-ground biomass, Total biomass and Plant height) for *Lotus corniculatus*. Significant correlations (Two-Tailed Test) are highlighted in bold.

		C:N	Above-ground biomass	Below-ground biomass	Total biomass	Plant height
N	Pearson Correlation	-.952**	-0.424	-0.577	-0.582	-0.047
	Sig. (2-tailed)	**0.000**	0.222	0.081	0.077	0.898
	N	10	10	10	10	10
C:N	Pearson Correlation		0.592	.652*	.709*	0.098
	Sig. (2-tailed)		0.072	**0.041**	**0.022**	0.787
	N		10	10	10	10
Above-ground biomass	Pearson Correlation			0.553	.823**	0.351
	Sig. (2-tailed)			0.097	**0.003**	0.320
	N			10	10	10
Below-ground biomass	Pearson Correlation				.928**	0.117
	Sig. (2-tailed)				**0.000**	0.746
	N				10	10
Total biomass	Pearson Correlation					0.237
	Sig. (2-tailed)					0.510
	N					10

For *Lotus corniculatus*, significant correlations were observed between various parameters. N and C:N presented a significant relationship ($P < 0.00$). Furthermore, the C:N was significantly linked with below-ground and total biomass ($P < 0.041$ and $P < 0.022$, respectively). Additionally, above-ground biomass was positively related to total biomass ($P < 0.003$) as well as below-ground was also related significantly to total biomass ($P < 0.00$) (Table 1).

In contrast, several associations did not reach significance. The correlations between N and above-ground, below-ground, and total biomass ($P = 0.222$, $P = 0.081$, and $P = 0.077$), along with plant height ($P = 0.898$) were not significant. Likewise, C:N was insignificantly related to above-ground biomass ($P = 0.072$) and plant height ($P = 0.787$). Finally, the comparisons among biomass measures yielded nonsignificant correlations between above-ground and below-ground biomass ($P = 0.097$), and plant height ($P = 0.320$), between below-ground biomass and plant height ($P = 0.746$), and between total biomass and plant height ($P = 0.510$) (Table 1).

Table 2 The table shows Pearson correlation coefficients between the response variables (Tissue N, C:N ratio, Above-ground biomass, Below-ground biomass, Total biomass and Plant height) for *Prunella vulgaris*. Significant correlations (Two-Tailed Test) are highlighted in bold.

		C:N	**Above-ground biomass**	**Below-ground biomass**	**Total biomass**	**Plant height**
N	Pearson Correlation	-.607*	-0.286	-0.163	-0.213	0.065
	Sig. (2-tailed)	**0.048**	0.394	0.632	0.529	0.850
	N	11	11	11	11	11
C:N	Pearson Correlation		0.560	0.456	0.537	0.429
	Sig. (2-tailed)		0.073	0.159	0.088	0.188
	N		11	11	11	11
Above-ground biomass	Pearson Correlation			0.398	0.595	.667*
	Sig. (2-tailed)			0.225	**0.045**	**0.025**
	N			11	11	11
Below-ground biomass	Pearson Correlation				.974**	.656*
	Sig. (2-tailed)				**0.000**	**0.028**
	N				11	11
Total biomass	Pearson Correlation					.739**
	Sig. (2-tailed)					**0.009**
	N					11

For *Prunella vulgaris,* several significant correlations emerged among the biomass measures. In particular, the relationship of N with C:N ($P < 0.048$), above-ground biomass with plant height ($P < 0.025$) and total biomass ($P < 0.045$) was significant. Additionally, there were strong positive correlations of below-ground biomass with total biomass ($P < 0.001$), and plant height ($P < 0.028$). Finally, total biomass was also significantly corelated with plant height ($P < 0.009$) (Table 2).

In contrast, other associations did not reach significance. The relationships of N with above-ground ($P = 0.394$), below-ground ($P = 0.632$), and total biomass ($P = 0.529$), and plant height ($P = 0.850$) were all nonsignificant. Similarly, the C:N was not significantly related to above-ground ($P = 0.073$), below-ground ($P = 0.159$), and total biomass ($P = 0.088$), or plant height ($P = 0.188$). Finally, the correlation between above-ground and below-ground biomass was not significant ($P = 0.225$) (Table 2).

Table 3 The table shows Pearson correlation coefficients between the response variables (Tissue N, C:N ratio, Above-ground biomass, Below-ground biomass, Total biomass and Plant height) for *Achillea millefolium*. Significant correlations (Two-Tailed Test) are highlighted in bold.

		C:N	**Above-ground biomass**	**Below-ground biomass**	**Total biomass**	**Plant height**
N	Pearson Correlation	-0.124	0.190	0.219	0.251	0.399
	Sig. (2-tailed)	0.659	0.498	0.434	0.367	0.140
	N	15	15	15	15	15
C:N	Pearson Correlation		0.117	-0.106	0.054	0.215
	Sig. (2-tailed)		0.678	0.707	0.848	0.442
	N		15	15	15	15
Above-ground biomass	Pearson Correlation			.750**	.882**	0.054
	Sig. (2-tailed)			**0.001**	**0.000**	0.848
	N			15	15	15
Below-ground biomass	Pearson Correlation				.963**	-0.136
	Sig. (2-tailed)				**0.000**	0.628
	N				15	15
Total biomass	Pearson Correlation					-0.072
	Sig. (2-tailed)					0.799
	N					15

The significant findings for *Achillea millefolium* were restricted to the relationships among biomass measures. In particular, the above-ground biomass significantly corelated to below-ground and total biomass ($P < 0.001$ for each), and below-ground biomass was also significantly related to total biomass with ($P < 0.00$) (Table 3).

In contrast, none of the relationships involving N or the C:N with growth parameters reached significance. The correlation between N and C:N was not significant ($P = 0.659$), and N showed no significant association with above-ground, below-ground, and total biomass ($P = 0.498$, *P* = 0.434, and *P* = 0.367, respectively), or plant height (*P* = 0.140). Similarly, the C:N did not significantly correlate with above-ground biomass (*P* = 0.678), below-ground biomass (*P* = 0.707), total biomass (*P* = 0.848), or plant height (*P* = 0.442). Moreover, the relationships between biomass and plant height were not significant, with plant height and above-ground biomass (*P* = 0.848), plant height and below-ground biomass and (*P* = 0.628), and plant height and total biomass (*P* = 0.799) all failing to reach significance (Table 3).

Table 4 The table shows Pearson correlation coefficients between the response variables (Tissue N, C:N ratio, Above-ground biomass, Below-ground biomass, Total biomass and Plant height) for *Plantago lanceolata*. Significant correlations (Two-Tailed Test) are highlighted in bold.

		C:N	**Above-ground biomass**	**Below-ground biomass**	**Total biomass**	**Plant height**
N	Pearson Correlation	-.831**	0.472	0.166	0.286	-0.129
	Sig. (2-tailed)	**0.000**	0.089	0.570	0.321	0.660
	N	14	14	14	14	14
C:N	Pearson Correlation		-.546*	-0.226	-0.355	-0.137
	Sig. (2-tailed)		**0.043**	0.438	0.213	0.642
	N		14	14	14	14
Above-ground biomass	Pearson Correlation			.738**	.879**	-0.085
	Sig. (2-tailed)			**0.003**	**0.000**	0.774
	N			14	14	14
Below-ground biomass	Pearson Correlation				.970**	-0.015
	Sig. (2-tailed)				**0.000**	0.959
	N				14	14
Total biomass	Pearson Correlation					-0.041
	Sig. (2-tailed)					0.889
	N					14

For *Plantago lanceolata*, several significant relationships were observed. Notably, with ($P < 0.00$), N was found to be significantly corelated with C:N , and the C:N ratio was significantly linked to above-ground biomass ($P < 0.043$). In addition, significant correlations among biomass measures were detected: above-ground and below-ground biomass, above-ground and total biomass, and below-ground and total biomass with P values ($P < 0.003$, $P < 0.00$, and $P < 0.00$, respectively) (Table 4).

On the other hand, the relationships between N and the various growth parameters above-ground ($P = 0.089$), below-ground ($P = 0.570$), and total biomass ($P = 0.321$), or plant height ($P = 0.660$) were not significant. Similarly, aside from its link with above-ground biomass, the C:N ratio did not show significant correlations with below-ground ($P = 0.438$) and total biomass ($P = 0.213$), or plant height ($P = 0.642$). Furthermore, plant height was independent of above-ground biomass ($P = 0.774$), below-ground biomass ($P = 0.959$) and the overall biomass ($P = 0.889$) (Table 4).

2.4 Discussion

2.4.1 Biomass

2.4.1.1 Above-ground biomass

In our study, we found no significant effect of N addition on above-ground biomass of *P. lanceolata* and *P. vulgaris*. Contrary to our observation, Stevens and Gowing (2014) found an increase in the above-ground biomass of *P. lanceolata* and *P. vulgaris*. Furthermore, for *A. millefolium*, our studies indicate no significant difference in above-ground biomass whereas Tatar et al. (2013) found that addition of N increased the above-ground biomass i.e. leaves and stem for *A. millefolium* group-related cultivar proa when N was applied at 0 kg ha^{-1}, 50 kg ha^{-1}, 100 kg.ha^{-}1 and 150 kg.ha^{-}1. Furthermore, Tilman (1986) also found a significant difference in biomass attained after 12 week of growth and the total soil nitrogen for *A. millefolium*. The reasons for differences are discussed in detail in section 4.1.3. In case of *L. corniculatus*, our studies indicate no significant effect of N addition to above-ground biomass. Zhao et al. (2022) studied the effect of nitrogen on above-ground biomass of *L. corniculatus* by addition of 200 mg N kg^{-1} by 0.079 mol L^{-1} NH_4Cl solution at 4 intervals separated by 10 days. N addition significantly decreased above-ground biomass.

2.4.1.2 Below-ground biomass

We found no significant effect of N deposition on below-ground biomass of *P. lanceolata*. Opposing to our results, Stevens and Gowing (2014) found an increase in below-ground biomass for *P. lanceolata*. For *P. vulgaris*, they found no significant effect of N deposition on below-ground biomass which is in direct agreement with our studies as we found no significant effect of N deposition in below-ground biomass of *P. vulgaris*. Our studies for *L. corniculatus* indicate no significant effect of N deposition on below-ground biomass. Similar to our study, Briggs (1991) found no significant effect of N addition on root biomass of *L. corniculatus*, indicating no change in below-ground biomass when N was applied in the form of ammonium nitrate, 0.03g as Low N and 0.13g as High N over 4.4 litre pots. Our studies indicate no significant effect of N deposition on below ground biomass of *A. millefolium*. Bai et al. (2015) showed that the addition of N at the rate of 20 kg N ha$^-$1 yr$^-$1 reduces the root biomass of forbs significantly by 58.9%.

2.4.1.3 Total Biomass

Our results clearly indicate no significant effect of N deposition on total biomass of *A. millefolium*. Our observations are backed by the results of Plassmann et al. (2009) who studied the effect of N deposition on total biomass of *A. millefolium* when they provided 7.5 kg N ha^{-1} yr^{-1} and 15 kg N ha$^-$1 yr$^-$1 for two years. They found no significant effect of N treatment on total biomass. Allison (2002) applied N in each growing season twice to give a total addition of 6 g N m^{-2} yr^{-1} to study the effects of N addition on various traits of *A. millefolium*. They reported an increase in the total biomass.

For *L. corniculatus,* we found no significant effect of N deposition on total biomass whereas Zhao et al. (2022) reported a significant decrease in total biomass when they studied the effect of N on total biomass of *L. corniculatus*. In case of *P. lanceolata*, total biomass was not affected by N deposition whereas literature indicates a significant difference in total biomass as a response to N deposition or limitation. For instance, Pankoke et al. (2015) added N in the form of nitrate and ammonium into the soil and observed that the total biomass of *P. lanceolata* is decreased on limited N supply. Our studies indicate no significant effect of N deposition of *P. vulgaris*. This is contrary to what Zhang et al. (2015) found for effect of N deposition of total biomass of forbs. They observed an increase in the total biomass of forbs when N was added in the form ammonium nitrate at the rate of 10 g m^{-2} yr^{-1}.

Compared to the aforementioned studies, only two of our results i.e. *P. vulgaris* belowground biomass, and total biomass of *A. millefolium* are in agreement with the literature. This deviation from previous studies (not only in case of biomass but also phenology, height and tissue chemistry) could be attributed to environmental stressors such as overwatering, heat waves impacting temperature in the greenhouse, and fungal infections affecting plant growth. Firstly, the plant were overwatered in couple of occasions and overwatering can lead to oxygen deficiency in the soil, hindering root respiration and nutrient uptake, including nitrogen (Swift et al., 2002). Only at the optimum level of air and water the plants uptake, release and consume the nutrients at their full capacity, a rise or fall in the water or air level can reduce the nutrient availability for plants (Wolf, 1999). Overwatering can cause volatilisation or leaching of the nutrients, thus available nutrients are reduced. This condition could limit the plants' ability to utilize added nitrogen effectively, thereby mitigating any growth benefits typically associated with N fertilization (Wolf, 1999). Secondly, during the time of the experiment there was a heatwave in the UK and the temperature in the greenhouse became very high for several days as a result the elevated temperatures during a heatwave in the greenhouse could have stressed the plants, affecting their physiological processes such as photosynthesis, water uptake, and nutrient absorption (Yu et al., 2019). High temperatures can accelerate water loss through transpiration, leading to dehydration and impaired growth, even in the presence of added nutrients like nitrogen (Feller and Vaseva, 2014). Moreover, a fungal infection was observed in some of *P. lanceolata*, fungal infections could have further compromised their health and growth. Fungal pathogens disrupt normal plant functions and affects it negatively (Zeilinger et al., 2016). In this scenario, the plants may have allocated more resources towards defence mechanisms against the fungal infection rather than utilizing added nitrogen for growth. Thus, while previous research mostly indicated increased growth with N addition, my results underscore the importance of considering environmental factors that may limit plant responses to nutrient inputs.

2.4.2 Plant Height

Our results measurement plant height of *L. corniculatus* indicates that there was no significant effect of N addition on the height. This is contrary to what others have found. For instance, Barta (1979) Performed a pot experiment to study the effect of N on *L. corniculatus*, N was applied in the form of 0.5mM ammonium nitrate, the resultant increase in the dry weight of shoot indicated an increase in shoot growth of *L. corniculatus* under greenhouse conditions. Furthermore, while studying the plant height for forbs, Fu and Shen (2016) observed an increase in the plant height owing to N addition.

Our results indicate no significant difference in plant height of *P. vulgaris* as a response to N addition. These results are contradicted by Chen et al. (2023a) who measured plant height of *P. vulgaris* as a response to N addition at three different levels i.e. 412.5, 825 and 1650 ammonium nitrate mg L^{-1} and 475, 950 and 1900 KNO_3 mg L^{-1}. They observed that lower N level led to greater plant heights of *P. vulgaris*.

When plant height of *P. lanceolata* was measured, it showed no significant effect of N addition. Literature contradicts our results as Hancock et al. (2013) found an increased plant height of *P. lanceolata* in response to fertilizer addition containing N.

Furthermore, N addition did not change the plant height for *A. millefolium* whereas Grainger and Turkington (2013) observed an increase in the plant height of *A. millefolium* when they added very high N at 17.5 g N/m^2/year to study the response of four different species *Mertensia paniculata, Epilobium angustifolium, A. millefolium,* and *Festuca altaica*.

Our results contradict most of the literature as we performed the pot experiment for specific species whereas the studies on our selected species conducted earlier were mostly field studies. Overheating in the glass house along with over watering might be the reason we didn't see any effect of nitrogen deposition on plant height.

2.4.3 Flowering and seeds

2.4.3.1 Total number of seeds

Our results indicate that N addition significantly promoted the number of seeds for *L. corniculatus.* This result supports previous findings, for example, Briggs (1991) studied how N fertilization can affect the seed number of *L. corniculatus* in a pot experiment. Nitrogen was applied in the form of ammonium nitrate, 0.03g as Low N and 0.13g as High N. N fertilization, as a result, the number of seeds increased. Moreover, Basto et al. (2015) performed an experiment involving the deposition of N at 35N kg ha^{-1} yr^{-1} and 140 N kg ha^{-1} yr^{-1}. They suggested a probable reduction in seed production of one grassland species. Our results observed that N addition did not significantly affect the number of seeds of *P. lanceolata,* which is disagree with the study of Basto et al. (2015).

2.4.3.2 Total number of flowers

We observed no significant effect of N addition to number of flowers for *L. conrniculatus.* Similarly, Nogueira et al. (2017) found no significant effect of N deposition on phenology of legumes. This result is opposed by the studies of Briggs (1991) who found an increase in the number of flowers for *L. corniculatus* as a response to N addition.

In our results the number of flowers of *A. millefolium* was increased. Similarly, Tatar et al. (2013) found an increase in the number of flowers of *A. millefolium* group-related cultivar proa as a response to N addition when N was applied at 0N, 50N, 100N, and 150N.

Our results indicate no potential effect of N addition to *P. lanceolata*. These results contradicts those of Leake and Lee (2007) who found that N deposition decreases the total number of flowers for forbs.

N deposition can seriously affect the flowering of plant especially by compromising habitat quality by reducing the seed bank. A decrease in total number of flowers as a response to N addition can lead to reduction of seed bank as flowers are the source of seed production. Hence, there is a significant relation between total number of flowers and total number of seeds as fewer flowers lead to fewer seeds (Leake and Lee, 2007). To study the effect of N deposition on competitive ability of *A. millefolium*, He et al. (2012) performed an experiment by adding 1 g N m^{-2} yr^{-1} N as low deposition rate and 4 g N m^{-2} yr^{-1} as high deposition rate of N. They found a decrease in population of *A. millefolium* and other forb (*Helianthus annuus L.*) as a response to N addition whereas the invasive competitive species *Centaurea stoebe* increased in population. Skogen et al. (2011) studied the effects of N deposition on legume population and found that it to be decreased as legumes are sensitive to N deposition whereas the competitive species increased in population. This shows that increased N deposition leads to lesser numbers of flowers which leads to lesser seed production and hence the seed bank declines resulting in lesser competitive ability of the species to survive.

2.4.4 Plant Tissue Chemistry

2.4.4.1 Tissue C

Our findings reveal that N deposition did not significantly influence the carbon (C) content within the tissues of *P. lanceolata* and *P. vulgaris*. These results are backed by the study of Pan et al. (2010) who found no change in tissue C concentration in fertilized and unfertilized plots for *P. lanceolata* and *P. vulgaris*. Similarly, Hancock et al. (2013) found no significant effect of N containing fertilizer on *P. lanceolata*. Furthermore, *A. millefolium* also indicated no significant effect of N addition on tissue carbon similar to the results of Pan et al. (2010) where no significant effect in tissue C concentration was seen in forbs. Finaly, tissue carbon concentration for *L. corniculatus* also remained the same after N addition. Similar to our results, Chen et al. (2018) also found no significant effect of N deposition on tissue C content of legumes.

2.4.4.2 Tissue N

A. millefolium showed no significant effect of N addition on tissue N content. This is backed by the study of (Stevens et al., 2006) who found no significant effect of N deposition on forbs *Galium saxatile*. They utilised a N deposition at a range of 6-36 N ha^{-}1 yr^{-}1, found that N deposition has no effect on tissue N content of forbs. On the other hand Lee et al. (2003) found contrasting results, they presented the effects of N deposition on *A. millefolium* and found an increase in the concentration of tissue N when nitrogen was added at the rates of + 0, 4, 8, 12, 16, or 20 g N m^{-2} yr^{-1} which were very high.

P. lanceolata showed a significant effect of nitrogen deposition to tissue N content in our experiment. Similarly, Pan et al. (2010) also found an increase in tissue N for *P. lanceolata*.

Whereas, Hancock et al. (2013) found no significant effect of N containing fertilizer on *P. lanceolata*.

In the case of *P. vulgaris,* there was no increase in N content of tissue. The study by Rhymes et al. (2018) opposes our results as they found an increase in N concentration in *P. vulgaris* when a ground water with increased N concentration was provided in dune slacks. Furthermore, Pan et al. (2010) also found an increase in tissue N% of *P. vulgaris* as a response to N addition at 20 g N m^{-2}.

Tissue chemistry of *L. corniculatus* showed no effect of N deposition on tissue N content. These results are backed by the findings of Cui et al. (2010) who observed no significant effect of N addition to legumes as legumes already have high N concentration in the tissue. Similarly, Chen et al. (2018) also reported the same result as no significant effect of N deposition was observed on tissue N content of legumes.

We found no significant difference in the tissue N concentration on *P. vulgaris***,** *A. millefolium* and *L. corniculatus* as a response to N addition which opposes the literature. As they performed long term experiment, whereas our experiment is the short-term pot experiment. Several studies have also failed to find a significant difference in tissue N concentration due N deposition, which is in support of our results.

Some plants could use N addition rapidly for growth. Thus, N doesn’t get accumulated in the tissues and as a result, there is no change in the tissue N concentration (Stevens et al., 2006). This is backed by the theory of optimal partitioning, which proposes that plants strive to balance nutrient exchange ratios to optimize the efficiency of their resource utilization (Bloom et al., 1985).

2.4.4.3 Tissue C:N

In the context of carbon to nitrogen (C:N) ratios within plant tissues, our investigation revealed that the C:N ratio of *A. millefolium* remained unaltered in response to N deposition. Contrary to what we found, DeKoning (2011) showed that the addition of N decreases C:N of *A. millefolium*. Furthermore, Novotny et al. (2007) also found a decrease in C:N of *A. millefolium* following exposure to nitrogen at a dosage of 4 g N m^{-}2 per annum categorized as high nitrogen, in contrast to a control condition of 0 g N m^{-}2 per annum denoted as low nitrogen.

Analysing the C:N ratio dynamics for *L. corniculatus*, our data indicated an absence of significant alterations as a response to addition of nitrogen whereas Novotny et al. (2007) showed a decrease in C:N of legumes as a response to N addition.

In the case of *P. lanceolata*, C:N exhibited a marked sensitivity to nitrogen, the C:N ratio of *P. lanceolata* was decreased significantly on N addition. This is backed by the studies of Li et al. (2016a) who found a decrease in the C:N of forbs following N addition. Similarly, C:N was also observed to be lowered for forbs as a result of N addition in a study by Shen et al. (2019).

In contrast to *A. millefolium*, *P. vulgaris* showed no effect of N addition to C:N. These results are opposed by Li et al. (2016a) and Shen et al. (2019) who reported a decrease in C:N of forbs due to N deposition.

The deviation in our studies might be due to overwatering. As described earlier, overwatering can lead to leaching of nutrients, hence, the plants could not use the added N effectively.

2.4.5 Phenology

2.4.5.1 Date of first flower

Adding N can impact the flowering in several ways, it can either advance, delay or neutrally affect the flowering time of plants (Cleland et al., 2006, Smith et al., 2012, Xia and Wan, 2013, Xi et al., 2015). Date of first flower opening in both of our forb species i.e. *A. millefolium* and *P. lanceolata* did not show significant effect of N deposition. Liu et al. (2017) supports these results as they found no significant effect of N deposition on first flowering time of a forb species *Trollius farreri*. Furthermore, Xia and Wan (2013) studied the response of five forb species to N addition. Out of these five, four species showed no significant effect of N deposition on flower timing. Additionally, Fremlin et al. (2011) found no significant effect of N deposition on phenology of *A. millefolium*.

The first flowering time of *L. corniculatus* legume also remained the same in our results whereas (Wang and Tang, 2019) found that the added N delayed the first flower timing of legumes as other factors such as mean annual temperature and mean annual rainfall also affect the phenology along with N deposition.

2.4.5.2 Date of first buds

Our studies presented that *A. millefolium* and *P. lanceolata* showed no effect on date of first buds as a response to N addition. Petraglia et al. (2014) supports our studies. They studied the effects of N fertilization, P fertilization and N+P fertilization on phenology of forbs and found that the N fertilization has no effect on date of formation of first buds of forb plant.

There was no significant effect of N deposition on date of first buds of *L. corniculatus*. Contrary to our results, (Wang and Tang, 2019) found that in response to N addition, budding happened later than expected i.e. the date of first buds delayed in legumes.

2.4.5.3 Seed Production

L. corniculatus was observed to show no significant effect of nitrogen deposition on seed production date whereas *P. lanceolata* showed a significant delay. Nogueira et al. (2017) studied the effects of N deposition and drought on different species of forbs and legumes. They found that drought delayed the plant phenology (i.e. flowering and development of seeds) of forbs and legumes whereas N addition had no effect on plant phenology of both species.

2.4.6 Pearson correlation coefficients between the response variables (Tissue N, C:N ratio, Above-ground biomass, Below-ground biomass, Total biomass and Plant height)

2.4.6.1 The relationship between the response variables for *L. corniculatus, P. vulgaris, A. millefolium* and *P. lanceolata*

In *Lotus corniculatus*, N addition significantly influenced the carbon to nitrogen (C:N) ratio, aligning with Peng et al. (2023), who reported a decrease in C:N values in legumes under increasing N levels (0 N to 32 N kg^{-1} m^{-2}). However, N addition showed no relation with above-ground, below-ground, and total biomass, or plant height. Zhao et al. (2022) observed a reduction in above-ground and total biomass following N enrichment (200 mg N kg^{-1} applied via NH_4Cl), while Briggs (1991) found no effect of nitrogen on root biomass, supporting our findings. In contrast, Barta (1979) and Fu and Shen (2016) reported increased shoot growth and plant height with nitrogen application, differing from our results.

The C:N ratio correlated with below-ground and total biomass but not with above-ground biomass or plant height. Since below-ground biomass contributes to total biomass, hence C:N was also significantly related to total biomass. Ludidi et al. (2007) reported no relation between shoot C:N and root dry weight in *Pisum sativum*, while root C:N depended on root dry weight, with no effect on shoot or root length.

Above-ground biomass showed no significant correlation with below-ground biomass or plant height, aligning with Ludidi et al. (2007). However, it was significantly related to total biomass. In contrast, Fornara and Tilman (2008) found a significant link between above-ground and below-ground biomass in legumes. The below-ground biomass was significantly correlated with total biomass but not with plant height, consistent with Ludidi et al. (2007), who reported that plant height depended only on nodule number. Additionally, total biomass and plant height showed no significant relationship as above-ground and below-ground biomass were insignificantly related to plant height.

In our study, *Prunella vulgaris* and *Plantago lanceolata* revealed that the added N was significantly corelated with C:N, while *Achillea millefolium* showed no significant relationship. These findings align with Peng et al. (2023), who reported a decrease in C:N ratio with N addition in forbs, except for *Achillea millefolium*. Additionally, we found no significant effect of N addition on the above-ground biomass of all three forbs. In contrast, Tatar et al. (2013) and Tilman (1986) observed increased biomass with N application, while Stevens and Gowing (2014) reported increased above-ground biomass for *Plantago lanceolata*.

Our study found no significant effect of N addition on the below-ground biomass of all three forbs species. These results align with Stevens and Gowing (2014) for *Prunella vulgaris* but contrast with their findings for *Plantago lanceolata*, where they reported an increase in below-ground biomass. Additionally, our results differ from Bai et al. (2015), who observed a 58.9% reduction in *Achillea millefolium* root biomass at 20 kg N ha^{-1} yr^{-1}.

Our findings show that forbs have no relationship between nitrogen and total biomass. This aligns with Zhang et al. (2015), who also found no effect of nitrogen on forb biomass, and with Plassmann et al. (2009), who reported no biomass change in *Achillea millefolium*. However, our results contrast with Allison (2002), who observed an increase in *Achillea millefolium* biomass with N addition, and Pankoke et al. (2015), who reported a decrease in *Plantago lanceolata* biomass in response to nitrogen supply.

Plant height was found to be independent of N addition for *Prunella, Achillea*, and *Plantago*. These findings contradict previous studies, as Chen et al. (2023a) reported greater plant height with lower N addition, while Grainger and Turkington (2013) and Hancock et al. (2013) found increased heights for *Achillea millefolium* and *Plantago lanceolata*, respectively, in response to N addition.

Plantago lanceolata showed a significant correlation between C:N and above-ground biomass, unlike *Prunella* and *Achillea*. C:N did not corelate with below-ground or total biomass for any species. In contrast, Balazs et al. (2022) conducted an experiment with 16 native grass and forb species across 36 plots per site, including control, monoculture, and polyculture treatments, and found that C:N was correlated with overall biomass.

Furthermore, C:N and plant height revealed no significant relation for any of our three species. In a five-year study, Peng et al. (2023) also observed that C:N plant height relationship was not significantly related for forbs in an alpine meadow.

Since N did not relate to overall biomass, below-ground biomass, above-ground biomass and plant height for all three species, hence C:N was also found to be insignificantly related to all the parameters except *Plantago lanceolata* which showed a relationship between C:N and above-ground biomass.

For *Achillea millefolium* and *Plantago lanceolata*, there was a clear correlation between above and below-ground biomass; *Prunella vulgaris* showed no such link. This result is in line with other research on forbs by Fornara and Tilman (2008), Gupta and Narayan (2011) and Cahill Jr (2003), all of which also revealed clear relationships between root and shoot biomass of forbs. Whereas *Prunella* showed a positive link between above-ground biomass and plant height, *Achillea* or *Plantago* did not show this reflecting species-specific allometric patterns (Anten and Hirose, 1998, Anten and Hirose, 1999). Finally, above-ground and total biomass across all three species was shown to be rather connected. Any increase in above-ground biomass immediately adds to total biomass as total biomass consists of both above and below-ground biomass, therefore producing a noteworthy correlation.

In all three species, below-ground and total biomass were significantly positively associated. Since below-ground biomass makes up part of overall biomass, any change in root biomass affects total biomass. In addition, *Prunella* revealed a substantial positive association of plant height with below-ground as well as total biomass. Since below-ground and above-ground biomass combine to form total biomass, total biomass was found to be related with plant length as above-ground biomass and below-ground biomass were strongly correlated with plant height. In contrast, *Achillea* and *Plantago* did not demonstrate significant relationships between total biomass and plant height.

2.5 Conclusion

The flowering pot experiment was conducted in a greenhouse to assess the response of *A. millefolium*, *L. corniculatus*, *P. lanceolata,* and *P. vulgaris* to nitrogen addition on plant growth, biomass, tissue chemistry, and phenology. The results indicated no significant difference in above-ground biomass, below-ground biomass, total biomass, and plant height across all four species. However, notable exceptions were observed in the total number of seeds and flowers for *L. corniculatus* and *A. millefolium*, respectively, where significant differences were recorded.

Plant tissue chemistry analysis revealed no significant difference in carbon content across all of the species. Similarly, nitrogen content and C:N ratios showed no significant difference in all species, except for *P. lanceolata*, which exhibited a significant difference in both N content and C:N ratios. This indicates a specific sensitivity of *P. lanceolata* to N additions in terms of tissue chemistry. Phenological observations, including the date of first buds, the date of the first flower opening, and the date of seed production, showed no significant differences for all of the species except for a significant difference in the date of seed production in *P. lanceolata*. These findings increase our understanding of plant-nutrient interactions and highlight the need for species-specific considerations in managing ecosystems and agricultural practices in the context of increasing N deposition.

3 The effect of long-term N deposition on heathland seed bank and vegetation

3.1 Introduction

Heathlands are usually acidic in nature. Heathlands are usually dominated by shrubs including *Calluna vulgaris, Vaccinium myrtillus* and *Erica cinerea*. Below the shrub layer grasses, sedges and mosses including *Deschampsia flexuosa, Carex caryophyllea* and *Hypnum jutlandicum* are common.

The issue of nitrogen deposition has drawn a lot of attention to heathlands. The addition of nitrogen to the environment frequently has positive effects on *Calluna* growth. *Calluna* growth readings were taken with three experiments that have demonstrated this over a range of 8, 7, and 4 years of time period (Uren et al., 1997, Power et al., 1998a, Carroll et al., 1999). Furthermore, nitrogen deposition increases the N content of *Calluna* (Pitcairn et al., 1995). The evidence gathered from these studies suggests that the increased nitrogen content in *Calluna* leaves makes the plant more susceptible to both biotic (living organisms) and abiotic (environmental) stressors. This increased vulnerability is evidenced by unfavorable nutrient ratios when comparing root-to-shoot proportions, and an elevated frequency of winter injury in the plant (Carroll et al., 1999, Hartley and Amos, 1999, Power et al., 1998b). The results suggest that while nitrogen additions can stimulate growth in *Calluna*, they may also have implications for the plant's capability to cope with stress and affect the overall ecosystem's nutrient dynamics.

The soil seed bank refers to the collection of seeds or vegetative propagules present in the soil, capable of regenerating natural vegetation. These seeds are typically released from mature plants and eventually end up on the soil surface. Germination can occur promptly or be delayed for an unspecified duration. Throughout this period, the seeds situated within or on the soil collectively form what is known as a soil seed bank (Fenner and Fenner, 1985).

The soil seed bank, an important component of ecosystem dynamics, plays a key role in allowing natural vegetation regrowth and community regeneration (Thompson and Fenner, 2000, Wang et al., 2009, Kiss et al., 2018). In the context of global environmental changes, understanding the impact of N deposition on soil seed banks is very important for restoration and conservation efforts. Importantly, the introduction of nitrogen has been found to influence seed germination dynamics within the soil seed bank, potentially leading to significant shifts in its composition and dynamics (Ochoa-Hueso and Manrique, 2010, Zhong et al., 2019, Plassmann et al., 2008) as supported by the studies performed by (Basto et al., 2015). Basto et al. (2015) observed a considerable decline in seed abundance and species richness in the acidic grassland soil seed bank under long-term N deposition, even after four years without N deposition, the soil seed bank showed little recovery. Similarly, in a different ecological context, investigations revealed a reduction in the Shannon–Wiener index, which accounts for seed richness and evenness, in the soil seed bank under a wheat-soybean rotation system due to nitrogen inputs (Pan et al., 2020). Another report also suggest a neutral impact of N deposition on the soil seed in a desert community (Schneider and Allen, 2012). They observed no significant influence from short-term N addition in a desert community on seed density and species richness within the soil seed bank.

Seed production is one of the inputs for seed banks. Negative impacts of N deposition on flowering in several communities have been recorded, which can lead to decrease seed production (Phoenix et al., 2012). Moreover, the addition of N may have an impact on the development of flowers as well as nectar secretion (Burkle and Irwin, 2010, Ceulemans et al., 2017). The reproductive success of self-incompatible species may also change as a result of changes in floral traits that influence how pollinators forage (Vaudo et al., 2022, David et al., 2019). Uren et al. (1997) analyzed the effect on N deposition on flowering in heathland habitat by adding ammonium sulphate for 1 year at 15.4 and 7.7 kg N ha^{-1} yr^{-1}. They found that flowering was highest with increased N addition.

In various vegetation types, a discrepancy has been noted between the species present in the seed bank and those thriving in the vegetation (Thompson and Grime, 1979, Smith and Kadlec, 1983, Pratt et al., 1984). Research suggests that N deposition can have a significant impact on the similarity between the seed bank and vegetation above-ground in heathland ecosystems (Basto et al., 2015, Southon et al., 2013). However, the similarity between seed bank and vegetation above-ground can be affected by other factors such as precipitation, temperature, and grazing (Hopfensperger, 2007, Osem et al., 2009, White et al., 2012). N deposition is expected to reduce the similarity between above-ground vegetation and below ground seed bank as demonstrated in Baso et al.`s study where plots receiving 140 kg N ha^{-1} yr^{-1} showed decreased similarity between seed bank and above-ground vegetation (Basto et al., 2015).

Hypothesis

This experiment employs a long-term N addition on two area of heathland in Bedworth and Ruabon. This study will investigate the effect of N deposition on the composition and structure of below-ground seed bank as well as how it relates to the species composition of the above-ground vegetation of a heathland.

The following are the study's hypotheses after a long-term N addition.

1. Experimental N addition will not reduce the species richness of the heathland seed bank but alter it’s species composition.
2. Experimental N deposition will reduce the similarity of the species composition of the seed bank compared to above-ground vegetation.

3.2 Methods

3.2.1 Study sites

Based on previous studies (Field et al., 2013), two heathland sites, Little Budworth Common and Ruabon Moor, were chosen to investigate the long-term impacts of atmospheric N deposition on seed banks.

The experiment sites were Budworth Common and Ruabon Moor, which are two areas of heathland, where long-term N studies have been conducted (Figure 20a, 20b and Figure 21a, 21b) (Field et al., 2013).

Little Budworth Common is a site located in Cheshire, north west England, at latitude 53°19’ N, 2°62’ W) and 70 ma.s.l. (Field et al., 2013) (Figure 20a). The experimental site was established in 1996, it is one of the longest-running experiments about the effects of N deposition on heathlands. The value of soil pH was 3.8 and soils are humo-ferric, sandy podzols with a thin layer of organic matter on top (Field et al., 2013). The majority of the plant community is made up of *Calluna vulgaris, Deschampsia flexuosa* and *Hypnum jutlandicum*.

The Ruabon Moor experiment is located in north Wales (53.03 °N, 3.15 °W) at an altitude of 480 m a.s.l (Field et al., 2013). The experiment was established in 1989 and it is also one of the longest-running experiments about the impacts of N deposition on heathlands. The value of soil pH was 3.9 and the soil type is ironpan stagnopodzol, with a 10 cm peaty organic surface horizon layered on top of a 5 cm *Calluna* litter layer (Field et al., 2013). The vegetation covering is dominated by *Calluna vulgaris*, with *Vaccinium myrtillus* interspersed, and the understory is dominated by the moss *Hypnum jutlandicum*.

Both sites are currently managed by Professor Simon Caporn and Dr Chris Field at Manchester Metropolitan University.

3.2.2 Experimental design

At Little Budworth Common, since 1996 16 plots (1 x 2 m) have been receiving N treatments of 20, 60, 120 kg N ha^{-1} y^{-1} of NH_4NO_3 or rainwater only (control plots), and the total annual rainwater recorded in 2007 was 780mm. Four replications of a fully randomised block design, with four plots in each block, were used to apply the treatments (Figure 20b, c).

At Ruabon Moor, since 1998 20 plots are 2 x 2 m, but only (1 x 2 m) were treated after 2002 of 10, 20, 40 and 120 kg N ha^{-1} y^{-1} of NH_4NO_3 or rainwater only (control plots), and the total annual rainwater recorded in 2007 was 987mm. In 1998 new plots were established, then in 2002 each subplot has divided in half and one half was kept for recovery studies and the other for N addition. All seed bank samples for this study were collected from N addition plots (Figure 21c).

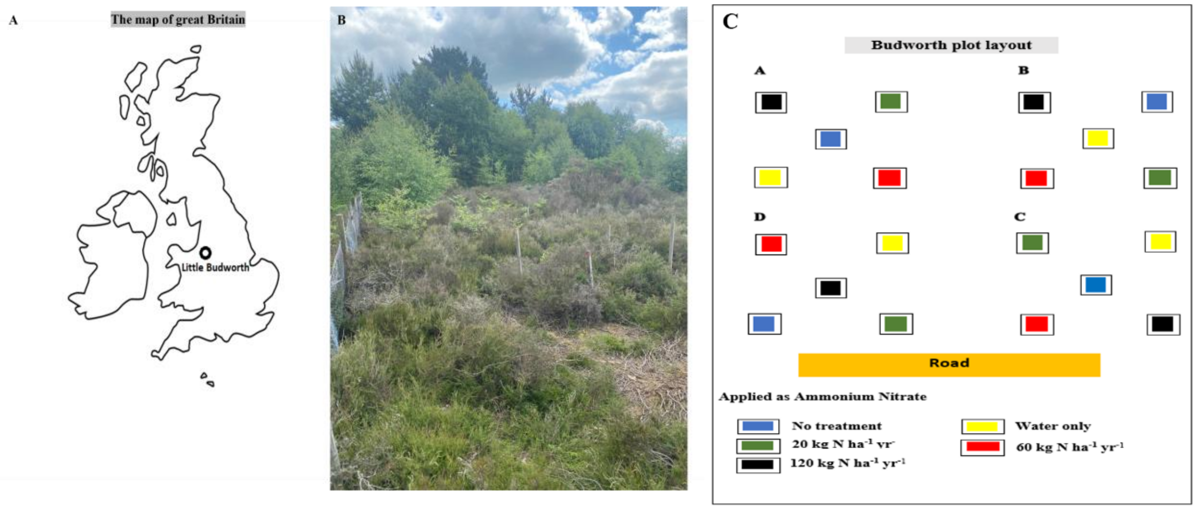

Figure 20 (a) The map of the Little Budworth Common study site, (b) the plots (c) the diagram shows the blocks A to D, each one of them includes four treatments, 120 kg N ha^{-1} yr^{-1} represented by black rectangle, 60 kg N ha^{-1} yr^{-1} represented by red rectangle, 20 kg N ha^{-1} yr^{-1} represented by green rectangle and the control plot represented by blue rectangle. The yellow rectangles in each block correspond to the control (water only).

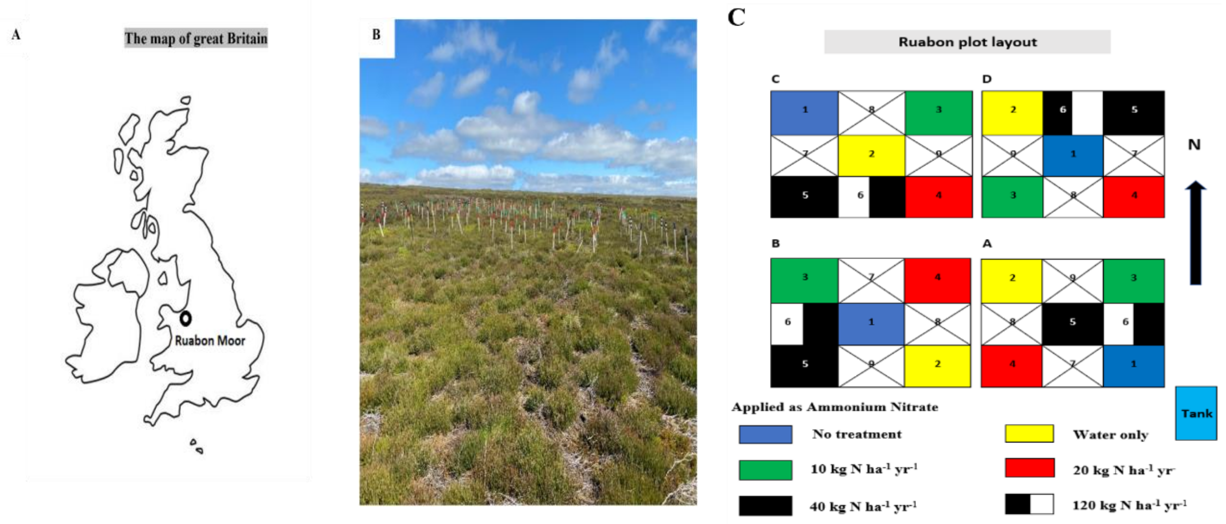

Figure 21 (a) The map of Ruabon Moor study site, (b) the plots (c) the diagram shows the blocks A to D, each one of them includes fife treatments, 120 kg N ha-1 yr-1 represented by black and white rectangle, 40 kg N ha-1 yr-1 represented by black rectangle, 20 kg N ha^{-1} yr^{-1} represented by red rectangle, 10 kg N ha^{-1} yr^{-1} represented by green rectangle and the control plot represented by blue rectangle. The yellow rectangles in each block correspond to water only.

3.2.3 Seed bank sampling and characterization

Inside each 1 x 2 m plot, one soil sample was collected randomly by using a metal frame (10 × 10 × 5 cm deep, 500 cm^3 volume) in June 2022 (at Budworth), and July 2022 (at Ruabon) homogenised by hand and collected in plastic bags. 35 soil samples in total were taken from the plots at both locations. At 40 °C, the soils were dry then sieved via 2 mm sieve to remove all rhizome, roots, vegetation, and coarse and fine soil components. Then, weed suppression fabric was used in each tray (50 cm × 25 cm) to line the trays to prevent seed loss through the holes in the bottom of the tray and filled up with 2 cm of potting compost. Each seed bank sample was spread out in a 1 cm layer across the whole tray. Additionally, to investigate seed contamination two trays were filled only with compost at one specific point inside the greenhouse. Every seed germination tray was set up on a bench and then maintained at Lancaster University in a greenhouse with a constant temperature of 25 °C. Trays were watered 2 - 3 times per week from above with tap water to maintain soil moisture. During the first two months, seedling emergence was recorded two days per week. The seedlings were tallied and removed soon after being identified to the species level. Plug trays were used to develop and flower the unidentified seedlings and facilitate identification. After identifying and removing the recently emerging seedlings from each tray, the soil was carefully mixed to promote the germination of any leftover seeds (Smith et al., 2002). After four weeks of not recording the emergence of new seedlings, no more germination controls were carried out, the germination trial was conducted during nine months.

3.2.4 Vegetation characterization

For both sites, the species composition of canopy vegetation was recorded in June and July 2022.

3.2.5 Statistical analysis

All the statistical analyses were conducted in Statistical Package for the Social Sciences (SPSS) version 27 (SPSS 27, Microsoft Corporation, released 15.0.1., 2020) (SPSS 27, 2020). Species composition analysis was carried out in R software (version 4.3.0; R Core Team, 2023) using the meta-MDS and the envfit functions in the vegan package (Oksanen J et al., 2022) for the non-metric multidimensional scaling (NMDS) ordination analysis and the ggplot2 package (Wickham et al., 2016) for figure plotting.

3.2.5.1 Seed bank data

The response variables were mean number of species, total mean number of seeds, mean number of shrub seeds and the mean number of *Calluna vulgaris* and the explanatory variable was the N treatment (nitrogen addition at rate of 0, 10, 20, 40, 60, 120 kg ha^{-1} yr^{-1}). For each block, the total number of species recorded in the seed bank and the mean of seed abundance were calculated. For the seed bank analysis was carried out with treatment as a fixed factor and the variables (mean number of species, total mean number of seeds, mean number of shrub seeds and the mean number of *Calluna vulgaris*) as a random factors. All variables were examined before analysis, and the normality was checked for parametric tests. For the data that were not normally distributed a non-parametric test was used. Using a Bray-Curtis distance as a measure of similarity matrices, a non-metric multidimensional scaling (NMDS) ordination was carried out to visualise changes in seed bank species composition between treatments. After confirming that the therapy was connected throughout the NMDS ordination, the NMDS ordination was fitted with the treatment as a factor.

3.2.5.2 Above-ground vegetation data

The response variables were the mean cover of species, the mean percentage of vegetation cover, the mean cover of shrubs and the mean cover of *C. vulgaris* and the explanatory variable was the N treatment (nitrogen addition at rate of 0, 10, 20, 40, 60, 120 kg ha^{-1} yr^{-1}). For each treatment, the total percentages of the vegetation cover of each block were added together and the mean percentage of the vegetation cover of each species was calculated by dividing the cover percentage of the species per block by the total cover percentage of all species per block. The significant value was set at p=0.05. The vegetation cover analysis was carried out with N treatment as a fixed factor and the variables (the mean cover of species, the mean percentage of vegetation cover, the mean cover of shrubs and the mean cover of *C. vulgaris*) as a random factors. All variables were examined before analysis, and the normality was checked for parametric tests. For the data that were not normally distributed a non-parametric test was used. Changes in above-ground species composition between treatments were analysed and displayed by using a NMDS ordination as described above.

3.2.5.3 Seed bank and above-ground vegetation data

An analysis using a non-metric multidimensional scaling (NMDS) ordination and a Bray-Curtis distance was used to determine whether the species composition of the above-ground vegetation and the seed bank were identical. Data from seed banks were transformed to relative abundance, and data from vegetation to relative cover, in order to prevent variations in sample scales. For each treatment, the seed bank relative abundance of each species was calculated by dividing the total number of seeds (the number of seedlings of the species that emerged from the seed bank) per block by the total number of seeds (the total number of seedlings of all species that emerged from the seed bank) recorded in every block. For each treatment, the relative vegetation cover of each species was calculated by dividing the cover percentage of the species per block by the total cover percentage of all species per block. It was verified whether treatment was correlated across the NMDS ordination, then treatment was fitted as a factor to the NMDS ordination. Furthermore, correlation analysis was used to analyse the relationship between above-ground vegetation species richness and the soil of the seed bank richness by using (SPSS 27, 2020).

3.3 Results

3.3.1 The long-term nitrogen deposition experiment at Little Budworth Common

3.3.1.1 Effects of the long-term nitrogen deposition on seed bank.

3.3.1.1.1 Seed bank richness and composition

Six species were recorded and 1650 seedlings emerged from seed banks for all treatments (Table 5). There was no significant effect of long-term N deposition on heathland seed bank richness at Little Budworth Common (ANOVA; F= 1.133; df= 3; *P* = 0.375). After 26 years of N addition, no differences in the mean number of species between treatments were recorded (Figure 22).

Table 5 Species composition and the mean number of *Calluna vulgaris* obtained from the seed bank under different N treatments and the P value of *Calluna* at Budworth.

Species	**120 N**	**60 N**	**20 N**	**Control**
Shrubs				
Calluna vulgaris	416	448	384	351
Forbs				
Cirsium vulgare	1	1	3	0
Rumex acetosella	9	0	25	0
Grasses				
Deschampsia flexuosa	3	3	0	0

Trees				
Betula pendula	1	0	3	1
Sedges				
Carex caryophyllea	0	0	1	0
Mean Number of *Calluna*	**104**	**112**	**96**	**87.75**
Standard deviation	**25.98**	**21.11**	**23.67**	**28.65**
P value of *Calluna*	***P* = 0.57**			

The highest mean number of *Calluna vulgaris* was in the 60 N treatment, while the lowest was in the control. However, there was no significant effect of N treatments on the mean number of *Calluna* (ANOVA; F= 0.695; df= 3; *P* = 0.57) (Table 5).

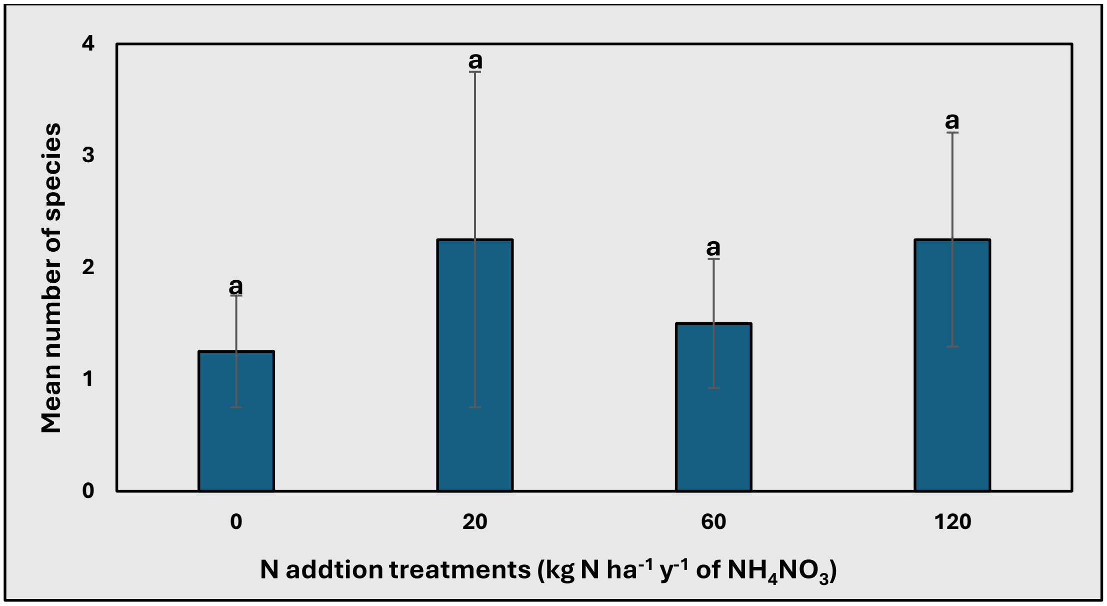

Figure 22 Mean number of species per sample germinating from seed banks in a heathland at Little Budworth Common, UK. Values are Mean ± SE, those sharing the same letters denoted no significant differences between the groups based on Tukey's HSD post-hoc test.

In addition, seed bank composition has not been significantly altered (treatment was not significantly correlated with the NMDS ordination structure, $r^2 = 0.14$, $P = 0.68$, Figure 23).

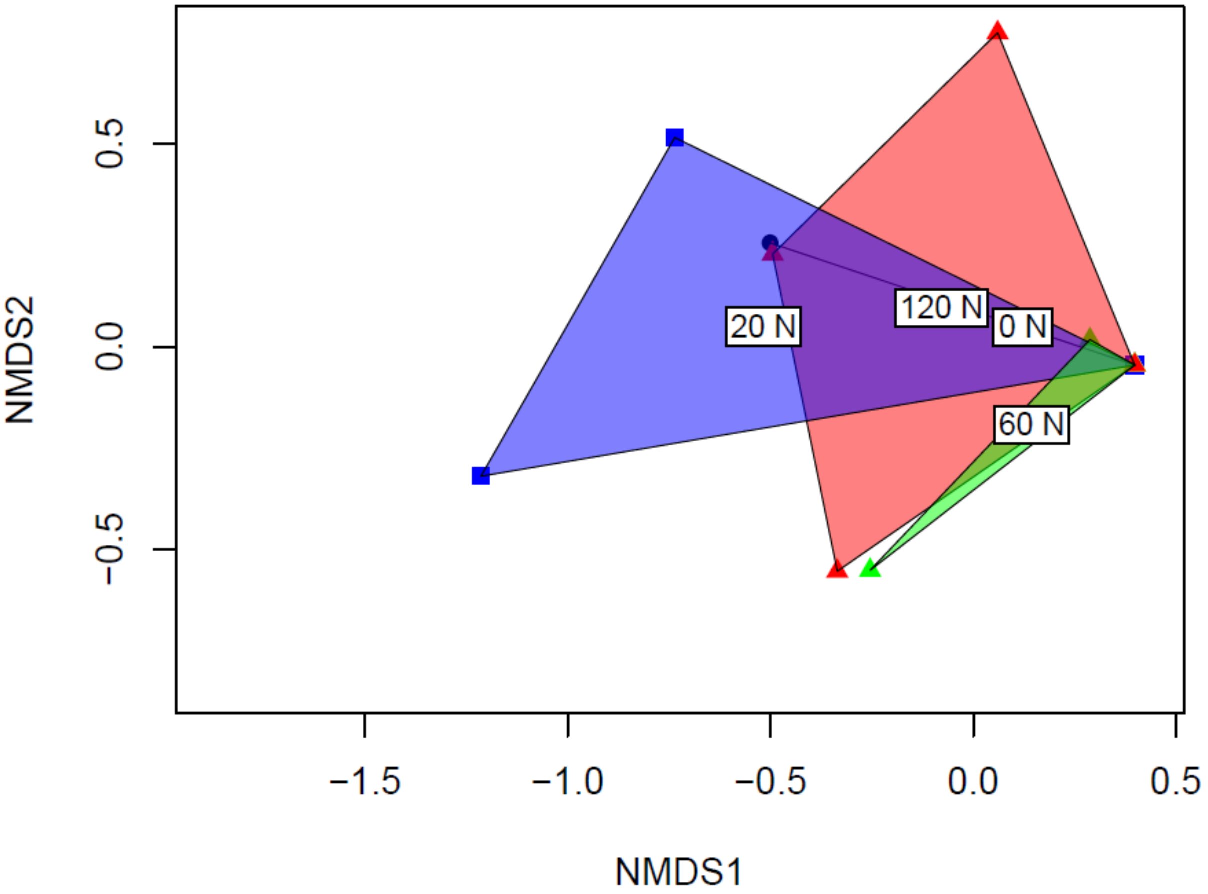

Figure 23 Two-dimensional non-metric multidimensional scaling (NMDS) ordination (stress = 0.068) of seed banks after 26 years of simulated N deposition at Little Budworth Common, UK. Treatments, as categorical variables (polygons), are represented by colours: black = control, blue = 20 N, green = 60 N and red = 120 N. Ordination is based on species abundance data; there were 4 plots in each treatment. Text shows centroids for N-treatment levels. The location of each ordination point shows the degree of similarity of this point to the others.

3.3.1.1.2 Seed bank abundance

The mean number of seeds from the seed bank that germinated was not significantly impacted by the N deposition (ANOVA; F= 0.627; df= 3; *P* = 0.611). The number of seedlings recorded in plots under all N addition treatments was higher than in the control plots, but it was not significant (Figure 24).

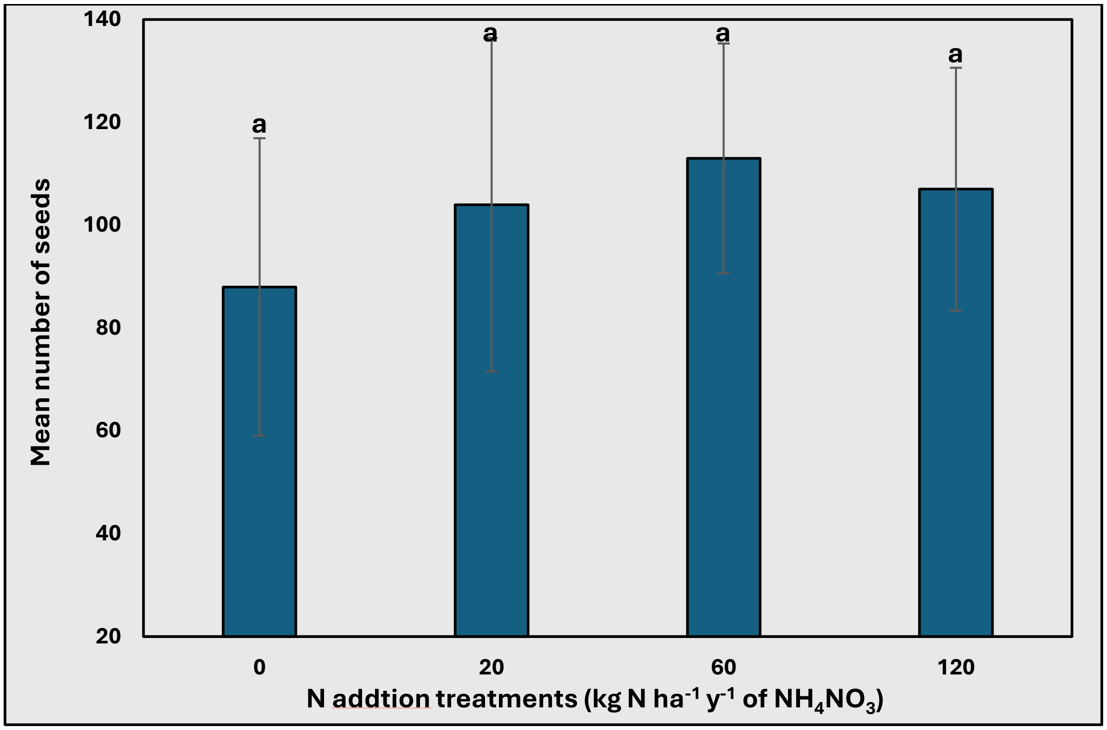

Figure 24 Mean number of seeds per sample germinating from seed banks in a heathland at Little Budworth Common. Values are Mean ± SE, those sharing the same letters denoted no significant differences between the groups based on Tukey's HSD post-hoc test.

3.3.1.1.3 Seed bank functional groups

Shrubs were the most abundant group recorded in the seed bank; the total number of seedlings that emerged from the soil in all treatments was 1599. Forbs seed abundance was 38 seedlings, Grass seed abundance was 6 seedlings, Tree seed abundance was 5 seedlings and Sedges seed abundance was 1 seedling.

3.3.1.1.4 *Calluna vulgaris* seed bank response

Calluna vulgaris was the most abundant species recorded in the seed banks; the total number of seeds was 1599. The highest number of *C. vulgaris* seedlings was found under the 60 N treatment (448), while the lowest was in the control (351). However, there was no significant effect of N treatments on *C. vulgaris* seed bank abundance (ANOVA; F= 0.695; df= 3; $P = 0.573$) (Figure 25).

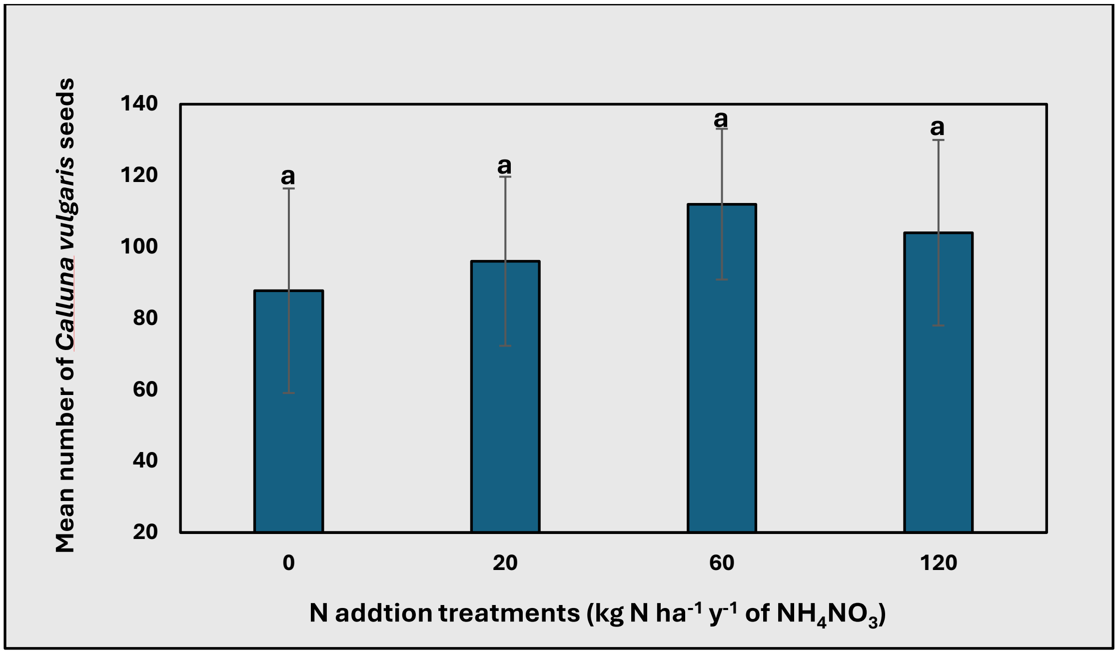

Figure 25 Mean number of Calluna vulgaris seeds per sample recorded in seed banks in a heathland at Little Budworth Common. Values are Mean ± SE, those sharing the same letters denoted no significant differences between the groups based on Tukey's HSD post-hoc test.

3.3.1.2 Effects of the long-term nitrogen deposition on above-ground vegetation.

3.3.1.2.1 Above-ground vegetation richness and composition

Overall, species richness was low; five species were recorded in all plots at Little Budworth Common (Table 6) and 1071 the total of cover percentage emerged from above-ground vegetation for all treatments. Above-ground vegetation richness did not change significantly by N deposition treatments (ANOVA; F= 1.759; df= 3; *P* = 0.208) (Figure 26).

Table 6 The cover percentage for five species recorded in the above-ground vegetation in the experimental plots (all N treatments), the mean cover percentage of *Calluna vulgaris* and the P value of *Calluna* at Budworth.

Species	**120 N**	**60 N**	**20 N**	**Control**
Shrubs				
Calluna vulgaris	115	225	245	155
Erica cinerea	135	17	45	55
Ulex europaeus	15	0	0	15
Ferns				
Pteridium aquilinum	30	0	10	0
Grasses				
Deschampsia flexuosa	8	0	1	0
Mean cover percentage of *Calluna*	**28.75**	**56.25**	**61.25**	**38.75**
Standard deviation	**11.08**	**15.47**	**17.96**	**20.56**
P value of *Calluna*	***P* = 0.05**			

The mean cover of *Calluna* in 20 N and 60 N was higher than in the 120 N and in control plots. However, these changes were not significant (ANOVA; F= 3.308; df= 3; $P = 0.05$) (Table 6).

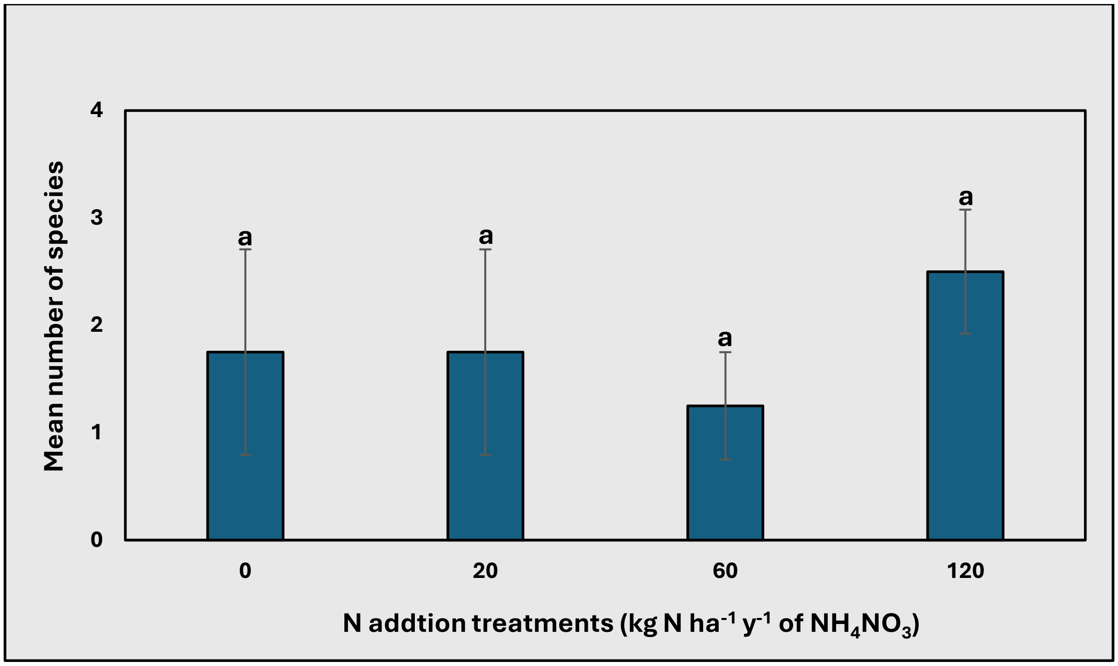

Figure 26 Mean number of species recorded in the above-ground vegetation in a heathland at Budworth Common. Values are Mean ± SE, those sharing the same letters denoted no significant differences between the groups based on Tukey's HSD post-hoc test.

Moreover, the long-term nitrogen deposition has not changed above-ground species composition (treatment was not significantly correlated with the NMDS ordination structure, $r^2 = 0.22$, $P = 0.38$, Figure 27).

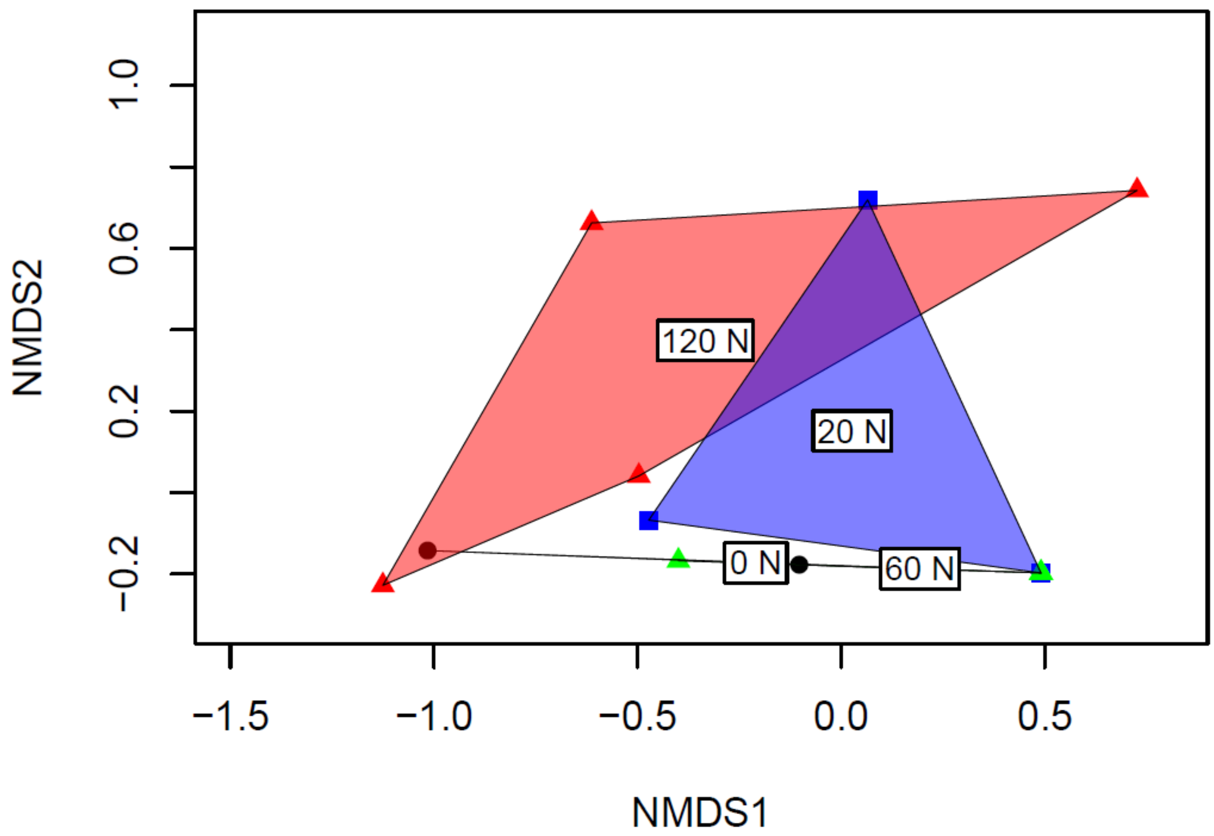

Figure 27 Two-dimensional non-metric multidimensional scaling (NMDS) ordination (stress = 0.049) of above-ground vegetation after the long-term N deposition at Little Budworth Common, UK. Treatments, as categorical variables (polygons), are represented by colours: black = control, blue = 20 N, green = 60 N and red = 120 N. Ordination is based on species abundance data; there were 4 plots in each treatment. Text shows centroids for N-treatment levels. The location of each ordination point shows the degree of similarity of this point to the others.

3.3.1.2.2 Above-ground vegetation cover

The long-term N deposition did not have a significant impact on above-ground cover (ANOVA; F= 0.774; df= 3; *P* = 0.53) (Figure 28). The lowest percentage of total cover was recorded in the control while the highest percentage of total cover was found in the 120 N treatment. The 20 N and 120 N treatments had nearly the same total vegetation cover, which was higher in plots receiving these treatments than in the control and 60 N plots. However, none of these differences were significant.

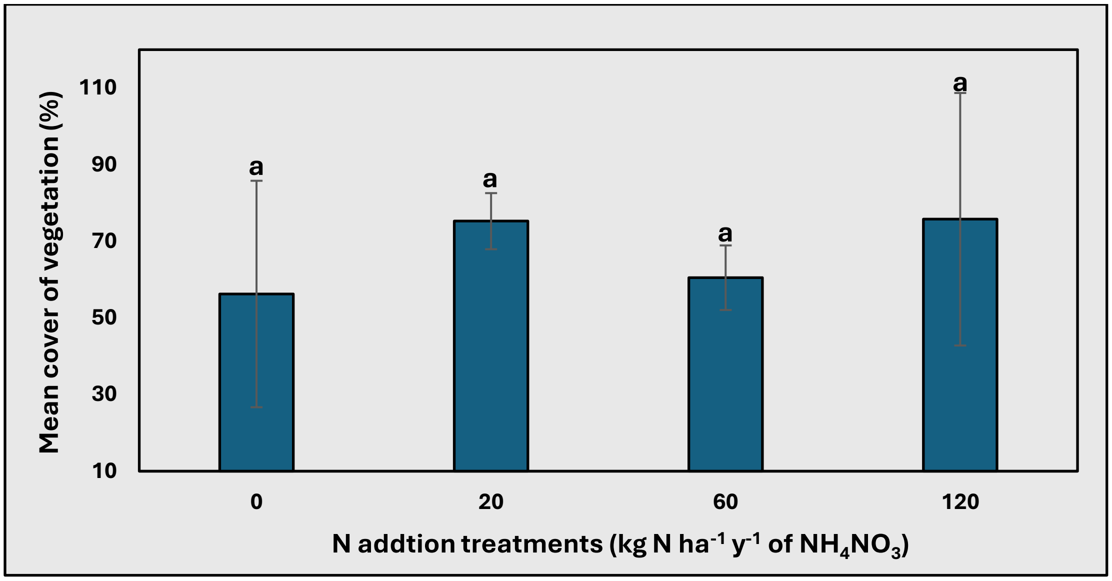

Figure 28 Mean percentage cover of above-ground vegetation, in a heathland at Budworth Common after 26 years of simulated N deposition. Values are Mean ± SE, those sharing the same letters denoted no significant differences between the groups based on Tukey's HSD post-hoc test.

3.3.1.2.3 Above-ground vegetation functional groups

Shrubs were also the dominant group in the above-ground vegetation. The total cover percentage of shrubs was 1022. Although the mean cover of shrubs under 20 N and 120 N treatments was higher than in the 60 N and the control treatments, between the treatments there were no significant differences (ANOVA; F= 0.391; df= 3; *P* = 0.762) (Figure 29).

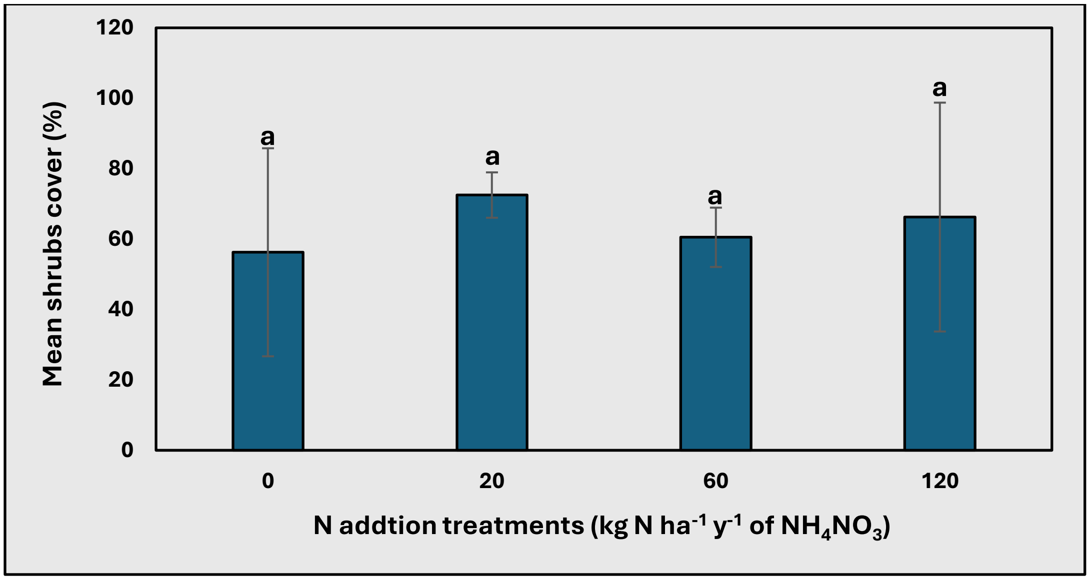

Figure 29 Mean percentage of shrub cover recorded in a heathland at Budworth Common under N addition treatments. Values are Mean ± SE, those sharing the same letters denoted no significant differences between the groups based on Tukey's HSD post-hoc test.

3.3.1.2.4 *Calluna vulgaris* above-ground vegetation cover

In the above-ground vegetation, *Calluna vulgaris* was also the dominant species. The cover of this species in plots receiving 20 and 60 kg N ha^{-}1 y^{-}1 was higher than in the plots receiving 120 N kg N ha^{-1} y^{-1} and in control plots. However, these changes were no significant (ANOVA; F= 3.308; df= 3; *P* = 0.057) (Figure 30).

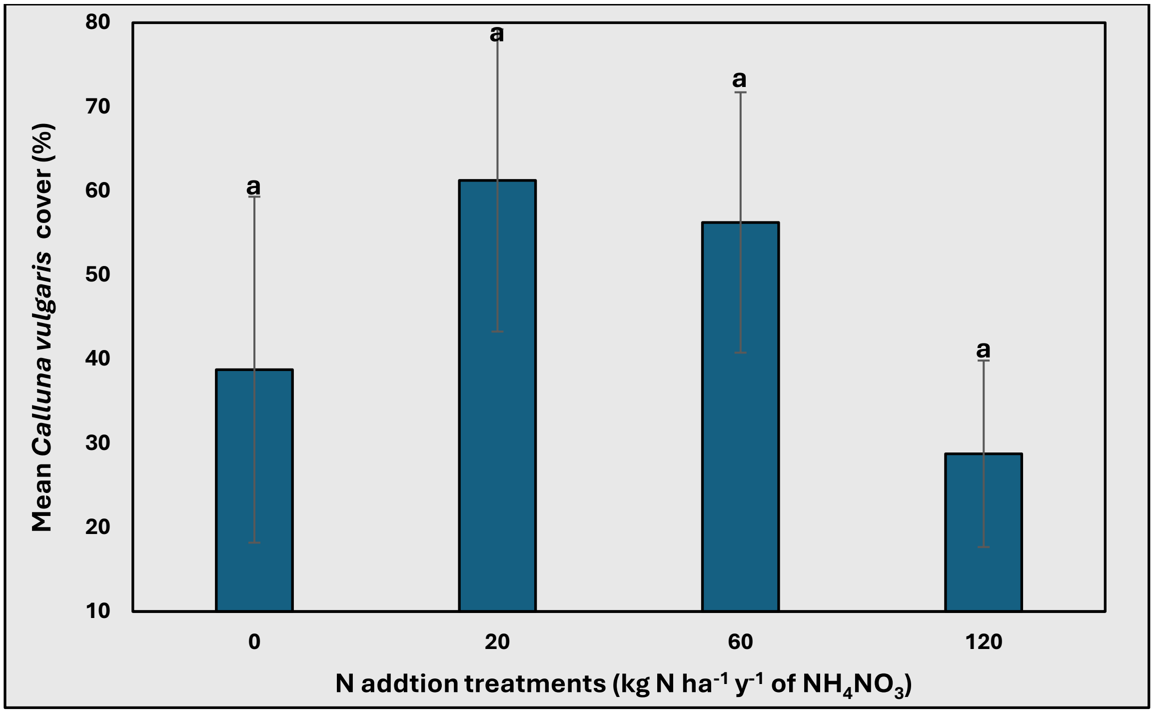

Figure 30 Mean percentage of *Calluna vulgaris* above-ground cover in each N treatment at Budworth Common. Values are Mean ± SE, those sharing the same letters denoted no significant differences between the groups based on Tukey's HSD post-hoc test.

3.3.1.3 The effect of the long-term nitrogen deposition on the similarity between the seed bank and the above-ground vegetation.

Relationship between vegetation and seed bank above- and below-ground species richness were not significantly correlated ($P = 0.461$). However, vegetation and seed bank species composition showed significant changes under the long-term nitrogen deposition (the correlation between N treatment and composition was significant but weak, $r^2 = 0.41$, $P = 0.047$, Figure 31).

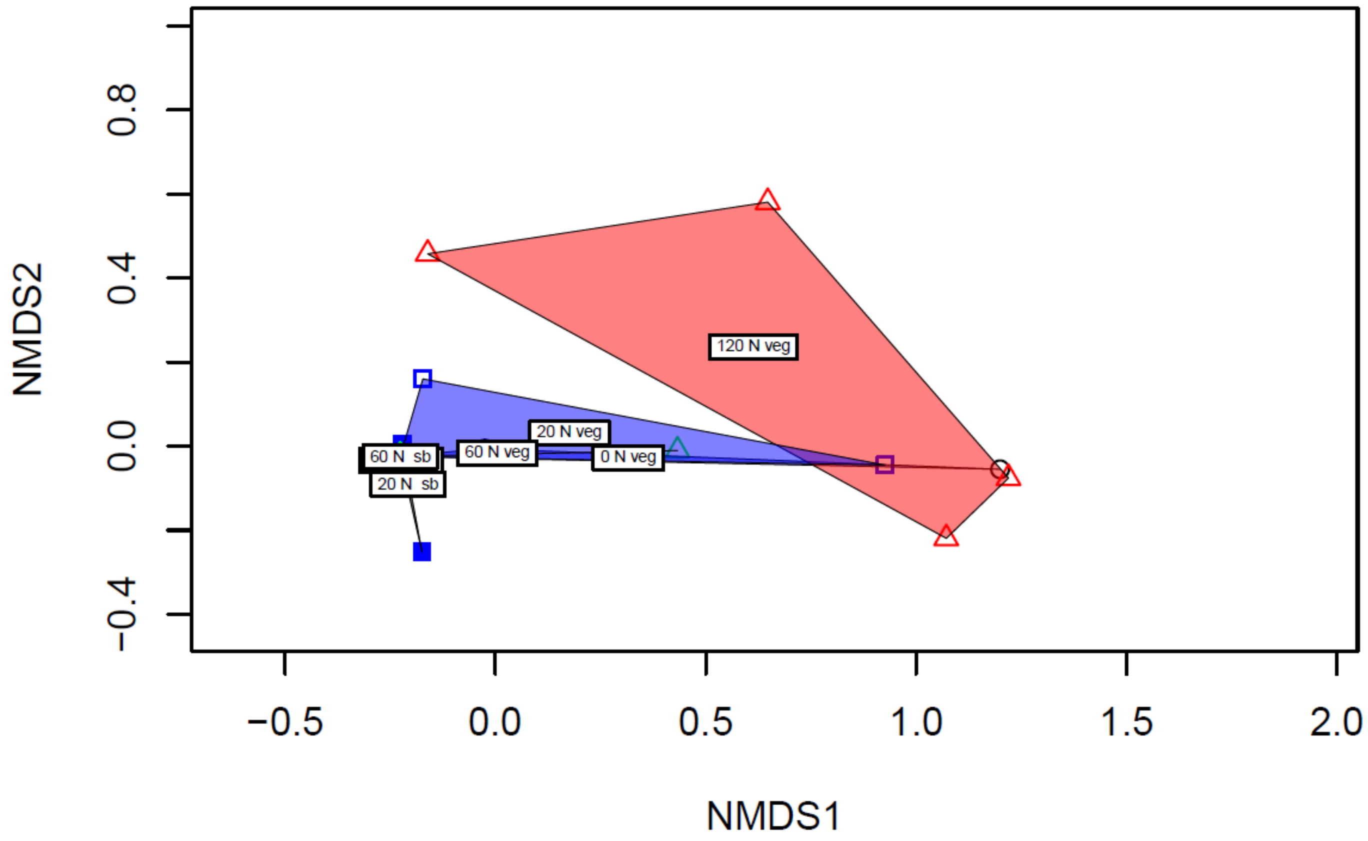

Figure 31 Two-dimensional nonmetric multidimensional scaling (NMDS) ordination (stress = 0.015) of seed banks and above-ground vegetation after 26 years of N addition at Little Budworth Common, UK. Closed symbols represent seed bank data and open symbols above-ground vegetation data. Treatments, as categorical variables (polygons), are represented by colours: black = control, blue = 20 N, green = 60 N and red = 120 N. Ordination is based on species abundance data; there were 4 plots in each treatment. Text shows centroids for N-treatment levels. The location of each ordination point shows the degree of similarity of this point to the others.

3.3.2 The long-term nitrogen deposition experiment at Ruabon Moor

3.3.2.1 Effects of the long-term nitrogen deposition on seed bank.

3.3.2.1.1 Seed bank richness and composition

Five species were recorded and 1157 seedlings emerged from seed banks for all treatments (Table 7). There was no significant effect of long-term N deposition on heathland seed bank richness at Ruabon Moor (ANOVA; F= 0.977; df= 4; *P* = 0.44). Although there was no significant difference between the treatments, the lowest mean number of species was recorded under the 40 N treatment and the highest was found in 10 N treatment but none of these differences were significant. After 24 years of N addition, no differences in the mean number of species between treatments were recorded (Figure 32).

Table 7 Species composition and the mean number of *Calluna vulgaris* obtained from the seed bank under different N treatments and the P value of *Calluna* at Ruabon.

Species	120 N	40 N	20 N	10 N	Control
Shrubs					
Calluna vulgaris	235	185	223	217	202
Forbs					
Cirsium vulgare	0	0	2	0	0
Gras					
Deschampsia flexuosa	7	0	12	11	9
Anthoxanthum odoratum	1	0	0	2	0

Rushes					
Juncus squarrosus	17	2	17	11	4
Mean Number of *Calluna*	**58.75**	**61.66**	**55.75**	**54.25**	**50.50**
Standard deviation	**2.50**	**2.88**	**1.50**	**5.67**	**1**
P value of *Calluna*	**$P < 0.00$**				

The mean number of *Calluna vulgaris* was the highest in 40 N while, the control plot was the lowest. Furthermore, 120 N treatments had more *Calluna vulgaris* than control, 10 and 20 treatments, while 10 and 20 N treatments had nearly same and there was significant difference in the mean number of *Calluna* between treatments (ANOVA; F= 6.409; df= 4; $P < 0.00$) (Table 7).

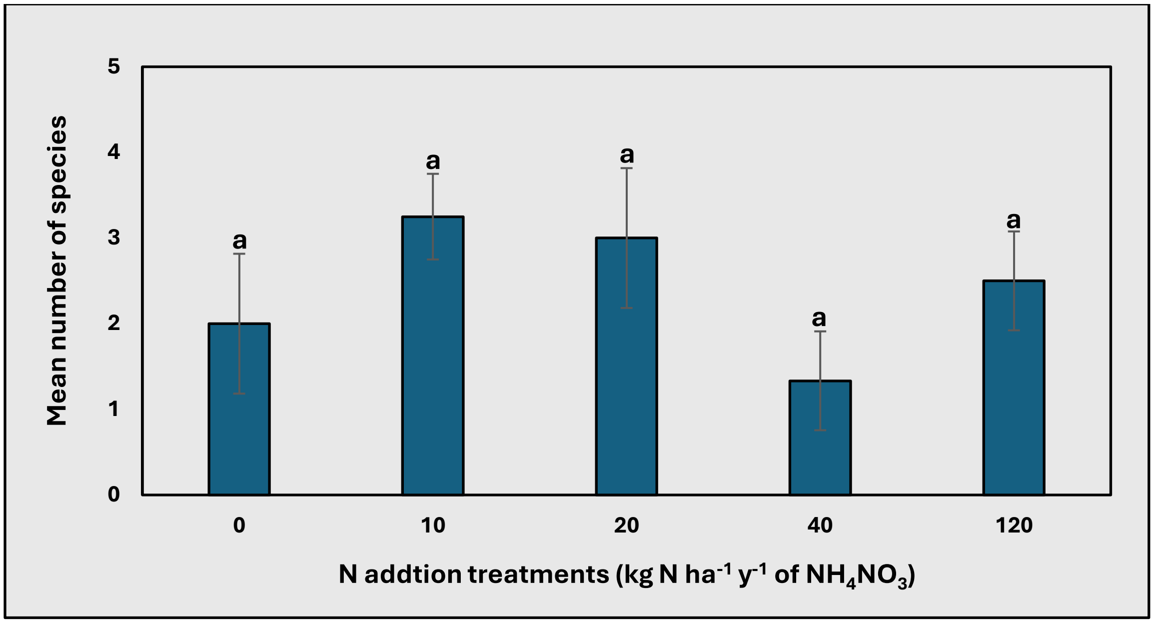

Figure 32 Mean number of species per sample germinating from seed banks in a heathland at Ruabon Moor, UK. Values are Mean ± SE, those sharing the same letters denoted no significant differences between the groups based on Tukey's HSD post-hoc test.

However, N deposition has changed seed bank composition (treatment was significantly but weakly correlated with the NMDS ordination structure, $r^2 = 0.43$, $P = 0.042$, Figure 33.

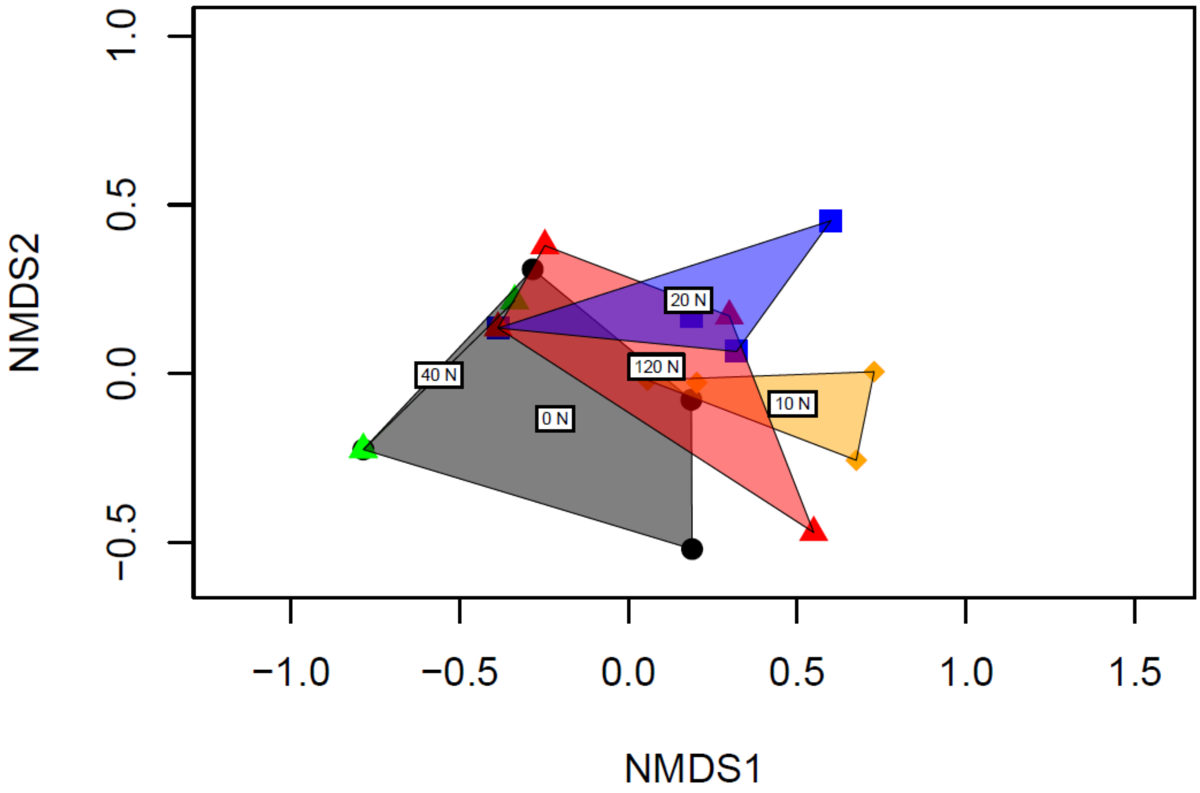

Figure 33 Two-dimensional non-metric multidimensional scaling (NMDS) ordination (stress = 0.074) of seed banks after 24 years of N addition at Ruabon Moor, UK. Treatments, as categorical variables (polygons), are represented by colours: black = control, orange = 10 N, blue = 20 N, green = 40 N and red = 120 N. Ordination is based on species abundance data; there were 5 plots in each treatment. Text shows centroids for N-treatment levels. The location of each ordination point shows the degree of similarity of this point to the others.

3.3.2.1.2 Seed bank abundance

N deposition had no significant effect on the mean number of seeds that germinated from the seed bank (ANOVA; F= 2.597; df= 4; *P* = 0.082), the lowest number of the seedlings was observed in the control while the highest number of seedlings was in the 120 N treatments, but this was not significant (Figure 34).

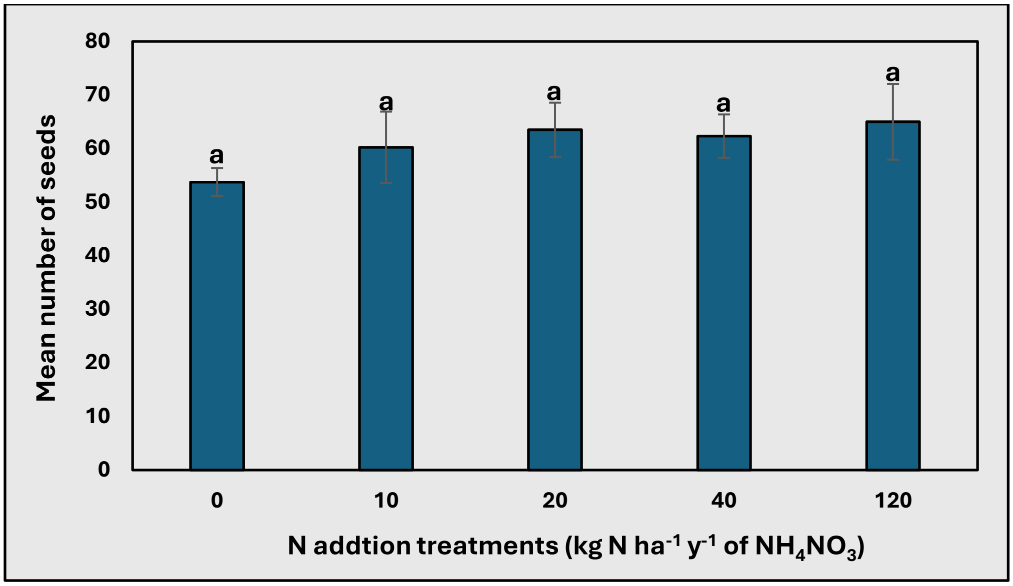

Figure 34 Mean number of seeds per sample germinating from seed banks in a heathland at Ruabon. Values are Mean ± SE, those sharing the same letters denoted no significant differences between the groups based on Tukey's HSD post-hoc test.

3.3.2.1.3 Seed bank functional groups

Shrubs were the most abundant group recorded in the seed bank; the total number of seedlings that emerged from the soil in all treatments was 1062. Rush seed abundance was 51 seedlings, Grass seed abundance was 42 seedlings, forbs seed abundance was 2 seedlings.

3.3.2.1.4 *Calluna vulgaris* seed bank response

Calluna vulgaris was the most abundant species in the seed bank. The number of seedlings increased significantly (ANOVA; F= 6.409; df= 4; $P < 0.004$) in soils receiving 40 kg N ha^{-1} per year. The highest number of seeds of Shrubs (245) were recorded in soils receiving 40 kg N ha^{-1} y^{-1} of NH_4NO_3, while the lowest was recorded in the control treatment (202). Moreover, for 120

N treatment had significantly more *Calluna vulgaris* seedlings than control, 10 and 20 treatments, while 10 and 20 N treatments had nearly same (Figure 35).

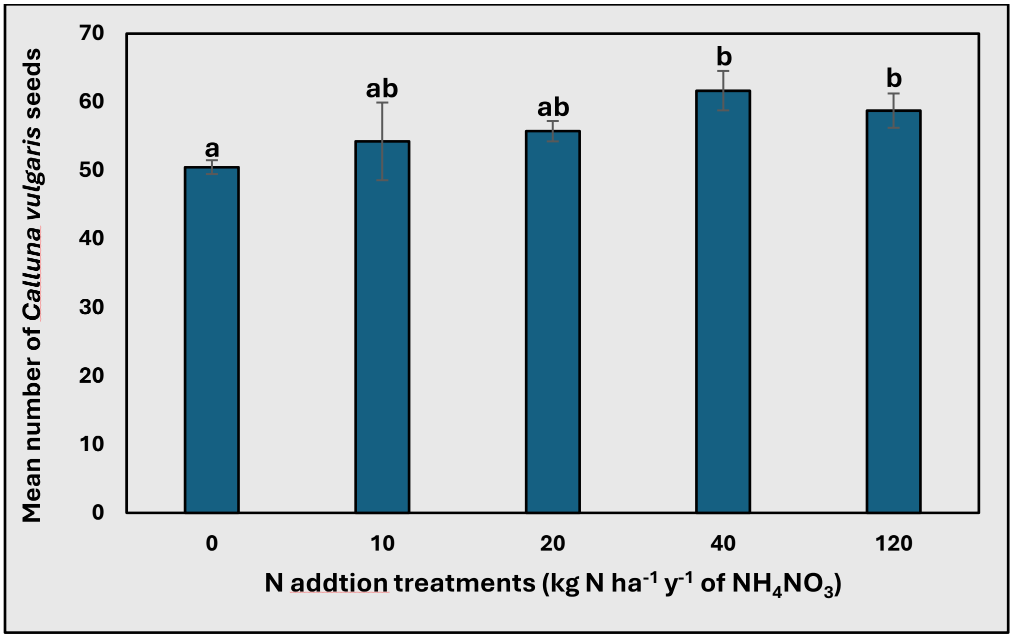

Figure 35 Mean number of *Calluna vulgaris* seeds per sample recorded in seed banks in a heathland at Ruabon Moor. The error bars show the standard error of the mean, those do not have identical letters denoted significant differences between the groups based on Tukey's HSD post-hoc test, with a significant level set P that less than 0.05.

3.3.2.2 Effects of the long-term nitrogen deposition on above-ground vegetation.

3.3.2.2.1 Above-ground vegetation richness and composition

Overall, species richness was low; four species were recorded in all plots at Ruabon Moor (Table 8) and 1517 the total of cover percentage emerged from above-ground vegetation for all treatments. Above-ground vegetation richness did not change significantly by N deposition treatments (ANOVA; F= 0.921; df= 4; *P* = 0.479) (Figure 36).

Table 8 The cover percentage for four species recorded in the above-ground vegetation in the experimental plots (all N treatments), the mean cover percentage of *Calluna vulgaris* and *Vaccinium myrtillus* and the p value of *Calluna and Vaccinium* at Ruabon.

Species	120 N	40 N	20 N	10 N	Control
Shrubs					
Calluna vulgaris	205	140	215	208	235
Vaccinium myrtillus	100	85	115	110	77
Erica cinerea	5	0	0	0	0
Rushes					
Juncus squarrosus	5	0	2	15	0
Mean cover percentage of *Calluna*	**51.25**	**46.66**	**53.75**	**52**	**58.75**
Standard deviation	**8.53**	**7.63**	**14.93**	**21.19**	**11.08**

Mean cover percentage of *Vaccinium*	**25**	**28.33**	**28.75**	**27.50**	**19.25**
Standard deviation	**14.71**	**16.07**	**10.30**	**9.57**	**10.27**
P value of *Calluna*	***P* = 0.84**				
P value of *Vaccinium*	***P* = 0.80**				

The mean cover of *Calluna vulgaris* in the control and 20 N treatment was higher than 120 N, 40 N and 10 N treatments. The lowest man cover of *Calluna* was found in 40 N treatment. However, there were no significant differences were found between the treatments (F= 0.348; df= 4; *P* = 0.841. For the mean cover of *Vaccinium,* 20 N and 40 N treatments were higher than the control plot and the treatments, while the control plot was the lowest, but the differences were not significant (ANOVA; F= 0.403; df= 4; *P* = 0.80) (Table 8).

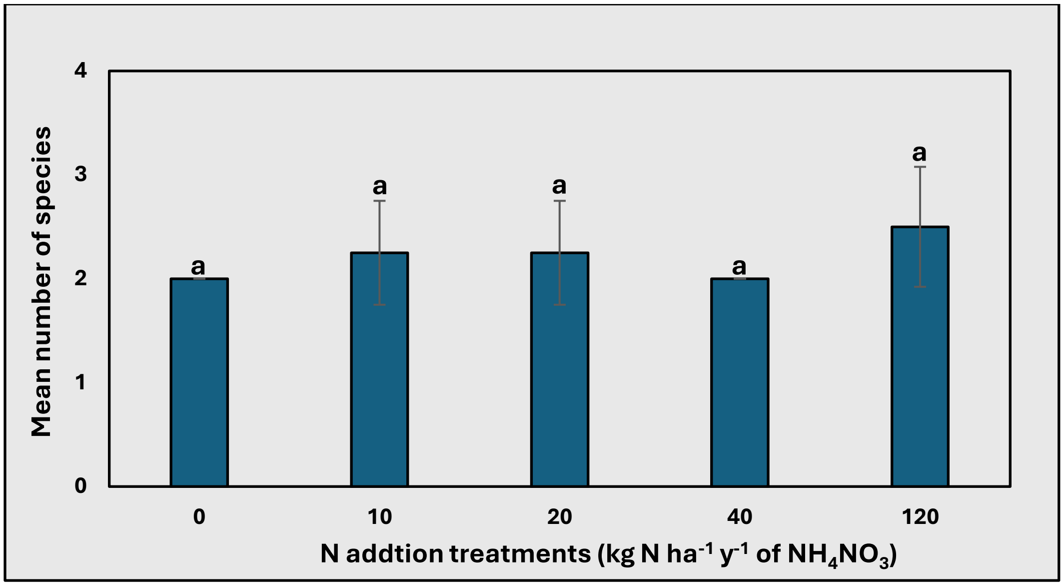

Figure 36 Mean number of species recorded in the above-ground vegetation in a heathland at Ruabon Moor. Values are Mean ± SE, those sharing the same letters denoted no significant differences between the groups based on Tukey's HSD post-hoc test.

Species composition was also not altered by any of the N treatments (treatment was not significantly correlated with the NMDS ordination structure, $r^2 = 0.14$, $P = 0.86$, Figure 37).

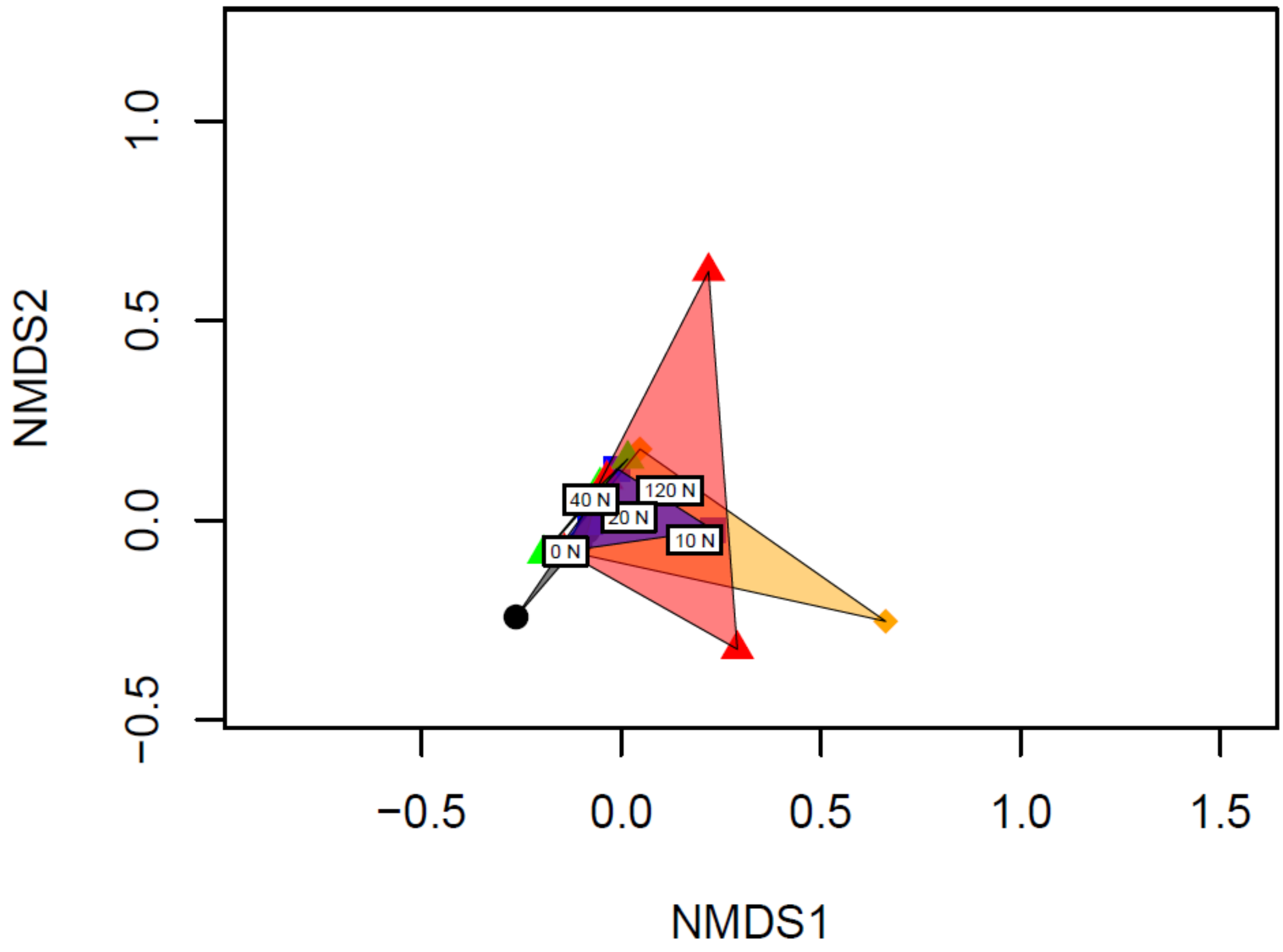

Figure 37 Two-dimensional non-metric multidimensional scaling (NMDS) ordination (stress = 0.025) of above-ground vegetation after 24 years of N addition at Ruabon Moor, UK. Treatments, as categorical variables (polygons), are represented by colours: black = control, orange = 10 N, blue = 20 N, green = 40 N and red = 120 N. Ordination is based on species abundance data; there were 5 plots in each treatment. Text shows centroids for N-treatment levels. The location of each ordination point shows the degree of similarity of this point to the others.

3.3.2.2.2 Above-ground vegetation cover

Above-ground cover was not significantly affected by the long-term N deposition (ANOVA; F= 0.261; df= 4; *P* = 0.898) (Figure 38). The lowest parentage of total cover was recorded in 40 N treatment while the highest percentage of total cover was found in 10 N treatment compared to the control. The 10 N treatment and 20 N treatments had nearly same total vegetation cover which was higher in plots receiving these treatments than in the control, 40 N and 120 N treatment. However, none of these differences were significant.

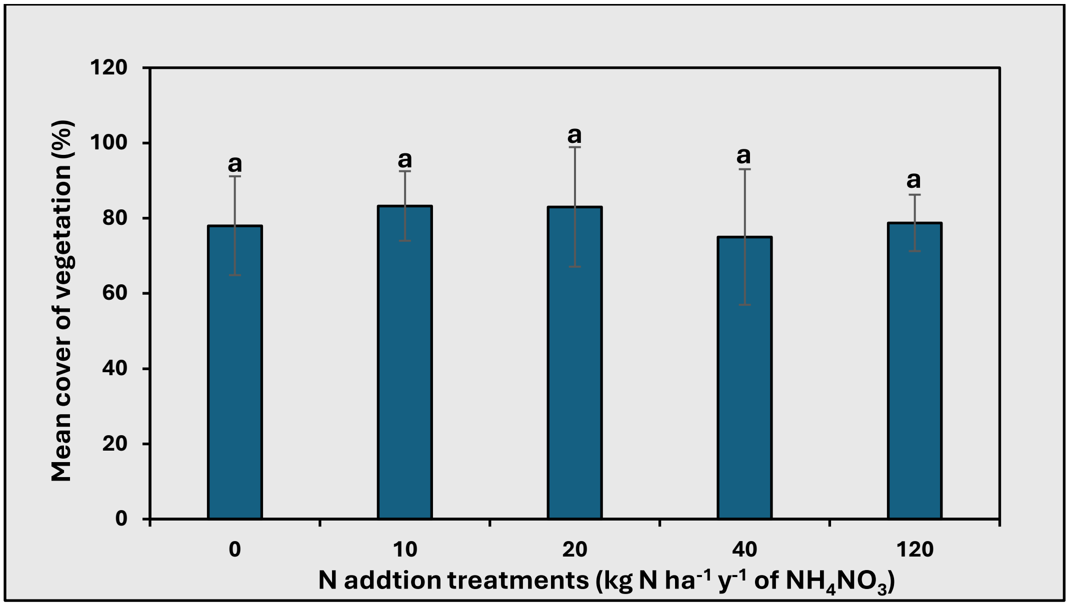

Figure 38 Mean percentage cover of above-ground vegetation, in a heathland at Ruabon Moor after 24 years of simulated N deposition. Values are Mean ± SE, those sharing the same letters denoted no significant differences between the groups based on Tukey's HSD post-hoc test.

3.3.2.2.3 Above-ground vegetation functional groups

Shrubs were also the dominant group in the above-ground vegetation. The mean cover of Shrubs shows that there was no significant difference found between the treatments (ANOVA; F= 0.145; df= 4; *P* = 0. 962). The total cover of shrubs was 1495. Between the treatments, there was no significant difference, the lowest mean cover of Shrubs was recorded under the 40 N treatment and the highest was found in 20 treatments but none of these differences were significant (Figure 39).

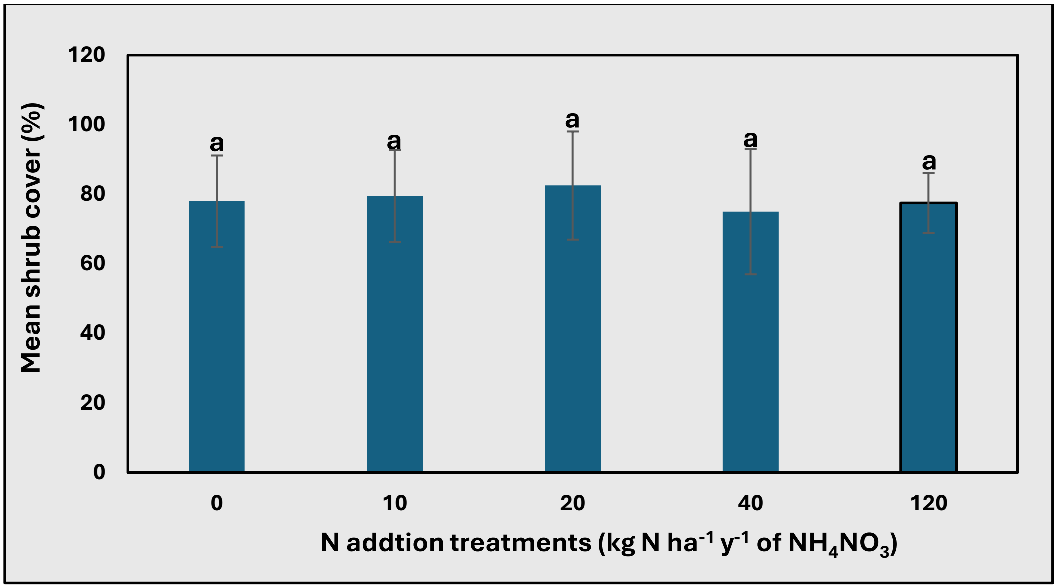

Figure 39 Mean percentage cover of shrubs recorded in a heathland at Ruabon Moor under N addition treatments. Values are Mean ± SE. Values are Mean ± SE, those sharing the same letters denoted no significant differences between the groups based on Tukey's HSD post-hoc test.

3.3.2.2.4 *Calluna vulgaris* above-ground vegetation cover

Calluna vulgaris was also the dominant species in the above-ground vegetation. The above data shows no significant differences between the treatments (ANOVA; F= 0.348; df= 4; *P* = 0.841).

The total cover of *Calluna* in the control and 20 N treatments was higher than 120 N, 40 N and 10 N treatments. The lowest total cover of *Calluna* was found in 40 N treatment but the data was highly variable which meant differences were not significant (Figure 40).

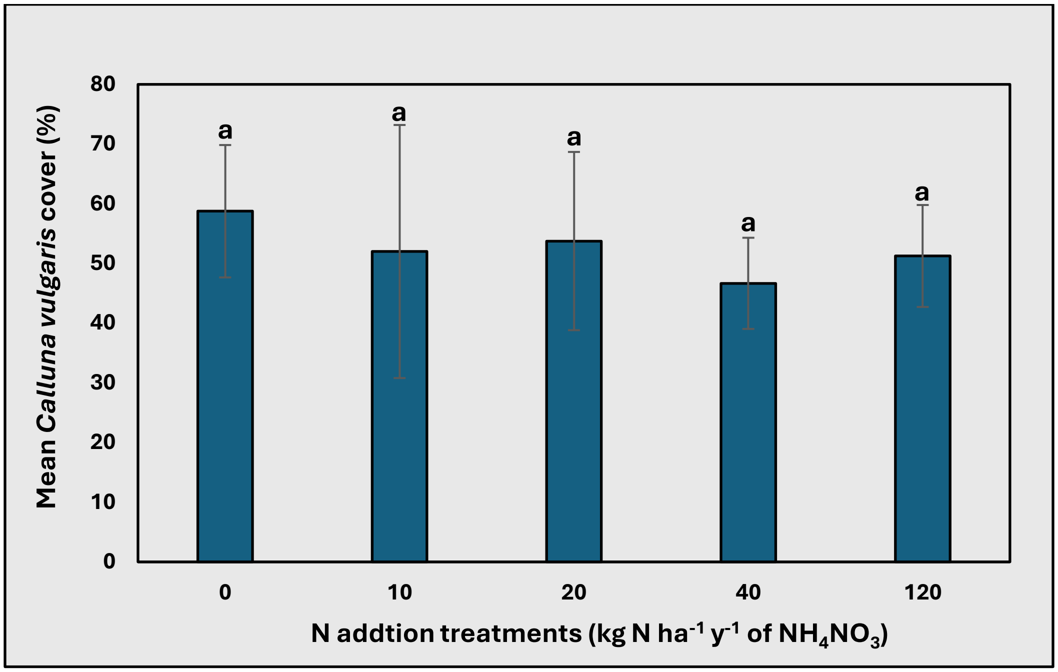

Figure 40 Mean percentage of *Calluna vulgaris* above-ground cover in each N treatment at Ruabon Moor. Values are Mean ± SE. Values are Mean ± SE, those sharing the same letters denoted no significant differences between the groups based on Tukey's HSD post-hoc test.

3.3.2.3 The effect of the long-term nitrogen deposition on the similarity between the seed bank and the above-ground vegetation.

Relationship between vegetation and seed bank above and below-ground species richness were not significantly correlated (*P* = 0.455). However, the species composition of the seed bank and the vegetation both showed significant changes under the long-term N deposition (the correlation between N treatment and composition was significant but weak, $r^2 = 0.72$, $P < 0.001$, Figure 41). There was a clear separation into two distinct groups along NMDS1 between seed bank and above-ground species composition indicating large and significant differences in composition caused by the long-term N deposition.

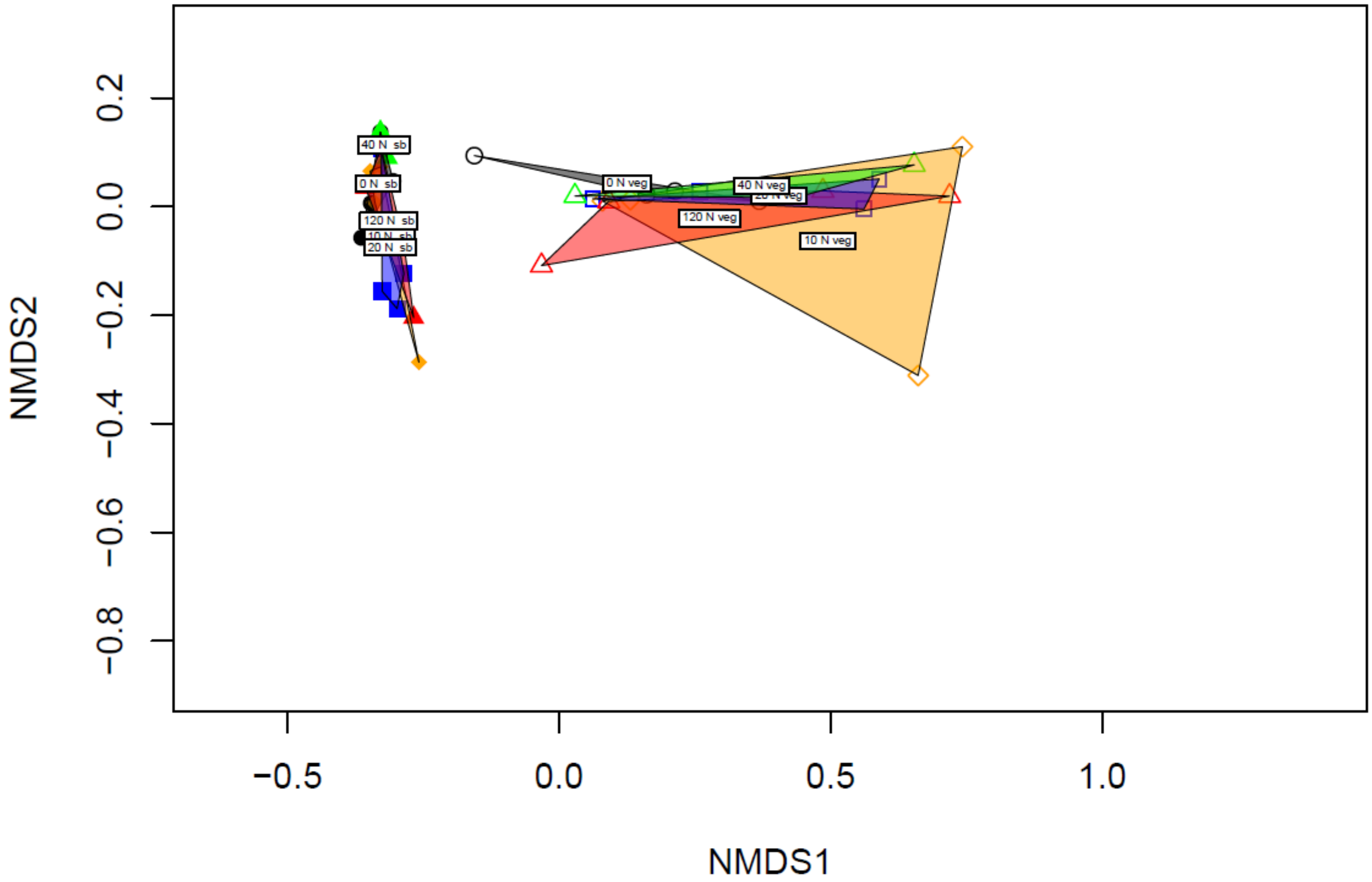

Figure 41 Two-dimensional nonmetric multidimensional scaling (NMDS) ordination (stress = 0.026) of seed banks and above-ground vegetation after 24 years of N addition at Ruabon Moor, UK. Closed symbols represent seed bank data and open symbols above-ground vegetation data. Treatments, as categorical variables (polygons), are represented by colours: black = control, orange = 10 N, blue = 20 N, green = 40 N and red = 120 N. Ordination is based on species abundance data; there were 5 plots in each treatment. Text shows centroids

for N-treatment levels. The location of each ordination point shows the degree of similarity of this point to the others.

3.4 Discussion

3.4.1 Seed bank

Overall, our study showed that experimental N addition did not reduce the total number of seeds and the species richness of the heathland seed bank and above-ground vegetation for Budworth Common and Ruabon Moor. However, N deposition has altered seed bank composition in Ruabon Moor.

The size of seed bank is controlled by various soil properties such as nutrient level and pH (Pakeman et al., 2012, Török et al., 2018, Zhang et al., 2019). Furthermore, soil acidification is induced by N deposition (Horswill et al., 2008) that has deep rooted effects on germination of seeds causing germination in selected species either directly (Deska et al., 2011) or indirectly by increasing the toxicity of soil through toxic metal accumulation (Abedi et al., 2013); (Osaki et al., 1997) or altering microbial compositions and growth (Rousk et al., 2011). Boateng (2021) studied the effect of N and P deposition in Budworth and Ruabon (Boateng, 2021). He described that the addition of nitrogen has an adverse effect on *C. vulgaris* competitiveness and also causes soil acidification, limiting other nutrients such as phosphorous. The characteristics of soils, as influenced by biological, geological, and chemical processes, are intricately tied to their pH levels. Heathlands typically exhibit an acidic pH (Aerts and Heil, 2013) which plays a crucial role in regulating the availability of minerals in the soil for plant absorption. When nitrogen-induced acidification occurs in already acidic heathland soils (Boateng, 2021), it can trigger a decrease in the presence of other nutrients due to the leaching of basic cations. This leaching process can result in elevated levels of H^+ and Al_3^+ at the absorption sites within the soil (de Graaf et al., 1998), causing the decrease in soil pH. At lower pH levels, the breakdown of organic matter slows down, and nitrification the conversion of ammonium to nitrate is hindered, leading to an accumulation of ammonium ions (NH_4^+) (de Graaf et al., 1998). Consequently, this accumulation can lead to higher concentrations of Al_3^+ and NH_4^+ compounds, along with an increased ratio of aluminum to calcium (Al_3^+/Ca_2^+ ratio).

The potential impact of N addition on seed bank density is dependent upon various factors. Increased nitrogen inputs can potentially lead to either an increase or decrease in seed bank density (Basto et al., 2015). This outcome depends on whether the increased inputs primarily result in increased seed production, leading to higher seed bank density, or if they cause diminished seed production, resulting in elevated losses from the seed bank. However, some studies suggest no significanct difference in the total number of seeds of seed bank. For instance, Schneider and Allen (2012), found no significant difference in seed bank density in a desert community after short term N deposition. Our results both at Budworth and Ruabon support these findings.

N deposition can also alter the species richness of soil seed bank. There are a few studies to support this effect in desert community (Schneider and Allen, 2012) and grassland communities (Basto et al., 2015) but there are no such experimental works in heathland. For instance, Basto et al. (2015) found that addition of nitrogen in a thirteen year long-term experiment reduced the species richness of seed bank in acidic grassland (Basto et al., 2015). Furthermore, no recovery was observed even after 4 years of stopping N deposition. In contrast, no effect of N addition on species richness in desert community was reported by (Schneider and Allen, 2012) in a short-term experiment. In our study, no significant difference was observed for species richness between treated and the control plots in Budworth and similarly for Ruabon, no significant difference was observed. This may be due to low interspecific competition owing to sparse above-ground vegetation as observed in other studies (Kinugasa et al., 2012).

The effect of N deposition on the composition of the seed bank is derived from its influence on the above-ground plants and the process of seed germination. The increased accessibility of nutrients to the parent plant may stimulate changes in seed production and quality, resulting in the creation of a higher number of bigger seeds with improved germination rates (Fenner, 1992, NAYLOR, 1993)**.** The plants grown from larger seeds under high nutrient conditions have been proven to be superior in germination rate to plants grown from smaller seeds (Stanton, 1985). However, in a study of N addition on understory species in a forest, Wang et al. (2021) found no significant difference in the composition of the seed bank between UAN25 (Understory plot with 25kg N ha^{-1} y^{-1}) and UAN50 (Understory plot with 50 kg N ha^{-1} y^{-1}) treatments. Our studies support these results as no significant difference was observed in species composition of seed bank in Budworth. Furthermore, significant differences can also be observed in some cases. For instance, Basto et al. (2015) found significant differences in species composition in grassland after N deposition. Our studies support these results as a significant difference in species composition of seed bank in Ruabon under treatment 40 N. However, some seeds such as the fruit of billberry are likely to be eaten so the seeds will be removed from the plot. Similarly, grasses such as *deschampsia* are grazed upon. N pollutants have the potential to reduce the seed bank by encouraging seed germination (Ochoa-Hueso and Manrique, 2010, Plassmann et al., 2008). N deposition further causes soil acidification affecting the seed germination (Horswill et al., 2008). The impact of soil acidification on seed germination is complex and varies among species. In some cases, it may directly promote germination, while in other instances, the effects may be indirect.

As a result, the composition of the seed bank is subject to change due to the factors associated with N deposition and soil acidification (Basto et al., 2015).

N deposition effects can also lead to changes in functional groups in the seed bank. For instance, in Ruabon, we recorded a significant increase in the number of shrub seeds in the plot receiving 40 N treatment. In some cases, no effect of N treatment have been recorded, for example, Wang et al. (2021) found in the forest ecosystem that N deposition has no significant effect on seed banks of shrubs. In our results for Budworth, there was no significant effect of N treatment on the number of shrub seeds. The difference in response between the two sites could be due to soil conditions, climate, or interaction with wild herbivores. Field et al., (2013) studied the soil exchangeable nitrogen, soil solution N and mineralization in response to N deposition at Budworth, Ruabon and Whim (Field et al., 2013). Similar to our result, they found the increment in shrub *C. vulgaris* as compared to Budworth owing to a uniform *C. vulgaris* canopy in Ruabon resulting in thick layer of litter. This thick layer, which is absent in Budworth, has the potential to hold N, reducing its leaching. Furthermore, species receive their main nutrients from humus layer in upland heath whereas this humus layer is non-existent in lowland heath, so plants have to obtain their nutrients from organic and mineral layers (Boateng, 2021).

The flowering of *C. vulgaris* has been enhanced at heathland locations with nitrogen doses ranging from 7.7 to 60 kg N ha^{-1} yr^{-1}, as documented in different studies (Power et al., 1995, Uren et al., 1997, Pilkington, 2003, Cawley, 2000, Britton and Fisher, 2008). These responses can be quite large; *C. vulgaris* flowering was observed to double in a lowland heath with a relatively modest nitrogen dose of 15.4 kg N ha^{-1} yr^{-1} (Power et al., 1995), and an increase of up to threefold was recorded at a nitrogen dose of 50 kg N per hectare per year in low alpine heath (Britton and Fisher, 2008). For Budworth, we found no significant increase in the number of seedlings of *C. vulgaris*.

In contrast, in Ruabon there was a significant increase in the number of seedlings in soils receiving 40 kg N ha^{-1} per year. However more flowering doesn't represent more germination of seeds as it is dependent on various other factors. Chapman and Bannister (1995) desribed the dependance of *Calluna Vulgaris* flowering on altitude, soil moisture percent, soil organic matter percent, and soil pH (Chapman and Bannister, 1995).

3.4.2 Above-ground vegetation

There are mixed results on how heathland ecosystems respond to N deposition. For instance, In the Netherlands, where there is high N deposition rate, particularly in its reduced form, there has been observed a rapid shift from heathland dominated by *Calluna* to heathland dominated by grasses has been observed (Bobbink and Heil, 1993). On the contrary, in other locations, community composition changes are hard to notice. Field experiments in the UK have shown temporary increases in grass dominance, as well as harmful effects on lichens and bryophytes (Barker, 2001). In Denmark, the addition of ammonium nitrate did not result in any noticeable effects on species composition or dominance (Riis-Nielsen, 1997). However, the reason for that which is increases in the pest species heather beetle. N deposition caused population explosions and they ate all of the heather.

However, broader observations from field surveys and assessments of changes over time in heathland extent and composition in the UK have indicated a potential link between increased N deposition rates and the decline of heathlands dominated by *Calluna* (Marrs, 1993, Rose et al., 2000). A recent analysis in UK focusing on bogs and heathland suggests that the spatial increase in the frequency of species that requires more N is associated with reduced N deposition, but not oxidized N (Smart et al., 2003) (Smart et al., 2003). For Budworth and Ruabon, we found no significant increase in the number of seedlings of *C. vulgaris* on above-ground cover.

In our study, we found that there was no effect of N deposition on species richness, species composition, total cover, total *Calluna vulgaris* cover and functional groups cover of above-ground vegetation as the lack of response to the high background levels of deposition and that the critical load is already exceeded. Power et al. (1998a) studied the impact of N addition on species composition in lowland heathland in a 7-year experiment with 0 N kg ha^{-1}, 53.9 N kg ha^{-1} and 100.1 N kg ha^{-1}. They observed no significant difference in species composition between the treatments. Our studies support these results as no significant difference was observed in species composition of vegetation in Budworth and Ruabon.

3.4.3 The similarity between the seed banks and above-ground vegetation

Our study showed experimental N deposition has reduced the similarity of the species composition of the seed bank compared to above-ground vegetation at Budworth and Ruabon. N deposition causes the similarity between the below-ground seed bank and above-ground vegetation to be changed. Eskelinen et al. (2023) found that the addition of nutrients to soil increases the similarity between seed bank and vegetation in grasslands. Another study showed opposite results, a decrease in the similarity between above-ground vegetation and belowground seed bank in the plots receiving 140 N (Basto et al., 2015). When above-ground and below-ground species composition were compared, the ordination analysis confirmed a difference between seed bank and above-ground vegetation communities. The differences might arise due to various reasons. Nitrogen-containing compounds can stimulate seed germination, and N pollutants may diminish the seed bank by promoting germination. N deposition also acidifies soils, potentially affecting seed germination and promoting pathogenic microorganisms, leading to seed mortality. Additionally, high N deposition can impact flowering and seed production, while increasing litter build-up may act as a barrier to seed entry into the seed bank, thus reducing the similarity between seed bank and above ground vegetation (Basto et al., 2015).

3.4.4 The role of seed bank in heathland restoration and management

The recovery of the ecosystem depends on various factors such as soil, habitat and hydrological conditions, history of deposition and reduction extent but there are only a few studies have addressed the recovery of heathlands after N addition (Stevens, 2016). Species composition during recovery can be significantly hindered by several factors. Insufficient N processing and failure of recovery by underground community may lead to high soil N levels which in turn decreases the plant species that are affected by N addition, thus their representation in seed bank is reduced (Bakker and Berendse, 1999). The absence of a viable seed source for declining species presents a challenge for their recolonization (Bakker and Berendse, 1999), especially given the widespread impact of N deposition across extensive areas. There are no studies that particularly focus on seed bank role in recovery of heathland. Seed banks have contributed to recovery from nitrogen deposition (Basto et al., 2015). According to Wang et al. (2013), soil seed banks are especially crucial for vegetation restoration in degraded and fragmented grasslands because soil seed banks play a critical role in the restoration of vegetation in degraded and fragmented grasslands. This is particularly important because, as the total amount of vegetation cover declines, the ability to obtain seeds from the surrounding vegetation is greatly reduced (Wang et al., 2013).

Our studies for Ruabon showed more abundant *C. vulgaris* seed bank as compared to *Deschampsia flexuosa, Cirsium vulgare, Anthoxanthum odoratum*, and *Juncus squarrosus* as most part of the seed bank was represented by *C. vulgaris*. Putwain and Gillham (1990) explained the significance of seed bank in heathland restoration. They created the soil seed bank by addition of *Calluna vulgaris, Erica cinerea and Erica tetralix* along with a few other heathland species and found that the above ground vegetation reasonably correlated with the seed bank species composition. For Budworth, *C. vulgaris* exhibits a substantial seed bank, suggesting that a negative degradation might not be anticipated. However, other species identified in our findings for Budworth, such as *D. flexuosa*, *C. vulgare, Rumex acetosella, Betula pendula*, and *Carex caryophyllea* are notably scarce. Consequently, their limited presence suggests they might not contribute significantly to increasing diversity on their own.

Enhancing diversity within this habitat would necessitate targeted management strategies. To foster greater diversity within this habitat, a focused management approach is essential, potentially involving the introduction of additional seeds. However, it is noteworthy that *C. vulgaris*, being the dominant species with a substantial seed bank.

Studies suggest that decreases in nitrogen deposition might not prompt an immediate restoration of heathland habitats (Power et al., 2006, Stevens, 2016). The continued impact on plant growth, phenology, and microbial activity, alongside the accumulation of nitrogen in the soil, may impede or obstruct the process of ecosystem recovery (Power et al., 2006, Edmondson et al., 2013).

The primary objective for site managers typically revolves around improving habitat suitability, often accomplished through the removal of above-ground vegetation biomass and litter (Borer et al., 2014). The management strategies that can be adopted in heathland are grazing, burning and cutting. Grazing has a small impact on N removal whereas it can promote habitat suitability. Furthermore, burning removes considerable amount of N but also has its negative impact. Finally, cutting is considered to be one of the best methods for N removal but promotes little to habitat stability (Jones et al., 2017).

4 The effect of nutrient addition and the disturbance in grassland habitat

4.1 Introduction

Grasslands, covering approximately 30% of land surface on Earth, are important ecosystems at a global level as they provide food through grazing animals and support a wide range of species (Bi et al., 2020). Between 2000 and 2013, grasslands globally have shown a significant increase in net primary productivity with 60.94% grasslands restored as a response to the climate change and human activities that increase soil nutrients (Liu et al., 2019). Specifically in the UK, climate change has shown positive effects on the productivity of grasslands (Fezzi et al., 2014). By 2050, this productivity is expected to rise further, with biomass reaching 72 million tons by 2050 due to atmospheric CO_2 emissions (Qi et al., 2018). In addition to CO_2, atmospheric N deposition (Storkey et al., 2015, Payne et al., 2011), as well as agricultural nutrient additions (Fountain et al., 2008) and disturbance through rotavating (Walker et al., 2004) have all been shown to affect various properties of grasslands in the UK.

The addition of t nutrients to grasslands results in improved productivity (Alhamad et al., 2012, Bedaso et al., 2022, Pallett et al., 2016) with an increase in plant biomass leading to competitive exclusion, decrease in colonization and a subsequent reduction in diversity of plants (Francksen et al., 2022). Grasslands thrive and support high diversity due to balance in nitrogen and phosphorus concentrations (Fay et al., 2015, Harpole et al., 2011). These elements along with various other factors define the vegetation that can be established in a place (Liu et al., 2021, Luo et al., 2020). Research has proven that species richness of grasslands generally declines with increasing levels of nitrogen (Bonanomi et al., 2006). Furthermore, above-ground biomass of grasslands has been observed to increase with N addition in the UK (Cole et al., 2008) as well as globally (Lee et al., 2010). Phosphorus further affects ecosystems through increased productivity (Phoenix et al., 2003) leading to increase in above-ground biomass as well as reduction in plant species composition (Willems et al., 1993). Li et al. (2016b) shows that P addition leads to an increase in above-ground and below-ground biomass in grasslands. Additionally, previous research has demonstrated that elevated phosphorus levels adversely impact biodiversity in grasslands (Oerlemans et al., 2012). This occurs due to a reduction in niche dimensionality and increased competition for light, which together lead to a decrease in species diversity (Li et al., 2022). Specifically, P addition has shown to decrease the number of forbs in grasslands (Wang et al., 2024). Much less attention has been directed towards other nutrients such as potassium, but these are also known to influence plant communities, including species diversity and composition (Zhao et al., 2013, Chen et al., 2022). Kirkham et al. (1996) has shown that the addition of phosphorus and potassium individually reduced species diversity and led to dominance by species such as *Holcus lanatus, Rumex acetosa,* and *Lolium perenne* in grasslands of the UK. In addition to N, P and K individually, and the mix of macronutrients has shown a decrease in species diversity in grasslands (Harpole and Tilman, 2007). Addition of nutrients has been reported to increase grass cover as compared to forbs cover (Song et al., 2011). In the UK grasslands, forb cover has been observed to reduce as a response to N deposition (Stevens et al., 2006).

Disturbances within grassland ecosystems can influence the dynamics of colonization and extinction, as well as shift the competitive interactions between plant species (Grime, 1977, Turner et al., 1998). Disturbances such as rotavation can affect the characteristics of soil through various ways including elevating pH levels (Dolman and Sutherland (1994), which may change the species composition, leading to reduced grass biomass and increased forb biomass (Schnoor et al., 2011). The use of rotavator as a disturbance has been applied in various studies (Klaus et al., 2017, Nath et al., 2022). Klaus et al. (2017) reported that seven months after disturbing the grassland with rotavator, an increase in seedling number and species richness was observed. Moreover, such disturbances of a mechanical nature, especially over broader areas, changed the community composition by activating seed bank of soil and decreasing the competitive pressures from existing species (Mackey and Currie, 2001). Edwards and Crawley (1999) found that soil disturbance by rotovation reduced percentage cover of grasses in grassland and Doerr et al. (1984) reported an increase in the forb cover as a result of soil disturbance involving the mechanical mixing of topsoil. Furthermore, disturbance also affects the light availability as Gendron and Wilson (2007) reported an increase in the light availability with disturbance in grasslands.

Disturbance also interacts with nutrient addition. Some studies suggest that the impact of soil disturbance on species richness varies with nutrient levels, while others not support this (Wilson and Tilman, 1991, Lavorel et al., 1999). For example, according to a study by Wilson and Tilman (2002), only low or no nutrient addition can change the species richness under the disturbance whereas high nutrient in the presence of disturbance had no impact. Furthermore, the interaction effect of disturbance and nutrient addition has also been shown to impact species composition. For instance, short lived species are favoured by soil disturbance by rototiller and nutrient addition (Wilson and Tilman, 1991). The perennial species frequency either decreases or remains unaffected with nutrient addition whereas in the presence of disturbance, it always decreases (McIntyre et al., 1995, Inouye et al., 1987, Wilson and Tilman, 1991). Seabloom et al. (2020) found that the soil disturbance by sward introduced an instant temporary increase in the above-ground biomass of plants owing to the short-term release of the trapped nutrients but this biomass subsequently reduced leading to an overall decrease in biomass. When the NPK macronutrient treatment was applied with disturbance, the biomass not only increased but also persisted for 23 years, demonstrating the long-term positive effect of nutrient addition. In UK grassland, Chalmers et al. (2005) showed that the soil disturbance with a rotary hoe decreased the above-ground biomass whereas N addition increased it. Effects on cover of different species of grassland were studied by Gendron and Wilson (2007) who found that the increased nitrogen levels led to a higher cover of *Bromus inermis* grass, but this cover decreased under soil disturbance (tilling of plots). Conversely, the cover of the grass *Agropyron cristatum* remained unchanged with nitrogen addition but decreased under disturbance conditions.

The effect of nutrient addition and disturbance and the interaction between them on the species composition in grassland habitat shows a knowledge gap and few related studies look at the interaction between nutrients addition and the disturbance. This experiment spanned four years, from 2021 to 2024. During the initial two years, no treatments were applied to provide baseline data. In the following two years, treatments involving nutrient addition (NPKµ) and soil disturbance were implemented. The disturbance treatment involved moving turfs and disturbing the topsoil to investigate the effects of nutrient addition, soil disturbance, and their interaction on species diversity, biomass, functional groups, and light availability in a grassland habitat.

The study's hypotheses are as follows:

1. Nutrient addition will reduce species richness in the grassland, alter its species composition and increase the biomass, leading to changes in plant diversity and productivity.

2. Nutrient addition combined with disturbance treatment will reduce forb biomass and cover, demonstrating an interaction between nutrient enrichment and mechanical disturbance.

3. The disturbance treatment will reduce the biomass of grass and alter species composition, impacting the overall ecological structure and function of the grassland.

4. Light availability will be increased by disturbance treatment, influencing the competitive dynamics and colonization patterns of plant species in the grassland habitat.

4.2 Methods

4.2.1 Site Description

The experimental site in Lancaster is located at Hazelrigg (54.013714° N, -2.77558° W) (Figure 42a). It is part of the network DRAGNet, which is a global experiment about the impact of nutrient addition and the physical disturbance on the plants and soil (DRAGNet, 2021). Background nitrogen deposition is 18.9 kg N ha^{-1} yr^{-1}. Fertilisers have not been added in the last twenty years though land use before this is not fully known. The experiment was set up in 2021, it has 12 plots with three replicated blocks with 5 x 5 m^2 dimensions separated by 1 m walkways. All the data for this study were collected from the designed plot for core sampling (2 x 2 m) see (Figure 42c). The disturbance treatment was rotavation to 15 cm depth. The disturbance treatment was applied by clipping all vegetation by using a Medium Duty Petrol Rotavator / Tiller. In mid-April 2023 and on the first of April 2024, nutrient treatments were applied: Control, NPKμ, Disturbance, Disturbance + NPKμ rate of 10 g m^{-2} y^{-1} of each nutrient. Nitrogen was added in the form of Urea, phosphorus in the form triple super phosphate, potassium in the form potassium sulphate. A micronutrient mix was added at a rate of 0.1 g m^{-2} y^{-1} in the first year only.

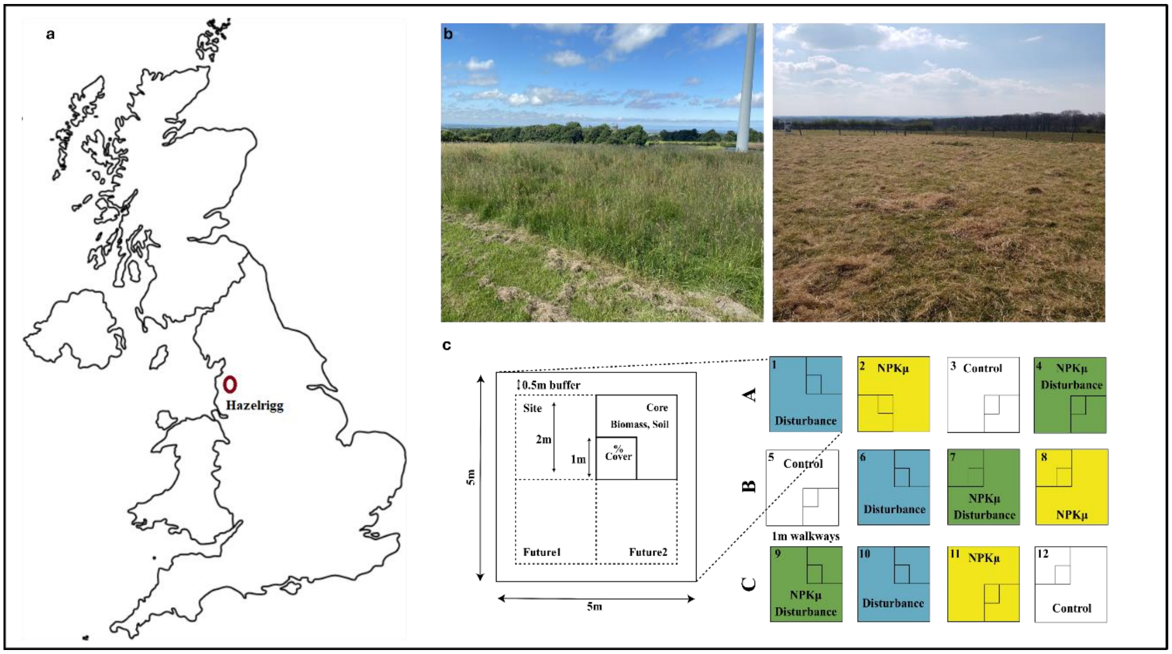

Figure 42 The map of the Hazelrigg study site, (b) the plots in the field site (c) the schematic diagram shows the blocks A, B, and C, each one of them includes four treatments: 1) 10 g m^{-2} yr^{-1} of NPKμ represented by a yellow square, 2) 10 g m^{-2} yr^{-1} of NPKμ Disturbance represented by green square, 3) Disturbance represented by blue square and 4) the control plots represented by white square.

4.2.2 Sampling methods

In July 2021 and 2022 data were collected prior to the addition of treatments to provide baseline data, while in July 2023 and 2024 post-treatment data collection was carried out for above-ground biomass, plant species composition, and light availability. Above-ground biomass was collected for all plant species rooted within a 0.2 m^2 area, made up of two 10 × 100 cm strips, clipped at ground level. Then, standing vegetation was separated into grass, forb, and dead litter. The biomass was measured and recorded after being dried for 48 hours at 60 °C. Cover was recorded for each plant species in each plot that rooted within a 1 m^2 quadrat located in the core sampling area of the plot, vegetation canopy was evaluated visually and was quantified to the closest 1 %. Species richness and the Shannon-Wiener diversity index were calculated (Wang et al., 2021). The availability of light was recorded by using a light meter (Sky Instruments Standard Fibre Optic Light Measuring System). For each subplot, two measurements of the ambient light were made: one above and one below the canopy. The ratio of PAR below and above the canopy was used to calculate light availability, the mean was recorded in 10 readings in each subplot. In April 2021 prior to the addition of treatments at Hazlerigg, outside each 1 x 1 m quadrat located in the core sampling area of the plot, four soil samples were collected from 12 plots before the addition of the treatments, one in each corner by using a metal frame (10 × 10 × 5 cm deep, 500 cm^3 volume). The samples were homogenised by hand and stored in plastic bags. These samples were used to assess the soil seed bank following methods as outlined in chapter 3.

4.2.3 Data analysis

Before analysis, every variable was examined, and the normality was checked. For the data that were not normally distributed a logarithmic transformation (log 10) was used. To investigate specifically if the total number of seeds, the species cover of functional group, species diversity, species richness, biomass and light availability were influenced by nutrient addition and disturbance. All the variable analyses were conducted by ANOVA with nutrient addition and disturbance treatments as a fixed factor and block as a random factor to consider whether the number of species in each block was different among treatments with Tukey's method used for post-hoc pairwise comparisons. For seed bank data, the response variable was total mean number of seeds, and the explanatory variable was the treatment (Control, NPKμ, Disturbance, Disturbance + NPKμ). The total number of species recorded in the seed bank was determined for each plot. Analysis of the data was conducted using SPSS version 27 (SPSS 27, Microsoft Corporation, released 15.0.1., 2020) (SPSS 27, 2020). The significance value was set at *P* = .05.

4.3 Results

4.3.1 Effects of nutrients addition and disturbance treatments on above-ground vegetation

4.3.1.1 Pre-treatments in 2021 and 2022

Table 9a and 9b shows all the variables measured. There were no significant differences between treatment plots in 2021 (See Table 9a). In 2022 there were no significant differences between any plots (See table Table 9b).

Table 9 The P value of the different variables for above ground vegetation under nutrients addition and disturbance treatment (NPKμ, NPKμ+Disturbance, Disturbance, Control) in 2021 and 2022 (over a two-year period).

a

Pre-treatment 2021	Species richness	Species diversity-Shannon winner index	Forb biomass (g)	Grass biomass (Log 10)	Total biomass (g)	Light availability %	Forb cover %	Grass cover %
NPKµ	1	0.602	0.981	0.833	0.944	0.243	0.142	0.206
NPKµ + Disturbance	0.199	0.643	0.944	0.924	0.919	0.588	0.128	0.363
Disturbance	1	0.602	0.981	0.833	0.944	0.243	0.142	0.206
Control	0.919	1	0.991	0.734	0.879	0.912	0.179	0.233

b

Pre-treatment 2022	Species richness (Log 10)	Species diversity-Shannon winner index	Forb biomass (g)	Grass biomass (g)	Total biomass (g)	Light availability %	Forb cover (Log 10)	Grass cover %
NPKµ	1	0.223	0.157	0.510	0.266	0.997	0.696	0.515
NPKµ + Disturbance	0.434	0.095	0.531	0.998	0.918	0.878	0.293	0.444
Disturbance	1	0.223	0.157	0.510	0.266	0.997	0.696	0.515
Control	0.890	0.300	0.134	0.886	0.494	0.854	0.523	0.491

4.3.1.1.1 Seed bank abundance in 2021

Sixteen species were recorded in the seedbank (Table 10). Treatment did not have a significant impact on the average number of seeds that germinated from the seed bank (ANOVA; F= 0.538; df= 3; *P* = 0.667).

Table 10 Total number of seedlings obtained from the seed bank under nutrient addition and disturbance at Hazelrigg.

Species	**NPKμ**	**NPKμ + Disturbance**	**Disturbance**	**Control**
Trifolium repens	0	0	0	2
Prunella vulgaris	1	0	0	0
Cirsium arvense	5	1	1	1
Rumex acetosa	4	3	0	1
Ranuculus repens	40	99	5	31
Urtica dioica	0	1	1	0
Plantago lanceolata	1	0	0	0
Agrostis capillaris	68	34	23	27
Anthoxanthum odoratum	1	4	2	1
Festuca rubra	16	1	1	16
Holcus lanatus	1	3	9	2
Epilobium angustifolium	0	0	1	1
Poa pratensis	1	5	0	0

Sonchus oleraceus	0	0	0	1
Veronica serpyllifolia	0	0	1	1
Rorippa Palustris	1	0	0	0
Total number of seedlings	**139**	**151**	**44**	**84**
Mean Number of seedlings	**1.54**	**1.48**	**1.06**	**1.20**
Standard deviation	**0.47**	**0.67**	**0.41**	**0.56**

4.3.1.2 Post-treatments in 2023

4.3.1.2.1 Plant species richness and diversity

There was no significant effect of treatments on species richness (ANOVA; F= 1.070; df= 3; *P* = 0.415) (Figure 43a). However, vegetation diversity did change significantly with nutrient and disturbance treatments (F= 4.436; df= 3; *P* < 0.041). The highest species diversity was found in disturbance plot, while the lowest was found in NPKµ + Disturbance treatment, and there was significant difference only between Disturbance and NPKµ + Disturbance treatments (F= 4.436; df= 3; *P* < 0.033) (Figure 43b).

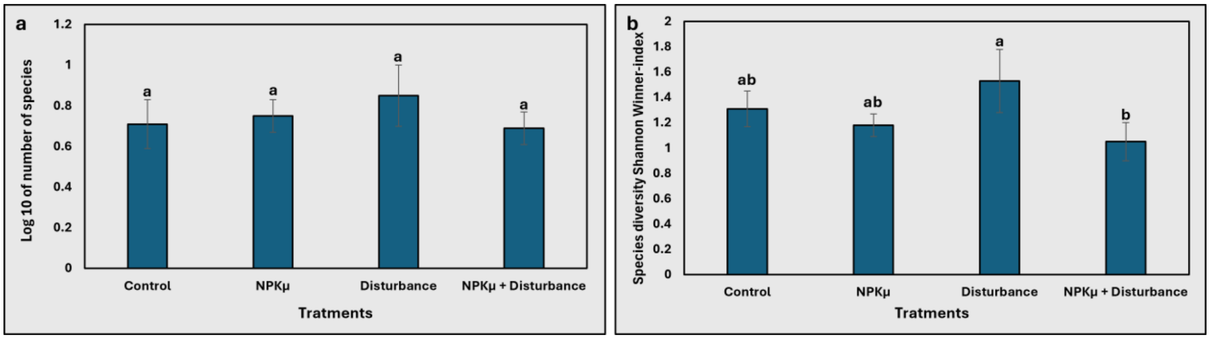

Figure 43 (a) The average of species richness, (b) Species diversity Shannon Winner-index for each treatment. Control, NPKμ, Disturbance and NPKμ+Disturbance; NPKμ indicates the nutrients addition (10 g m^{-2} yr^{-1} of each nutrient). The error bars show the standard error of the mean, those do not have identical letters denoted significant differences between the groups based on Tukey's HSD post-hoc test, with a significant level set P that less than 0.05.

4.3.1.2.2 Above-ground vegetation biomass

Total biomass, total biomass of forbs and total biomass of grasses did not differ significantly between treatments (ANOVA; F= 1.155; df= 3; *P* = 0.385, F= 3.924; df= 3; *P* = 0.054 and F= 0.096; df= 3; *P* = 0.960, respectively) (Figure 44a, 44b and 44c). However, total biomass of forbs was found significantly higher in Disturbance and NPKμ+Disturbance than in the control plots, while NPKμ was found the lower than other treatments, and there was significant difference only between NPKμ and Disturbance treatments (F= 3.924; df= 3; *P* = 0.042) (Figure 44b).

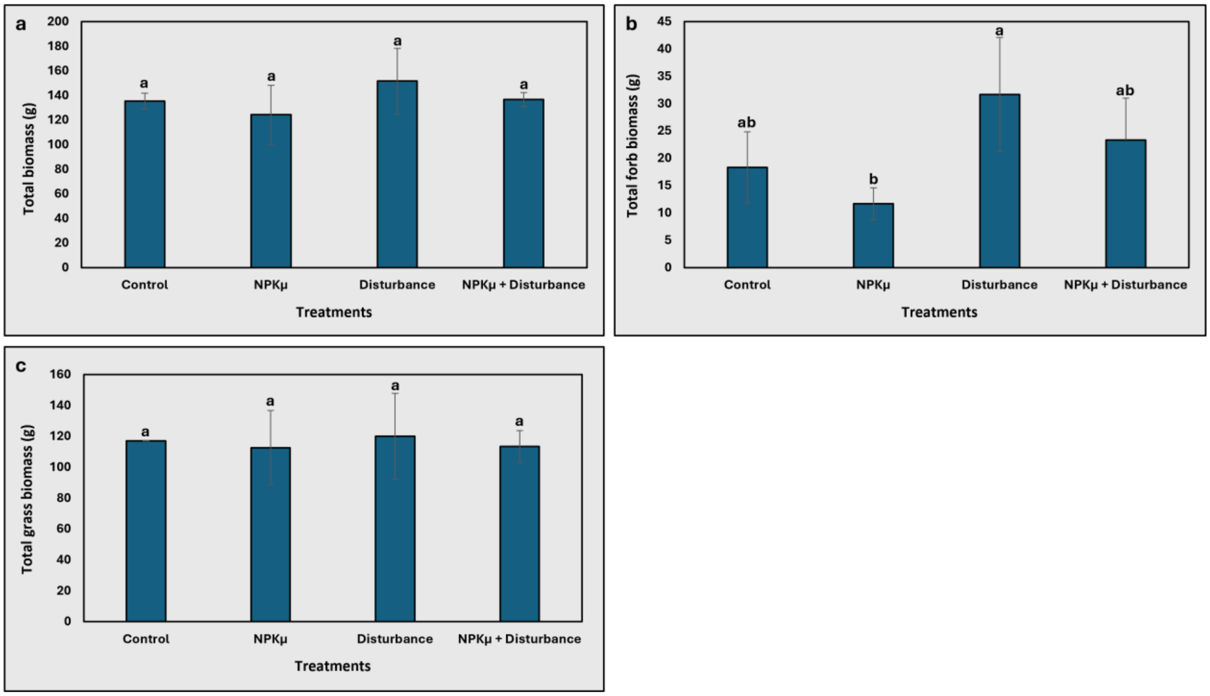

Figure 44 (a) The average of total biomass, (b) total forb biomass, (c) total grass biomass. The error bars show the standard error of the mean, those do not have identical letters denoted significant differences between the groups based on Tukey's HSD post-hoc test, with a significant level set P that less than 0.05.

4.3.1.2.3 Light availability

Light availability was not significantly different across the treatments (ANOVA; F= 0.202; df= 3; *P* = 0.892). The average of the light availability is quite constant throughout the treatments (Figure 45).

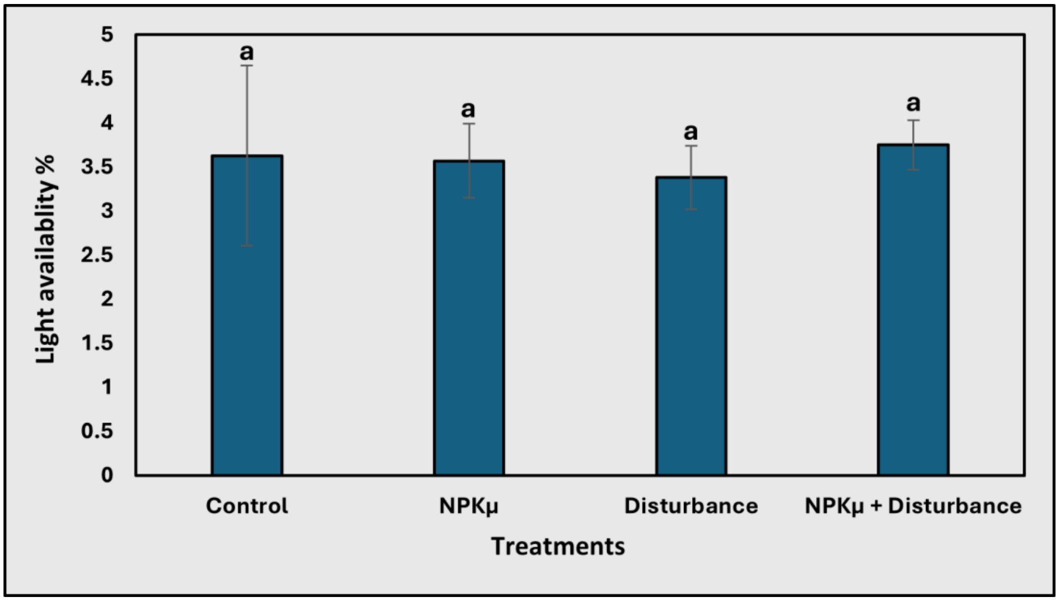

Figure 45 The average of light availability for each treatment presented by percentage. Values are Mean ± SE, those sharing the same letters denoted no significant differences between the groups based on Tukey's HSD post-hoc test.

4.3.1.2.4 Above-ground vegetation functional group

Although the mean cover of grass under (NPKμ + Disturbance) and NPKμ treatments was higher than in disturbance and the control plot, there were no significant differences were noted between the treatments (ANOVA; F= 2.861; df= 3; $P = 0.104$) (Figure 46b). However, the mean cover of forbs was significantly higher in disturbance than in NPKμ+ Disturbance, while NPKμ + Disturbance was the lower than the control and other treatments, and there was significant difference between Disturbance and NPKμ+ Disturbance treatments (F= 5.300; df= 3; $P < 0.039$) (Figure 46a).

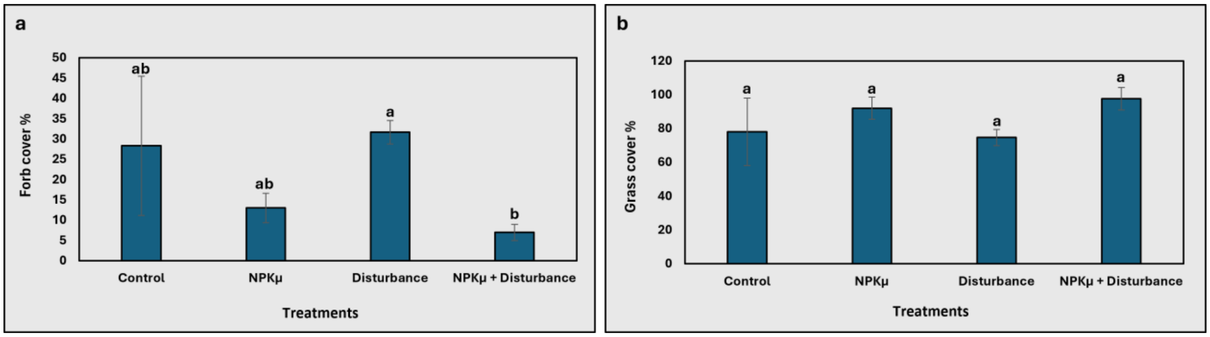

Figure 46 (a) Mean percentage cover of forbs, and (b) grasses recorded in a grassland at Hazelrigg under nutrients addition and disturbance treatments. The error bars show the standard error of the mean, those do not have identical letters denoted significant differences between the groups based on Tukey's HSD post-hoc test, with a significant level set P that less than 0.05.

4.3.1.3 Post-treatments in 2024

4.3.1.3.1 Plant species richness and diversity

There were no significant effect of treatments on species richness (ANOVA; F= 0.175; df= 3; *P* = 0.910) (Figure 47a). However, the nutrient and disturbance treatments had a considerable effect on vegetation diversity (F= 4.407; df= 3; *P* < 0.042). The highest species diversity was found in control plot, while the lowest was found in NPKμ + disturbance treatment, and there was significant difference between the control and NPKμ + disturbance treatments (F= 4.407; df= 3; *P* < 0.027) (Figure 47b).

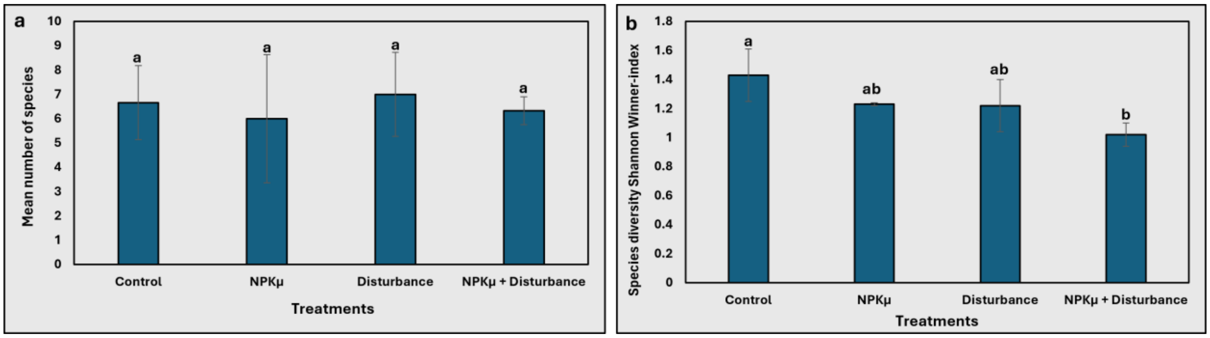

Figure 47 (a) The average of species richness, (b) Species diversity Shannon Winner-index for each treatment. Control, NPKµ, Disturbance and NPKµ+Disturbance; NPKµ indicates the nutrients addition (10 g m^{-2} yr^{-2} of each nutrient). The error bars show the standard error of the mean, those do not have identical letters denoted significant differences between the groups based on Tukey's HSD post-hoc test, with a significant level set P that less than 0.05.

4.3.1.3.2 Above-ground vegetation biomass

Treatments showed significant differences in total biomass, forbs biomass, and grass biomass (ANOVA; F= 35.207; df= 3; $P < 0.001$, F= 7.152; df= 3; $P < 0.012$ and F= 18.785; df= 3; $P < 0.01$, respectively), (Figure 48a, 48b, 48c). There was a significant decrease in the total biomass in the disturbance and NPKµ + disturbance treatments in comparison to the control (F= 35.207; df= 3; $P < 0.001$ and F= 35.207; df= 3; $P < 0.001$, respectively). Moreover, there was a significant difference between NPKµ + disturbance and NPKµ treatments (F= 35.207; df= 3; $P < 0.01$). Furthermore, a significant difference was seen between the disturbance and NPKµ + disturbance treatments (F= 35.207; df= 3; $P < 0.046$) (Figure 48a). The control plot had the highest total biomass of forb, whereas NPKµ and NPKµ + disturbance treatments had the lowest total biomass of forb, and there was significant difference between the control and NPKµ (F= 7.152; df= 3; $P <$

0.019), as well as the control and NPKμ + disturbance (F= 7.152; df= 3; $P < 0.014$). The highest total biomass of grass was recorded in the control plot and NPKμ, while the lowest biomass was observed in the disturbance treatment, and there was significant difference between the disturbance and NPKμ (F= 18.785; df= 3; $P < 0.00$) , between the disturbance and the control ($P < 0.00$), between NPKμ + disturbance and NPKμ (F= 18.785; df= 3; $P < 0.028$), and between NPKμ + disturbance and the control (F= 18.785; df= 3; $P < 0.014$) (Figure 48c).

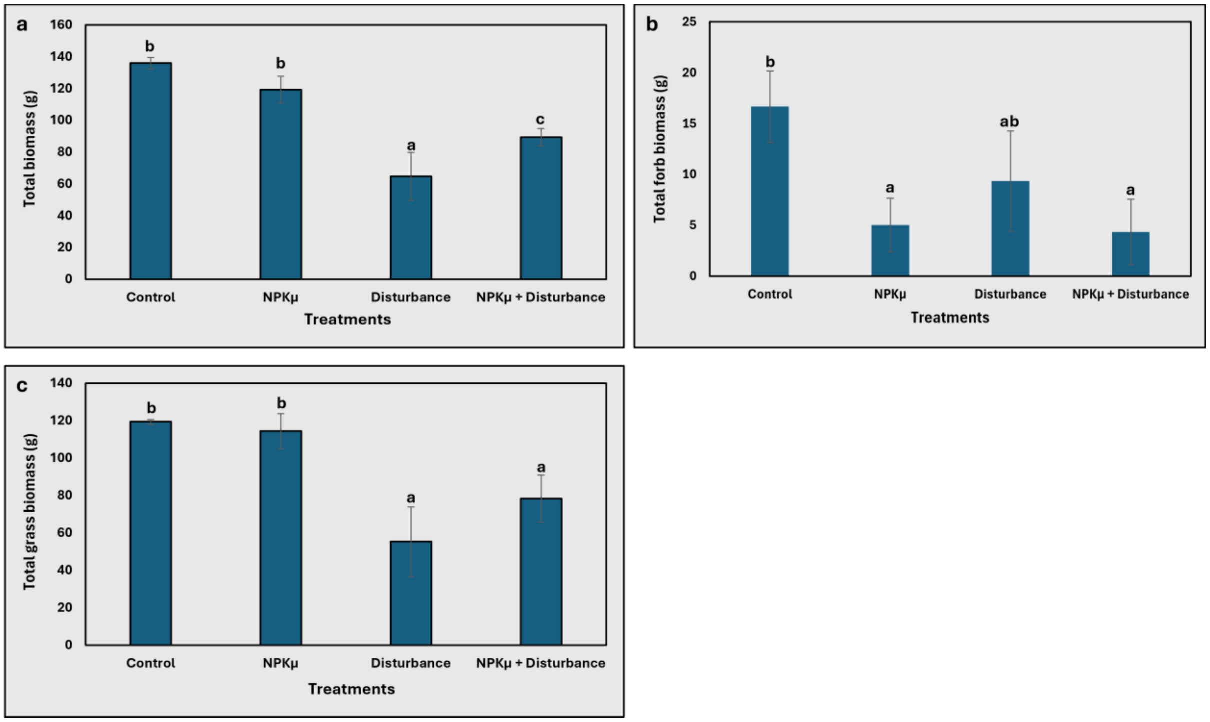

Figure 48 (a) The average of total biomass, (b) total forb biomass, (c) total grass biomass. The error bars show the standard error of the mean, those do not have identical letters denoted significant differences between the groups based on Tukey's HSD post-hoc test, with a significant level set P that less than 0.05.

4.3.1.3.3 Light availability

There was significant effect of treatments on Light availability. Light availability was significantly higher in disturbance than in the control and the treatments, and there was significant difference between the disturbance and the treatments (ANOVA; F= 7.204; df= 3; $P < 0.012$), (Figure 49).

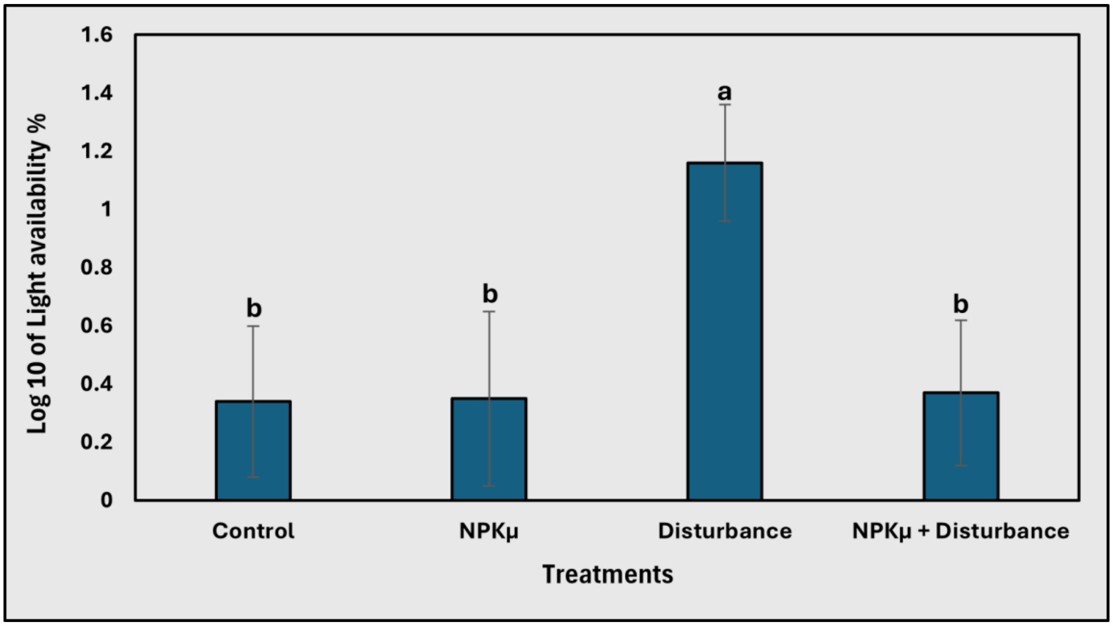

Figure 49 The average of light availability for each treatment presented by percentage.

4.3.1.3.4 Above-ground vegetation functional group

Although the p-value showed a significant difference in the mean grass cover between the treatments (ANOVA; F= 4.451; df= 3; $P < 0.041$) (Figure 50b), no significant difference was observed between the control plot and the treatments (F= 4.451; df= 3; $P = 0.056$, F= 4.451; df= 3; $P = 0.056$, F= 4.451; df= 3; $P = 0.609$ and F= 4.451; df= 3; $P = 0.068$, respectively). The mean cover of forbs was significantly higher in disturbance than in the treatments and the control plot, while NPKμ + Disturbance was the lower than the control and the treatments, and there was significant difference between disturbance and NPKµ+ Disturbance treatments (F= 4.592; df= 3; $P < 0.045$) (Figure 50a).

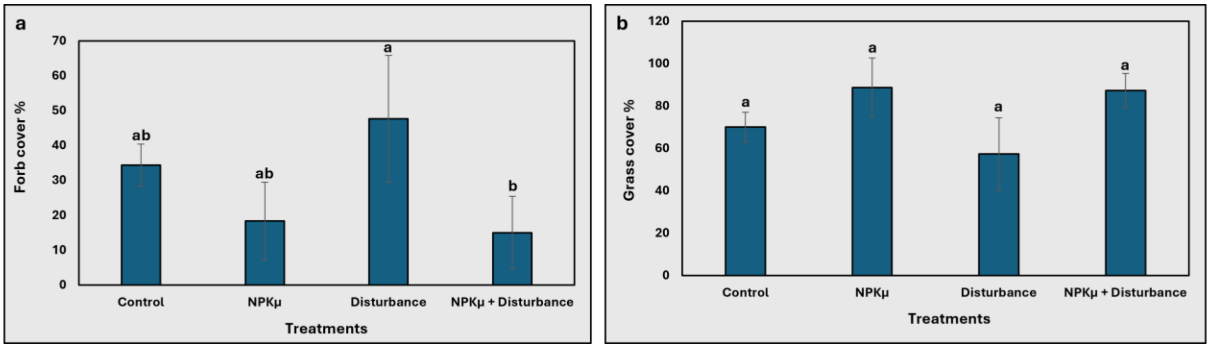

Figure 50 (a) Mean percentage cover of forbs, and (b) grasses recorded in a grassland at Hazelrigg under nutrients addition and disturbance treatments. Values are Mean ± SE. The error bars show the standard error of the mean, those do not have identical letters denoted significant differences between the groups based on Tukey's HSD post-hoc test, with a significant level set P that less than 0.05.

4.4 Discussion

4.4.1 Pre-treatment data (2021-2022)

During the pre-treatment phase of research (2021–2022), no nutrient additions or disturbance treatments were applied to the grassland plots. Therefore, it would be expected that untreated plots will naturally maintain their baseline ecological states. In such a case, the species composition remains rather stable in grassland habitats, and that there is no significant alteration in the biomass distribution in the absence of external interventions, for instance, added nutrients or other forms of disturbances (Gross et al., 2005). Community diversity and composition are, in that case, determined by local processes alone (Gross et al., 2005).

4.4.2 Post-treatment data (2023-2024)

4.4.2.1 Nutrient addition

Over the two-year period, nutrient addition had no significant impact on species diversity. In contrast to our study, Oerlemans et al. (2012) found that the addition of N fertilizer lead to a decrease in species diversity of grassland in UK owing to the competition between the species. Fertilization has been shown to alter the structure of plant communities, giving an advantage to species that grow quickly and are efficient at acquiring resources as well as to plants with higher productivity and moisture (Boch et al., 2021). Increased competition for light in dense, fertilized areas particularly disadvantages smaller species, causing their exclusion (Suding et al., 2005, Clark et al., 2007). Various studies have shown that the species diversity decreases with nutrient addition to grasslands (Hobbs and Huenneke, 1992, Rajaniemi, 2002, Baer et al., 2003). In contrast to the previous studies, species richness and diversity did not show any significant change in either year. This suggests that nutrient addition did not lead to the loss or introduction of species. This may be due to the already low species richness and the abundance of nutrient demanding species within the community. The already low value of species richness prior to nutrient addition has limited the potential for additional species loss under fertilization (Benizri and Amiaud, 2005, Francksen et al., 2022).

Another reason could be that there were already high nutrient levels present in the soil. Nutrient poor systems tends to be impacted more by nutrient addition as compared to nutrient rich systems (Brooks, 2003).

The impact of nutrient addition on total above-ground biomass was not significant in either year. The weaker response observed in either years may be due to initially high soil fertility, which meant that additional nutrients did not significantly affect biomass. However, to study the full extent of effect nutrient addition effect, more than 5 years are required (Fay et al., 2015). Various studies have shown that the addition of nutrients lead to increase in total aboveground biomass in grasslands (Li et al., 2022, Hobbs et al., 1988). Fay et al. (2015) found that the annual net primary productivity measured as above ground biomass of plants increased each year with NPK treatment. Hobbs et al. (1988) found four times increase in the total aboveground biomass of plants in as a response to fertilizer addition in grassland. Additionally, grass biomass has also been shown to increase following the nutrient addition as observed in various studies (Humbert et al., 2016, Hobbs et al., 1988). In contrast to the previous works, there was no significant effect of nutrient addition on grass biomass in our site. Our site did however, show a decrease in forb biomass across both years, suggesting that forbs were competitively suppressed by the nutrient-favoured grasses. This reduction in forb biomass aligns with the non-significant trend of decreased diversity and highlights the difficulties forbs face in nutrient-rich environments. The experiment performed by Hobbs et al. (1988) also supports our results as they found that the forb biomass reduces with nutrient addition.

Despite the reduction in biomass our site shows no significant effect of nutrient addition on forbs. This is supported by the results of Pillay et al. (2021) who found that the nutrient addition did not increase percentage forb cover significantly. In addition to forb cover, our results indicate that the grass cover also remained stable in both of the years, this may be due to the difficulty of assessing small variations in grass cover when it is already so high. Song et al. (2011) opposes our results as they reported an increased grass cover with the addition of nitrogen, although their species richness exhibited minimal variation across different N treatment levels. Additionally, Gendron and Wilson (2007) also found an increase in *B.inermis* grass cover when measured after 11 years of nutrient treatment.

Light availability was unaffected in both the years. Since plant cover remained stable, the structure and density of the vegetation did not change significantly. Since the canopy structure and density were similar before and after nutrient addition, the amount of sunlight that reached the lower layers of the ecosystem or the ground level also remained the same. In the second year, there was a slight but not significant decrease in light availability. This is in agreement with the studies of Borer et al. (2014) who found that nutrient addition limits the light availability by competitive exclusion of inferior species (Dybzinski and Tilman, 2007, Hautier et al., 2009).

4.4.2.2 Disturbance via rotavation

Rotavation led to a significant increase in species diversity in the first year but no significant change was recorded in the second year. This suggests that the disturbance disrupted the dominance of certain species, creating opportunities for a wider range of species to establish and thrive. By breaking up the soil and existing plant cover, rotavation likely opened up niches for less dominant species to take root, enhancing diversity (Schnoor and Olsson, 2010). Rotavation alters the community composition by stimulating the soil seed bank and reducing the competitive pressure from established species (Mackey and Currie, 2001). Previously, Schnoor et al. (2011) has shown that the rotavation can lead to change in species composition in grasslands. Another study by Schnoor et al. (2015) has shown that the functional diversity increased as a result of rotavation disturbance leading to the appearance of specialist species in rotavated plots. The community quickly shifted back to the initial phase in the second year, possibly because the site was already a nutrient rich site and nutrients mitigated the effects of disturbance.

The species richness remained unchanged in both years which means that the total number of species present did not change. The same species were present before and after the disturbance, but their proportions in the ecosystem changed in the first year. Supporting our results, Schnoor et al. (2011) also found no effect of rotavation disturbance on species richness of grassland. While rotavation did not significantly affect total biomass in 2023, it led to a significant decrease in 2024. It could be that the soil disturbance had affected the growth of the plants, leading to a reduction of their cumulative biomass over time, as they could not recover from repeated soil disturbance. This is in alignment with similar findings from Gendron and Wilson (2007), who postulated that aboveground biomass in the presence of disturbance that involved tilling 100% cover would reduce. Grass biomass did not differ in 2023 but decreased substantially in 2024. Therefore, it might be hypothesized that grasses, mainly dominant in the plant community under undisturbed conditions, were less capable of maintaining their biomass under the continued disturbance by rotavation. The disruption to the soil and plant roots likely weakened grasses, reducing their dominance. Similar results were reported by Schnoor et al. (2011) with a decrease in biomass for the grasses due to the disturbance through rotavation.

Forb cover also increased significantly in both years, further supporting the idea that rotavation promoted forb growth. As the soil was disturbed, forbs likely colonized the newly available space, expanding their cover in the ecosystem. The increase in forb cover with disturbance has been shown by Doerr et al. (1984) who found that the mechanical mixing of topsoil led to an increase in annual forb canopy cover with 4th year of disturbance showing the maximum mean forb canopy cover. Grass cover remained very high and stable in both the years. This is in contrast to Doerr et al. (1984) who found a decrease in perennial grasses cover after disturbance. Additionally, findings of Edwards and Crawley (1999) also found an increase in the grass cover after rotavation disturbance in grassland. Forb biomass also increased significantly in the first year, indicating that forbs positively responded to the rotavation, but showed no significant change in second year. Our results are supported by Schnoor et al. (2011), who also found out that the rotavation disturbance lead to an increased biomass of forbs. Unlike in 2023, forb biomass did not increase in 2024, which could be due to the exhaustion of the seed bank or the forbs having reached their carrying capacity in the disturbed environment.

Light availability increased significantly in 2024, but not in 2023. This coincides with the decrease in grass biomass in 2024. As grass decreased due to rotavation, more light penetrated to the lower layers of vegetation, benefiting forbs and other species that require more light. Gendron and Wilson (2007) supports our results as they found an increase in light availability of grassland after disturbance.

4.4.2.3 Interaction of nutrients and disturbance

The combined effects of nutrient addition and rotavation resulted in a significant decrease in diversity across both years. The overall decrease in species diversity suggests that although disturbance alone boosted diversity, the nutrient addition favoured certain dominant species. This counteracted the positive effects of disturbance, ultimately resulting in an overall reduction in species diversity. Species richness remained unchanged under the combined treatment, indicating that the interaction of nutrients and rotavation did not affect the total number of species present. Instead, the combined treatment influenced the balance and abundance of species without altering species numbers. Wilson and Tilman (2002) found that in the presence of high nutrients as well as the disturbance by rototiller, there was no change in the species richness of the grassland. The interaction had no significant effect on total biomass in 2023 but led to a decrease in 2024. Chalmers et al. (2005) has shown that the addition of nitrogen increases the aboveground biomass whereas the rotary hoe disturbance reduced it. This suggests that, in our results, the species present may play an important role in the outcome of the experiment. Grass biomass remained stable in 2023 but decreased significantly in 2024 under the combined treatment. Nutrient addition increases grass biomass (Hobbs et al., 1988) whereas the disturbance decreases it (Schnoor et al., 2011, Edwards and Crawley, 1999). This indicates that while nutrients supported grass growth in our results, the disturbance from rotavation eventually undermined this effect, leading to a decline in grass biomass. Nutrient addition combined with disturbance treatment will reduce forb biomass and cover. For the grass cover, our results showed that the grass biomass remained unaffected in the nutrient presence as well as the disturbance separately. There could also have been an interaction with weather conditions in the individual years.

Disturbance typically increases forb cover (Doerr et al., 1984), while nutrient addition tends to reduce it (Stevens et al., 2006). Our results show that, the nutrient addition offsets the positive effect of disturbance on forbs, leading to a net decrease in forb biomass and cover. In 2023, light availability remained unchanged; however, it significantly decreased in 2024 under the combined treatment, indicating that the effects of nutrient addition were more dominant than those of disturbance. Although grass biomass decreases due to the combined effects of disturbance and nutrient addition, the overall vegetation structure may still become denser due to the proliferation of other competitive species that can effectively reduce light availability.

Ecosystems with higher nutrient availability recover more rapidly from disturbances compared to those with lower nutrient levels (Seabloom et al., 2020). In our context, this means that the application of fertilizer following a disturbance can lead to a more rapid return to pre-disturbance conditions, as plants have the resources needed to regrow and recolonize the disturbed area more efficiently. Considering the poor species richness and dominance of a few species, the resilience of the community might be more dependent on the nutrient levels. High nutrient levels could lead to quicker recovery of the dominant species, potentially reinforcing their dominance and suppressing the recovery of less competitive species. This could result in a faster recovery but also in a less diverse community post-disturbance.

5 Soil recovery in a long-term nitrogen addition experiment

5.1 Introduction

N deposition is a major threat to ecosystems (Shi et al., 2018). The global causes of elevated N deposition include fossil fuel combustion, changes in land-use patterns, biomass burning and use of nitrogen-based fertilizers (Verma and Sagar, 2020). N deposition is responsible for changes in the nitrogen status of the soil, including microbial biomass nitrogen, total mineral nitrogen and soil pH. This results in changes in soil dynamics such as the rate of soil nitrogen leaching, nitrogen mineralization, nitrification and ammonification (Verma and Sagar, 2020), which in turn leads to changes in plant diversity, composition and carbon storage capacity. N deposition is found to be among key factors influencing climate change and is a major modulator of the climate change being experienced globally, and hence a threat to the well-being of humans (Verma and Sagar, 2020, Carter et al., 2017).

Rockström et al. (2009) recommended a maximum limit for the N cycle be fixed at 35 Tg N yr^{-1}, with an allowance preserved for agricultural purposes and industrial fixation of N_2 as part of boundary pillars. The seven planetary boundary pillars operate in a safe space for humanity based on the biophysical processes that maintain the stability of the earth. According to Rockström et al. (2009), it is essential to address biogeochemical processes as a boundary pillar. The boundary pillar links the biogeochemical processes within the earth's ecosystem and specifically the nitrogen cycle because it will help address the issue of environmental change.

Rates of N deposition have been declining in some parts of Europe, as a result of a wide range of policies aimed at reduction of industrial emissions (Fowler et al., 2007). Over several decades, from the 1990s to 2020, there has been a notable decline in industrial emissions within the countries listed in the EU28 (Dirnböck et al., 2018). NO_x components have significantly reduced by more than 50% compared to emissions before the 1990s (Dirnböck et al., 2018). The European Environment Agency EEA (2018) reported that at 41%, the transport sector was responsible for the highest proportion of NO_x emissions. A 44% reduction in NO_x emissions was recorded between 1990 and 2011 within the 33 EEA countries (except Turkey, Cyprus, Luxembourg, and Malta). In comparison, NH_3 reduced at a slower rate of below 30% between 1990 and 2015 (Dirnböck et al., 2018).

An understanding of how ecosystems recover from N is important. One approach to gain understanding of the impact of both industrial and agricultural emission reductions is the use of continuous monitoring and assessment of ecosystems following the cessation of N additions (Bowman et al., 2018). Bowman et al. (2018) used an experiment that increased N deposition rates by 0, 20, 40, and 60 kg N ha^{-1} yr^{-1} for nine years. After this time, they ceased additions and monitored for nine years. It was established that the experiment resulted in an increase in nitrification rates, a decrease in soil pH and elevated magnesium (Mg^{2+}), aluminium (Al^{3+}) and manganese (Mn^{2+}) levels. During the 12 years where they implemented the cessation of N deposition, there was an increase in microbial abundance (Bowman et al., 2018). Another approach includes the transplant of plants from polluted environments to less polluted environments. Granath et al. (2009) conducted their study in *Sphagnum balticum* and *Sphagnum fuscum* to establish their photosynthetic responses. The two species were transplanted within northern Europe from north, with a N deposition rate of 2.8 kg N ha^{-1} yr^{-1}, to south, where the N deposition was 14.9 kg N ha^{-1} yr^{-1}. The results indicated that *S. balticum* was more sensitive to N deposition than *S. fuscum*. Another approach involves cleaning rainfall of nitrogen to reduce the rates of deposition. A project implemented in five forested areas in Germany, Denmark, Sweden and the Netherlands under the NITREX project (Wright et al., 1995) aimed to establish the risks of nitrogen saturation in coniferous forests. The researchers added 35 kg NH_4NO_3-N ha^{-1} yr^{-1} in 5% extra water per week, to the ambient of 12 kg N ha^{-1} yr^{-1} and established that within the first two weeks of the experiment, elevated concentrations of nitrate runoff occurred. This was an indication that N saturation can be achieved within a short time. Moreover, a threshold of 12 kg N ha yr^{-1} was brought about by the concentration of nitrogen, which was a significant factor causing nitrate leaching (Wright et al., 1995). Stevens et al. (2012) observed that 15 years after treatments ended at rates of 25, 50, 100, and 200 kg N ha^{-}1 yr^{-}1 for eight years at Tadham Moor, the amounts of nitrates and ammonium were dramatically decreased. Therefore, microbial biomass and total nitrogen increased after habitat recovery (Stevens et al., 2012) . In a review of N recovery experiments Stevens (2016) suggested that recovery of soil chemistry occurs more quickly than recovery of the plant community.

5.1.1 The effect of nitrogen forms on recovery

Soil chemistry is altered following N deposition and this has been shown to occur as a result of the alteration of the pH of the soil and an increase the available N forms (Houdijk et al., 1993). Furthermore, N deposition has been shown to change the soil ratio of NH_4^+:NO_3^-, resulting in changes in plant performance. For instance, a high NH_4^+:NO_3^- ratio results in NH_4^+ accumulation. This affects cation uptake, resulting in detrimental effects on plants. The diversity of these forms of nitrogen compounds, including NH_4NO_3, $NaNO_3$ $(NH_4)_2SO_4$, NH_4Cl, and urea, influence the species' recovery depending on their movement over time (Phoenix et al., 2012). The influence of the combination of these N deposition compounds has been found to be crucial in the recovery role of different species or nutrients that might have been lost (Gaudnik et al., 2011). There has been very little research into recovery from the addition of different nitrogen forms, which is the focus of this chapter.

5.1.2 The effect of mowing on recovery from nitrogen deposition

The practice of mowing could play an important role in the recovery plan for grasslands. Mowing has the potential to both remove N from the ecosystem and to alter ecosystem properties. While Bobbink et al. (1998) pointed to the role of mowing on the plants' intrinsic sensitivity, Collins et al. (1998) indicated that although mowing was in many circumstances used as a way of harvesting hay in grassland, it ultimately played a role in preserving plant diversity within the N deposition context. In relation to nitrogen saturation, removing the biomass available in the ecosystem plays a key role in arresting the eutrophication effects. Allowing light penetration to the grasslands provides an essential condition for the performance of the ecosystems (Storkey et al., 2015).

Hypothesis

This study uses a long-term experiment in Norway. This experiment received N from 2008-2014 and has been in recovery since 2015. The focus of this study is the impact of N deposition on soils from 2015 to 2020. During experiments at the Norwegian site in 2013-2015, Rogers (2019) found that soil pH differed considerably between treatments, with pH in the N70-C0 and N70-RED treatments lower than in the N0-C0 and N70-OXI treatments, and N70-RED likewise lower than N35-C0. The total N content of the soil (kg m^{-2}) did not differ significantly between treatments. Furthermore, there were no significant differences in total C concentration (kg m^{-2}) across treatments. In the N35-C0 and N70-C0 treatments, the mean C:N ratios of the very fine fraction were marginally lower (~0.7) than in the N0-C0 treatment. No significant differences were found in microbial biomass C (mg m^{-2} for 10 cm depth, $P = 0.82$) between treatments (Rogers, 2019).

The hypotheses of the current study are, that after five years of recovery:

1- There will be no observable difference in soil chemistry between control and N addition plots.

2- No difference between the oxidised (N70-OXI) and reduced (N70-RED) treatments would be visible.

3- There will be no significant difference between the mowed plots and the plots without mowing.

5.2 Methods

5.2.1 Site Description

The experimental site is located in Revne (Fusa municipality, Hordaland), Norway at 60° 09 29 N, 5° 44 31.5 E) (Figure 51a). Background nitrogen deposition is 6.1 Kg N ha^{-1} yr^{-1}. It consists of 60 plots that include 5 replicated blocks; each block has 12 plots with 2x2 m^2 dimension, separated by 1 m walkways (Figure 51b and 51c). One plot from each block was not in use. Nitrogen was added at rate of 70 kg N ha^{-1} $year^{-1}$ in three forms: reduced N (as NH_4Cl/ N70-red), oxidised N (as $NaNO_3$/ N70-ox) or a combination of the two as NH_4NO_3 (N70). There was also a 35 kg N ha^{-1} $year^{-1}$ treatment applied as NH_4NO_3 (N35). Control plots (N0) received only deionized water (Dorland et al., 2013). All plots received an annual biomass cut in July (cut to 0, 1 and 2 cm above soil level) and cutting plots received a second cut in the late summer (C1). N additions were carried out from 2008 to 2014 and afterwards plots were left to recover.

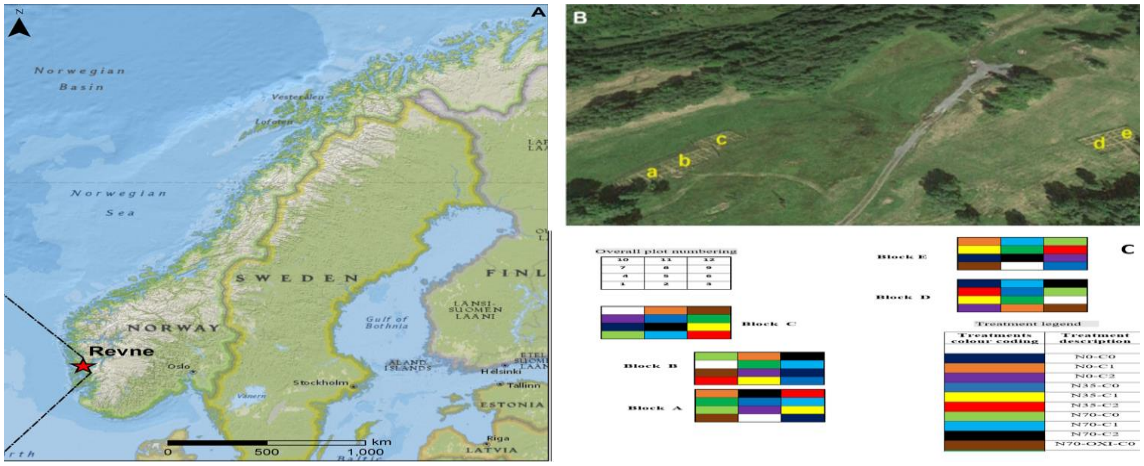

Figure 51 (a) The location of study area in Revne, (b) the plots from west to east A to E, and (c) schematic overview of blocks and treatments (Rogers, 2019).

Table 11 The table demonstrates the overview of treatments code and the forms of the treatment in Revene.

Treatment code	The forms of the treatment
N0, N35, N70	N dose of 0, 35 or 70 kg NH_4NO_3/ha/yr
N70-RED	N dose of 70 kg NH_4/ha/yr
N70-OXI	N dose of 70 kg NO_3/ha/yr
C0	No cutting
C1, C2	Cutting 1 and 2 per year

5.2.2 Sampling Methods

One sample from each replicate was collected in 2020 by Amy Eycott, the coordinator of the field experiments, from each plot (up to 10 cm depth, 5.2 cm diameter) using an auger. They were homogenised by hand and stored in plastic bags.

5.2.3 Soil Analysis

To determine soil moisture, 10 g of fresh soil was dried at 105 ºC for 24 hours. Microbial biomass was determined using chloroform fumigation (Vance et al., 1987). 5 g of fresh soil was used for fumigated and non-fumigated soils. Soils were fumigated with chloroform in a desiccator at 25 ºC for 24 hours. 25 ml of 0.5 M K_2SO_4 (the pH of the K_2SO_4 was corrected to within 6.8-7 using NaOH) was added to the fumigated and unfumigated soils. Both the fumigated and unfumigated soils were shaken on the orbital shaker for 30 minutes at 180 rpm, centrifuged for 5 minutes at 3000 rpm, then filtered with a Whatman No 42 (2.5 μm) filter. Total carbon was determined using a total organic carbon (TOC) analyser (Shimadzu, TOC-5000A), and the value of the result for the fumigated sample was subtracted from the result for the unfumigated one to give microbial biomass carbon (C). For nitrate and ammonium extracts, 5 g of fresh soil was weighed and put in extraction bottles with 25 ml of 2 M KCl. They were shaken by orbital shaker for one hour then filtered through Whatman No 42 filters. NH_4^+ and NO_3^- were analysed by using an auto analyser (Bran Luebbe, AutoAnalyzer3). For TOC 5 g of fresh soil was used, with 25 ml of 0.5 M K_2SO_4 (the pH of the K_2SO_4 was corrected to within 6.8-7 using NaOH). It was shaken for 30 mins at 180 rpm, left to settle for 10 minutes then filtered using a Whatman No 42 filter. TOC extracts were analysed using an auto analyser (Shimadzu, TOC-5000A). Soil pH was measured using Metteler Toledo Seven Compact with a ratio of 25 ml distilled water (Allen, 1989). For total C and N soils were analysed using an auto analyser (elementar, vario EL III), by using Dumas method to measure the concentrations of the element content for carbon and nitrogen and C/N ratios in the soil samples.

5.2.4 Data analysis

Data analysis was carried out using SPSS version 27 (SPSS 27, Microsoft Corporation, released 15.0.1., 2020) (SPSS 27, 2020) with N treatment as a fixed factor and block as a random factor. All variables were examined before analysis, and the data were logarithmically or square root converted to achieve variance normality and homogeneity respectively. For post-hoc pair-wise comparisons of all treatments, Tukey's technique was utilised (SPSS 27, Microsoft Corporation, released 15.0.1., 2020) (SPSS 27, 2020) to determine whether there were differences between N treatments for each variable (both between N addition treatments and the control plots and between N addition treatments). The significant value was set at $P = 0.05$.

5.3 Results

5.3.1 Soil pH

For each treatment, the average pH of the soil was calculated pH. Results show no significant difference between merged, separated and cutting treatments (ANOVA; F= 1.664; df= 10; P = 0.120, F= 1.685; df= 4; P = 0.168 and F= 2.003; df= 2; P = 0.145, respectively) (Figure 52a, 52b and 52c). In the merged treatments both combined N and reduced N had lower pH than the control and oxidised N treatments, except N35-C0 which was lower than control but higher than N70-OXI-CO although these differences were not significant. In the separate treatments N35 which was slightly lower than control and N70-OXI-CO but higher than N70 and N70-RED. In the cutting treatments, the control had the highest mean pH while C1 had the lowest, but no significant difference was observed between the testaments (Figure 52).

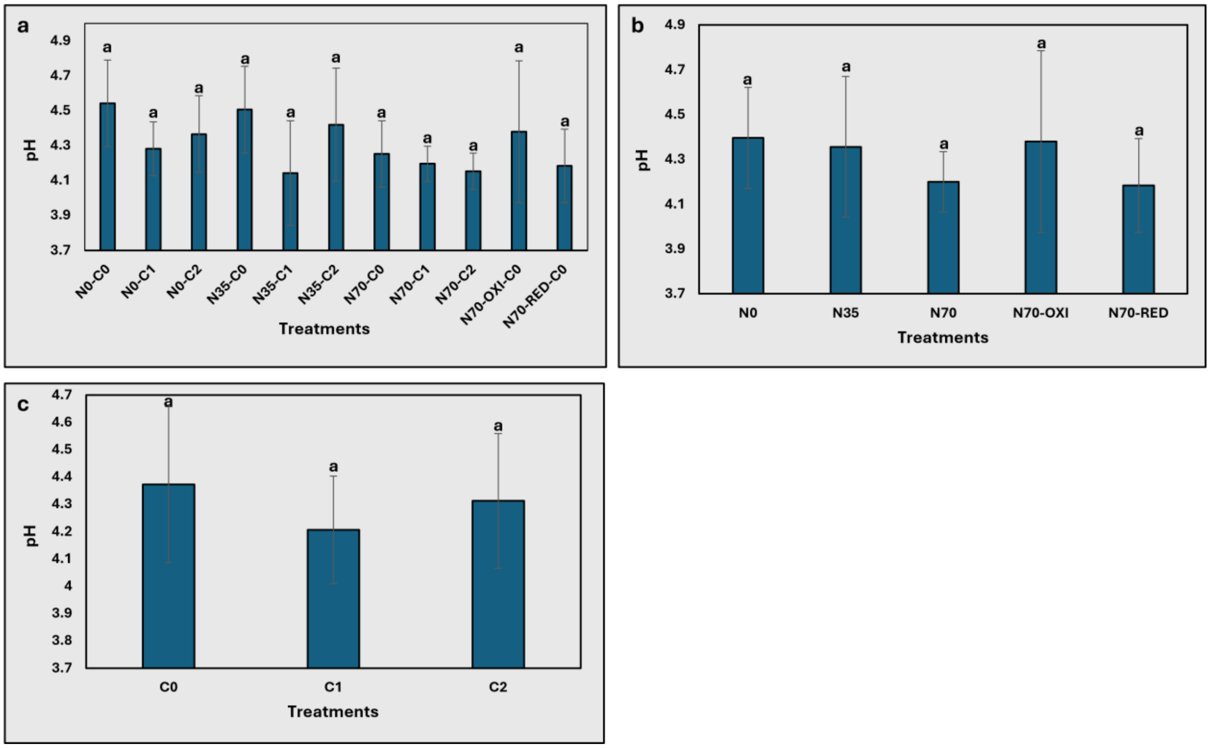

Figure 52 pH average of the soil (a) for merged treatment, (b) for separated treatment and (c) for cutting treatment. N0, 35 and 70 indicate the previous N additions (control, 35 kg N ha^{-1} yr^{-1}, or 70 kg N ha^{-1} yr^{-1}), OXI and RED the N form and C the frequency of cutting. Values are Mean ± SE, those sharing the same letters denoted no significant differences between the groups based on Tukey's HSD post-hoc test.

5.3.2 Nitrogen concentration

There was no significant difference in soil N concentration between merged, separated and cutting treatments (ANOVA; F= 0.742; df= 10; $P = 0.681$, F= 0.224; df= 4; $P = 0.924$ and F= 0.495; df= 2; $P = 0.612$, respectively) (Figure 53a, 53b and 53c). The merged and separated treatments show that the highest level of N content was in N70-C0 compared to the control, although this treatment has high variability and the difference is not significant. In contrast, reduced and oxidised treatments had nearly same value of N content which was lower than N70-C. In the cutting treatments C2 was higher than the control and C1 but no significant difference was found between the treatments (Figure 53).

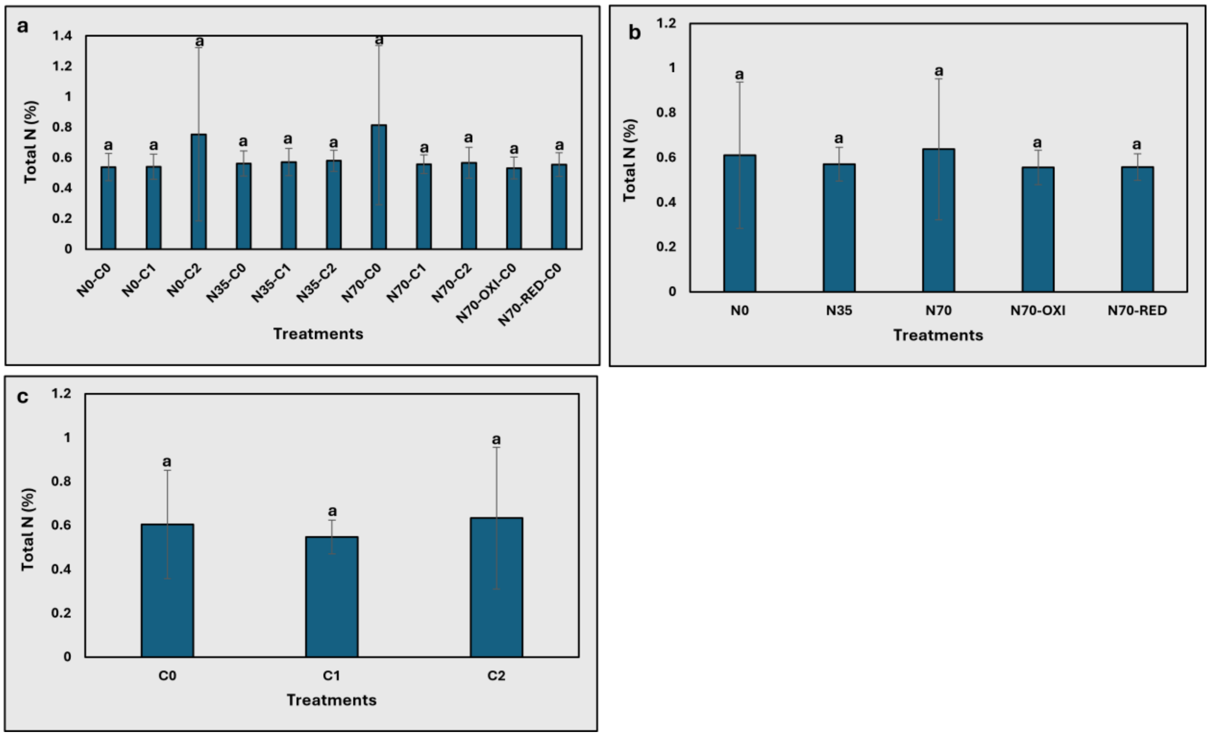

Figure 53 The contents of the total soil N in (a) merged treatment, (b) separated treatment and (c) cutting treatment presented by percentage. Values are Mean ± SE, those sharing the same letters denoted no significant differences between the groups based on Tukey's HSD post-hoc test.

5.3.3 Carbon concentration

For soil C, no significant difference was observed in merged, separated and cutting treatments (ANOVA; F= 0.757; df= 10; *P* = 0.668, F= 0.133; df= 4; *P* = 0.970 and F= 0.717; df= 2; *P* = 0.493, respectively) (Figure 54a, 54b and 54c).

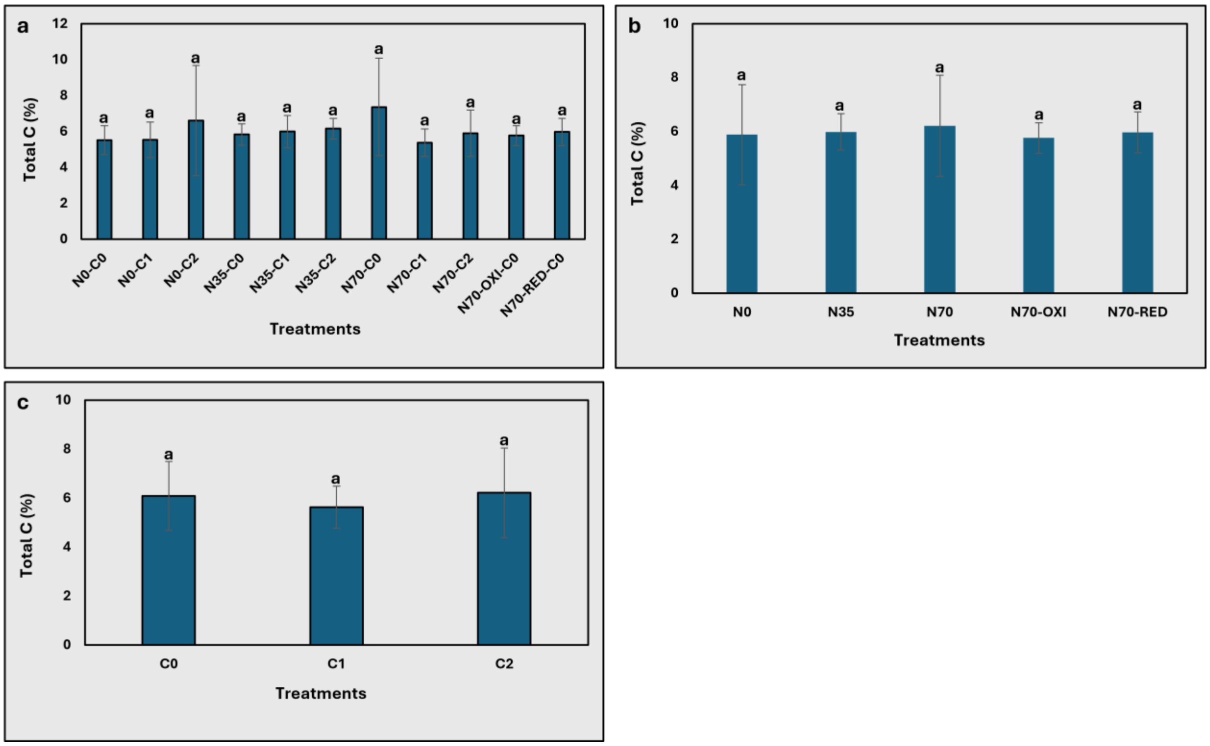

Figure 54 The contents of the total soil C in (a) merged treatment, (b) separated treatment and (c) cutting treatment presented by percentage. Values are Mean ± SE, those sharing the same letters denoted no significant differences between the groups based on Tukey's HSD post-hoc test.

5.3.4 C:N ratios

There was no significant difference between merged, separated and cutting treatments has been observed in C:N ratios (ANOVA; F= 0.452; df= 10; *P* = 0.911, F= 0.820; df= 4; *P* = 0.519 and F= 0.071; df= 2; *P* = 0.932, respectively) (Figure 55a, 55b and 55c). The data are very consistent across the treatments (Figure 55).

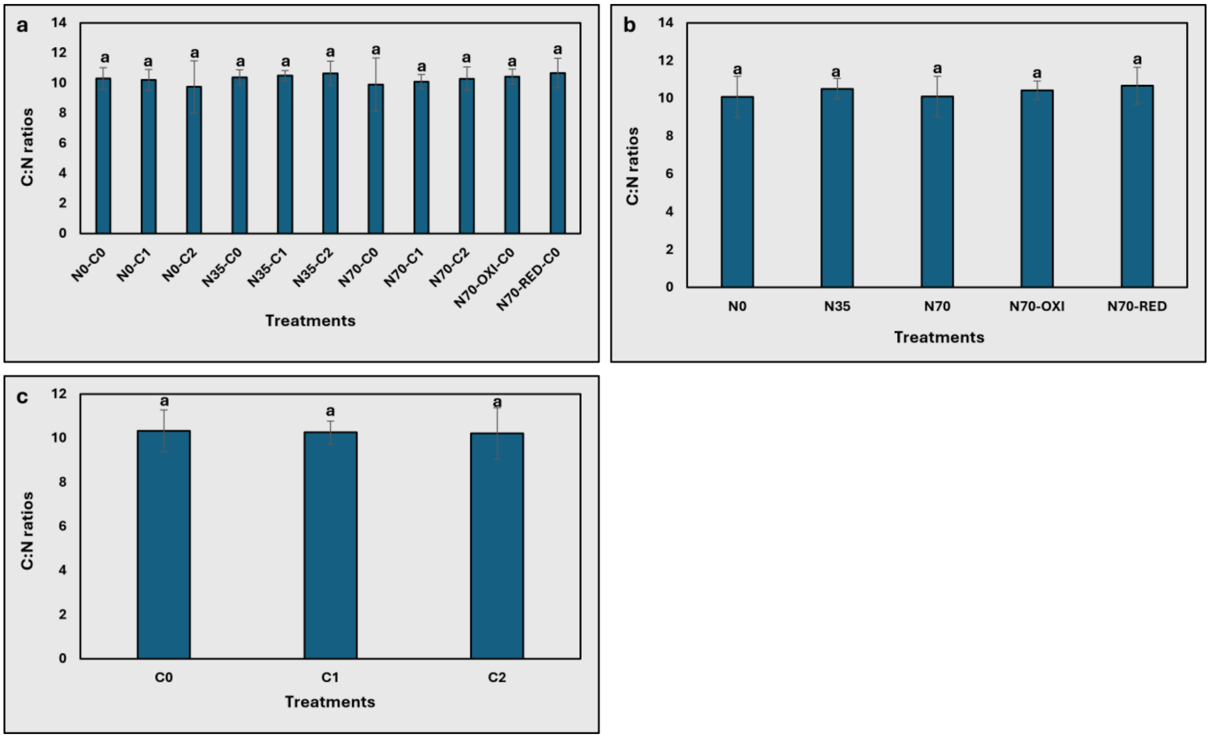

Figure 55 The total ratios of C:N for (a) merged treatment, (b) separated treatment and (c) cutting treatment. Values are Mean ± SE, those sharing the same letters denoted no significant differences between the groups based on Tukey's HSD post-hoc test.

5.3.5 NH_4^+ concentration

The above data show no significant difference between the merged, separated and cutting treatments (ANOVA; F= 0.613; df= 10; *P* = 0.794, F= 0.174; df= 4; *P* = 0.951 and F= 0.990; df= 2; *P* = 0.379, respectively) (Figure 56a, 56b and 56c). In the merged treatments the highest concentration of NH_4^+ was found in N70-C0 in the all treatments and the control (N0-C0). While the lowest concentration of NH_4^+ was in N70-C1, reduced and oxidised treatments were also less than the control (N0-C0). In addition, NH_4^+ concentration in N35-C1 was higher than in N70-C1 but lower than in N0-C0. However, none of these differences were significant or of a large magnitude. Furthermore, NH_4^+ concentration was higher in N70-OXD-C0 than N70-RED-C0. In C0 treatments NH_4^+ concentration was the highest while the lowest concentration of NH_4^+ was in C1 but the difference was also not significant (Figure 56).

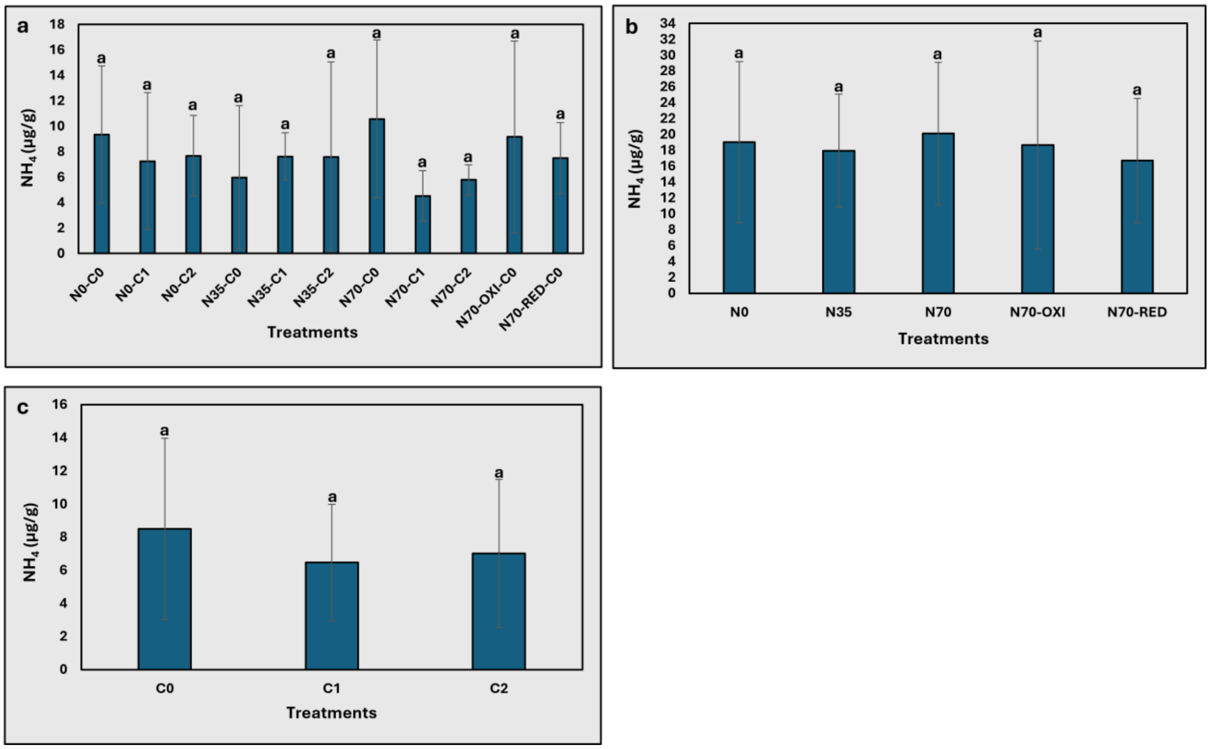

Figure 56 The average of NH_4 concentration for (a) merged treatment, (b) separated treatment and (c) cutting treatment. Values are Mean ± SE, those sharing the same letters denoted no significant differences between the groups based on Tukey's HSD post-hoc test.

5.3.6 NO_3^- concentration

The data for NO_3^- show no significant difference between merged, separated and cutting treatments (ANOVA; F= 0.433; df= 10; *P* = 0.922, F= 0.274; df= 4; *P* = 0.893 and F= 0.469; df= 2; *P* = 0.628, respectively) (Figure 57a, 57b and 57c). In the merged the highest NO_3^- was in N70-C2 treatment comparing to all treatments and the control (N0-C0). And the lowest NO_3^- were found in N35-C2 and N70-C0. Although the concentration of NO_3^- in N70-OXD-CO was higher than N70-RED-C0, it was still lower than N70-C2. In addition, the combined (N35-C1), (N70-C1) were higher than reduced, oxidised and the control. As with NH_4^+ none of these differences were significant or of a large magnitude. In the separated treatments, the concentration of NO_3^- in N70-OXD was higher than the control and the treatments. However, none of these differences were significant (Figure 57).

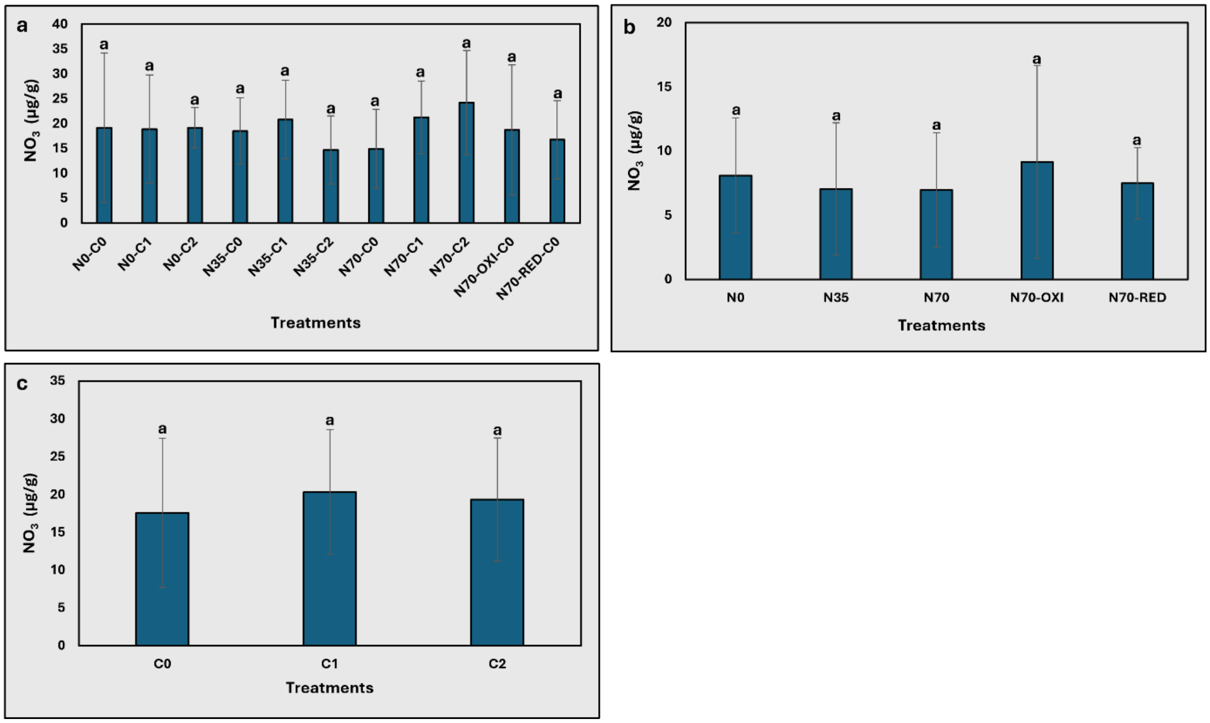

Figure 57 The average of NO_3 concentration for (a) merged treatment, (b) separated treatment and (c) cutting treatment. Values are Mean ± SE, those sharing the same letters denoted no significant differences between the groups based on Tukey's HSD post-hoc test.

5.3.7 TOC

The data show no significant difference between merged, separated and cutting treatments for TOC (ANOVA; F= 0.296; df= 10; $P = 0.978$, F= 0.588; df= 4; $P = 0.673$ and F= 0.229; df= 2; $P = 0.796$, respectively) (Figure 58a, 58b and 58c). In the merged treatments the total organic carbon in N35-C0, N0-C2 and N0-C1 treatments were higher than the control (N0-C0). N70-OXD-CO treatment were lower than N70-RED-C0 and the control (N0-C0). Furthermore, reduced and oxidised were lower than the control and lower than combined N70-C0 and N35-C0 but samples were quite variable and differences were not significant (Figure 58). C2 treatment was higher than C0 and C1 but there was no significant difference noted between the treatments (Figure 58).

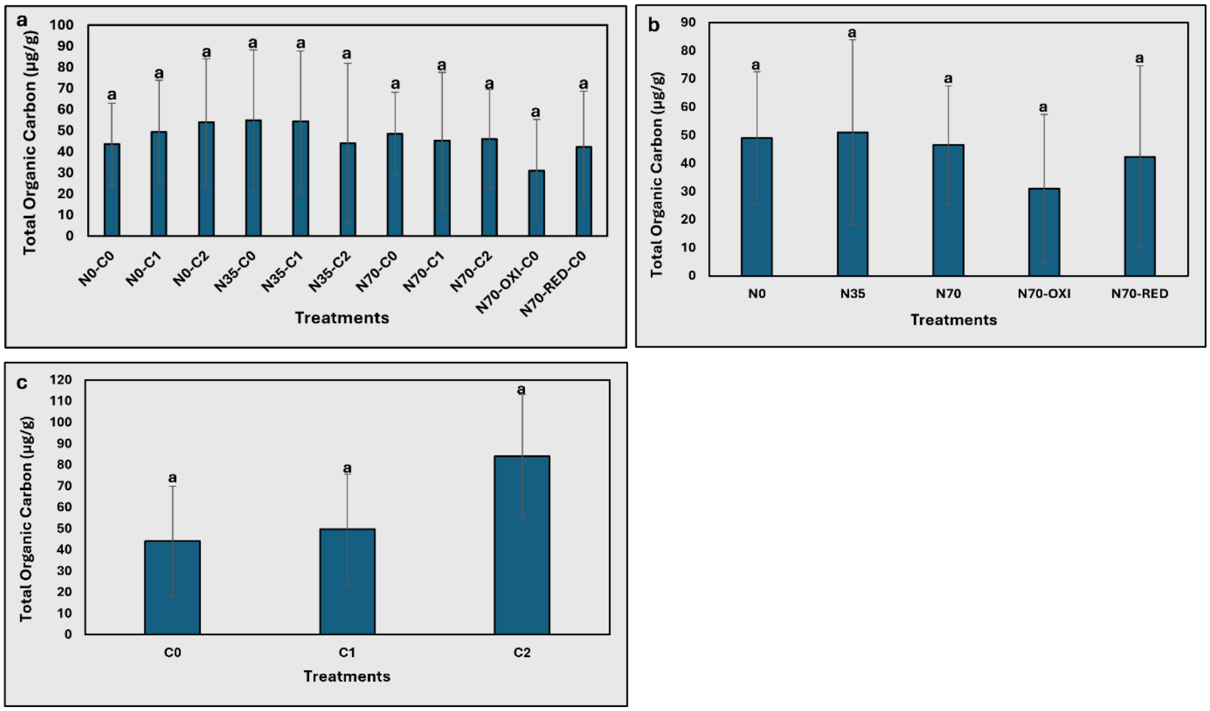

Figure 58 The average of the total organic carbon for (a) merged treatment, (b) separated treatment and (c) cutting treatment. Values are Mean ± SE, those sharing the same letters denoted no significant differences between the groups based on Tukey's HSD post-hoc test.

5.3.8 Microbial biomass C

The results of microbial biomass C show no significant difference between merged, separated and cutting treatments (ANOVA; F= 0.738; df= 10; *P* = 0.685, F= 0.326; df= 4; *P* = 0.859 and F=

0.577; df= 2; *P* = 0.565, respectively) (Figure 59a, 59b and 59c). In the merged treatments the highest microbial biomass C was found in the control which was slightly higher than N70-C0. On the other hand, oxidised N was more than N35-C0, N35-C1 and N35-C2, while N70-RED-C0 and oxidised N were less than N70-C0 but differences were not significant, and variability was high (Figure 59). The results of microbial biomass C are very consistent among the separated and cutting treatments (Figure 59b and 59c).

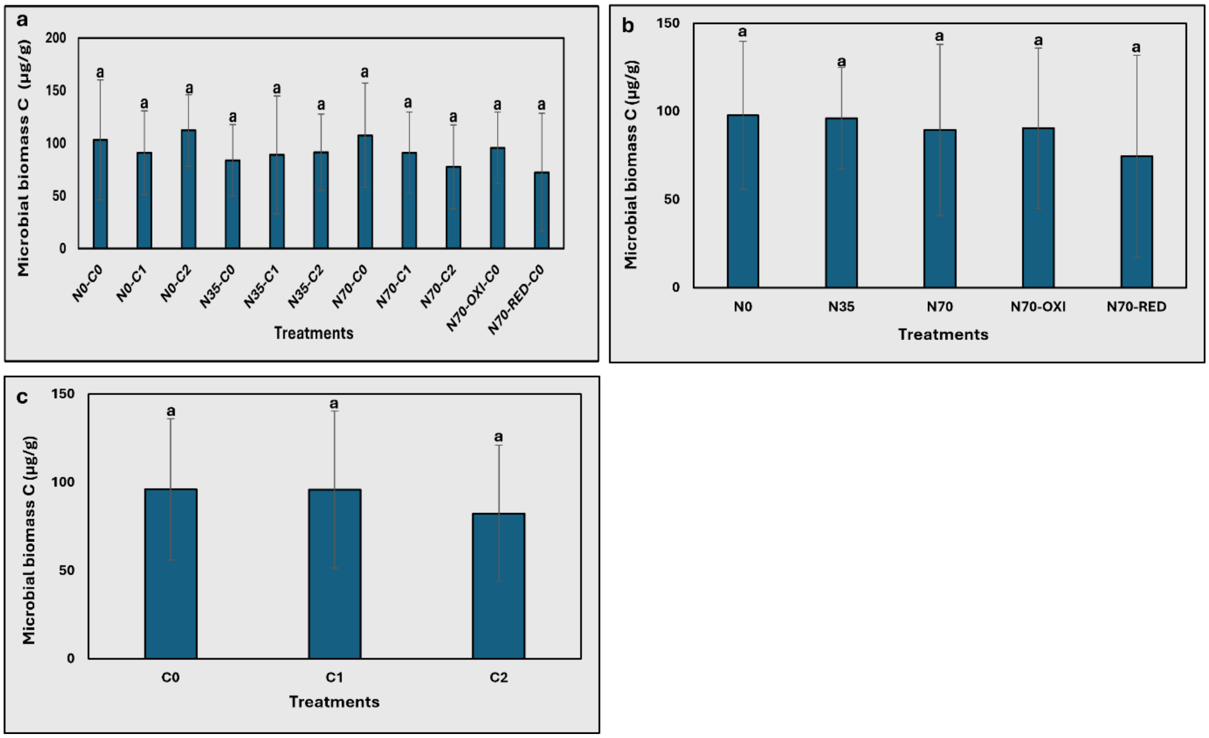

Figure 59 Microbial biomass C for (a) merged treatment, (b) separated treatment and (c) cutting treatment. Values are Mean ± SE, those sharing the same letters denoted no significant differences between the groups based on Tukey's HSD post-hoc test.

5.4 Discussion

5.4.1 The amount and form of N addition

The main purpose of this study was to determine whether the soil recovered from N addition during the study period from 2015 to 2020. Rogers (2019) provides data from immediately before the N additions were stopped. Her results (*P* = 0.11) taken during the end phase of soil treatment are consistent with the current study, where there was also no significant difference in the total soil N concentration between N addition plots and control samples (*P* = 0.68). There was also no significant difference for the plots treated with N70-OXI and N70-RED. Again, similarly to Rogers (2019), the N concentration at various depths of soil had not differed. This indicates that there was no effect of the N treatments on soil N concentration. However, the difference in the mean values for N measurements between the Rogers (2019) study and the current study suggests that the soil had recovered from the nitrogen addition during the time span of 5 years (2015-20) (Figure 60). These findings are supported by Clark et al. (2009) whose studies were focused on Minnesota's prairie grassland treated with N added at rates of 10N, N20, N34, N54, N95, N170 and 270 Kg N ha^{-1} yr^{-1} for ten years. Since there was no appreciable difference in the N pools in the treatment and control samples, this could suggest a high rate of N loss from the system through such processes as leaching. Although there was no significant difference in N concentrations either at the end of the experiment of Rogers (2019) or after the recovery period of this study, the current study found that the mean of N concentration was consistently higher than in Rogers's study, which could be a result of continued input from background deposition (Figure 60).

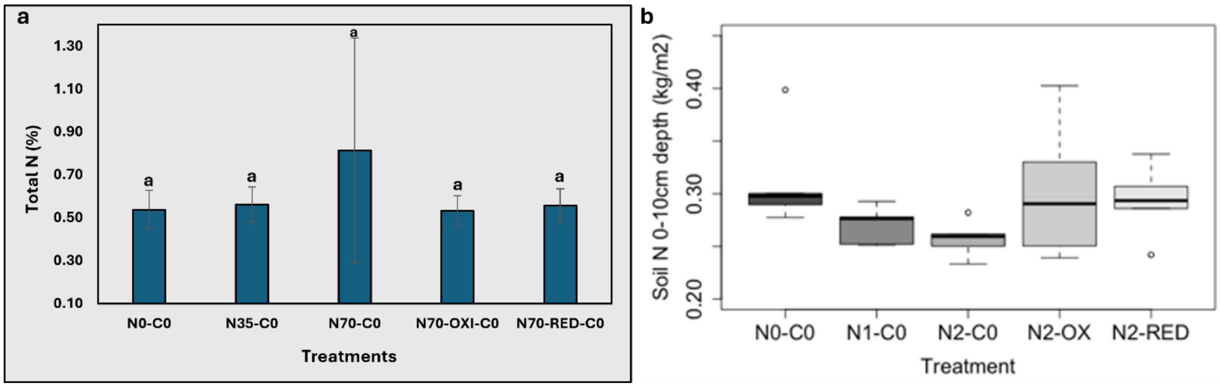

Figure 60 (a) The means of total N for this study, (b) N means for Rogers (2019) study.

Furthermore, the difference in C:N ratios between treatment plots and control plots were not significant ($P = 0.91$). Rogers (2019) found some difference in this in N35-C0 and N70-C0 plots, but it was not significant ($P = 0.21$) which possibly indicates some recovery from N addition. Other studies have found recovery in C:N ratios. Olff et al. (1994), examined four fields where the addition of N had been ceased for 2, 6, 19, and 45 years. They noted that there was no significant difference in C:N ratio between these four fields during the recovery period.

According to Rogers (2019), there was also no significant increase in the ammonium concentration between the plots. The present findings also show no significant difference in NH_4^+ concentrations between treatment and control samples ($P = 0.79$). This indicates that there was no NH_4^+ accumulation in the soils of the N addition plots. According to Rogers (2019), the overall N content of the soil was not significant, but it was observed to be the strongest in the N70-RED treatment as it contained more nitrogen in the form of ammonium for the plants to be assimilated (O'sullivan et al., 2011) studied nitrogen recovery at Wardlow Hay cop acidic and limestone grassland and also found no difference in N. They took four plots treated with different N concentrations, N0, N35, N70, and 140 kg N ha^{-1} yr^{-1} (NH_4NO_3) for a time span of eleven years, during which there was a significant increase in ammonium concentration. After cessation of N addition, recovery studies were carried out which indicate that after four years of recovery time, there was no significant difference in ammonium concentration between the treatment and control plots, indicating that N had recovered ($P = 0.05$).

In the current data, while NO_3^- concentrations differed slightly between treatment and control samples, the differences were not significant ($P = 0.92$). The fact that there was no significant difference in NO_3^- concentrations between the N plots and the control plots means the soils had recovered from the prior N deposition.

5.4.2 The effect of N addition on soil pH

Rogers (2019) found considerable variation in soil pH between the treatment plots ($P < 0.001$), showing that the pH of the soil was strongly affected by the N additions. This experimental study of soil pH revealed that there was no significant difference between the treatments ($P = 0.12$). No significant difference in soil pH between the treatment and the control indicates that the soil had recovered more from N addition. Soil pH has declined consistently across the plots since (Rogers, 2019) took her measurements, which could indicate continued acidification from background deposition **(**Figure 61**).** Stevens et al. (2012) also found no significant difference in soil pH while studying the soil chemistry for recovery after fifteen years of N addition cessation when 0, 25, 50, 100, 200 kg N ha^{-1} yr^{-1} had been applied between 1986 and 1994 to a neutral grassland in Somerset (Stevens et al., 2012). According to the results of Stevens et al. (2012), the N35 and N70 plots showed no significant difference between most of the soil parameters, which is consistent with the results of the current study. Recovery was clearly seen in other pools of soil N including soil pH.

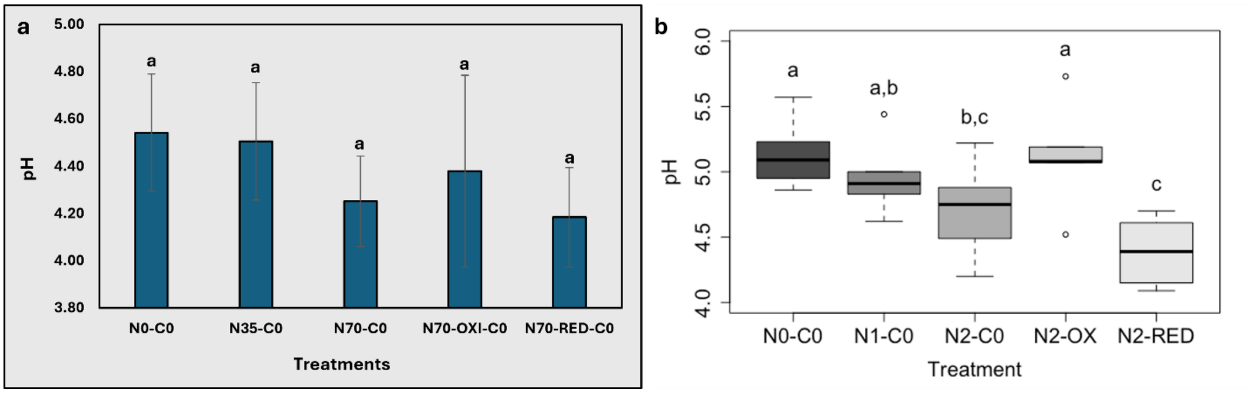

Figure 61 (a) The means of pH for this study, (b) pH means for Rogers (2019) study.

5.4.3 The effect of N addition on soil C

In terms of soil carbon concentration, the results of the current study (*P* = 0.66) align with Rogers' findings (*P* = 0.08) as no significant difference was reported between treatment plots. The lack of a significant difference on total biomass C may be attributed to the possibility that the biomass at the site was limited by phosphorous. Furthermore, the findings of the current study are also supported by the results obtained by Stevens et al. (2012), which also showed a lack of significance in total carbon (*P* = 0.21).

While comparing the microbial biomass C content between the treatment and control samples, no significant difference was reported in the results (*P* = 0.93). Although the differences were not significant, N0-C2 had the highest microbial biomass C, which was slightly higher than N0-C0. Oxidised N was more than N35-C0, N35-C1 and N35-C2 while N70-RED-C0 and oxidised N were less than N70-C0. Rogers's findings also show a lack of significant difference in microbial biomass C between treatment and control plots (*P* = 0.82). Stevens et al. (2012) results show no significant difference in soil pH (*P* = 0.36), in alignment with this study (*P* = 0.12).

Overall, there was no observable difference in soil chemistry between control and N addition plots. Rogers (2019) found relatively few impacts of the N addition treatments but those she did find are no longer apparent.

5.4.4 Mowing and potential for recovery

Mowing and biomass removal has the potential to play an important role in ecosystem recovery (Storkey et al., 2015). Mowing and biomass removal could be used to improve habitat suitability and remove N from the system, which is typically accomplished by removing above-ground vegetation biomass and litter, and this would lead to opening the canopy and raising light levels below (Borer et al., 2014). In the present study, there was no difference between the control and treatment plots and therefore no additional benefit from mowing. It is certainly possible that there could be a benefit for vegetation and species composition (Storkey et al., 2015), however further research would be needed to assess this.

5.5 Conclusion

These findings show that there was no significant difference between the control and N treatment plots across all the soil parameters, including soil pH, nitrogen concentration, carbon concentration, C:N ratios, NH_4 concentration, NO_3 concentration, TOC, and microbial biomass C. Overall, there was no observable difference in soil chemistry between control and N plots. Rogers (2019) examined the soils in 2015 and found relatively few impacts of the N additions on soil chemistry. Therefore, other than soil pH which was significantly different showing that the pH has recovered, results do not indicate recovery in Rogers' study. Additionally, no difference between the oxidized (N70-OXI) and reduced (N70-RED) treatments was visible, as hypothesised.

Therefore, after five years of recovery, the N addition plots had recovered from the effects of N addition. Also, there was no significant difference between the mowed plots and the plots without mowing, and therefore the additional mowing was not necessary for recovery at this site. However, there were some signs that background N deposition may be continuing to impact on the site since pH has declined and total N has increased since the 2015 survey.

6 General discussion

6.1 Summary

The thesis focuses on the effects of atmospheric N deposition on grassland and heathlands in terms of plant biomass, flowering, and phenology, as well as long-term responses of the seed bank. It also seeks to examine the interactive effects of nutrient addition and disturbance on the structure of the species composition and investigates grassland soil recovery from N deposition in relation to what function mowing plays in that recovery process.

Chapter 2 examined the effects of N deposition on plant traits measuring biomass, flowering, and phenology in a controlled experiment conducted with selected grassland species. It discussed how N addition affected the above-ground biomass, below-ground biomass, total biomass, plant height, number of flowers and seeds, plant tissue chemistry, and phenology. No significant impact of N addition was found on biomass across the species, contrasting with previous studies that reported increases in biomass under N deposition. Plant height also remained unaffected, likely due to environmental stressors like overwatering and a heatwave during the experiment. Despite limited effects on growth, reproductive traits showed species-specific responses, with *L. corniculatus* showing increased seed production and *A. millefolium* producing more flowers. Tissue chemistry remained largely unchanged, except for *P. lanceolata,* which exhibited a significant increase in nitrogen content and a decrease in its C:N ratio. Phenological observations, including the timing of first buds and flowers, showed no major changes, although *P. lanceolata* experienced delayed seed production. The chapter concluded that while N deposition had minimal effects on biomass and growth, it significantly influenced reproductive traits and tissue chemistry in some species, underscoring the importance of species-specific responses in understanding ecosystem impacts.

Chapter 3 examined the effects of long-term N deposition on the seed bank and above-ground vegetation of heathlands at Budworth and Ruabon. Results showed that while N deposition did not reduce species richness or total seed density, it altered species composition at Ruabon. Soil acidification from N deposition likely influenced seed germination and nutrient availability, favoring species like *Calluna vulgaris.* However, despite increased flowering of *C. vulgaris,* the number of seeds germinating from the seed bank was not always higher. Above-ground vegetation showed no significant changes in species richness or composition, likely because N deposition levels were already high. N deposition reduced the similarity between the seed bank and above-ground vegetation at both sites. The chapter concluded that while *C. vulgaris* dominates the seed bank, other species like *D. flexuosa* are scarce, indicating that management strategies, such as seed introduction, may be necessary to restore diversity and support ecosystem recovery after N deposition.

Chapter 4 addressed the interactions between nutrient addition (NPK) and mechanical disturbance (rotavation) treatments and their impacts on grasslands in terms of species composition, biomass, and diversity. An experiment was set up at Lancaster University to determine which aspects of nutrient enrichment and disturbance control competitive dynamics between grasses and forbs. Pre-treatment observations showed stable species composition and biomass without external interventions. Nutrient addition had little impact on species richness, diversity, or biomass, likely due to high soil fertility, which limited further ecological changes, but forb biomass decreased, indicating that grasses gained a competitive advantage in nutrient-rich conditions. Rotavation, however, increased species diversity in the first year by disrupting dominant species, allowing less competitive species to establish. This effect was temporary, as the ecosystem quickly reverted to its original state by the second year, despite further disturbance. Biomass declined, especially for grasses, while forbs expanded in response to the disturbance. The interaction of nutrients and disturbance led to a decrease in species diversity and biomass, with dominant species benefiting from the combined treatments. Light availability increased as grass biomass decreased, allowing more light to reach the lower layers of vegetation. The chapter concluded that while nutrient addition alone had minimal effects, rotavation temporarily increased diversity, but the combination of both treatments reduced diversity and favored dominant species.

Chapter 5 focused on exploring the effects of N deposition on soil recovery in a long-term experiment in Norway from 2015 to 2020, examining nitrogen levels, carbon content, C:N ratios, pH, and the role of mowing. It concluded that there was no significant difference between control and N-treated plots for soil pH, nitrogen concentration, ammonium (NH_4^+), nitrate (NO_3^-), carbon, or microbial biomass, indicating that the soil had recovered from nitrogen addition. The findings, consistent with earlier studies like Rogers (2019), showed no visible differences between oxidized (N70-OXI) and reduced (N70-RED) nitrogen treatments, supporting the conclusion that soil recovery was complete. Additionally, the study found no significant impact of mowing on recovery, suggesting that mowing was unnecessary for soil recovery. However, a continued influence of background N deposition was noted, with slight declines in soil pH and increases in nitrogen since the 2015 survey. In summary, the chapter confirmed that after five years, the soil had recovered from nitrogen addition without needing further intervention, except for monitoring background deposition.

6.2 Seed production and seed banks

Chapter 2 highlights that N addition, has varied effects on flowering across species. For example, while *Achillea millefolium* exhibited an increase in the number of flowers due to N addition, *Plantago lanceolata* showed no significant effect on flowering. These differences in flowering directly influence seed production, as the number of flowers often correlates with the number of seeds produced (Leake and Lee, 2007). Consequently, species that respond positively to N addition in terms of flowering are likely to have a better chance of regeneration and contribute more seeds to the seed bank (Leake and Lee, 2007). The species less responsive to N addition may contribute fewer seeds and thus decline both in the seed bank and aboveground vegetation (Phoenix et al., 2012).

Chapter 3 emphasizes the importance of seed banks as reservoirs of genetic material that could contribute to the recovery of plant communities after disturbances (Thompson and Fenner, 2000, Wang et al., 2009, Kiss et al., 2018). The soil seed bank acts as a buffer against the impact of environmental changes by storing seeds when the conditions become unfavorable. Therefore, it supports local re-establishments of plant populations following events like nutrient addition and/or physical disturbance through regeneration process.

When nutrient addition favours certain species, particularly grasses, the variety of seeds entering the seed bank decreases. Over time, this can deplete the seed bank, making it less resilient to future disturbances. The effects of a depleted seed bank over time were observed in long-term studies, where N deposition resulted in reduced species richness in seed banks as dominant species outcompete others and reduce seed input from less competitive species (Basto et al., 2015).

The observed seed bank depletion can be related to several factors. Nitrogen-containing substances can boost the germination of seeds across various species, suggesting that nitrogen pollution might deplete seed bank reserves by promoting germination (Ochoa-Hueso and Manrique, 2010, Plassmann et al., 2008). Furthermore, N deposition contributes to soil acidification, which complicates seed germination either directly or indirectly by raising toxic metal levels, affecting microbial populations and growth. It can also encourage the growth of pathogenic microorganisms, major contributors to seed death in soil, thereby increasing seed loss (Basto et al., 2015). Negative effects on flowering due to N deposition could lead to lower seed production, and excessive nitrogen levels might result in litter accumulation, hindering seeds from joining the seed bank (Phoenix et al., 2012).

The relationship between flowering traits and seed bank dynamics is crucial for understanding ecosystem resilience. Flowering traits related to the timing and number of flowers, depend on environmental factors such as the availability of nutrients (Cleland et al., 2006, Smith et al., 2012, Xia and Wan, 2013, Xi et al., 2015). These traits, in turn, partly determine the number of seeds produced and stored in the seed bank. In species-poor communities, where relatively few species contribute most of the seeds entering the seed bank, even slight disruption to flowering can have great impacts on the composition of the seed bank as reduced flowering of a species can decrease its seed production (Phoenix et al., 2012), which further makes up the seed bank. For instance, in species like *Lotus corniculatus* and *Achillea millefolium*, which showed a positive response to N addition in terms of seed production, the seed bank may be dominated by these species. This could lead to reduced diversity in the seed bank, making the ecosystem less resilient to disturbances that favor a few species. On the other hand, species like *Plantago lanceolata*, which did not show significant changes in seed production, may become underrepresented in the seed bank in comparison to other species, potentially reducing their future presence in the plant community.

One of the reasons we selected *Plantago lanceolata* was because it exhibited a negative response to N deposition in Stevens et al. (2004). The decline observed in that study was possibly related to reductions in flowering under elevated N conditions. However, our results contrast with those of Leake and Lee (1997) and Basto et al. (2015) which may be due to external factors like overwatering and heatwaves that were explained earlier. Additionally, changes in flowering phenology are important because they influence interactions with pollinating insects. When environmental drivers, such as nutrient availability or climate events, alter phenology, it can lead to phenological asynchronicity (Dixon, 2003, Rafferty and Ives, 2011) . This misalignment can disrupt plant-pollinator interactions, affecting seed production and ultimately impacting seed bank dynamics.

6.3 Disturbance

Disturbance, especially when combined with nutrient addition, plays a critical role in shaping plant communities by influencing both the above-ground vegetation (flowering and biomass) and the below-ground seed bank (Turkington et al., 1993, Gross et al., 2005). Chapter 2, which involved sowing the seeds of four forbs (*Achillea millefolium*, *Lotus corniculatus*, *Plantago lanceolata*, and *Prunella vulgaris*), provided details about how nutrient addition affects flowering success and, by extension, seed production. Chapter 3 further explored how nitrogen addition can deplete or activate the seed bank. Chapter 4, the interaction between nutrient addition and disturbance was found to significantly impact forb cover and biomass. The addition of nutrients typically favors fast-growing species, often leading to an increase in grass cover at the expense of forbs (Humbert et al., 2016, Hobbs et al., 1988). However, when combined with disturbance, such as rotavation, the competitive balance shifts, allowing forbs to re-establish in the newly disturbed areas showing that the disturbance dominated the nutrient addition impact (Schnoor et al., 2011, Edwards and Crawley, 1999).

Chapter 4 shows that the seed bank is already dominated by forbs including *Ranunculus repens* and *Agrostis capillaris*. The disturbance promoted the forb growth. Our data from 2023 and 2024 clearly show significant increases in forb cover and forb biomass under the influence of disturbance, which overshadowed the suppressive effects of nutrient addition.

Disturbance without adequate recovery time could deplete the seed bank as seeds germinate without sufficient replenishment from flowering plants (Leder et al., 2017). Disturbance directly affects seed bank dynamics by triggering seed germination and reducing the seed bank's stored reserves (Luzuriaga et al., 2005). This depletion was observed in our study as explained in chapter 3, where the seed bank appeared to be less capable of supporting recovery in the second year of disturbance.

Literature supports the idea that while disturbances (such as grazing or burning) can activate the seed bank they can also lead to its depletion if not managed carefully (Leder et al., 2017). Over time, without sufficient input from flowering plants, the seed bank may lose its ability to support diverse plant communities. Chapter 3 highlighted the importance of maintaining a healthy seed bank for ecosystem recovery. Disturbances can deplete the seed bank if there is insufficient seed input from flowering plants, particularly in species-poor communities where a few dominant species drive ecosystem dynamics. This was reflected in our study on grassland ecosystem, where the combination of nutrient addition and disturbance led to a reduction in seed bank diversity, as the competitive advantage given to certain species limited the variety of seeds entering the seed bank.

6.4 Recovery

Recovery from N deposition is a critical process that influences ecosystem function, particularly in grassland and heathland habitats. Prolonged exposure to elevated N levels alters soil chemistry, species composition, and plant traits, often leading to increased biomass in nutrient-responsive species and suppression of more sensitive plants (Boch et al., 2021). As N inputs decrease or cease, ecosystems can begin to recover, though the rate and extent of recovery depend on various factors including the duration and intensity of N deposition, as well as the specific characteristics of the ecosystem (Stevens, 2016). Our data in chapter 5 showed no significant difference between the control and N treatment plots across all the soil parameters, including soil pH, nitrogen concentration, carbon concentration, C:N ratios, NH_4 concentration, NO_3 concentration, TOC, and microbial biomass C. This reveals that the soil had recovered from any nutrient addition impacts.

One of the primary concerns regarding recovery from N deposition is the re-establishment of pre-deposition soil nutrient balances. N deposition often leads to soil acidification and nutrient imbalances (Basto et al., 2015, Horswill et al., 2008), which can persist long after N inputs are reduced or stopped. These changes in soil chemistry can delay or inhibit the recovery of plant species as soil acidification has deep rooted effects on seed bank which are the source of recovery (Deska et al., 2011). Additionally, the accumulation of nitrogen in soils can promote the growth of competitive, fast-growing species that may outcompete less nutrient-demanding species, even after N inputs decline (Boch et al., 2021, Humbert et al., 2016, Hobbs et al., 1988).

In terms of vegetation recovery, species that were suppressed under high N conditions may slowly return as soil nitrogen levels decrease. However, the recovery process is often slow and can be incomplete. For example, heathland species like *Calluna vulgaris*, which are sensitive to N deposition (Boateng, 2021), may struggle to regain dominance in areas where nutrient-demanding grasses have established. This recovery is not just limited to above-ground vegetation; below-ground seed banks are also affected by N deposition. Long-term N deposition can reduce the abundance and diversity of the seed bank Basto et al. (2015), which limits the capacity for regeneration once N levels decline.

The interaction between recovery from N deposition and plant traits, such as flowering time and seed production, is particularly important. N deposition has been shown to alter phenological traits, including delayed flowering and reduced seed production in sensitive species (Briggs, 1991, Tatar et al., 2013). As N levels decrease, some of these traits may revert to their original state, but recovery is not guaranteed. Species that produced more flowers and seeds in response to nutrient addition were better positioned to recover after N addition ceased, as their seeds could quickly germinate and establish new plants.

Disturbance also interacts with nutrient dynamics in ways that can either aid or hinder recovery from N deposition (Seabloom et al., 2020, Bach et al., 2010). Disturbance can create opportunities for nutrient redistribution and plant regeneration (Schnoor and Olsson, 2010), but when combined with high nutrient inputs, it may exacerbate the competitive advantage of fast-growing species, further delaying recovery. Conversely, as nutrient levels decline, disturbance may help in restoring diversity by allowing suppressed species to re-establish. Thus, while the focus of recovery in this study is on N deposition, the potential role of disturbance in facilitating or complicating recovery should not be entirely overlooked.

6.5 Future work

Future experiments could explore the effects of varying nutrient levels on flowering traits across different species. Although this study targeted a set of particular forbs, extending this to a broader suite of species that differ in sensitivities to nutrient addition would consider allowing more comprehensive conclusions about how nutrient availability affects flowering and seed production. Additionally, exploring how flowering is affected by interactions of environmental factors, such as temperature and water availability with the nutrient levels could help in understanding the potential resilience in grassland plant species to changing climate conditions.

The research on seed banks revealed several knowledge gaps that warrant further investigation. One critical area is the impact of N deposition on seed bank dynamics across different habitats. This study has focused solely on heathlands; future studies should investigate how N deposition affects seed banks in other ecosystems, such as forests, and wetlands where there are particular knowledge gaps. Understanding how seed banks respond to long-term N addition in diverse habitats would help in developing conservation strategies tailored to specific ecosystems. Additionally, studies on the recovery potential of seed banks after N deposition, including the role of seed bank diversity in supporting ecosystem resilience, would be valuable.

Other studies have indicated that long-term studies are very important in understanding the implications of nutrient inputs on grassland ecosystems (Phoenix et al., 2012). While the present study provided short-term effects of nutrients, extending it into a longer period would have comprehensively contextualized how nutrient addition influences temporal changes in species composition, biomass, and ecosystem functions. Long-term studies could also explore the cumulative impacts of repeated nutrient inputs and disturbances, shedding light on the threshold levels of nutrient addition that ecosystems can tolerate before experiencing irreversible changes.

Future work on the Norwegian experiment (Chapter 5) could focus on detailed vegetation analysis to assess the long-term effects of N deposition on plant communities in the region. This could involve monitoring changes in species composition, biomass, and soil health over time, with particular attention to how different functional groups (e.g., grasses, forbs, and shrubs) respond to N addition. Additionally, research could investigate the potential for recovery in these ecosystems by examining the role of seed banks and the resilience of vegetation after cessation of N inputs.

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