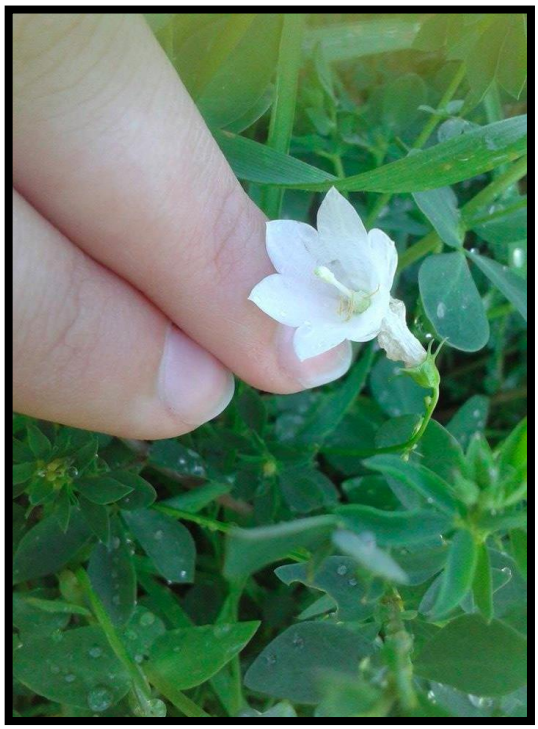
Carbon storage in grasslands: the impact of atmospheric nitrogen pollution



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September 2016

Declaration

I herewith declare that this thesis is my own work, and that it has not previously been presented to obtain a degree in any form.

Thesis word length is 40,254, and therefore does not exceed the permitted maximum.

Isabel Beltrán Rogers,
Lancaster University, September 2016

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Abstract

Atmospheric nitrogen deposition is a major threat to biodiversity and ecosystem service provision around the globe. It is known that nitrogen enrichment affects various chemical and biological processes involved in carbon cycling and storage in soil. This is especially significant as soil carbon storage is an essential form of climate change mitigation. However, there is a lot of uncertainty regarding the impact of nitrogen accumulation on terrestrial carbon storage. Determining the impacts of nitrogen addition on soil carbon is crucial to our understanding of how soil can be managed as a carbon sink. Evidence suggests that the chemical form of nitrogen may affect how grasslands respond to nitrogen enrichment. In addition, nitrogen has both direct and indirect (via plant community change) effects on carbon. In order to understand how nitrogen affects carbon storage, these different effects must be disentangled.

By using two seven-year field nitrogen addition experiments, a microcosm incubation, and a two-year mesocosm study, this thesis aimed to investigate the effects of nitrogen addition on carbon cycling and storage in acid grasslands. Results show that reduced nitrogen is likely to have the strongest long-term effects on carbon storage, due to decreases in soil pH and potentially adverse effects of ammonium accumulation. Moreover, nitrogen addition was found to have a negative effect on soil respiration, possibly via nitrogen-enhanced carbon and phosphorus limitations, as well as possible effects of nitrogen-induced acidification. Results also suggest that nitrogen addition may have different direct and indirect effects on soil carbon. Indirect effects, driven by plant community change, strongly influenced inputs of fresh carbon to soil. However, direct effects of nitrogen could alter the storage of older, mineral-associated soil carbon. Finally, this thesis highlights the need for more long-term (over ten-year) studies in order to determine the true effects of nitrogen on soil carbon storage.

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Chapter 1: General Introduction

General Introduction

1.1 Why is carbon important?

In a world where the expression 'climate change' has become part of a popular nomenclature, the significance of carbon (C) is not easily overlooked. Global warming and the mitigation of greenhouse gas emissions are priority issues worldwide, and have come to be perceived as focal points in our understanding of future conditions on earth. A large variety of policies, measures, instruments and approaches developed over the years are available to national governments in order to limit the emission of greenhouse gases (Metz, 2007). These include regulations and standards, taxes and charges, tradable permits, voluntary agreements (VAs), and subsidies and incentives such as direct payments or tax reductions (Metz, 2007). Due to its pressing nature, the subject of climate change remains high on the international political agenda. This is evidenced by the occurrence of high profile events such as the annual United Nations Framework Convention on Climate Change. This conference was attended by 189 Parties, and aimed to provide the basis for concerted international action to mitigate climate change and to adapt to its impacts (Blobel et al., 2006).

Pre-industrial concentrations of carbon dioxide (CO_2) in the atmosphere were around 280 ppm, considerably less than the 402 ppm recorded in 2016 (Dlugokencky and Tans, 2016). Combustion of fossil fuels has rapidly increased the atmospheric concentration of CO_2 over the last 250 years, and continues to do so to this day. Between 1970 and 2004 for example, total annual anthropogenic greenhouse gas emissions rose by 70% (Solomon, 2007). A major consequence of this is that global temperatures are also increasing. From 1956 to 2005, global temperatures increased on average by 0.13°C per decade, and eleven out of the twelve years between 1995 and 2006 had been ranked among the twelve warmest years on record since 1850 (Pachauri and Reisinger, 2007). Recently, researchers have predicted that within the next two decades, half of the world's population will regularly experience regional summer mean temperatures that exceed those of the historically hottest summer (Mueller et al., 2016). This temperature increase has led to numerous different effects including changes to terrestrial biological systems (the behaviour of many species of plants and animals), human health problems and sea level rise (average of 3.1 mm per year between 1993 and 2003) (Pachauri and Reisinger, 2007).

Depending on the different emission scenarios used, estimates for atmospheric CO_2 concentrations in 2100 range between 730 and 1090 ppm, and the resulting range of global mean temperature changes between 1990 and 2100 is estimated at 1.4 to 5.8°C (Solomon, 2007). Consequences of global warming are likely to be more severe in sensitive regions such as the poles. Average Arctic temperatures have increased at almost twice the global average rate in the past 100 years (Pachauri and Reisinger, 2007), as demonstrated by the decreasing extent of Arctic sea ice (Stroeve et al., 2007) and the thawing of large areas of permafrost observed over the years (Schuur et al., 2008). Although once again, estimates depend on the emission scenarios used, models predict that during the last two decades of the 21st century, annual mean surface air temperature change in the arctic could be as high as 8°C (Solomon, 2007). In addition, patterns of precipitation are also very likely to change, causing more extreme weather events leading to increased frequency of floods and droughts (Solomon, 2007). It must be noted however, that any predictions of this kind involve a huge amount of uncertainty (Stainforth et al., 2005).

If we wish to minimise the consequences of climate change, then it is necessary to moderate the volume of C being released into the atmosphere. It is widely understood that the combustion of fossil fuels acts as the primary source of emitted C (as CO_2) (Metz, 2007), and that therefore it is beneficial to either implement methods that would restrict C emissions during such processes, promote efficient use of fossil fuel derived energy, or to invest in alternative power supply systems. However, the planet also possesses its own mechanisms for extracting C from the atmosphere. Two methods include absorption by the oceans, and sequestration by terrestrial ecosystems.

Only 45% of all current anthropogenic CO_2 emissions remain in the atmosphere. Of the remaining 55%, 30% is taken up by terrestrial ecosystems and 25% by the oceans (Erisman et al., 2011). Globally, terrestrial systems contain approximately 2100 Gt of C (De Deyn et al., 2008), of which around 80% is stored in soils (Bardgett and Wardle, 2010). It is this ability of soil to act as a major C sink that makes it essential to climate change mitigation. Nonetheless, we still know remarkably little about what factors regulate the fluxes of C to and from soil (Bardgett and Wardle, 2010), which is why it is so important that research be carried out to try to understand and thus protect soil C storage.

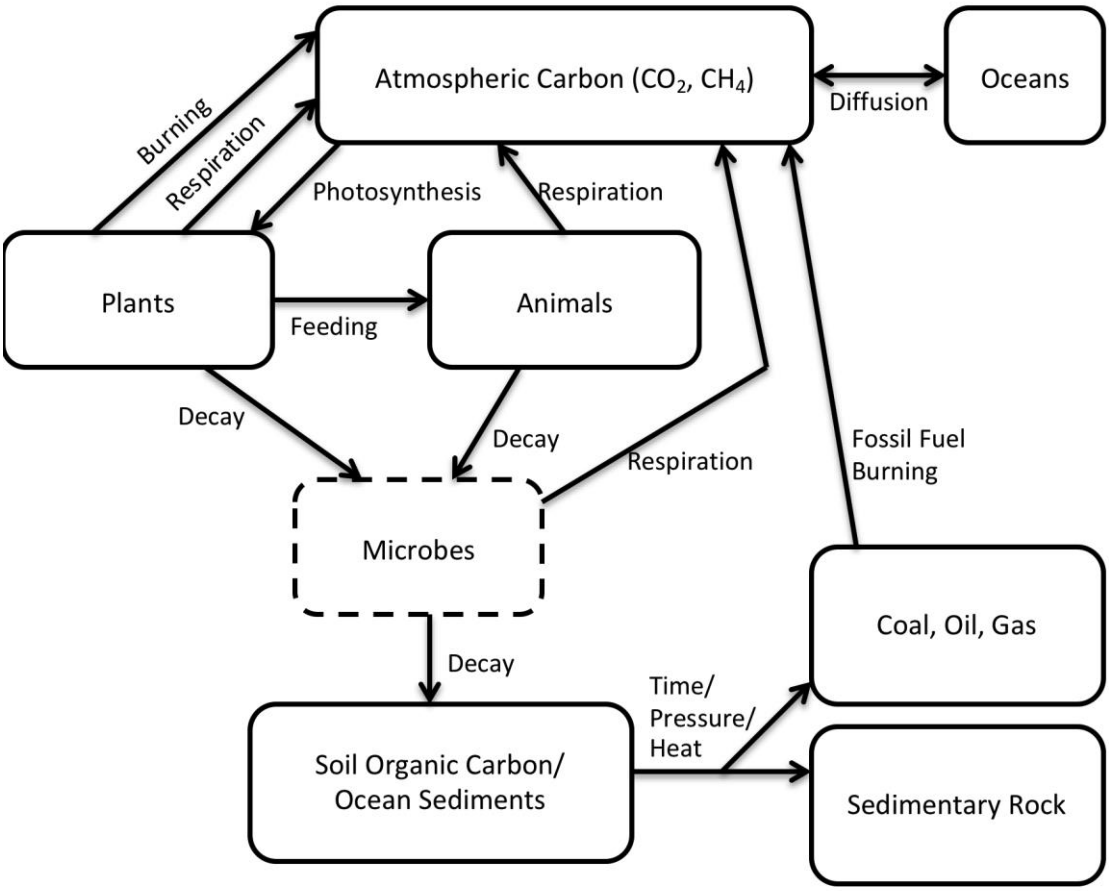

Figure 1.1: The Carbon Cycle. Carbon, as CO_2, is taken out of the atmosphere via photosynthesis by plants, or by diffusing into the oceans. Plants store C in their tissue, which can be passed to animals when they feed, or released into the atmosphere if the plants are burned. Living plants and animals respire CO_2 into the atmosphere, and when they die, microbes decompose their tissue, while also respiring CO_2 or CH_4. Leftover organic C is stored in the soil or at the bottom of the oceans. Over time, soil organic C and ocean sediments are buried, thus applying pressure and heat, and slowly turning them into sedimentary rocks or fossil fuels. Humans now burn these fossil fuels, releasing old carbon deposits back into the atmosphere as CO_2.

1.2 Why has reactive nitrogen input increased and why is this important?

Nitrogen (N) is highly abundant as di-nitrogen gas (N_2), which makes up 78% (by volume) of the Earth's atmosphere (Galloway et al., 1995). However, N is naturally limited in its reactive forms, which include oxidised and reduced N compounds such as nitrate, nitrite, ammonia, ammonium, nitric acid and organic N compounds (Bobbink et al., 2010). It is only in these

various reactive, or 'fixed' forms that N is useable to most plants and animals (Ashman and Puri, 2002). N is essential to plant growth (Erisman et al., 2011), and as a result, crop production is commonly limited by N availability. Processes such as lightning, wildfires and biological N_2 fixation by organisms like cyanobacteria and *Rhizobium* in legumous plants provide ecosystems with a constant input of reactive N (Ashman and Puri, 2002). However, natural N fixation alone is not enough to fully sustain modern human agricultural practices.

By the end of the 19th century, increased demand for food to support a growing human population created an unprecedented demand for reactive N that could not be met by traditional practices (Sutton, 2011). Since the invention of the Haber-Bosch process in the 1950s however, Man has been able to manufacture relatively cheap N fertiliser from N_2 gas on an industrial scale (Gorman, 2013). The Haber-Bosch process has allowed the human population to expand beyond the soil's natural means. In fact, the European Nitrogen Assessment (Sutton, 2011) describes the invention of the Haber-Bosch process as "the greatest single experiment in global geo-engineering that humans have ever made". Without the cheap production of fertiliser for agriculture, humanity could never have produced enough food to sustain the growth of our population to what are now over 7 billion people (Sutton, 2011).

However, agricultural practices (including animal husbandry) have proven to be wasteful and inefficient, and every year a copious amount of N is lost to the environment via processes such as surface runoff, leaching, and volatilisation (Galloway et al., 2008; Sylvester Bradley, 1993). In the year 2000 it was estimated that 100 Tg of reactive N were released each year from N fertilizer spread on farmlands around the world (Fields, 2004). In 1996 the IPCC defined a default amount of applied N that is lost through leaching and runoff in agriculture of 30% (Houghton, 1997). If this is the case, then the amount of reactive N released from agricultural leaching and runoff could be as high as 30 Tg per year.

Compared with pre-industrial levels, global production of reactive N has more than doubled (Galloway et al., 1995; Manning et al., 2006; Sutton, 2011), and human processes now convert more N_2 into reactive N than is produced naturally in the entire world (Erisman et al., 2011; Galloway and Cowling, 2002; Rockstrom et al., 2009). Fossil fuel combustion also contributes to the global N cycle by producing large amounts of oxidised forms of N, which also end up in the environment via atmospheric N deposition (Galloway et al., 1994; Galloway et al., 2008; Holland et al., 2005; Howarth, 1998).

The amount of synthetically produced reactive N is now so large that it has altered the global N cycle, leading to a series of serious environmental issues. Such problems include nitrate pollution (and thus eutrophication) of waters, emissions of nitrous oxide (a powerful greenhouse gas), NO_x and ammonia to the atmosphere (creating human health problems), and N accumulation in terrestrial systems (Galloway et al., 1995; Rockstrom et al., 2009). This N accumulation is causing massive impacts on biodiversity and ecosystem processes worldwide (Bobbink et al., 2010; Bobbink et al., 1998; Galloway et al., 2003).

Policies have been put into place in order to restrict the amount of reactive N being released into the environment. The main sources of N pollution as recognised by governmental bodies are combustion (NO_x by industry, power plants and traffic), waste waters (dissolved and particulate N in discharges by industry and households) and agriculture (NH_3 and N_2O to air, NO_3 to groundwater and dissolved and particulate N to surface waters) (Sutton, 2011). The severity of the effects of N pollution depend on the duration, total amount, and N form of the inputs, as well as the sensitivity of both the biotic and abiotic components of the affected ecosystems (Bobbink et al., 2010). Policies are therefore designed to address different sources, N compounds, regions and receptors (Sutton, 2011). Important policies that deal with N pollution include the 2008 Directive on Industrial Emissions concerning Integrated Pollution Prevention and Control (IPPC), the 2001 National Emission Ceilings Directive, the 2008 Ambient Air Quality Directive, the 2000 Water Framework Directive, the 1991 Urban Waste Water Directive, the 1991 Nitrates Directive and the 2006 Groundwater Directive (Sutton, 2011).

Nonetheless, these measures are arguably not enough. Although evidence for decreases in oxidised N emissions suggests a relative success of current policies (Carslaw et al., 2016; Li et al., 2016b; Skalska et al., 2010), N critical loads are still exceeded across the globe, and the ecological status of many systems (especially regarding eutrophication) still remains below the set targets (RoTAP, 2012; Sutton, 2011). Moreover, while emissions of oxidised N have decreased in recent years, efforts to mitigate the release of reduced N have not been so successful (Li et al., 2016b). Emissions of ammonia from agriculture have been the least affected by environmental policies (Sutton, 2011), and projections for future N deposition predict an increase in the proportion of reduced N pollution (Dennis et al., 2010; Engardt and Langner, 2013). It is expected that global N deposition rates will increase another two- or threefold before reaching a plateau (LeBauer and Treseder, 2008). However, Rockström *et al.* (2009) suggest that artificially produced reactive N input should be reduced to 35 million

tonnes per year, around a quarter of all current emissions in order to avoid major impacts from this pollution.

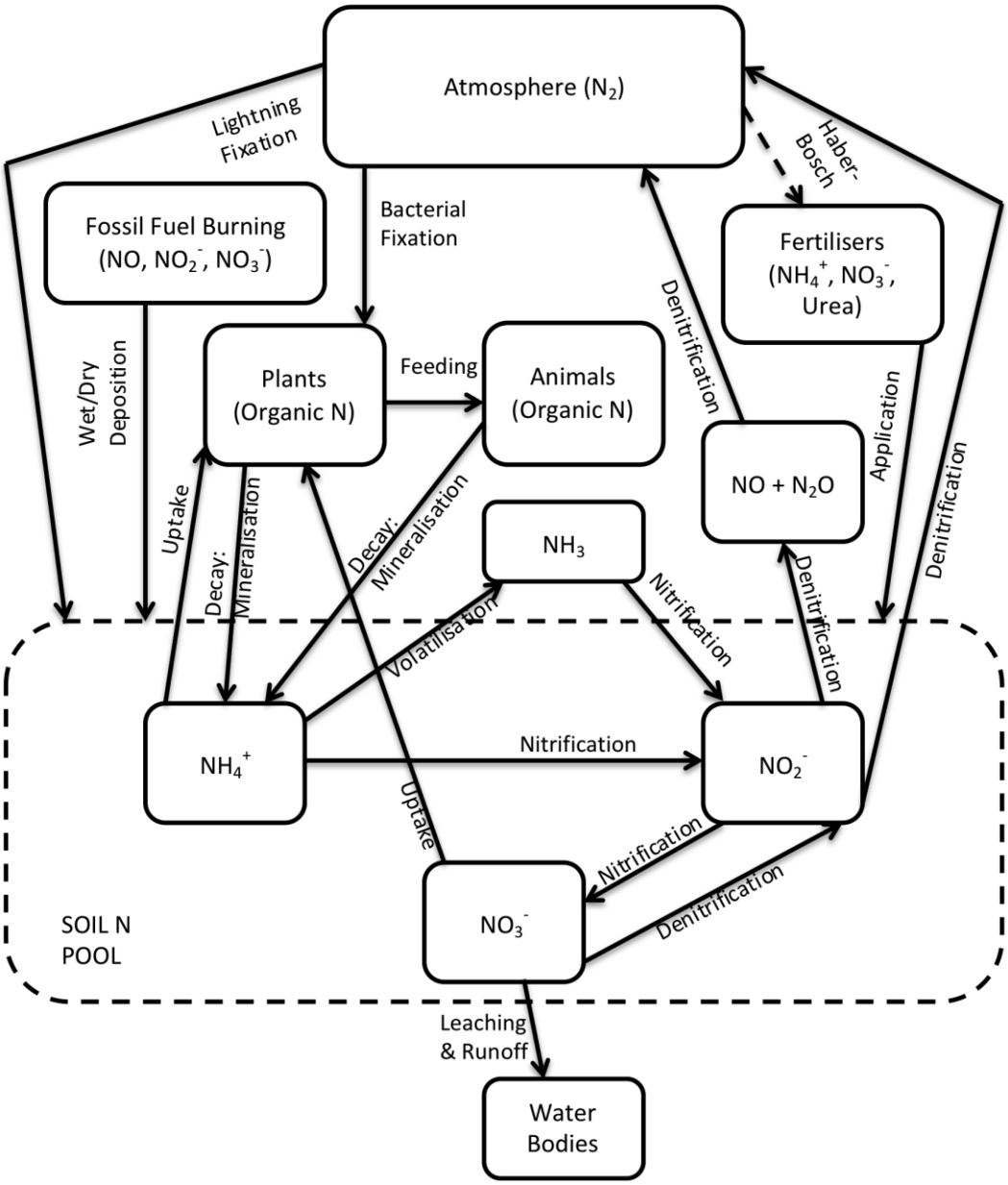

Figure 1.2: The Nitrogen Cycle. Natural processes such as bacterial fixation (by both symbiotic and non-symbiotic bacteria) and lightning turn N_2 gas into available forms of N (NH_4^+ and NO_3^-). These can be taken up by plants, then eaten by animals, and stored in plant and animal tissue as organic N. When they die, plant and animal tissue is decomposed by bacteria, which mineralise organic N into NH_4^+. NH_4^+ can then either be taken up by plants again, volatilised into NH_3 gas, or it can undergo nitrification by nitrifying bacteria, turning it into NO_2^-, then NO_3^-, which can then be taken up by plants or leached from soil into water bodies. NO_3^- can also undergo denitrification by denitrifying bacteria, turning it back into NO_2^-, then NO and N_2O, and then N_2. Human burning of fossil fuels and manufacture of artificial fertilisers is releasing huge quantities of reactive N into the environment.

In recent years, researchers have acknowledged the importance of considering the links between increased reactive N levels and the C cycle. Although this field is still full of uncertainty, knowledge of how reactive N addition may affect C cycling, and thus climate change is rapidly expanding. As a key component of amino acids (Barrett, 1985), the 'building blocks' of all proteins, N is essential for the functioning of all living things. Consequently, N addition is known to affect the growth, activity and community composition of plants and soil microorganisms through various direct and indirect pathways (Bardgett et al., 1999; Bardgett and Wardle, 2010; LeBauer and Treseder, 2008; Rousk et al., 2011; Stevens et al., 2010). This in turn affects how C is stored and cycled in the soil. It is this alteration of the terrestrial CO_2 sink that is especially significant (Erisman et al., 2011). Since the soil's ability to store C is vital for climate change mitigation, any change to the amount of C stored in soil could have substantial global consequences. Early research projected that N deposition would increase the potential for C storage in terrestrial ecosystems due to additional photosynthetic conversion of CO_2 into organic C (Galloway et al., 1995). Estimates for the total amount of C potentially stored as biomass due to N addition ranged from 0.2 all the way up to 9 Pg yr^{-1} (Galloway et al., 1995). However, studies have also shown that other consequences of N fertilisation can include increased decomposition rates (Fenn, 1991; Hunt et al., 1988), which can therefore potentially offset the amount of C stored due to increased plant growth. To this day, estimates of how much C sequestration can be stimulated by N addition vary enormously (Erisman et al., 2011), thus highlighting the need for further research in this field of study.

1.3 The effects of nitrogen deposition on carbon cycling in soil

The effects of increased reactive N input on C cycling in soils are intricate, varied, and often occur at different spatial and temporal scales. Consequently, it is exceedingly challenging to determine the full nature and extent of these effects, let alone to quantify their exact impacts on C pools. It is also difficult to trace whether one process or interaction is directly connected to another, especially since a lot is still uncertain about how soil biota functions (Coleman, 2011). The task of differentiating between the effects of N deposition on C cycling is further complicated by the existence of both direct and indirect effects. For example, soil acidification caused by N addition has a direct inhibitory effect on plant growth (Asner et al., 2001), however, acidification can also cause plant community change through the exclusion of plant species not tolerant of acidic conditions (Bobbink et al., 2010), thus leading to

indirect effects on soil C via differences in plant traits. In order to manage soils effectively, it is important to recognise which processes contribute most to changes in C pools, and therefore which to focus on when determining management schemes. A few studies (Manning et al., 2006; Manning et al., 2008) have championed the idea that decoupling direct and indirect effects of N deposition on C cycling is crucial to understanding exactly how the soil system functions when it is exposed to increasing levels of reactive N. Nonetheless, in order to effectively decouple different effects of N addition requires heavily synthetic methods, and thus remains a problem when attempting to relate findings to 'real world' situations. In addition, it is difficult to know whether any short-term effects will be similar to the long-term effects of N deposition on C cycling in soils. However, long term experiments over one to two decades have shown that thresholds for significant N effects may be lower with increased duration of treatment (Bobbink et al., 2010).

1.3.1 Direct effects

1.3.1.1 Nitrogen effects on carbon mediated by soil chemistry

N addition causes an increase in rhizosphere acidity (Bardgett et al., 1999; Houdijk et al., 1993; Hruska et al., 2012; Zhang et al., 2012), especially after the nitrification of ammonium in weakly buffered systems (Bobbink et al., 1998). In a meta-analysis carried out by Lu *et al.* (2011a), it was found that N addition significantly decreased soil pH by 3.5% across various ecosystems. A high degree of acidity leads to numerous problems. If soil pH falls below 5.5 for example, it can lead to high concentrations of Al^{3+} in solution (Ashman and Puri, 2002), which can in turn inhibit cell division and root elongation in plants (Ashman and Puri, 2002). Decreased soil pH can also make other potentially toxic metals soluble, and render essential nutrients such as phosphorous insoluble (Ashman and Puri, 2002). Consequentially, N-induced acidification can cause decreases in plant productivity (Asner et al., 2001), thus reducing the amount of C stored as plant biomass. In addition, several threatened plant species prefer higher soil pH and lower levels of Al^{3+} (Bobbink et al., 1998). Changes in pH also affect the soil's microbial community. Many fungi thrive under slightly acidic conditions, while bacteria prefer a higher soil pH (Ashman and Puri, 2002). Lu *et al.* (2011a), suggest that increased acidity may limit microbial biomass growth, thus potentially restricting C accumulation in this particular soil pool.

N deposition can also cause chemical changes in the soil that can be detrimental to the development of both plants and soil microbes (de Vries et al., 2012; Houdijk et al., 1993). This could lead to reduced C uptake and storage in these two system components. A change in the balance between ammonium and nitrate has been known to affect the performance of several plant species, including rare grassland species such as *Arnica montana*, *Cirsium dissectum* and *Thymus serpyllum* (Bobbink et al., 1998; Houdijk et al., 1993). High NH_4^+/NO_3^- ratios (ammonium accumulation) can be detrimental to such plant species due to an effect on cation uptake, especially if mineralisation rates are low (Bobbink et al., 1998). In addition, N leaching caused by high N inputs can capture and transport cations, leading to base cation impoverishment (Asner et al., 2001; Horswill et al., 2008). This in turn can be exasperated by soil acidification, which may indirectly lead to further exchangeable base cation losses (Bobbink et al., 2010; Houdijk et al., 1993). Increased N availability has also been shown to create an imbalance between N and other essential nutrients such as Mg and P, leading to detrimental changes in plant nutrition (Drenovsky and Richards, 2004; Lomsky et al., 2012; Mellert et al., 2004; Prietzel et al., 2008). Lomský *et al.* (2012) examined young Norway spruce (*Picea abies*) stands in the Jizera Mountains suffering from chronic N deposition, and found that their needles contained disturbed N:Mg and N:P ratios. It was determined that the trees were taking up too much N and too little Mg and P, thus causing Mg and P deficiency and stunting the trees' growth.

1.3.1.2 Nitrogen effects on carbon mediated by plant growth and tissue quality

As plant growth is often limited by reactive N availability (Bardgett and Wardle, 2010; LeBauer and Treseder, 2008), its addition normally causes an increase in plant primary productivity (and thus CO_2 fixation), especially in nitrophilic species (Bobbink et al., 1998; Erisman et al., 2011; Foster and Gross, 1998; Galloway et al., 1995; Gough et al., 2000; Shaver et al., 2001). The subsequent C accumulation via biomass gain is demonstrated in numerous studies (Gough et al., 2000; Kinugasa et al., 2012; LeBauer and Treseder, 2008; Shaver et al., 2001; Vitousek et al., 1997); however, the magnitude of this response to N input varies greatly. In their meta-analysis, Lu *et al.* (2011a) warn that although N addition usually stimulates plant growth, it is unclear whether this can lead to net C storage in soil. Indeed, the issue of how much C storage is actually brought about by N stimulation of biomass gain is still highly controversial. This debate is exemplified by the stand-off between Magnani *et al.* (Magnani et al., 2007) and de Vries *et al.* (2008) in Nature, where the former

argue that for every kg of N deposited, the forest ecosystems studied (temperate and boreal) sequestered an average of 470 kg of C. However, the latter group say that this is a massive over-estimation of the amount of C uptake, and that the actual value is more likely to be around 30-70 kg C per kg N deposited. A follow-up paper by Sutton *et al.* (2008) conducted a re-analysis of Magnani *et al.* (2007)'s findings, stating that when dry as well as wet N deposition is accounted for, along with the effects of intersite climatological differences, actual C retention values are indeed likely to be a lot smaller than suggested. A later paper by de Vries *et al.* (2009) also emphasised this idea by reporting that calculations made using data collected from field experiments as well as ecosystem model outputs indicated a total C sequestration range of 5-75 kg C/kg N deposition for forests and heathlands, with a most common range between 20 and 40 kg C/kg N.

C uptake and storage via plant growth cannot be considered without the inclusion of C loss through decomposition. N is known to accelerate rates of litter decomposition (Wang et al., 2010) due to increased litter quality. N deposition can lead to the improvement of litter quality by lowering the C:N ratio in plant tissue (Manning et al., 2008). Substrate quality has long been considered a critical factor in determining rate of decay (Melillo et al., 1982). Detritus with a higher N concentration decomposes more easily, leading to faster turnover of plant material (Martinez et al., 2013). Although C release due to N-enhanced decomposition could potentially offset C uptake via increased plant growth, this is not the case in many situations. In their Ecotron experiment, Manning *et al.* (2006) demonstrate that after five plant generations, net ecosystem productivity (NEP) increased under high N deposition, and that this was enough to offset the loss of C through augmented decomposer activity. However, this was a highly synthetic study using only a limited number of plant species, thus lacking a good range of plant functional diversity. Research carried out in the field under more life-like conditions has presented different results. Morecroft *et al.* (1994) for example, examined calcareous and acidic grasslands in the Peak District, UK, and found that N-induced increases in plant productivity had a negative effect on overall C storage, as growth was limited by other nutrients and environmental factors. Also, in a study by Soudzilovskaia *et al.* (2005) conducted on lichen-rich alpine tundra in the north-west Caucasus, Russia, an increase in vascular plant biomass caused by N (and P) fertilisation was counterbalanced by a large decrease in the lichen population, resulting in no overall change in aboveground biomass.

Higher quality plant tissue is also more palatable to herbivores (Bardgett and Wardle, 2010), which can increase the impacts of grazing on plant vitality as well as trampling. In contrast,

higher rates of grazing may also lead to greater investment in belowground growth by plants (Bardgett and Wardle, 2010), therefore increasing vital belowground C inputs. Nonetheless, research has shown that intensive grazing has a negative effect on soil C storage (van der Wal et al., 2007).

Root biomass is also affected by changing N inputs to soils. When plants have access to a large supply of N, they tend to allocate most C uptake to aboveground rather than belowground growth (Beets and Whitehead, 1996). This is because less effort (decreased C cost) is needed in order to collect nutrients (i.e. N) from the soil, but more energy is required to increase foliage area so as to out-compete other fast-growing species for light (Beets and Whitehead, 1996; Wang and Feng, 2005). Therefore, when N fertilisation occurs, a reduction in plant root biomass can occur (Ringwall et al., 2000). This phenomenon is observed in Bardgett *et al.* (1999), where N addition to soil collected from temperate upland grasslands in North Wales caused a decrease in the root biomass of all plant species investigated (*Lolium perenne*, *Agrostis capillaris*, *Holcus lanatus* and *Festuca rubra*). Less allocation of C to roots is also known to lead to decreased root exudation (Johnson, 1993), which is an important source of C for soil microbes. Reduced allocation of C to belowground growth is especially significant because direct C input to the rhizosphere is critical for soil C sequestration (Balesdent and Balabane, 1996). This importance is stressed in the meta-analysis of Lu *et al.* (2011a), where it is stated that soil organic C (SOC) storage across the studies considered was significantly correlated with the quantity of belowground organic matter inputs, but not with aboveground input. On the other hand, a study by Manning *et al.* (2006) found that after five generations, plants adapted to high N availability had a greater root biomass than those adapted to low N inputs. This result denotes the importance of considering different timescales when assessing the impacts of N deposition on C storage. However, it must be noted that in this experiment plant communities were manipulated in order to artificially create species compositions similar to what could probably occur naturally over time in a high N environment. So even though this method enabled Manning *et al.* to accelerate the effects of time, one must be wary of how synthetic this study was.

1.3.1.3 Nitrogen effects on carbon mediated by soil microorganisms

It has been suggested that N addition may have a direct inhibitory effect on the soil community (Bardgett et al., 1999). Indeed, a meta-analysis by Treseder (2008) reveals that microbial biomass declined 15% on average under N fertilisation, and that this decline was

more evident in studies of longer durations and with higher total amounts of N added. This negative effect of N on microorganisms is strongest for decomposers and mycorrhizal fungi, and occurs through the repression of enzyme activity and the build-up of recalcitrant and toxic compounds (Bardgett et al., 1999). Enzymes mediate the decomposition of litter, the breakdown of OM and the mineralisation of N and P (Suding et al., 2008). Consequently, interference with the production of these enzymes could potentially deregulate key processes linked to the cycling of C. N enrichment is known to have direct and differential impacts on extracellular enzymes involved in decomposition processes (Bardgett and Wardle, 2010). According to Lu *et al.* (2011a), N fertilisation tends to accelerate the degradation of easily degradable litter, but may slow the decomposition of recalcitrant litter through the stimulation or repression of different sets of microbial extracellular enzymes. This view is supported by Xu *et al.* (2004), who state that N additions significantly accelerate the decomposition of light soil C factions, but stabilise soil C compounds in heavier, mineral-associated factions. Saiya-Cork *et al.* (2002) also find that N deposition can enhance litter decomposition, but reduce soil organic matter degradation through its effects on extracellular enzymes. However, studies have shown that N addition can enhance, reduce, or have no effect on decomposition rates (Keeler et al., 2009; Manning et al., 2006; Xu et al., 2004). A possible explanation for this divergence in results is that perhaps the effects of N on decomposition vary over time. It has been suggested that N deposition may initially accelerate plant decay, but slow it down during its later stages, particularly during the decomposition of lignin (Berg and McClaugherty, 2008; Xu et al., 2004). This idea is expressed in the findings of Magill and Aber (1998), whose long-term (seven-year) N-addition experiment resulted in decreased decomposition rates and increased lignin accumulation in the litter collected from treated plots. If this is the case, then it is possible that C would accumulate in the soil over time.

1.3.2 Indirect effects

1.3.2.1 Nitrogen effects on carbon mediated by plant community change

Recently, attention has been drawn to the effects of N deposition on plant community change. Research has shown that N accumulation is the main driver of changes to plant species composition across a whole range of different ecosystem types (Bobbink et al., 2010). In the UK and in Europe for example, it is known that species richness has been

significantly reduced by long-term, chronic N deposition (Stevens et al., 2004; Stevens et al., 2010). As N is a growth limiting nutrient, its addition to the environment can favour species that are better suited to high nutrient levels (Stevens et al., 2004; Wang and Feng, 2005). These nitrophilic species are usually fast-growing and thus rapidly dominate other species, causing species diversity loss and leading to changes in plant community structure over time (Bobbink et al., 2010; Galloway et al., 1995; Gough et al., 2000). In the Park Grass experiment at Rothamsted, UK, which has been running since 1856, N addition has caused a significant reduction in species diversity due to dominance by a few grasses as well as soil acidification (Bobbink et al., 1998).

Bryophytes and forbs tend to be competitively excluded by grasses under N fertilisation (Bobbink et al., 1998; De Deyn et al., 2011a). The susceptibility of mosses is also stressed in Bobbink *et al.* (1998), who state that due to N addition, many characteristic mosses (and lichens) have disappeared from calcareous grasslands. It is these slower growing species however, that are probably best for ecosystem C sequestration. This is because tissue from these plants usually decomposes more slowly and is a poor food source for soil microbes (De Deyn et al., 2011a). Moss-rich vegetation for example, has a slow rate of C respiration and a high C:N ratio. Therefore, it is possible that mosses play a key role in C sequestration in grasslands, especially during times when vascular plant growth ceases (autumn-early spring) (De Deyn et al., 2011a). Altered plant communities may also lead to changes in C allocation to different organic matter factions. In their investigation, Manning *et al.* (2006) found that while plant composition had no significant effect on C storage in the fine particulate organic matter (POM) fraction (500-53 µm), C storage in the mineral-associated POM fraction (<53 µm) was reduced under a community adapted to high N input. This is particularly significant as the mineral POM fraction is known to have longer residence times (Puget et al., 2000).

Timescales must also be considered when making assumptions about N effects on C storage mediated by changes in plant species composition. In some cases, N deposition may not have an immediate effect on plant community composition, but can instead cause sudden shifts in species dominance after an initial 'resilience period'. This is exemplified by Suding *et al.* (2008), who carried out a long term (six-year) field study on an alpine moist-meadow in Colorado, USA. In their experiment, N addition caused the sudden decline of *Geum rossii*, one of the two dominant species present at the site, after a four-year resilience period. This in turn facilitated the other dominant species, *Deschampsia caespitosa* via competitive release, causing a community-wide drop in diversity. Moreover, this change in species composition also decreased overall aboveground biomass, as *Deschampsia caespitosa* was

not able to grow enough to make up for the loss of *Geum*'s biomass due to phosphorus limitation.

Increased evapotranspiration caused by N-induced plant growth can significantly lower soil moisture content and water through-flow (Manning et al., 2006). Microorganisms are sensitive to changes in soil moisture, and this phenomenon has been linked to a decrease in decomposer activity (Manning et al., 2006). On the other hand, increased plant growth can also induce higher rates of microbial activity through greater litter production (Hernandez and Hobbie, 2010; Manning et al., 2006). Plant community change can also exacerbate any effects on decomposition rates caused by the improvement of litter quality (as previously mentioned in section 1.3.1.2), thus potentially leading to the acceleration of C cycling. As nitrophilic species slowly dominate over slow-growing plants, they can produce larger quantities of high quality litter, stimulating microbial activity (Manning et al., 2008). However, this may not be the case overall, as the size and activity of the soil microbial community is known to be lower under high-fertility conditions (Bardgett et al., 1999).

1.3.2.2 Nitrogen effects on carbon mediated by changes in the soil microbial community

Alterations to plant species composition can also bring about changes in the soil microbial community structure, potentially even altering microbial functional diversity (Hartmann et al., 2009; Zak et al., 2003). In an experiment by Bardgett *et al.* (1999) on temperate upland grassland soil, microbial activity showed greater responses to individual plant species than to N additions. This was due to plant-specific differences in the amount and patterns of root exudation, as well as changes in nutrient competition between the plants and rhizosphere microbes. Root exudation is also an important source of C input to soil (5–33% of daily photoassimilate), especially in actively growing plants (De Deyn et al., 2008). Consequently, any alterations made to this process can have large impacts on C storage. Smith *et al.* (2008; 2003) for example, showed that cessation of mineral fertilizer use coupled with the seeding of target plant species in managed grassland sites led to enhanced species richness (primarily through an increase in legumes) as well as an increase in soil fungi and the abundance of fungi relative to bacteria. When De Deyn *et al.* (2011b) carried out the same grassland restoration treatments, they found that these procedures also enhanced soil C and N storage. In addition, the treatments reduced the activities of key enzymes involved in the degradation of recalcitrant organic matter. This suggests that fungi may be especially important for C sequestration in soil. It also implies that ecosystem management that

promotes fungi over bacteria, such as the cessation of fertilizer application, could promote C sequestration by soil (De Deyn et al., 2011a).

Arbuscular mycorrhizal fungi (AMF) are a key component of the soil community. AMF symbiotically colonise plant roots, forming associations with 80% of plant species, and are found in nearly every habitat in the world (Treseder and Allen, 2000). Chitin, which is not readily decomposed, can contribute up to 60% of fungal cell walls. AMF also produces glomalin, a compound that can account for 30-60% of C in undisturbed soils (Treseder and Allen, 2000). Mycorrhizal fungi can act as N 'miners' for the plants that they have a symbiotic relationship with (Hobbie et al., 2013). Therefore, when N availability is raised, plants allocate less C to these fungi (Treseder, 2004) as they are no longer dependent on them for N collection. This reduced investment causes alterations in the community of mycorrhizal fungi (due to C limitation), which can have important consequences for C storage in live hyphal tissue and/or its residual soil organic matter (OM) due to variances in the chitin content and growth rate of different mycorrhizal groups (Treseder and Allen, 2000). In addition, increased N availability may also increase the turnover rates of fungal tissue (Treseder and Allen, 2000).

A decline in the fruiting bodies of ectomycorrhizal (ECM) fungi has already been documented in European forests (Cairney and Meharg, 1999). Also, studies have shown that species composition of ECM fungal communities on root tips in different forest systems shift upon long-term N fertilisation (Johnson, 1993; Taylor and Alexander, 1990). The same can occur with AMF communities, as was recognised by Egerton-Warburton and Allen (2000). They analysed a natural N deposition gradient in southern California coastal sage scrub and found that spores of *Scutellospora* and *Gigaspora* species became less prevalent with increasing N deposition, while spores of certain *Glomus* species (e.g. *G. aggregatum*, *G. leptotichum* and *G. geosporum*) proliferated under the same conditions. An example of the repressive effects of N addition on AM fungi is documented by Klironomos *et al.* (1997). This study found that AM hyphal lengths increased with CO_2 concentrations under a low N treatment, but not under high N. In a meta-analysis by Treseder (2004), mycorrhizal abundance decreased by a mean of 15% under N fertilisation. However, there are other studies where the same does not occur. In their experiment using hybrid poplar saplings, Lussenhop *et al.* (1998) found that under elevated atmospheric CO_2 levels, arbuscular mycorrhizal root mass was twice as great when in high-N soil. Changes in mycorrhizal fungi communities have been shown to have significant impacts on C accumulation. In Manning *et al.* (2006), high N treatments and high N plant communities decreased C storage in the mineral-associated fraction of POM.

They suggest that this was perhaps mediated by AM fungi, as they produce glycoproteins, and were significantly less abundant in plant communities adapted to high N deposition.

It must be noted that changes in soil microbial structure caused by increased N availability can facilitate plant community shifts through soil feedback mechanisms. This is exemplified by the study of Suding *et al.* (2008). As mentioned in section 1.3.2.1, N addition in this experiment caused the sudden decline of *Geum rossii* after a 4-year resilience period. The authors propose that a shift in the soil microbial population may have led to this abrupt change via the elimination of *Geum*'s associated microbial feedbacks. It is suggested that high N availability, and the resulting decrease in belowground C allocation, may have decreased microbial biomass and activity, thus making *Geum* more vulnerable. Such shifts in plant species may have important consequences for C storage in soils (section 1.3.2.1), thus making this relationship between plants and their associated microorganism communities especially significant.

1.4 Emphasis on: Grasslands

Temperate grasslands occupy an area of around 15×10^6 km^2, or 11% of the earth's land surface (Chapin et al., 2001). Grasslands occur in almost all continents (Chapin et al., 2001) and over a wide range of temperate zones where seasonal droughts and fire, or regular removal of aboveground plant biomass by grazing or mowing prevent forest development (De Deyn et al., 2008).

Grasslands can harbour high levels of plant species diversity (Michalcova et al., 2014). The grasslands of Pampa in southern South America for example, contain more than 400 species of grass alone (Chapin et al., 2001). This of course does not include the multitude of microorganisms present in grassland soils, an area which is still greatly understudied.

Relatively few studies concerning the effects of N deposition on soil C cycling have been carried out on grasslands. Most research so far has been focused on forest ecosystems, due to large potential for C accumulation in woody tissues. However, grasslands are also massive potential C sinks (Soussana et al., 2007; Ward et al., 2016; Xu et al., 2004). In grassland systems, most C accumulation occurs in soil. Soil C pools are a lot larger than those in aboveground vegetation due to high belowground C allocation, lack of persistent woody structures aboveground, and generally higher decomposability of shoot than root tissue (De Deyn et al., 2008). In addition, grasslands have an exceptionally high microbial biomass C

pool (Ashman and Puri, 2002). Temperate grasslands have high soil organic C concentrations, often exceeding that of temperate forests (De Deyn et al., 2008), and should therefore be studied in much greater depth.

1.5 Thesis objectives

It is clear that human alteration of the N cycle can have important effects on C cycling in terrestrial ecosystems. This in turn can affect the potential for C storage in these systems, an essential form of climate change mitigation. The overall aim of this thesis was to tackle some of the large uncertainties in how N enrichment affects ecosystems' ability to store C, specifically by investigating the effects of N addition on acidic grasslands.

This thesis was funded by Natural Environment Research Council, and used two seven-year N addition experiments to investigate how N enrichment affected C and N cycling and storage in species rich acid grassland systems. In addition, a two-year mesocosm experiment was used to investigate the differences between the direct and indirect (via plant community change) effects of N deposition on grassland C cycling and storage. This thesis consists of a methods chapter followed by four experimental chapters designed to address the following questions:

(1) How N addition affected C and N pools in a Norwegian acidic grassland (Chapter 3);

(2) How N addition affected soil respiration and temperature sensitivity in a Norwegian acidic grassland (Chapter 4);

(3) How low levels of N addition affected C and N pools in a Welsh acidic grassland (Chapter 5); and

(4) How plant community composition affected the impacts of N addition on C cycling and storage in grassland mesocosms (Chapter 6).

Chapter 7 provides a summary of the key findings from each experimental chapter, their implications, and the potential for future work in this field of study.

Chapter 2: Methods

Methods

2.1 Field site descriptions

2.1.1 Norway (Chapters 3 and 4).

The experimental site is located in Revna (Fusa municipality, Hordaland), Norway. The site was chosen due to its relatively low background N deposition (ca. 6.1 kg N ha^{-1} yr^{-1}, see table 2.1), and is classified as a species-rich *Nardus* grassland (Violion caninae alliance (Schwickerath, 1944)). Vegetation is dominated by grasses such as *Agrostis capillaris*, *Anthoxanthum odoratum*, and *Festuca rubra*. The dominant bryophyte is *Rhytidiadelphus squarrosus*, and some common forbs present include *Cardamine pratensis*, *Leontodon autumnalis, Ranunculus acris*, *Ranunculus repens* and *Rumex acetosa*.

At the start of the experiment in 2007, average species richness was 14.9 species per 2 × 2 m plot for vascular plants and 2.7 for bryophytes (Dorland et al., 2013). The soil is shallow (ca. 13 cm), with a pH in the range 5.0-5.5. The grassland was traditionally managed for haymaking with annual mowing, and grazing by sheep in spring and autumn until 2005 when grazing was discontinued. Prior to the year 2000, low levels of fertiliser were applied. The field was probably used for potato and arable farming before ca. 1940. See table 2.1 for a summary of site and meteorological characteristics.

Table 2.1: Summary of site and meteorological characteristics. Table adapted from Dorland *et al.* (2013). The meteorological data are averages over the period 1971-2000. (1) Average values at weather station at Bergen (http://retro.met.no). (2) Data from http://www.climatedata.eu. (3) Calculated using EMEP-based IDEM models (Pieterse et al., 2007).

Site Characteristics	
Coordinates	60° 09' 29.6'' N 5° 44' 34.6'' E
Altitude (m asl)	160
Average background N-deposition (kg^{-1} ha^{-1} yr^{-1}) (3)	6.1
Climate Characteristics (1)	
Mean annual temperature (°C)	6-8
Mean maximum daily temperature (°C)	9.6
Mean minimum daily temperature (°C)	4.2
Mean annual rainfall (mm)	1773.4
Mean annual sun hours	1186 (2)
Mean number of rainfall days (>1mm)	202 (2)
Soil Characteristics	
Bedrock	Green schist/mica schist
Soil depth (cm)	13
Mean Olsen P (g m^{-2} for 10 cm depth)	0.020
Mean Total P (g m^{-2} for 10 cm depth)	19.71

2.1.2 Wales (Chapter 5)

The experimental site is located in Trefor (Gwynedd, Wales), UK. The site was chosen due to its relatively low background N deposition (ca. 9 kg N ha^{-1} yr^{-1}, see table 2.2), and is classified as a species-rich *Nardus* grassland (Violion caninae alliance (Schwickerath, 1944)). It consists of a dense sward, with vegetation dominated by grasses such as *Agrostis capillaris*, *Agrostis vinealis*, and *Festuca ovina*. The dominant bryophytes are *Rhytidiadelphus squarrosus* and *Pseudoscleropodium purum*, and some common forbs present include *Hieracium umbellatum*, *Hypochaeris radicata*, *Potentilla erecta*, and *Rumex acetosa*.

At the start of the experiment in 2007, average species richness was 16.6 species per 2 × 2 m plot for vascular plants and 3.0 for bryophytes (Dorland et al., 2013). The site sits on a slight slope at the top of sea cliffs, and has a soil pH in the range 5.0-5.5. The grassland is a former heathland, and has been managed for extensive grazing of sheep for many years. See table 2.2 for a summary of site and meteorological characteristics.

Table 2.2: Summary of site and meteorological characteristics. Table adapted from Dorland *et al.* (2013). The meteorological data are averages over the period 1971-2000. (1) Calculated using CBED (RoTAP, 2012), (2) Average values for weather station 'Valley' (http://www.metoffice.gov.uk).

Site Characteristics	
Coordinates	52° 59' 55.2" N 4° 25' 57.5" W
Altitude (m asl)	40
Average background N-deposition (kg^{-1} ha^{-1} yr^{-1}) (1)	9
Climate Characteristics (2)	
Mean annual temperature (^{o}C)	9.5-10.5
Mean maximum daily temperature (^{o}C)	13.1
Mean minimum daily temperature (^{o}C)	7.5
Mean annual rainfall (mm)	827.9
Mean annual sun hours	1621.4
Mean number of rainfall days (>1mm)	140.9
Soil Characteristics	
Soil depth (cm)	15 - 65

2.2 Experimental design and sampling procedures

2.2.1 Norway (Chapter 3)

The experiment at Revna, Norway was set up in 2007. 2 x 2 m plots were set up in a randomised block design in five replicate blocks. Blocks were set up at least 2 m apart with 1 m between plots. Plots were fenced to exclude grazing by red deer. N treatments consisted of 0 kg N ha^{-1} yr^{-1} (control, N0-C0), 35 kg N ha^{-1} yr^{-1} as NH_4NO_3 (N1-C0), and 70 kg N ha^{-1} yr^{-1} as NH_4NO_3 (N2-C0), $NaNO_3$ (N2-OX) and NH_4Cl (N2-RED). N was applied in solution using watering cans five times per year during the growing season (May-September). N addition was ceased at the end of 2014. All plots received an annual biomass cut in July, and all cut vegetation was removed.

Aboveground vegetation and soil sampling in Revna, Norway were carried out in July 2013. For each plot, all vascular plants and bryophytes were identified to species level. Biomass samples were collected from 50 x 50 cm subplots (cut to 3 cm above soil level) and separated into grasses, forbs, bryophytes and litter. A soil core (15 cm depth, 5.2 cm diameter) was taken from each plot. Subsamples were taken from 0-5, 5-10 and 10-15 cm depth, and the remaining 0-10 cm depth soil was sieved to 2 mm. These samples were used

to determine pH, total C and N content, C and N content of different soil fractions, soil microbial biomass C and N, fungal and bacterial biomass, and C and N content at different depths. In July 2015 additional soil (0-10 cm depth) was collected from each plot in Revna, Norway using an auger (3 cm diameter). This soil was sieved (2 mm), and analysed for dissolved organic C (DOC), dissolved organic N (DON), plant available N and N mineralisation rate.

2.2.2 Microcosm (Chapter 4)

Intact soil cores (10 cm height, 3.2 cm diameter) were taken from the field site in Revna, Norway in July 2014. Four cores were taken from each experimental plot, making four sets of twenty-five cores. No vegetation was included. Soil water content was adjusted by placing the cores on a sand table at -50 cm suction with cling film on top to stop water loss via evaporation. Cores were weighed daily in order to determine the water equilibration point, which was reached after 2.5 weeks.

One set of cores was spiked with P by adding NaH_2PO_4 dissolved in deionised water. N1-C0 cores received 0.95 g NaH_2PO_4, and N2-C0, N2-OX and N2-RED cores received 1.90 g NaH_2PO_4. Control cores in this set were not spiked with P. Instead, 0.92 g NaCl was added in order to account for the Na in the NaH_2PO_4 added to the other cores. The amount of P added to each core was based on the work of Harpole and Suding (2011), and was intended to remove any P limitation.

Each core was placed into a 1 L Kilner jar fitted with a septa. Three sets of jars were then placed at either 10, 16 or 22^{o}C with the lids slightly open to allow for some air flow. The set spiked with P was placed at 16^{o}C. All cores were incubated in the dark. The cores were left to equilibrate to their incubation temperatures for four days before gas sampling was initiated. Two gas samples were collected from each jar on the 5th, 7th and 15th day of incubation. The first sample (T_0) was taken as soon as the jar lids were shut, and the second sample (T_1) was taken 6 hours later. Each time, 10 ml of gas was taken using a syringe to pierce the septa. Gas samples were analysed using a gas chromatograph (PerkinElmer instruments, AutoSystem XL; FID and ECD at 350°C) in order to determine CO_2 and N_2O concentrations.

2.2.3 Wales (Chapter 5)

The experiment at Trefor, Wales was set up in 2007. 2 x 2 m plots were set up in a randomised block design in five replicate blocks. Blocks were set up at least 2 m apart with 1 m between plots. Plots were fenced to exclude grazing by sheep. N treatments consisted of 0 kg N ha^{-1} yr^{-1} (control, N0-C0), 4.3 kg N ha^{-1} yr^{-1} as NH_4NO_3 (N1-C0), 8.6 kg N ha^{-1} yr^{-1} as NH_4NO_3 (N2-C0), and 4.8 kg N ha^{-1} yr^{-1} as NH_4Cl (N2-RED)[1]. N was applied in solution using watering cans four times per year during the growing season (April-August). All plots received an annual biomass cut in July, and all cut vegetation was removed.

Aboveground vegetation sampling in Trefor, Wales was carried out in July 2013. For each plot, all vascular plants and bryophytes were identified to species level. Biomass samples were collected from 50 x 50 cm subplots (cut to 3 cm above soil level). Soil sampling in Trefor, Wales was conducted on two separate dates. In May 2013 a soil core (15 cm depth, 5.2 cm diameter) was taken from each plot. Subsamples were taken from 0-5, 5-10 and 10-15 cm depth, and the remaining 0-10 cm depth soil was sieved to 2 mm. These samples were used to determine pH, total C and N content, fungal and bacterial biomass, and C and N content at different depths. In September 2014 additional soil (0-10 cm depth) was collected from each plot using an auger (3.4 cm diameter) and sieved to 2 mm. These samples were used to determine the C and N content of different soil fractions, soil microbial biomass C and N, dissolved organic C (DOC), dissolved organic N (DON), and plant available N. CO_2 flux measurements were taken at Trefor, Wales on four different days in 2013 (in May, June, September and October) using infrared gas analysers (IRGAs).

2.2.4 Mesocosm (Chapter 6)

The experiment consisted of nine monoculture treatments (M1-9, one for each species used) and six different species mixture treatments (S1-6), as well as a bare soil control treatment (B). Each of these treatments received two levels of N addition: 0 and 35 kg ha^{-1} yr^{-1}. This level of N addition was chosen because it represents the top end of current atmospheric N deposition in the UK (RoTAP, 2012). Every treatment was replicated three times.

[1] Originally, N1-C0 was supposed to receive 35 kg N ha^{-1} yr^{-1}, and N2-C0 and N2-RED were supposed to receive 70 kg N ha^{-1} yr^{-1}, but a miscalculation at the beginning of the experiment meant that the N doses applied were actually much lower.

The criteria for plant species selection was a) that the species must belong to the species-rich *Nardus* grassland community (Violion caninae alliance (Schwickerath, 1944)) and b) that the species must be identified in literature as being positively associated with either high or low levels of N deposition. One grass, two forb and one legume species were selected for both the high and low N communities. The high N community species consisted of *Festuca ovina, Leontodon hispidus, Achillea millefolium* and *Trifolium repens* (De Deyn et al., 2009; Grime and Hunt, 1975; Rodwell, 1997; Stevens et al., 2011a). The low N community species were *Anthoxanthum odoratum, Plantago lanceolata, Campanula rotundifolia* and *Lotus corniculatus* (De Deyn et al., 2009; Grime and Hunt, 1975; Rodwell, 1997; Stevens et al., 2011a; Stevens et al., 2004). It was also decided that the grass species *Agrostis capillaris* would be present in all species mixtures due to its central role in defining the acid grassland community being studied.

Table 2.3 describes the design for each species mixture treatment, and table 2.4 shows how many plants of each species were used for each treatment. Treatments differed in their N community prevalence (dominated by either high or low N-associated species), in the number of species present (either 5 or 9 species), and in their functional group evenness (either dominated by grass species or not). The treatments designed to be dominated by grasses were planted with twice as many grass as non-grass individuals (see table 2.4).

Table 2.3: Design for each species mixture treatment.

	HIGH species number	LOW species number	
HIGH N community prevalence		S1 – High N species, grasses not dominant	HIGH functional group evenness
	S3 – Mixture, grasses dominant	S2 – High N species, grasses dominant	LOW functional group evenness
LOW N community prevalence	S6 – Mixture, grasses not dominant	S4 – Low N species, grasses not dominant	HIGH functional group evenness
		S5 – Low N species, grasses dominant	LOW functional group evenness

Table 2.4: Number of plants of each species used for each species mixture treatment.

	Treatment S1	Treatment S2	Treatment S3	Treatment S4	Treatment S5	Treatment S6
A. capillaris	9	12	11	9	12	6
F. ovina	8	12	11	-	-	5
L. hispidus	6	4	3	-	-	2
A. millefolium	7	4	3	-	-	2
T. repens	6	4	2	-	-	2
A. odoratum	-	-	2	8	12	6
P. lanceolata	-	-	2	7	4	5
C. rotundifolia	-	-	1	6	4	4
L. corniculatus	-	-	1	6	4	4
SUM	36	36	36	36	36	36
Grasses:non-grasses	17:19	24:12	24:12	17:19	24:12	17:19

Seeds were sown in John Innes no. 2 compost in August 2013, transplanted into plug trays in September and October 2013 and planted into 36 x 36 x 38 cm (LxWxH) planter pots in November 2013. The pots were filled with a soil made from two parts soil (pH = 6.93; NH_4^+ = 0.22 ppm; NO_3^- = 3.35 ppm), one part peat (pH = 3.83). Each pot also contained a bottom layer of gravel (around 8 cm deep) in order to maintain a good level of drainage. The pots were kept inside unheated solar domes until April 2014, when they were moved to an open-air enclosure at the Lancaster University Field Station (54°00'51.2"N 2°46'27.0"W, N deposition = 19.32 kg N ha^{-1} yr^{-1} (2012-2014 data, APIS)). The pots were arranged into three replicate blocks using a randomised layout. N was applied as NH_4NO_3 dissolved in 0.5 L deionised water every two weeks from April to July 2014 and from March to June 2015 (eight applications per year). Mesocosms were weeded throughout the experiment in order to maintain the original species mix. In June and September 2014, aboveground vegetation was cut 5 cm above soil level and removed in order to simulate the typical management of grasslands and meadows in the UK, which tend to be harvested in the summer and grazed in the autumn.

CO_2 flux measurements of the mesocosm pots were conducted in May 2015 using infrared gas analysers (IRGAs). Aboveground vegetation sampling was conducted in July 2015. Plants were cut at soil level and were separated by species. Roots were sampled in July 2015 by taking three cores (5.3 cm D, 10 cm H) from each pot. Soil sampling was conducted in July 2015. Three cores (5.3 cm D, 10 cm H) were taken from each pot and sieved to 2 mm.

2.3 Vegetation analysis methods

2.3.1 Aboveground biomass

Aboveground biomass was determined by weighing plant matter after drying at either 70^{o}C for 48 hours (Chapter 3), or 55^{o}C for 72 hours (Chapters 5 and 6).

2.3.2 Litter bags

Litter bags (5x5 cm, 1 mm nylon mesh, filled with ~1 g pre-weighed vegetation taken from the Trefor, Wales 2013 control plots' biomass harvest) were installed in Trefor, Wales on the 19th of May 2014. The bags were secured onto the soil surface of each plot, and then left until they were collected on the 10th of September 2014. Root intrusions were carefully removed with tweezers, and remaining litter biomass was determined after drying at 60^{o}C for 48 hours.

2.3.3 Root ingrowth cores

Root ingrowth cores were installed in Trefor, Wales on the 19th of May 2014. Soil for the ingrowth cores was collected with a 15 cm high, 5 cm diameter core. The soil was then sieved (4 mm) in-situ and carefully re-packed into a custom-made ingrowth core (15 cm length, 5 cm diameter, 1 mm nylon mesh). This mesh core was then placed back into the hole from which the soil was removed in order to allow roots to grow back into the core. Cores were carefully collected on the 10th of September 2014, using a sharp knife to cut around the core in the ground in order to sever the roots that had grown into the core before pulling it out.

2.3.4 Root biomass

Roots were carefully washed with warm tap water using either a 200µm sieve (root ingrowth cores, Chapter 5), or a 710µm sieve (root samples, Chapter 6) in order to prevent root loss during washing. Root biomass was determined by weighing washed root matter after drying at either 60^{o}C for 48 hours (Chapter 5), or 55^{o}C for 48 hours (Chapter 6).

2.3.5 Vegetation C and N

Aboveground vegetation C and N concentrations in Chapter 3 were determined by placing 4 mg dried, homogenised plant material into tin cups and then combusting in a CNS elemental analyser (Model EA NA1500; Carlo Erba Instruments, Milan, Italy). Aboveground vegetation, litter and root C and N concentrations in Chapters 5 and 6 were determined by placing 15 mg dried, homogenised plant material into tin cups and then combusting in a CN analyser (elementar, vario EL III).

2.3.6 Vegetation P

Vegetation P concentrations were determined by digesting 200 mg dried, homogenised samples in sealed Teflon vessels in an Ethos D microwave lab station Terminal 20 (Milestone Pharmatech, New Brunswick, NJ, USA) with 4 ml nitric acid (65% v/v) and 1 ml hydrogen peroxide (30% v/v), and then analysing the digests using Inductively Coupled Plasma emission spectrophotometry (ICP, Iris Intrepid II; Thermo Fisher Scientific, Waltham, MA, USA).

2.4 Soil analysis methods

2.4.1 Bulk density

Bulk density was determined using metal cores 6 cm deep and 5.8 cm in diameter. The soil collected was dried at 105^{o}C for 24 hours and weighed. To calculate bulk density, the dry mass of the soil collected in the core was divided by the volume of the core. If any large (>0.2 cm) stones were found in the bulk density samples, they were removed, and their volume (calculated via water displacement) was subtracted from the volume of the core.

2.4.2 Soil pH

Soil pH was determined by placing 10 g sieved fresh soil (0-10 cm depth) in 25 ml of deionised water, mixing thoroughly on an orbital shaker for 30 minutes, leaving for 12 hours and then measuring at the soil-water interface using a Mettler Toledo, Seven Compact pH meter (Allen, 1989).

2.4.3 Soil C and N

Total soil C and N content (0-10 cm depth), and soil C and N content at different depths (0-5, 5-10 and 10-15 cm) were determined by placing 30 mg dried (55^{o}C for 48 hours), ground soil into tin cups and then combusting using a CN analyser (elementar, vario EL III).

2.4.4 Soil fractionations

Physical soil fractionations were conducted using either air-dried (Chapter 3), or fresh (Chapters 5 and 6) soil (0-10 cm depth) according to the method of De Deyn *et al*. (2011b). The soil was separated into three size fractions: coarse, 2mm-200 µm; fine, 200-50 µm; and very fine, 50-0.45 µm. 1 L of deionised water was poured into a large bowl with a 200 µm sieve inside. 10 g dry weight-equivalent of soil was placed onto the sieve and gently broken up using a spatula. Once the soil had been sufficiently broken up, what was left on the sieve was transferred into a beaker. The total volume of deionised water in the beaker was made up to 40 ml and the beaker was then sonicated for 10 minutes using a sonic bath. In the meantime, the content of the bowl was poured into a large beaker, making sure to rinse all soil particles in the bowl into the beaker. A 50 µm sieve was then placed into the bowl and the contents of the large beaker was poured over it, again making sure all soil particles were transferred. The 200 µm sieve was then placed on top of the 50 µm sieve and the contents of the sonicated beaker was poured over both sieves. The soil left on both sieves was then rinsed into two separate pre-weighed beakers and dried at 40^{o}C for 48 hours. The remaining content of the bowl was then poured into a 2 L volumetric flask and deionised water was used to make up the volume of water to 2 L. This liquid was then thoroughly mixed and a 150 ml subsample was filtered through a pre-weighed 0.45 µm cellulose filter over a Buchner funnel. The filter (along with the soil collected on it) was then dried at 40^{o}C for 48 hours and the filtrate was analysed for dissolved organic C (DOC) content on a total organic carbon

analyser (Shimadzu, TOC-5000A). The three dried solid fractions were then ground up using a ball mill and analysed for C and N content by placing 30 mg soil into tin cups and then combusting using a CN analyser (elementar, vario EL III). Unused cellulose filters were also ground and analysed for C and N content in the same way in order to calculate the C and N content of the 50-0.45 µm fraction (as this was ground up along with the filter).

2.4.5 DOC and DON

Dissolved organic C and N (DOC and DON) were measured using fresh soil (0-10 cm depth). 35 ml of deionised water was added to 5 g of soil, mixed on an orbital shaker for 10 minutes, and then filtered through a Whatman No. 1 filter. DOC was measured by running some of the filtrate on a total organic carbon analyser (Shimadzu, TOC-5000A). 3 ml of 0.165M potassium persulphate was then added to 1 ml of the filtrate in a McCartney bottle, and autoclaved at 127^{o}C for 20 minutes. The original filtrate and the digested filtrate were then analysed for NH_4^+ and NO_3^- content using an auto analyser (SEAL Analytical, AA3). In order to determine DON, the total N content of the original filtrate was subtracted from the total N content of the digested filtrate.

2.4.6 Plant available N and Mineralisation rate

Plant available N and potential actual N mineralisation rate were determined using fresh soil (0-10 cm depth). 25 ml 1M KCl was added to 5 g of soil, mixed on an orbital shaker for 1 hour, and filtered through a Whatman No. 1 filter. Plant available N was determined by analysing the filtrate for NH_4^+ and NO_3^- content using an auto analyser (SEAL Analytical, AA3). Another 5 g of soil was weighed into a plastic bottle that was covered with a piece of polythene secured with a rubber band, and incubated at 24(+/-1)^{o}C for 14 days. This incubated soil was then extracted with KCl and analysed for NH_4^+ and NO_3^- content as described above. N mineralisation rate was calculated by subtracting the plant available N from the N content of the incubated soil.

2.4.7 Olsen P

Olsen P was determined using air-dried soil (0-10 cm depth) according to the method of Olsen *et al.* (1954). 100 ml of 0.5M $NaHCO_3$ was added to 5 g of soil, mixed on an orbital shaker for 30 minutes, and filtered through a Whatman No. 42 filter. The filtrate was then analysed for PO_4^{3-} content using an auto analyser (SEAL Analytical, AA3).

2.4.8 Total soil P

Total P was measured using the method of Allen (1989). 0.20 g of ground, air-dried soil (0-10 cm depth) was weighed into a digest tube. A pinch of anti-bumping granules was added before adding 4.4 ml of a pre-mixed digest reagent (0.42 g selenium, 14 g lithium sulphate, 350 ml hydrogen peroxide, 420 ml concentrated sulphuric acid). The digest tube was then slowly heated to 350^{o}C in a heating block and left for 4 hours until the digest had cleared. Once cooled, 1 ml of the digest was diluted with 5 ml of deionised water and filtered using a Whatman No. 44 filter. The filtrate was then analysed for PO_4^{3-} content using an auto analyser (SEAL Analytical, AA3).

2.4.9 Soil microbial biomass C and N

Soil microbial biomass C and N were measured using fresh soil (0-10 cm depth), via the chloroform fumigation method described by Vance *et al.* (1987). 5 g of soil from each sample was weighed into beakers and placed inside a vacuum desiccator lined with slightly damp paper towels. A beaker containing amylene stabilised chloroform and a few boiling chips was placed in the desiccator, which was then evacuated until the chloroform started to boil. The samples were left in the evacuated desiccator at room temperature for 18-24 hours. The desiccator was then opened in order to remove the paper towels and the chloroform beaker before the desiccator was re-evacuated and vented six times in order to remove any chloroform from the soil samples. These fumigated soil samples were then transferred into plastic bottles. Another 5 g of the original un-fumigated soil samples were weighed into plastic bottles. 25 ml of 0.5M K_2SO_4 was added to each soil sample (both fumigated and un-fumigated), mixed for 30 minutes on an orbital shaker, and filtered through a Whatman No. 1 filter. In order to determine the total dissolved C content of each sample, some of the filtrate was analysed using a total organic carbon analyser (Shimadzu, TOC-5000A). In order

to determine the total dissolved N content of each sample, 3 ml of 0.165M potassium persulphate was added to 1 ml of the filtrate in a McCartney bottle, autoclaved at 127^{o}C for 20 minutes, and analysed for NH_4^+ and NO_3^- content using an auto analyser (Bran Luebbe, AutoAnalyzer3, Chapter 3; SEAL Analytical, AA3, Chapters 5 and 6). Microbial biomass C and N were calculated by subtracting the C and N contents of the un-fumigated soil from the C and N contents of the fumigated soil.

2.4.10 PLFAs

Fungal and bacterial biomass were determined using phospholipid fatty acid (PLFA) analysis, using the Bligh and Dyer method (1959) adapted by White *et al.* (1979) and described by Bardgett *et al.* (1996). This method consists of three stages.

Stage 1 – Extraction:
1.5 g of ground, freeze-dried soil (0-10 cm depth) was weighed into a plastic, $CHCl_3$ -rinsed 50 ml tube. 1.5 ml of citrate buffer (15.76 g of citric acid in 450 ml MilliQ water, adjusted to pH4 using NaOH pellets, and made up to 500 ml), 1.9 ml $CHCl_3$, 3.8 ml MeOH and 2 ml Bligh and Dye ($CHCl_3$:MeOH:citrate buffer, 1:2:0.8 v/v/v) were added to the soil (in this order). The tubes were capped and vortexed for one minute, left to separate for 2 hours, vortexed again for one minute, then centrifuged at 650 RCF for 10 minutes. The supernatant was then transferred to a glass, $CHCl_3$ -rinsed 40 ml tube using a glass Pasteur pipette. The soil pellet was washed again using 2.5 ml of Bligh and Dye, vortexed for one minute, centrifuged at 650 RCF for 10 minutes, and the supernatant was transferred again as above. 3.1 ml of $CHCl_3$ and 3.1 ml of citrate buffer were added to the glass tube, vortexed for one minute and left overnight to separate. The following day, 3 ml of the lower phase was transferred into a 15 ml $CHCl_3$ -rinsed glass tube using a glass Pasteur pipette, and evaporated under a stream of N_2 in a sample concentrator.

Stage 2 – Lipid fractionation:
An isolute column was placed in a holder above a metal tray and activated with 2.5 ml of $CHCl_3$. The dry lipid material from stage 1 was then dissolved in 0.5 ml $CHCl_3$, vortexed for 5 seconds, and then transferred immediately to the column using a glass Pasteur pipette. This step was then repeated three times. Once all the lipid material had been transferred, 5 ml of $CHCl_3$ was added to the column to elute the neutral lipids. Then, 20 ml of acetone was added

to the column in 5 ml portions in order to elute the glycolipids. Once all the acetone had run to waste, a MeOH-rinsed 15 ml glass tube was placed under the column. 5 ml of MeOH was then added to the column in order to elute the phospholipids into the tube. Once the column had stopped dripping, the solvent in the tubes was evaporated under a stream of N_2 in a sample concentrator with the heating block on at 40^{o}C.

Stage 3 – Mild alkaline methanolysis

The dried sample from stage 2 was dissolved in 1 ml MeOH:toluene (1:1 v/v), and 1 ml 0.2M KOH (dissolved in MeOH) was added before the tube was capped and incubated in a water bath at 37^{o}C for 15 minutes. Then 2 ml hexane:$CHCl_3$ (4:1 v/v), 0.3 ml 1M acetic acid and 2 ml milliQ water were added, and the tube was vortexed for 1 minute and centrifuged at 650 RCF for 5 minutes. The upper (organic) phase was then transferred to a hexane-rinsed 15 ml glass tube using a glass Pasteur pipette. The lower layer was then washed with 2 ml of hexane:$CHCl_3$ (4:1 v/v), vortexed, and centrifuged as before, and again the upper phase was transferred to the 15 ml glass tube. Finally, 30 µl of a C13 standard (5 mg in 10 ml hexane) and 30 µl of a C19 standard (5 mg in 10 ml hexane) were carefully added to the tube and the now spiked phase was evaporated under a stream of compressed N_2 in a sample concentrator. 150 µl of hexane was then added to the tube, vortexed for 1 minute, transferred to a GC vial insert using a glass Pasteur pipette, and evaporated under a stream of N_2 in a sample concentrator. This was repeated five times in order to ensure complete sample transfer to the GC insert. The sample was then re-suspended in 25 µl of hexane for immediate analysis on a gas chromatograph (Agilent Technologies, 6890N).

The fatty acids 18:2ω6,9 and 18:1ω9 were chosen to represent fungal fatty acids, and i15:0, a15:0, 15:0, i16:0, 16:1ω7, i17:0, a17:0, 7,cy-17:0, 18:1ω7 and 7,8cy-19:0 to represent bacterial fatty acids (De Deyn et al., 2011a). Total PLFA was used as a measure of active microbial biomass (Bardgett and McAlister, 1999).

2.5 CO_2 flux analysis methods

2.5.1 Chapter 5

Net CO_2 flux and respiration rate measurements were made with portable infrared gas analysers (IRGAs; 2 minute closure time; EGM-4, PP Systems) using a light and dark chamber method (Ward et al., 2013). The chambers were made from plastic bell cloches secured to PVC rings. The dark chambers were covered with a dark plastic in order to block out all light. These custom-built light and dark chambers (30 cm diameter, 35 cm high, volume of 19,000 cm^3) included 5L tedlar sample bags to maintain a stable pressure inside the chamber throughout sampling. Base rings (10 cm high, 30 cm diameter) were installed at 5 cm depth in each plot in April 2013 so that the chambers could be placed over them during sampling (secured and made air-tight using rubber rings). The IRGAs were coupled with a PAR sensor (ACS009, PP Systems) for use with the light chamber, and with a soil temperature probe (STP-1, PP Systems) for use with the dark chamber. Air temperature was recorded (Tinytag, View 2) and three soil moisture readings were also taken from the area around the chamber in each plot using a moisture probe (Delta-T Devices, HH2).

Before statistical analysis, the IRGA data was checked for outliers and corrected for collar area, enclosure volume and air temperature. Net primary productivity (NPP) was measured as the net CO_2 flux obtained with the transparent chamber, and gross respiration as the flux when the chamber was darkened to exclude light.

2.5.2 Chapter 6

Net CO_2 flux and respiration rate measurements were made with portable infrared gas analysers (IRGAs; 2 minute closure time; EGM-4, PP Systems) using a light and dark chamber method (Orwin et al., 2014). The chambers were made with Liteglaze acrylic sheet (92% light transmission) fitted to a polypropylene base. The dark chambers were covered with a dark plastic in order to block out all light. These custom-built light and dark chambers (36x36 cm base, 35 cm high, volume of 46,800 cm^3) were designed to attach to the rims of the plant pots using clamps, and included 5L tedlar sample bags to maintain a stable pressure inside the chamber throughout sampling. The IRGAs were coupled with a PAR sensor (ACS009, PP Systems) for use with the light chamber, and soil temperature measurements were taken

with a probe (Tinytag, View 2) for use with the dark chamber. Air temperature was recorded at the site's weather station (Hazelrigg weather station) and three soil moisture readings were also taken from each pot using a moisture probe (Delta-T Devices, HH2).

Before statistical analysis, the IRGA data was checked for outliers and corrected for pot area, enclosure volume and air temperature. Net primary productivity (NPP) was measured as the net CO_2 flux obtained with the transparent chamber, and gross respiration as the flux when the chamber was darkened to exclude light.

2.6 Data analysis methods

2.6.1 Chapter 3

Data were analysed using R version 3.2.4 (R Core Team, 2016). All variables were tested for normality, and log or logit transformations were applied, as required, prior to analysis. One-way ANOVAs (using block as a random effect) with Tukey post-hoc tests were used to determine whether there were differences between N treatments (both between N addition treatments and the control plots and between N addition treatments) for each variable.

2.6.2 Chapter 4

CO_2 and N_2O fluxes were calculated by subtracting concentrations at T_0 from those at T_1. Q_{10} values were calculated using the following first-order exponential equation:

$Q_{10} = (C_2/C_1)^{10/(T_2-T_1)}$

Where T_1 and T_2 denote the temperatures (10°C and 22°C) at which the incubations were conducted, and C_1 and C_2 represent the CO_2 fluxes at those temperatures.

Data were analysed using R version 3.2.4 (R Core Team, 2016). All variables were tested for normality, and log transformations were applied, as required, prior to analysis. Two-way ANOVAs (using N treatment and temperature/P addition as fixed effects, and block and incubation day as random effects) with Tukey post-hoc tests were used to determine whether there were differences between N treatments (both between N addition

treatments and the control plots and between N addition treatments) for gas flux data. One-way ANOVAs (using N treatment as a fixed effect and block and incubation day as random effects) were used to determine whether there were differences between N treatments for Q_{10} data.

2.6.3 Chapter 5

Data were analysed using R version 3.2.4 (R Core Team, 2016). All variables were tested for normality, and log or logit transformations were applied, as required, prior to analysis. One-way ANOVAs (using block as a random effect) were used to determine whether there were differences between N treatments for each variable. IRGA data was analysed using mixed model ANOVAs with the 'lme4' package in R.

2.6.4 Chapter 6

Data were analysed using R version 3.2.4 (R Core Team, 2016). All variables were tested for normality, and log transformations were applied, as required, prior to analysis. Data were analysed using mixed model ANOVAs with the 'nlme' package in R (using block as a random effect). Tukey post-hoc testing was conducted on significant monoculture data using the 'lsmeans' package in R.

For the monoculture treatments, the independent variables used were 'plant species' (species identity) and 'N addition' (0 or 35 kg N ha^{-1} yr^{-1}). Degrees of freedom were 31, except for the fungal:bacterial and the Gram positive:Gram negative bacterial ratios, which had 26 degrees of freedom (due to missing data) and NPP and respiration, which had 33 and 32 degrees of freedom (due to extra explanatory variables). For the species mixture treatments, the independent variables used were 'community prevalence' (dominated by species associated with either high or low N deposition), 'species number' (either 5 or 9 species), 'evenness' (either dominated by grasses or not), and 'N addition' (0 or 35 kg N ha^{-1} yr^{-1}). Degrees of freedom were 29, except for the fungal:bacterial and the Gram positive:Gram negative bacterial ratios, which had 27 degrees of freedom (due to missing data) and NPP and respiration, which had 28 and 27 degrees of freedom (due to extra explanatory variables).

Chapter 3: Investigating the effects of atmospheric nitrogen deposition on carbon and nitrogen pools in a Norwegian acidic grassland

Investigating the effects of atmospheric nitrogen deposition on carbon and nitrogen pools in a Norwegian acidic grassland

3.1 Abstract

Since the 1950s, global emissions of reactive forms of nitrogen have become so large that atmospheric nitrogen pollution is now a major issue facing ecosystems worldwide. Grasslands are one such system threatened by nitrogen deposition. Nitrogen enrichment is known to affect various plant, microbial and soil processes (and their interactions) in grasslands, which can have consequences for carbon and nitrogen cycling. Although these effects are well researched, studies often report contrasting results. This is a problem especially regarding the effects of nitrogen inputs on long-term carbon storage. It is not clear for example, how nitrogen pollution may affect grasslands' ability to sequester and store carbon, and therefore mitigate climate change. In addition, there is evidence to suggest that the chemical form of nitrogen may affect how grasslands respond to N enrichment. This is especially important as experts predict that reduced nitrogen forms are likely to dominate future nitrogen pollution. Current research on possible differences between the effects of oxidised and reduced nitrogen forms is limited. Using a seven-year nitrogen addition experiment in Norway, this study examines the effects of different forms of nitrogen on acid grassland carbon and nitrogen pools.

Our results show that although nitrogen addition did not significantly alter the amount of aboveground biomass produced at this site, it did decrease the C:N ratio of the vegetation. Decreased soil fungal:bacterial ratio in the NH_4Cl plots compared with control plots indicates that reduced nitrogen addition changed the microbial community in these plots by favouring bacteria over fungi. Moreover, a trend for lower total PLFA biomass suggests that nitrogen addition may have reduced the active microbial biomass at this site. Although 70 kg ha^{-1} yr^{-1} of nitrogen addition as NH_4Cl and NH_4NO_3 decreased soil pH, no changes were observed in overall soil carbon and nitrogen pools for any treatment. However, there was a decrease in DOC in all the 70 kg N ha^{-1} yr^{-1} treatments and a decrease in very fine fraction carbon in the 70 kg ha^{-1} yr^{-1} NH_4NO_3 treatment, suggesting that changes to soil carbon pools may have begun occurring. Increased vegetation N:P ratios indicate that this system may be phosphorous limited, and so perhaps this could be limiting the effects of nitrogen addition. Overall, results suggest that this acid grassland is more resilient to nitrogen enrichment than

previously thought. However, it must be noted that this experiment was only run for seven/eight years, which may not be enough time for long-term effects of nitrogen addition to be observed.

3.2 Introduction

It is estimated that human activities now generate more reactive N (N_r) than is produced naturally in the entire world (Erisman et al., 2011; Galloway and Cowling, 2002; Vitousek et al., 1997). The main sources of atmospheric N_r pollution include the combustion of fossil fuel, the dominant source of oxidised forms of N, and agricultural activities such as the use of artificial N fertilisers and animal husbandry, the dominant sources of reduced forms of N. Atmospheric N deposition has become a major problem for some semi-natural ecosystems. In these systems N can limit plant growth, so large inputs of N_r deposition can increase productivity (Erisman et al., 2011; Gough et al., 2000; Morecroft et al., 1994; Shaver et al., 2001). N addition also tends to lower plant tissue C:N ratios, which leads to the production of higher quality (i.e. more readily decomposable) litter (Manning et al., 2008). Increasing the amount of N_r available to plants in grasslands is also known to alter the species composition of grassland communities (Bobbink et al., 2010). N addition promotes dominance by fast-growing species such as grasses (Stevens et al., 2009), as these species are better able to profit from the extra N availability. This can lead to lower species richness and plant community change (Bobbink, 1991; Bobbink et al., 1998; Stevens et al., 2004; Stevens et al., 2010). Consequently, competition under high N levels switches from belowground competition for nutrient acquisition to aboveground competition for light (Hautier et al., 2009; Stevens et al., 2011a). Increased N availability and plant community changes also have an impact on soil microorganisms. Higher N levels are known to favour bacteria over fungi (Kirchmann et al., 2013), and dominance by fast-growing plant species may encourage changes to the soil microbial community via plant-specific differences in the release of root exudates and alterations in nutrient competition between plants and microorganisms (Bardgett et al., 1999).

N deposition can also alter soil chemistry. N_r is known to increase available forms of N and lower soil pH (Houdijk et al., 1993). Moreover, depending on the form of N most prominent in the deposition at a site, N addition can change the NH_4^+:NO_3^- ratio in the soil. Studies have shown that a change in this ratio can affect the health and performance of some plants, and

that high NH_4^+:NO_3^- ratios (NH_4 accumulation) can be detrimental due to an effect on cation uptake (Bobbink et al., 1998; De Graaf et al., 2009; Houdijk et al., 1993; van den Berg et al., 2005). Plant species are also known to display preferential uptake of NH_4^+ or NO_3^- (van den Berg et al., 2016). Despite strong reasons to expect that reduced and oxidised N forms may differ in their impacts (Stevens et al., 2011b), research into the differences between the effects of reduced and oxidised N is still relatively limited. Nonetheless, differential effects of N form are set to become an important issue, as projections for future N deposition predict an increase in the proportion of reduced N pollution (Dennis et al., 2010). This is due to changes in land use, technology, and arguably most importantly, policy. Although relatively successful policies have been put into place in order to restrict the amount of N_r being released into the environment, most of these are designed to curb emissions from fossil fuel combustion (oxidised N). Reduced N pollution largely comes from agricultural sources, and it is this area of industry that has been the least affected by environmental N policies (Sutton, 2011).

Grasslands are important carbon (C) sinks (Tian et al., 2016b). Temperate grasslands store an estimated 12.3% of global C, making them the third largest global store of C in soils and vegetation after wetlands and boreal forests (Royal Society, 2001). Consequently, grassland C storage plays a significant role in climate change mitigation, and it is vital that grasslands maintain their ability to sequester and store C. Although the impact of N deposition on grassland processes is well researched, it is less clear how these changes might affect the ability of grasslands to store C (Lu et al., 2011b). Studies examining the effects of N addition on C storage often report contrasting results. In some systems, N addition has been shown to significantly increase soil C (Hyvonen et al., 2008; Pregitzer et al., 2008), whereas in other systems, the opposite is observed (Khan et al., 2007; Mack et al., 2004). Estimates for C sequestration are highly variable (even within the same ecosystem type) and they typically depend on the geographical location of the site studied, as well as how long the system has been exposed to N. Erisman *et al.* (2011) compared different studies on the effects of N addition on C sequestration (aboveground biomass and soil organic matter), and found that most studies ranged between 35 and 65 kg C sequestered per kg N. In another study, a meta-analysis conducted by Lu *et al.* (2011b), it was found that although N addition did significantly increase plant C pools, it did not change C storage in forests and grasslands (in both organic horizon and mineral soil). Though a lot of research supports the idea of higher C sequestration due to N-induced increases in primary productivity (LeBauer and Treseder, 2008; Magnani et al., 2007; Zaehle et al., 2010), these increases may not lead to greater soil

C storage. Higher litter quality could potentially increase soil organic matter (SOM) decomposition rates (Gong et al., 2015), which could increase the rate of C turnover. N-induced changes to plant and microbial communities may also exacerbate these processes by further altering the amount and quality of the litter produced (Hossain et al., 2010). In addition, it has been suggested that the way in which N alters decomposition rates may differ for different SOM fractions, as these vary in the energy required for their breakdown (Neff et al., 2002).

This study aimed to evaluate the effects of N addition on the C and N pools of acid grasslands, and to determine whether this has any implications for long term C storage. In addition, this study aimed to ascertain whether any effects of N fertilisation on C and N pools differ with the application of varying forms of N. This was accomplished by studying a replicated seven-year N addition experiment in Revna, Norway, an area with low background N deposition.

It was hypothesised that:

1. The N content of both the plant biomass and the soil would increase with N addition, and that these increases would be highest for plots receiving the most N (70 kg ha^{-1} yr^{-1}).
2. N addition would increase C present as plant biomass, but that this would not cause an increase in soil C content due to a decrease in plant biomass C:N ratios and changes to soil microbial activity and community composition.
3. If soil C were to decrease, these changes would be most apparent in the treatments containing high levels of ammonium (N2-RED and N2-C0) due to decreases in soil pH and potential negative effects of high ammonium levels in soil.
4. N addition would increase the proportion of bacteria in the microbial community (lower fungal:bacterial ratio).
5. Species richness was projected to decrease with N addition, as grasses were expected to begin dominating the plots.

In order to test these hypotheses, various soil and vegetation samples were collected from the experimental site and analysed in depth in the laboratory.

3.3 Results

3.3.1 Soil pH

Results show that soil pH (Figure 3.1) was significantly different between treatments ($p<0.001$). pH was lower in the high combined N and reduced N treatments (henceforth referred to as N2-C0 and N2-RED respectively) than it was in the control and oxidised N treatments (N0-C0 and N2-OX respectively). In addition, N2-RED was also significantly lower than the low combined N treatment (N1-C0).

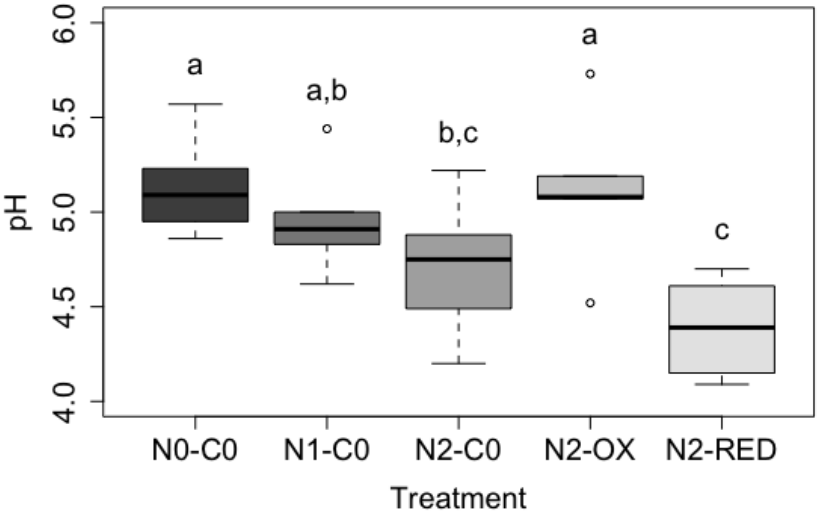

Figure 3.1: Soil pH for each treatment. Letters above boxplots represent significant differences ($p<0.05$) between treatments as measured using a Tukey post-hoc test.

3.3.2 N pools

Both aboveground and belowground N pools were examined in order to test the hypothesis that N addition would increase the amount of N present in vegetation and in the soil. Aboveground biomass N (total, grass, forb and bryophyte as g m^{-2}) was not found to be significantly different between treatments ($p=0.58$, 0.38, 0.77 and 0.53 respectively), although a non-significant trend for increased total biomass N can be observed. However, when total aboveground biomass N concentration was analysed (mg g^{-1}, Figure 3.2c), there were significant differences between treatments ($p<0.001$). N2-C0, N2-OX and N2-RED all had significantly higher biomass N (mg g^{-1}) than the control plots (N0-C0). In addition, biomass N (mg g^{-1}) in N2-RED was also significantly higher than in N1-C0. Microbial biomass N (mg m^{-2} for 10 cm depth) was not significantly different between treatments ($p=0.41$). Soil

total N content (kg m^{-2}) was also not significantly different between treatments. This was true for total N (0-10 cm depth, p=0.11, Figure 3.2d) and for total N at different depths (0-5 cm, p=0.68, 5-10 cm, p=0.41, 10-15 cm, p=0.25). No significant difference between treatments was observed for the N content of the coarse, fine or very fine soil fractions, both as kg m^{-2} (p=0.28, 0.88 and 0.64 respectively) and as proportions of total N stocks (p=0.09, 0.76 and 0.33 respectively). Soil dissolved organic N (DON, mg m^{-2} for 10 cm depth), available N (mg m^{-2} for 10 cm depth) and N mineralisation rate (mg m^{-2} $week^{-1}$ for 10 cm depth) were not significantly different between treatments (p=0.56, 0.92 and 0.69 respectively).

3.3.3 C pools

To test the hypothesis that N addition would increase vegetation C, but not soil C, both aboveground and belowground C pools were studied. Aboveground biomass C (total, p=0.32, grass, p=0.19, forb, p=0.26, and bryophyte, p=0.59, as g m^{-2}) and microbial biomass C (mg m^{-2} for 10 cm depth, p=0.82) were not significantly different between treatments. Soil C content (kg m^{-2}) was also not significantly different between treatments. This was true for total C (0-10 cm depth, p=0.08, Figure 3.2b) and for C at different depths (0-5 cm, p=0.49, 5-10 cm, p=0.49, 10-15 cm, p=0.27). Coarse (2mm-200 µm) and fine (200-50 µm) soil fraction C contents were not significantly different between treatments, both as kg m^{-2} (p=0.21 and 0.76 respectively) and as proportions of total C stocks (p=0.20 and 0.71 respectively). However, very fine (50-0.45 µm) soil C was significantly lower in the N2-C0 treatment than it was in N0-C0, both as kg m^{-2} ($p<0.05$) and as a proportion of the total C stock ($p<0.05$). Soil dissolved organic C (DOC, mg m^{-2} for 10 cm depth) was significantly different between treatments ($p<0.01$). DOC was lower in the N2-C0, N2-OX and N2-RED treatments than it was in N0-C0 (Figure 3.2a).

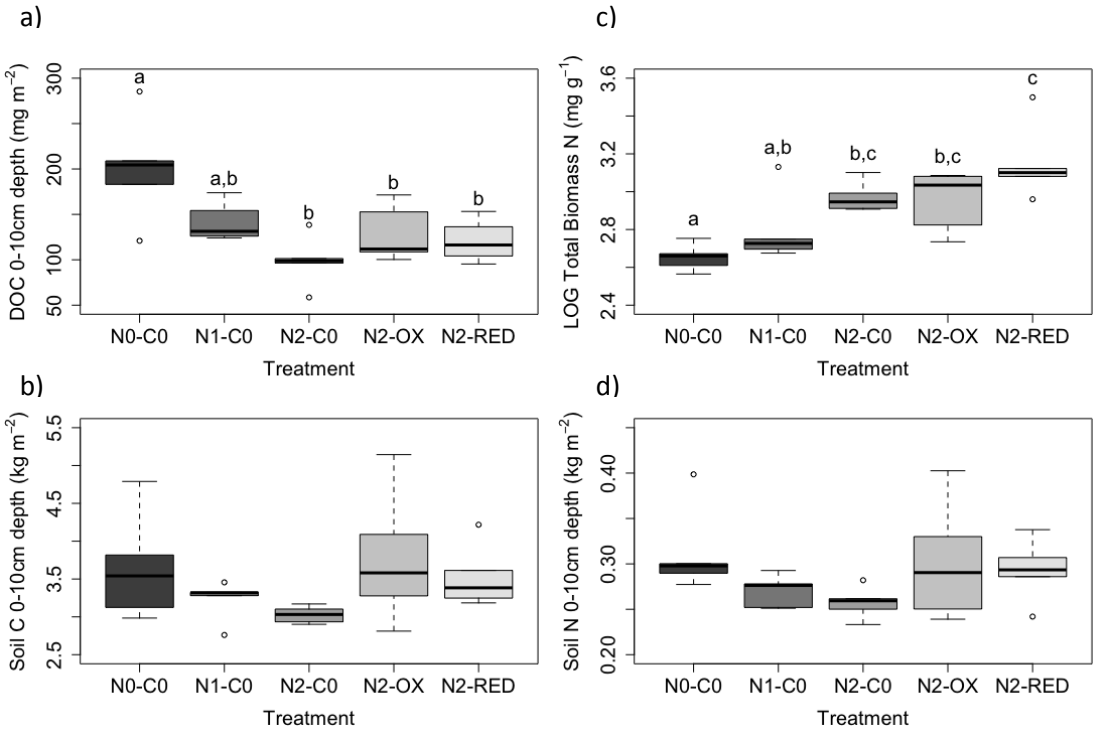

Figure 3.2: DOC (a), total soil C (b), log total biomass N in mg/g (c) and total soil N (d) for each treatment. Letters above boxplots represent significant differences ($p<0.05$) between treatments as measured using a Tukey post-hoc test.

3.3.4 C:N ratios

C:N ratios were tested in order to assess the quality of the organic matter present in this system, and therefore the potential for N-induced changes to microbial activity and decomposition rates. Total aboveground biomass C:N ratios (Figure 3.3a) were significantly different between treatments ($p<0.001$). Biomass C:N ratios were lower in N2-C0, N2-OX and N2-RED than they were in N0-C0 (and N2-C0 and N2-RED were also lower than N1-C0). Grass biomass C:N ratios (Figure 3.3b) were significantly lower ($p=<0.001$) in N2-C0, N2-OX and N2-RED than they were in N0-C0 and N1-C0. Forb biomass C:N ratios (Figure 3.3c) were significantly lower ($p=<0.01$) in N2-RED than they were in N0-C0 and N1-C0, and bryophyte biomass C:N ratios (Figure 3.3d) were significantly lower ($p=<0.01$) in all N treatments when compared to N0-C0. Microbial C:N ratio ($p=0.96$), total soil C:N ratios (0-10 cm, $p=0.21$, 0-5 cm, $p=0.74$, 5-10 cm, $p=0.08$, and 10-15 cm depth, $p=0.18$) and soil fraction C:N ratios (coarse, $p= 0.27$, fine, $p=0.23$, and very fine, $p=0.62$) were not significantly different between treatments. However, the mean C:N ratios of the very fine fraction were slightly lower (~0.7) in the N1-C0 and N2-C0 treatments compared with N0-C0.

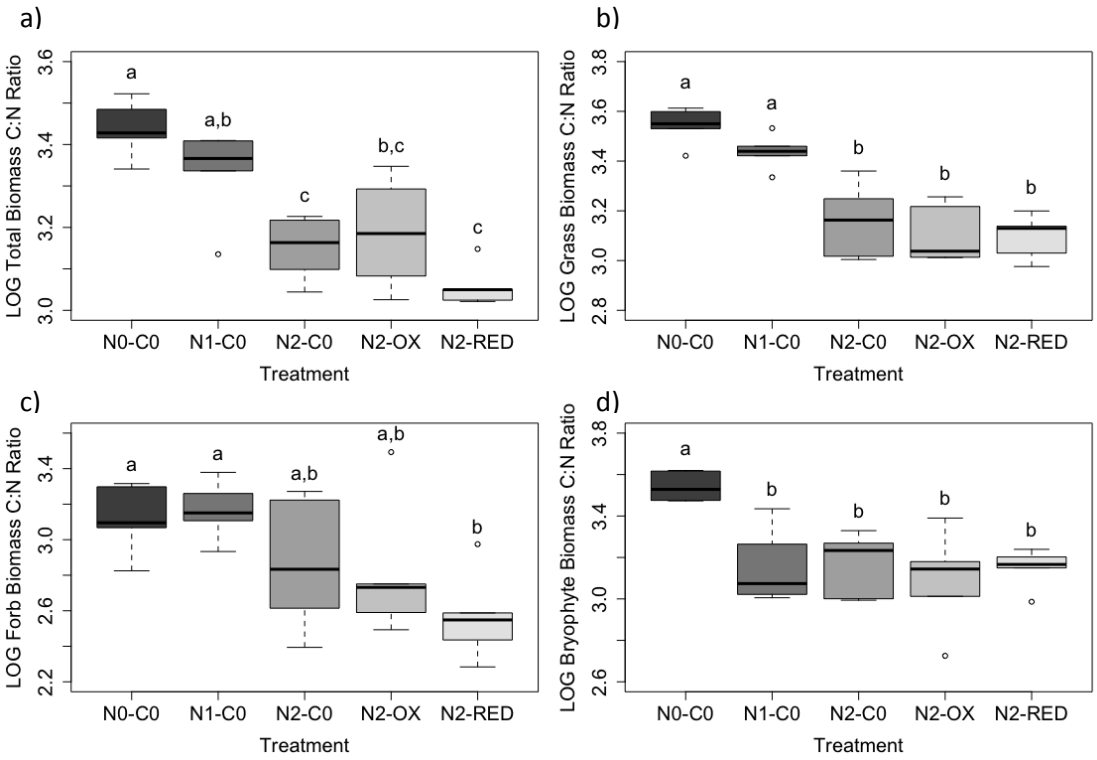

Figure 3.3: Log C:N ratios for total (a), grass (b), forb (c) and bryophyte (d) biomass for each treatment. Letters above boxplots represent significant differences ($p<0.05$) between treatments as measured using a Tukey post-hoc test.

3.3.5 Plant and microbial community structure

Plant community structure was studied in order to test the hypothesis that N addition would lead to an increase in the dominance of grasses, thus accelerating C and N cycling at this site. Total aboveground biomass (g m^{-2}) was not significantly different between any treatments ($p=0.06$, Figure 3.4). However, there was a trend for total biomass to be lowest in the N2-RED plots. Grass, forb, bryophyte and litter biomass (as proportions of total biomass) were also not significantly different between treatments ($p=0.13$, 0.55, 0.58 and 0.18 respectively). Nonetheless, some trends could be observed: N2-C0 had the lowest mean proportion of grass biomass, and all 70 kg N ha^{-1} yr^{-1} treatments (especially N2-RED) had higher mean proportions of litter biomass than N0-C0. Species richness was not found to be significantly different between treatments ($p=0.27$); however, mean values decreased by around 2 species when comparing the control plots (N0-C0) to the N2-CO and N2-OX plots (and 1 species loss for N2-RED plots).

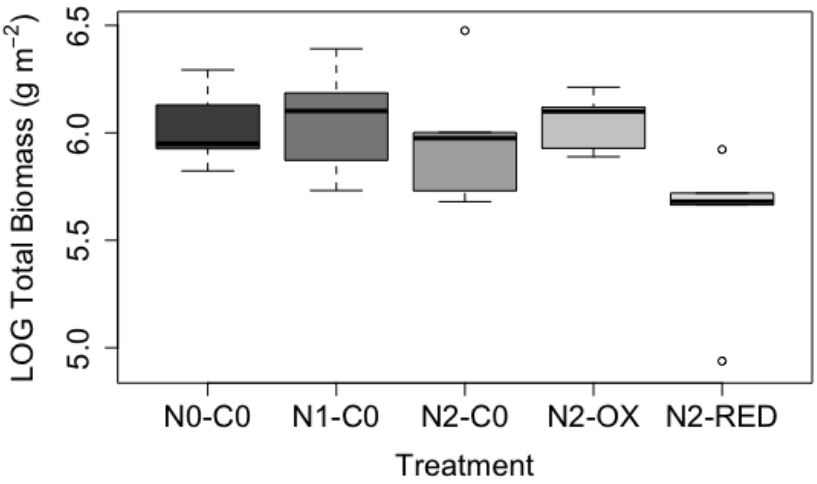

Figure 3.4: Log total aboveground biomass for each treatment.

Total, grass, forb and bryophyte aboveground biomass P (g m^{-2}) were not significantly different between treatments (p=0.18, 0.19, 0.56 and 0.69 respectively). However, grass and forb P potentially show slight trends to decrease with N addition. Total and grass aboveground biomass N:P ratios (Figure 3.5) were significantly different between treatments (p<0.05 and p<0.001 respectively). Total biomass N:P ratios were significantly higher for N2-C0 and N2-RED than for N0-C0. Grass aboveground biomass N:P ratios for N2-C0, N2-OX and N2-RED were significantly higher than that of N0-C0 (and N2-OX was significantly higher than N1-C0). Forb and bryophyte aboveground biomass N:P ratios were not significantly different between treatments (p=0.18 and 0.09 respectively).

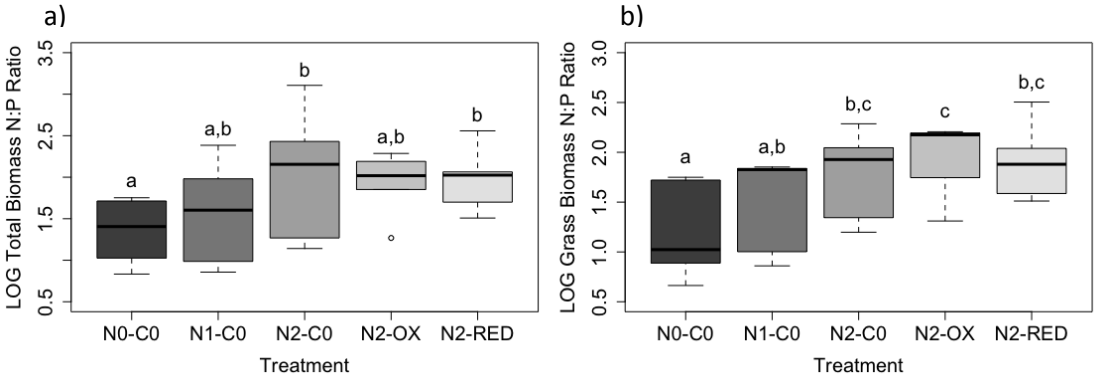

Figure 3.5: Log N:P ratios for total (a) and grass (b) aboveground biomass for each treatment. Letters above boxplots represent significant differences (p<0.05) between treatments as measured using a Tukey post-hoc test.

Microbial community structure was studied in order to test the hypothesis that N addition would increase the proportion of bacteria compared with fungi in the soil. Total PLFA biomass (µg g^{-1} dry soil, Figure 3.6a) was not significantly different between treatments (p=0.17). However, there was increased variability (higher standard deviation) in all N

addition treatments compared to N0-C0, and there was a trend of lower total PLFAs with N addition (especially for N2-RED). The same trends were observed for total fungal (p=0.14), total bacterial (p=0.23), Gram positive (Gram+, p=0.24) and Gram negative (Gram-, p=0.22) bacterial PLFAs. The soil fungal:bacterial ratio (Figure 3.6b) was significantly different between treatments (p<0.05). The fungal:bacterial ratio was significantly lower for N2-RED than it was for N0-C0 and N2-C0, and although the Gram+:Gram- ratio was not significantly different between treatments (p=0.66), variability did increase with N addition.

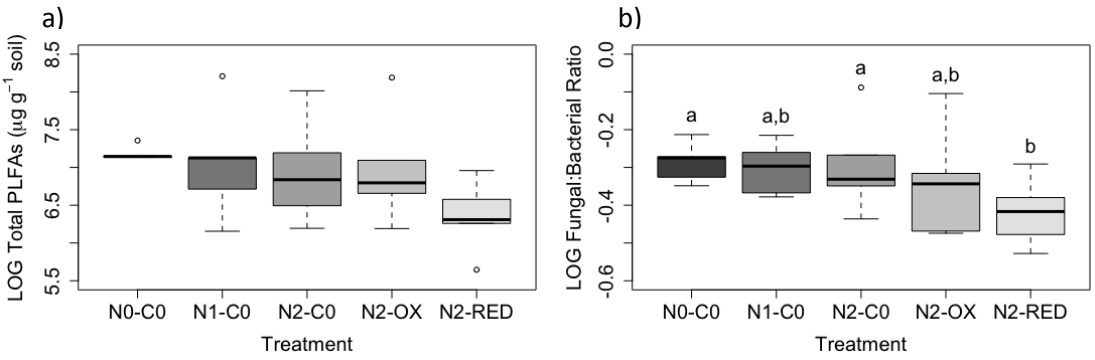

Figure 3.6: Log total PLFA biomass (a) and log fungal:bacterial ratio (b) for each treatment. Letters above boxplots represent significant differences (p<0.05) between treatments as measured using a Tukey post-hoc test.

3.4 Discussion

3.4.1 Effects of N addition on N pools

N addition significantly increased the aboveground biomass N concentration of all three high N (70 kg ha^{-1} yr^{-1}) treatments, a trend that had already been apparent in a previous study of this site (Dorland et al., 2013). This effect was strongest in the N2-RED treatment. The reason why plants growing under ammonium chloride addition had the highest N concentration may be the fact that NH_4^+ requires lower energy outputs than NO_3^- in order to be assimilated (Bloom et al., 1992). Biomass N concentration of the N1-C0 treatment on the other hand, did not significantly differ from the control vegetation except in bryophyte biomass. N1-C0 plots received half the amount of N addition of the other three N treatments, thus implying that 35 kg^{-1} ha^{-1} yr^{-1} of N was not enough to alter the chemistry of vascular plants at this site. As N addition did not seem to alter any other N pools in the N1-C0 treatment either, this could indicate high levels of N loss from the system perhaps due to leaching. Substantial N leaching coupled with the annual removal of plant biomass containing increased amounts of N could

also explain why soil N content (total, at different depths, in different fractions, plant-available and DON) did not significantly differ between N treatments. In addition, plant-available N and DON were sampled in the first year after N addition was terminated, and therefore this was another likely cause for the lack of difference in these variables between treatments. Phoenix *et al.* (2003) conducted a study of an acid grassland in the White Peak area of Derbyshire and found that only up to 38% of the simulated additional N deposition (35 or 140 kg N ha^{-1} yr^{-1} as NH_4NO_3) was accumulated in this system. They found that the major fluxes of N loss from this grassland were via the removal of aboveground biomass and through N leaching, and that both the losses through biomass removal and leaching were increased by N addition. It must be noted that the acid grassland studied by Phoenix *et al.* received very high levels of background N deposition (35+ kg^{-1} ha^{-1} yr^{-1}), whereas the site in Revna, Norway receives around 6 kg^{-1} N ha^{-1} yr^{-1}. However, a recent study by Tian *et al.* (2016a) suggests that grassland ecosystems are more susceptible to N saturation by chronic atmospheric N deposition, so perhaps even low levels of deposition can have an impact over time.

Despite increases in biomass N, there was no significant increase in the overall vegetation N pool because there were no significant differences in the amount of biomass at each treatment. Ammonium chloride may even have had a slight detrimental (although not significant) effect on the amount of aboveground biomass in the N2-RED plots. This could potentially be due to adverse effects of soil acidity, or perhaps toxicity effects of ammonium accumulation (de Graaf et al., 1998; Lucassen et al., 2003). Lack of plant growth despite an increase in plant biomass N concentration suggests that N is not limiting at this site. Similar results were reported by Carroll *et al.* (2003) when they studied acid grassland plots receiving 35, 70 and 140 kg N ha^{-1} yr^{-1} as NH_4NO_3 and 140 kg N ha^{-1} yr^{-1} as $(NH_4)_2SO_4$ over a period of six years. In this study, N addition did not affect plant growth, however, it did increase shoot tissue N, especially in the $(NH_4)_2SO_4$ treatment. Carroll *et al.* also propose that in the long-term, accumulation of ammonium ions in the soil could lead to possible toxic effects on plant growth.

3.4.2 Effects of N addition on C pools

Contrary to expectations, no significant difference was found in total biomass C between treatments. This lack of effect of N addition on biomass C is unexpected, as many studies

report a positive effect of N fertilisation on plant growth (Baer and Blair, 2008; LeBauer and Treseder, 2008; Semmartin and Oesterheld, 2001; Zhang et al., 2015). It is possible that N addition did not have a significant effect on plant biomass C because of N-induced P limitation at this site. Several studies describe the importance of N and P co-limitation as a constraint on primary production (Carroll et al., 2003; Harpole et al., 2011; Phoenix et al., 2003; Tampere et al., 2015). Elser *et al.* (2007) for example, not only found widespread evidence for strong positive synergistic effects of combined N and P enrichment, but also that fertilisation with only N or P quickly induced limitation by the other nutrient. Indeed, N:P ratios for total aboveground biomass were significantly higher for N2-C0 and N2-RED treatments compared with control plots, and grass N:P ratios were significantly higher for N2-C0, N2-OX and N2-RED treatments. These decreases in the amount of P compared to N in plant biomass along with the fact that extractable soil plant-available P was very low at this site (see table 2.1 in methods) support the idea that P limitation may be limiting the effects of N addition (Phoenix et al., 2012). However, it must be noted that the short Norwegian growing season coupled with annual biomass removal and potentially high leaching may also have had an effect on how aboveground biomass responded to N addition.

N addition decreased the C:N ratio of all the dominant functional plant groups in all the high N treatments, thereby increasing the quality of the litter produced. This effect was strongest in the N2-RED treatment. The N1-C0 treatment did not significantly differ from the control vegetation except in the bryophyte biomass C:N ratio. Litter quality is known to affect decomposability (Nicolardot et al., 2001), which has implications for C cycling. However, due to the annual biomass removal carried out at this site, litter is of limited importance in this system. Nonetheless, although not significantly different, the mean proportion of litter biomass in all 70 kg N ha^{-1} yr^{-1} treatments increased compared to control plots, which could be indicative of either earlier senescence (as samples were collected in July), or perhaps increased late summer growth the previous year.

There was no significant difference in soil C content between treatments. This was true for total soil C, as well as C at different depths. Large changes in soil C after seven years of experimental N addition were not expected, as studies have shown that N addition is likely to have only minor effects on grassland soil C storage (Lu et al., 2011a). Nonetheless, it was surprising that no differences were found for N2-RED and N2-C0 treatments, where soil pH, plant C:N ratios and fungal:bacterial ratios in N2-RED were negatively affected by N inputs. It was expected that the effects of lower pH and higher quality litter might change the soil

microbial community and thus alter rates of organic matter decomposition (Allison et al., 2013; Pietri and Brookes, 2009). However, even in N2-RED, where lower fungal:bacterial ratio suggests a change in the microbial community of these plots, no significant changes to C pools were observed, thus implying that overall decomposition rates remained unaltered. Several studies have suggested that N addition may not always have an effect on decomposition rates, and can actually inhibit litter decomposition. A study by Aerts *et al.* (2003) showed that long-term nutrient addition did not lead to increased decomposition rates in grasslands. They suggest that N is more likely to affect ecosystem C balance via effects on litter production rather than by affecting decomposition rates. Peng *et al.* (2014) report that high rates of N addition (both reduced and oxidised forms) to a semi-arid temperate grassland inhibited litter mass loss after one and two years. They propose that N addition can exhibit neutral or inhibitory effects on grassland litter decomposition, as any stimulatory effect of N on the decomposition of labile C may be offset by direct N suppression of the decay of more recalcitrant organic matter. Peng *et al.* suggest that such inhibitory effects of N on recalcitrant litter are not caused by changes to biomass C and N concentrations. Instead, potential underlying mechanisms could include N addition induced C limitation for microbial degradation, reduced microbial diversity and activity, and suppression of certain lignin-degrading enzymes. They also add that N enrichment can produce recalcitrant compounds by reacting with litter components. At Revna, there is an additional possibility that any potential effects of N on soil C were affected by P limitation constraints on primary productivity. Also, as previously mentioned, the annual biomass harvest may have negated the effects of reduced biomass C:N ratios on C cycling at this site.

Coarse and fine soil fraction C did not show any significant differences between treatments. However, the very fine (mineral-associated) soil fraction C content of the N2-C0 treatment was significantly lower than that of the control plots. As the mineral fraction is associated with the longest soil C residence times, these results imply that over the course of 7 years, 70 kg/ha/yr of N deposition as ammonium nitrate may have negatively affected the soil's capacity for long-term C storage. If this is the case, then changes could be observed in the soil C pool if the experiment were to run for more years. This finding is contrary to the work of Neff *et al.* (2002), who state that while long-term (10+ years) N addition accelerates the decomposition of light soil C fractions, it also stabilises soil C compounds in the mineral-associated fractions of an alpine dry meadow. They speculate that this may have been due to some plant material moving directly into stabilized, mineral-associated SOM pools. However, Manning *et al.* (2006) also reported a decrease in C storage in the mineral-associated soil

fraction with N deposition (44 kg ha^{-1} yr^{-1}), which they thought could have been due to changes in arbuscular mycorrhizal fungi (which were less abundant under high N deposition). It must be noted though, that this experiment was a highly artificial mesocosm study, and may therefore be less comparable to a natural system. Although no differences were detected in total soil C pools, soil DOC was significantly lower in N2-C0, N2-OX and N2-RED treatments compared with control plots, despite it being measured in the first year after N addition was terminated. While DOC is known to decrease at lower pH (Evans et al., 2012), this cannot be the reason for the observed decreases in DOC, as N2-OX did not exhibit lower soil pH. Instead, perhaps lower DOC is indicative of a decrease in available substrate C. This might suggest a possible C limitation, which could help to explain why N did not increase plant biomass. N addition has been shown to decrease the root C pool in grasslands (Zeng et al., 2010). Therefore, it might be possible that a C limitation could have been at least partly prompted by decreased root exudation.

3.4.3 Effects of N addition on plant and microbial community structure

There was no indication of any significant changes to plant community structure at this site, as none of the biomass proportions of the different functional groups showed any significant changes. This was not the case in a previous study of this site, where the N2-RED treatment had been found to reduce forb biomass (Dorland et al., 2013). Even species richness was not found to have changed significantly with N addition, although N2-C0 and N2-OX lost two species on average. These findings were unexpected, as many studies report a negative effect of N enrichment/deposition on grassland plant species richness and diversity (Dupre et al., 2010; Humbert et al., 2016; Roth et al., 2013; Stevens et al., 2004). However, effects of N addition in areas with low background N deposition can take many years to become apparent, and are likely to be cumulative (Dupre et al., 2010).

Although there were no observable changes to plant biomass, N2-RED displayed a significant decrease in the fungal:bacterial PLFA ratio. This indicates that the microbial community in these plots may have shifted to become more dominated by bacteria. Bacteria are known to favour the decomposition of high quality litter (Lange et al., 2014). This would be expected to have negative implications for soil C storage, as unlike fungi, bacteria tend to promote faster C turnover rates due to their shorter life cycle and preference for more labile C (Moore et al., 2003), but no differences in soil C pools were observed. A decrease in

fungal:bacterial ratios with increased N addition is also reported in the work of de Vries *et al.* (2006), and was mainly attributed to a decrease in fungal biomass. Despite not being significant, there were trends for decreasing total, fungal and bacterial PLFAs with N deposition (especially for N2-RED). This suggests that N deposition may be reducing the active microbial biomass at this site, thus possibly reducing the potential for decomposition of organic matter. These results are in contrast with those of Manning *et al.* (2008), who found that N deposition positively affected total, fungal and bacterial PLFAs in their mesocosm experiment. However, Rousk *et al.* (2011) examined the long-term N fertilisation Park Grass experiment and found that total, fungal and bacterial PLFAs were lower with high N application. They suggested this could potentially be due to reduced soil organic carbon quality. Johnson *et al.* (1998) also report that 7 years of N addition may reduce microbial biomass and activity in P-limited grasslands. Other measures of microbial biomass and activity (microbial biomass C and N, and N mineralisation rate) were not significantly different between treatments, but these measures are less likely to pick up on subtle changes to the microbial community at this site.

Although results suggest that the microbial communities in the N2-RED plots may have been altered, this did not result in any changes to total soil C pools. This could indicate that changes to the microbial communities had a neutral effect on organic matter decomposition rates. It is interesting to note that although soil pH was significantly lower in both the N2-RED and N2-C0 treatments than in control plots, the fungal:bacterial ratio was only significantly lower in N2-RED. N2-RED plots had the lowest mean soil pH (4.39), but this was not significantly different from the soil pH of the N2-CO plots (4.71). Therefore, the lack of change in the fungal:bacterial ratio in the N2-CO plots could indicate that perhaps it was the high level of ammonium addition of the N2-RED treatment, rather than the pH change, that caused an increase in bacteria relative to fungi. The significance of N form over that of pH for grassland processes has been described in the work of van den Berg *et al.* (2016), who propose that N form may have a more important role than pH in driving the relationship between N deposition and plant species richness across various UK ecosystems.

3.5 Conclusions

N addition affected some aspects of vegetation and soil chemistry, as well as microbial community structure. Biomass C:N ratios of N2-C0, N2-OX and N2-RED, soil pH of N2-C0 and

N2-RED and the fungal:bacterial ratio of N2-RED all decreased with N fertilisation. However, despite these alterations, there were no significant differences in soil (and vegetation) total C and N pools between treatments. The lack of change observed in soil C and N could be due to nutrient co-limitation restrictions on primary productivity, N leaching, or perhaps simply because the experiment had not been running long enough for significant changes to soil C to occur. Indeed, decreases in soil DOC in all three high N treatments, and lower very fine fraction C in N2-C0 could indicate that changes were beginning to occur.

Although most effects observed were strongest for the N2-RED treatment, there is no evidence to suggest that there were significant differences in the effects of reduced N compared to oxidised N deposition on overall C and N pools. However, given that N2-RED was the only treatment that displayed a significant change in the microbial community, this could potentially lead to effects on C and N cycling and storage over longer periods of N addition (decades).

Although few changes were observed after seven years of N addition, other studies have shown that even low levels of N can have impacts over longer periods of time (Phoenix et al., 2012). The large declines in soil pH with reduced N additions in particular could have important consequences in the long term, especially for plant community composition and therefore the indirect effects of N on C storage (Kleijn et al., 2008).

Chapter 4: Seven years of nitrogen addition reduce CO_2 emissions from Norwegian acidic grassland soil

Seven years of nitrogen addition reduce CO_2 emissions from Norwegian acidic grassland soil

4.1 Abstract

Due to increasing human consumption of fossil fuels, artificial fertilisers and animal-derived products, atmospheric nitrogen (N) deposition has become a major problem for ecosystems worldwide. It is known that the carbon (C) and N cycles are very closely linked. As a major C sink, soil is essential for climate change mitigation. Determining the impacts of N addition on soil C storage is crucial to furthering our understanding of how soil can be managed as a C sink.

In this study we wanted to examine the effects of N addition on soil respiration and temperature sensitivity. In 2007, a replicated N-addition experiment was set up on a species-rich, acid grassland site in Revna, Norway. Until 2014, N was added in three doses (0, 35 and 70 kg N ha^{-1} yr^{-1}) and three forms (NaNO3, NH4Cl and NH4NO3). In July 2014, intact soil cores were collected from Revna; three sets of cores were incubated in jars at either 10, 16 or 22^{o}C. A fourth set was spiked with phosphorous (P) and incubated at 16^{o}C. Gas samples were collected on the fifth, seventh and fifteenth day of incubation. Carbon dioxide (CO_2) and nitrous oxide (N_2O) concentrations were determined using a gas chromatograph.

Overall CO_2 fluxes were found to be lower for N treatment cores compared with control cores ($p<0.001$), suggesting that N addition may have inhibited soil microbial activity at this site. This could be due to N-enhanced C limitations, which may be constraining decomposition. Although N_2O emissions and temperature sensitivity did not significantly differ between treatments, P addition caused a significant increase in CO_2 (and to some extent N_2O) fluxes. This indicates that the effects of N addition may be mitigated by P availability. This study shows that grassland soil C pools may be more resilient to N addition than previously thought. However, multiple nutrients must be considered when studying the impacts of N pollution and climate change on soil C storage.

4.2 Introduction

Since the 1950s, human influence has had a profound impact on the global nitrogen (N) cycle. Due to activities such as artificial fertiliser production, fossil fuel combustion and large-scale livestock farming, human processes now account for the conversion of more N_2 into reactive N (N_r) than is naturally produced in the world per year (Erisman et al., 2011; Galloway and Cowling, 2002). This extra N_r production is causing serious environmental problems, one of which is N accumulation in terrestrial systems (Galloway *et al.*, 1995; Rockstrom *et al.*, 2009). Due to atmospheric deposition, volatilised N from farms, cities and roads is transported to many ecosystems that would normally be N limited. This N enrichment is having a major impact on biodiversity and soil processes (Bobbink et al., 2010; Bobbink et al., 1998; Galloway et al., 2003).

In recent years, researchers have acknowledged the importance of considering the links between increased N_r levels and the carbon (C) cycle. Although this field is still full of uncertainty, knowledge of how N_r addition may affect C cycling, and thus climate change is rapidly expanding. N is known to affect the growth, activity and community composition of plants and soil microorganisms through various direct and indirect pathways (Bardgett et al., 1999; Bardgett and Wardle, 2010; LeBauer and Treseder, 2008; Rousk et al., 2011; Stevens et al., 2010). This in turn can affect how C is stored and cycled in the soil. It is this potential change in the terrestrial C sink that is especially significant (Erisman *et al.*, 2011). Only 45% of all current anthropogenic CO_2 emissions remain in the atmosphere. Of the remaining 55%, 30% is taken up by terrestrial ecosystems and 25% by the oceans (Erisman *et al*., 2011). Globally, terrestrial systems contain approximately 2100 Gt of C (De Deyn *et al*., 2008), of which around 80% is stored in soils (Bardgett and Wardle, 2010), thus making soil essential to climate change mitigation.

Depending on how N may be affecting soil C storage, it could have substantial consequences for climate change. Early research projected that N deposition would increase the potential for C storage in terrestrial ecosystems due to additional photosynthetic conversion of CO_2 into organic C (Galloway *et al*., 1995). Estimates for the amount of C potentially stored as biomass due to N addition ranged from 0.2 to 9 Pg Yr^{-1} (Galloway et al., 1995). However, research has shown that in some circumstances, other consequences of N fertilisation include increased decomposition rates (Fenn, 1991; Hunt et al., 1988), which could offset the amount of C stored due to increased plant growth.

As temperatures rise, it is expected that soil respiration, and therefore soil CO_2 emissions, will increase (Schlesinger and Andrews, 2000). However, the combined effects of warming plus N addition on C loss via CO_2 emissions may not be so straightforward. Graham *et al.* (2014) reported that soil warming (3^oC) and N addition (50 kg ha^{-1} yr^{-1}) increased soil respiration by 41% and 12% respectively in an experimental tussock grassland. In addition, these effects were additive under combined warming and nitrogen addition. Conversely, Tao *et al.* (2013) found that although temperature (10^oC increase) had a positive effect on CO_2 production in wetland soil, N addition (0.1, 0.2 and 0.5 mg N g^{-1} sample) had the opposite effect. This contrast in the effects of N on respiration could be due to differences in the two habitats studied, especially differences in water availability and plant communities, but it exemplifies the need for further research in this area.

Another important greenhouse gas emission that can be altered by N addition is nitrous oxide (N_2O). Although the volume of N_2O emitted worldwide is much smaller than that of CO_2, N_2O has a global warming potential 310 times greater than that of CO_2 (IPCC, 1996). N_2O is created via the process of denitrification, which is when denitrifying bacteria (mostly anaerobic) reduce nitrate (NO_3^-) to nitrite (NO_2^-), then to N_2O and nitric oxide (NO), then finally to molecular nitrogen (N_2). N addition to soil can lead to increased emissions of N_2O (Clayton et al., 1997; Davidson, 2009; Galloway et al., 2003), thus adding to climate forcing. Furthermore, temperature rises are predicted to increase soil emissions of N_2O due to enhanced soil respiration (Smith, 1997).

The rate at which soil CO_2 emissions increase with temperature could also be influenced by N addition. Again, contrasting results have been published regarding the effect of N fertilisation on soil temperature sensitivity. In their work on an alpine meadow, Zhang *et al.* (2014) found that two years of N addition (9.2 g and 2.3 N m^{-2} yr^{-1}) caused a significant decrease in Q_{10} (the factor by which the rate of reaction changes as temperature increases by 10^oC) compared to the control during the non-growing season. However, no such difference was found during the growing season. Hu *et al.* (2010) found that although N addition (50, 100 and 150 kg N ha^{-1} yr^{-1}) reduced soil respiration, it increased the temperature sensitivity of the soil in a northern subtropical deciduous broadleaf forest. Cusack *et al.* (2010) also found that N fertilization (50 kg N ha^{-1} yr^{-1}) significantly increased the temperature sensitivity of slowly cycling C pools in two tropical forests.

Anthropogenic N_r creation is expected to increase as global populations rise, so assuming they reach a peak in around 2050, this will mean increasing Nr pollution for at least another

35 years (Galloway and Cowling, 2002). This increase, coupled with potential temperature rise could have severe consequences. Moreover, although current policies have been successful in reducing the emission of oxidised forms of N, a lot less has been achieved for reduced N (Sutton et al., 2011). This could mean that in the future, the proportion of NO_x to NH_y could change. This is significant because there is evidence to suggest that different forms of N have differential effects on plant and soil processes (Stevens et al., 2011b). Nutrient co-limitation must also be considered when assessing the effects of N deposition on C cycling in soil. Several studies have described the importance of N and P co-limitation as a constraint on primary production (Carroll et al., 2003; Elser et al., 2007; Harpole et al., 2011; Phoenix et al., 2003; Tampere et al., 2015). If P is a constraint to the effects of N on plant growth, then this could affect the microbial community and therefore soil respiration. It is crucial that research is conducted into soil responses to different nutrient additions as well as temperature rise in order to deal with all this uncertainty.

This study aimed to evaluate whether seven years of N addition have changed respiration rates, N_2O production and temperature sensitivity of soil from a Norwegian acid grassland. In addition, the experiment examined whether any effects on gas fluxes differed with the application of varying forms of N, as well as with the addition of P.

It was hypothesised that:

1. N addition would increase soil respiration (CO_2 emission) due to increased microbial activity.
2. N addition would increase N_2O emissions from soil due to soil N accumulation.
3. N addition would increase the temperature sensitivity (Q_{10}) of the soil due to changes in the microbial community (higher proportion of bacteria, see Chapter 3).
4. P addition would further increase both CO_2 and N_2O emissions, as it would remove any constraints on decomposition caused by P limitation.

In order to test these hypotheses, various intact soil cores were collected from a long-term N addition experiment, incubated at different temperatures and gas samples were collected over two weeks.

4.3 Results

4.3.1 Effects of long term N addition on CO_2 and N_2O fluxes

4.3.1.1 CO_2 fluxes

On days 5 and 7 the mean amounts of CO_2 released were 0.55 and 0.54 mg CO_2-C kg^{-1} hr^{-1} respectively, but by day 15 the quantity of CO_2 released had slightly decreased to a mean of 0.47 mg CO_2-C kg^{-1} hr^{-1}. Nonetheless, CO_2 fluxes were not significantly different between sampling days (p=0.05). CO_2 fluxes significantly increased with temperature (p<0.001), with mean CO_2 emissions being 0.31, 0.40 and 0.85 mg CO_2-C kg^{-1} hr^{-1} at 10, 16 and 22°C respectively. Overall, CO_2 fluxes were significantly different between N treatments (p<0.001). N0-C0 (control) cores exhibited the highest overall CO_2 fluxes (mean = 0.72 mg CO_2-C kg^{-1} hr^{-1}). The second highest fluxes generally came from the N2-OX cores, followed by the N1-C0 cores, and finally the N2-C0 and N2-RED cores (mean = 0.58, 0.51, 0.41 and 0.37 mg CO_2-C kg^{-1} hr^{-1} respectively). CO_2 fluxes were significantly lower in N1-C0, N2-C0, N2-OX and N2-RED compared with N0-C0 cores (p<0.001, <0.001, <0.01 and <0.001 respectively; Figure 4.1). In addition, CO_2 fluxes were lower in N2-RED cores compared with N1-C0 and N2-OX cores (p<0.01 and <0.001 respectively), and in N2-C0 compared with N2-OX (p<0.001; Figure 4.1). There were no significant interactions between N treatment and temperature (p=0.879).

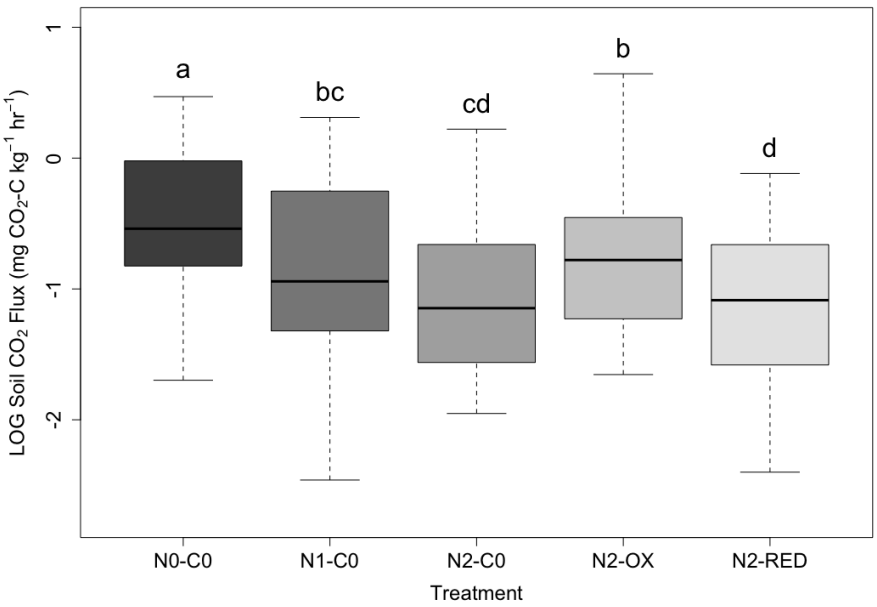

Figure 4.1: Log soil CO_2 fluxes for each treatment across all incubation days (5, 7 and 15) and temperatures (10, 16 and 22°C). Letters above boxplots represent significant differences (p<0.05) between field N addition treatments as measured using a Tukey post-hoc test.

4.3.1.2 N_2O fluxes

N_2O emissions were significantly different between sampling days ($p<0.001$). Mean N_2O emissions were 10.81 and 9.72 µg N_2O-N kg^{-1} hr^{-1} on days 5 and 7 of the incubation respectively, but had decreased to 3.33 µg N_2O-N kg^{-1} hr^{-1} by day 15. N_2O emissions significantly increased with temperature ($p<0.001$). Mean N_2O emissions were 2.81, 8.86 and 12.20 µg N_2O-N kg^{-1} hr^{-1} at 10, 16 and 22°C respectively. N_2O emissions were not significantly different between N treatments ($p=0.81$; Figure 4.2). Mean N_2O emissions were 8.10, 7.04, 7.73, 7.74 and 9.16 µg N_2O-N kg^{-1} hr^{-1} for N0-C0, N1-C0, N2-C0, N2-OX and N2-RED respectively. However, there was a significant interaction between treatment and temperature for N_2O emissions ($p<0.05$).

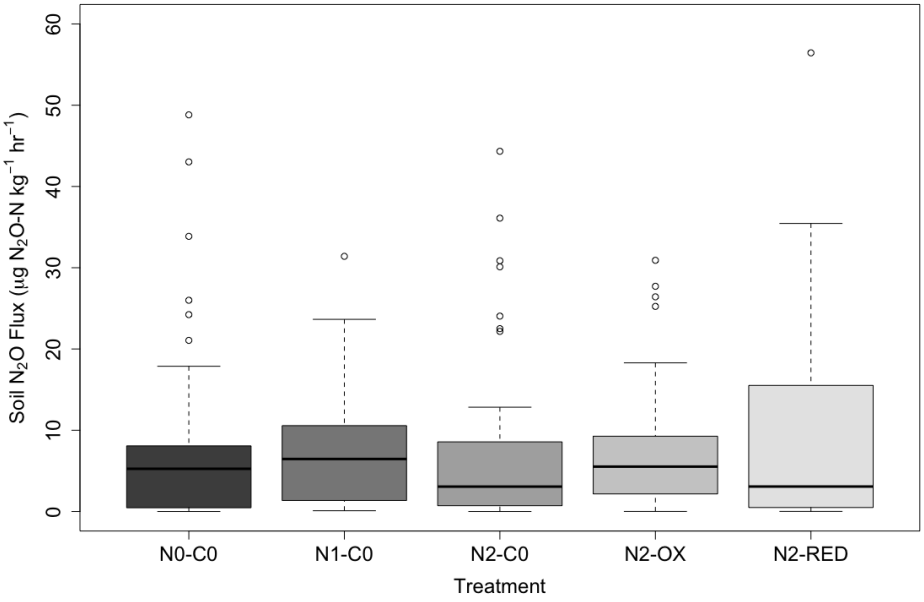

Figure 4.2: Soil N_2O fluxes for each treatment across all incubation days (5, 7 and 15) and temperatures (10, 16 and 22°C). Circles represent outliers.

4.3.2 Effects of long term N addition on temperature sensitivity

There were no significant differences between the Q_{10} values of the different N treatments ($p=0.28$). Mean Q_{10} values calculated from the CO_2 fluxes of cores incubated at 10 and 22°C were 2.22, 2.45, 2.59, 2.49 and 2.92 for N0-C0, N1-C0, N2-C0, N2-OX and N2-RED respectively. However, there was a very slight (non-significant) trend for increasing Q_{10} values with N addition (Figure 4.3).

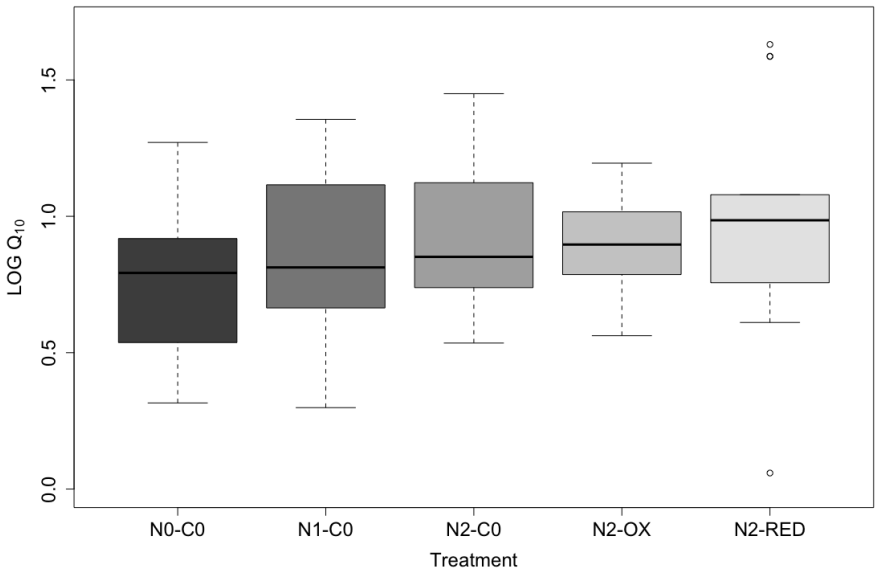

Figure 4.3: Log Q_{10} values for each treatment calculated from cores incubated at 10 and 22^{o}C across all incubation days (5, 7 and 15).

4.3.3 P effects on CO_2 and N_2O fluxes

4.3.3.1 CO_2 fluxes

All cores that were spiked with P emitted significantly more CO_2 than the cores that were incubated at the same temperature but did not receive any P ($p<0.001$; Figure 4.4). Control cores with added NaCl did not significantly differ from control cores incubated at the same temperature, but without NaCl ($p=0.74$; Figure 4.4). Mean CO_2 emissions of cores spiked with P (and control cores spiked with NaCl) were 0.68, 0.99, 0.86, 0.75 and 0.85 mg CO_2-C kg^{-1} hr^{-1} for N0-C0, N1-C0, N2-C0, N2-OX and N2-RED respectively. CO_2 emissions of cores spiked with P were significantly higher for N1-C0, N2-C0 and N2-RED than for control cores ($p<0.001$, <0.01 and <0.01 respectively; Figure 4.4). Mean CO_2 emissions of cores incubated at 16^{o}C that did not receive any P were 0.60, 0.42, 0.28, 0.38 and 0.31 mg CO_2-C kg^{-1} hr^{-1} for N0-C0, N1-C0, N2-C0, N2-OX and N2-RED respectively. CO_2 emissions of cores incubated at 16^{o}C that did not receive any P were significantly lower for N1-C0, N2-C0, N2-OX and N2-RED compared with control cores ($p<0.05$, <0.001, <0.05 and <0.001 respectively; Figure 4.4). There was a significant ($p<0.001$) interaction between P addition and N treatments for CO_2 fluxes.

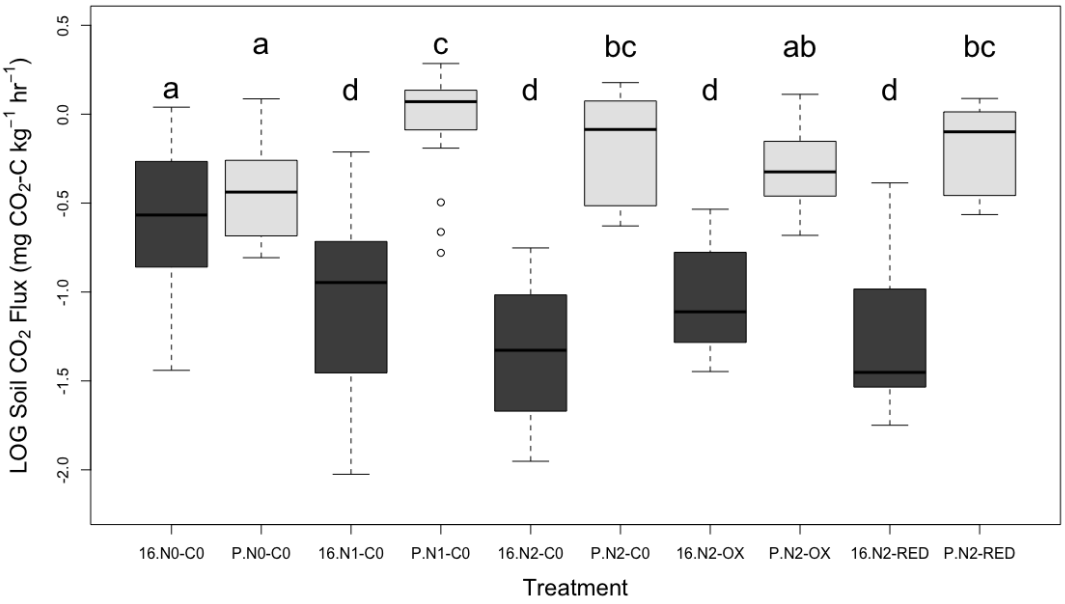

Figure 4.4: Log soil CO_2 fluxes for each treatment across all incubation days (5, 7 and 15). Dark grey boxes represent cores incubated at 16°C, while light grey boxes represent cores incubated at the same temperature, but spiked with P (except for N0-C0, which was spiked with NaCl). Letters above boxplots represent significant differences ($p<0.05$) between treatments as measured using a Tukey post-hoc test.

4.3.3.2 N_2O fluxes

Mean N_2O emissions of the cores spiked with P (and control cores spiked with NaCl) dropped from 37.54 and 37.18 µg N_2O-N kg^{-1} hr^{-1} on days 5 and 7, to 6.79 µg N_2O-N kg^{-1} hr^{-1} on day 15. In contrast, the mean N_2O emissions of cores incubated at 16°C that did not receive any P remained consistent throughout the incubation period (8.32, 9.75 and 8.50 µg N_2O-N kg^{-1} hr^{-1} on days 5, 7 and 15 respectively). Due to this, N_2O emissions from days 5 and 7 were analysed separately from day 15.

On days 5 and 7, mean N_2O emissions of cores spiked with P (and control cores spiked with NaCl) were 24.42, 27.74, 29.68, 70.76 and 34.20 µg N_2O-N kg^{-1} hr^{-1} for N0-C0, N1-C0, N2-C0, N2-OX and N2-RED respectively. On days 5 and 7, mean N_2O emissions of cores incubated at 16°C that did not receive any P were 9.50, 8.25, 6.03, 12.53 and 8.89 µg N_2O-N kg^{-1} hr^{-1} for N0-C0, N1-C0, N2-C0, N2-OX and N2-RED respectively. Although overall N_2O emissions on days 5 and 7 were significantly higher in cores spiked with P compared with cores incubated at the same temperature ($p<0.001$), the only treatment that showed a significant increase in N_2O emissions with P addition was N2-OX ($p<0.001$; Figure 4.5a). Due to this, there was a significant interaction between P addition and N treatment ($p<0.01$).

All significant effects of P addition and N treatment on N_2O emissions were lost by day 15 of the incubation (Figure 4.5b).

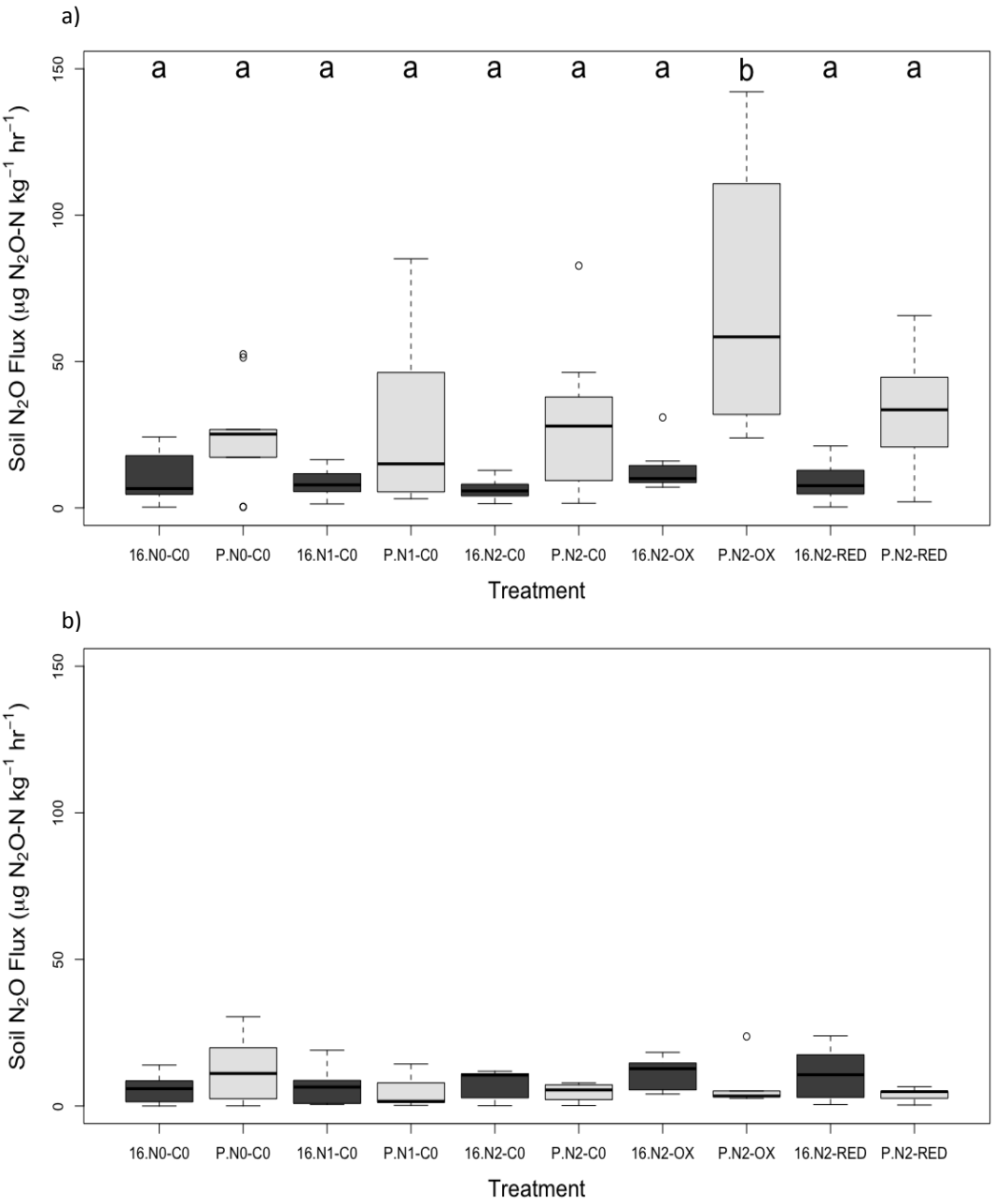

Figure 4.5: Soil N_2O fluxes for each treatment on days 5 and 7 (a) and on day 15 (b) of the incubation. Dark grey boxes represent cores incubated at 16^{o}C, while light grey boxes represent cores incubated at the same temperature, but spiked with P (except for N0-C0, which was spiked with NaCl). Letters above boxplots represent significant differences ($p<0.05$) between treatments as measured using a Tukey post-hoc test.

4.4 Discussion

4.4.1 Effects of N addition on CO_2 fluxes

N addition led to a significant decrease in soil CO_2 fluxes, indicating that N may have suppressed microbial activity at this site. The negative effect of N application on microbial respiration has been documented in several N fertilization studies (Burton et al., 2004; DeForest et al., 2004; Fisk and Fahey, 2001; Fog, 1988; Knorr et al., 2005; Liu and Greaver, 2010; Olsson et al., 2005; Pregitzer et al., 2008; Ramirez et al., 2010; Treseder, 2008). In addition, Ramirez *et al.* (2010) tested the effect of different forms of inorganic N addition (NH_4NO_3, KNO_3, NH_4Cl, $(NH_4)_2SO_4$, $Ca(NO_3)_2$) on microbial respiration in grassland soil. As in our study, they found that all N forms had a negative effect on microbial respiration. However, Ramirez *et al.* did not find differences in the magnitude of the decrease between N forms. Contrastingly, in this study the treatments receiving high levels (70 kg N ha^{-1} yr^{-1}) of reduced N (N2-RED and N2-CO) showed a larger decrease in respiration than the treatment receiving only oxidised N (N2-OX). This suggests that high levels of ammonium addition may have had an especially detrimental effect on soil microbial activity at this site. Interestingly, the pattern observed for CO_2 fluxes was the same as that of soil pH at this site (pH was lowest in N2-RED followed by N2-C0, and highest in N0-C0 and N2-OX, see Chapter 3), which suggests that CO_2 fluxes may have been affected by reduced N-induced acidification. Indeed, Chen *et al.* (2016) conducted a long-term (12 year) N addition experiment on a semi-arid grassland and found that reductions in microbial respiration were more strongly controlled by N-induced acidification than by N availability.

Although microbial biomass C and N were not significantly different between treatments at this site (see Chapter 3), there was a non-significant trend for decreased total PLFA biomass with N addition, especially in the N2-RED treatment (see Chapter 3). As total PLFA biomass is regarded as a measure of active microbial biomass (Bardgett and McAlister, 1999), this helps to support the idea that N addition (especially reduced N) may be suppressing microbial activity. There was also a trend for decreased fungal:bacterial ratio with N addition at this site, however N2-RED was the only treatment that displayed a significantly lower fungal:bacterial ratio (see Chapter 3). This suggests that N addition (especially reduced N) may have caused a shift in the microbial communities at this site, favouring bacteria over fungi. Ramirez *et al.* (2012) propose that N addition decreases microbial respiration by changing bacterial community composition, thus yielding communities that are less capable of decomposing more recalcitrant soil C pools. In their study of the Park Grass experiment,

Rousk *et al.* (2011) also note a decrease in the fungal:bacterial PLFA ratio with N fertilisation, as well as a decrease in respiration with increased N addition. However, they attribute this to a decrease in soil organic carbon (SOC) availability, and state that the negative impact of N on soil respiration was largely unrelated to changes in the actively growing microbial population, and instead C availability directly influenced soil respiration. Eberwein *et al.* (2015) also state that C availability regulated the respiration response of their soil incubations to N and temperature. Indeed, despite no significant differences between the total soil C contents of the different treatments (see Chapter 3), soil at the Revna site showed a significant decrease in DOC with N addition (see Chapter 3). This could indicate that N fertilisation has reduced available SOC at this site, thus potentially driving the decrease in soil microbial respiration. Reduced microbial respiration with increased N fertilisation has been attributed to the suppression of the activity of enzymes required for the mineralisation of recalcitrant litter fractions (Berg and McClaugherty, 2008; Fog, 1988), resulting in an increased proportion of low-quality C in the SOC.

4.4.2 Effects of N addition on N_2O fluxes

N addition did not lead to any significant changes to soil N_2O fluxes. This may have been due to the lack of N accumulation in the soil at this site, as demonstrated by the lack of significant change in soil N pools between treatments at this site (see Chapter 3). It is also possible that most N_2O emissions occurred soon after the field N application, and therefore were not captured by this lab incubation. Clayton *et al.* (1994) for example, found that N_2O fluxes peaked five days after N fertilisation. Conversely, the absence of an effect of N addition on N_2O production may have, like CO_2, been constrained by SOC availability. In their laboratory soil incubations, Liang *et al.* (2015) found that labile C as well as reactive N additions regulated both N_2O and CO_2 fluxes due to controls on decomposition rates. In addition, Bouwman *et al.* (2002) report that N_2O emissions increase with N application rates as well as with higher soil organic C content.

4.4.3 Effects of N addition on temperature sensitivity

Despite a very slight trend for increasing Q_{10} with N addition, N fertilisation did not lead to any significant changes to the soil's temperature sensitivity. Similarly, Zhu *et al.* (2016) found that N addition did not affect the temperature sensitivity of soil respiration in a semi-arid grassland. In addition, Qian *et al.* (2016) found that seasonality, rather than N addition, influenced the temperature sensitivity of microbial respiration in soil from five different

systems (including a grassland). It is possible that the effect of N addition on temperature sensitivity may have been constrained by SOM availability. Karhu *et al.* (2014) collected soils along a climate gradient from the Arctic to the Amazon to investigate how microbial community-level responses control the temperature sensitivity of soil respiration. They found that the strongest enhancing responses were observed in soils with high C:N ratios. In addition, Gershenson *et al.* (2009) found that increased substrate availability had a significant positive effect on the temperature sensitivity of soil respiration. It is also possible that temperature sensitivity effects may have been constrained by P limitation, as available P at this site was very low (mean of 0.020 g m^{-2} for 10 cm depth). A study by Elser *et al.* (2007) for example, found widespread evidence that fertilisation with only N or P quickly induced limitation by the other nutrient.

4.4.4 Effects of P addition on CO_2 and N_2O fluxes

P addition significantly increased soil CO_2 production for every N treatment. This result strongly suggests that the soil at this site is P-limited, and that this limitation is potentially mitigating the effects of N addition on CO_2 emissions. If this is truly the case, it has implications for the C-storing capacity of acid grasslands. Indeed, some studies have put forth the idea that increased N deposition has created an imbalance between N and P, and has led to a shift from N to P limitation in some ecosystems (Penuelas et al., 2012; Vitousek et al., 2010). However, in contrast to our results, Li *et al.* (2010) found that N and P additions to a semi-arid grassland did not affect soil respiration. Peng and Thomas (2010) also found that P addition reduced microbial respiration in a northern hardwood forest subjected to N deposition. N0-C0 cores spiked with NaCl did not differ significantly from the N0-C0 cores incubated at the same temperature, but with no NaCl. This shows that the NaCl added as part of NaH_2PO_4 probably did not have any effects on the soils' response to P. The significant interaction between P addition and N treatments for CO_2 fluxes suggests that there may be implications for temperature sensitivity when N and P are both added to this site.

P addition did not significantly change soil N_2O production for all treatments except for N2-OX. In tropical soils however, where P is often a limiting nutrient, P addition has been found to reduce N_2O emissions by increasing respiratory efficiency (Mori et al., 2016). When P was added to N2-OX cores, it significantly increased N_2O emissions compared with N2-OX cores incubated at the same temperature. This could indicate that denitrification may have been the most important process for N_2O production in the soil from this site. Indeed, in their experiment, Anderson and Levine (1986) found that the highest N_2O production came from a

denitrifying bacterial species, and Firestone *et al.* (1980) found that increasing concentrations of nitrate enhanced production of N_2O relative to molecular nitrogen during denitrification in soils. Increased N_2O emissions with N and P enrichment in acid grasslands could have implications for climate forcing, as N_2O is a powerful greenhouse gas (Lashof and Ahuja, 1990). However, the variability for these fluxes was very high, and the effect was lost after 15 days of incubation. Overall, results suggest that the effects of N addition on N_2O emission at this site might be constrained by P availability, but that this is likely to depend on the N form. In addition, N_2O emissions may peak quite soon after P addition.

4.5 Conclusions

Seven years of N addition have had a significant negative impact on soil respiration rates. This reduced microbial activity is probably due to a combination of N-enhanced C and P limitations, as well as possible effects of N-induced acidification. It must be noted that treatments containing high levels of ammonium tended to display the strongest reductions in CO_2, which could become important if reduced forms of N do become dominant in the future. Although N_2O emissions and temperature sensitivity did not significantly differ between treatments, there is a possibility that this might not be the case if P was not a limiting factor. This study shows that it is important to consider multiple nutrients when accessing the impacts of N deposition on C and N cycling in grassland soils.

Chapter 5: Investigating the effects of low levels of nitrogen deposition on carbon and nitrogen pools in a Welsh acid grassland

Investigating the effects of low levels of nitrogen deposition on carbon and nitrogen pools in a Welsh acid grassland

5.1 Abstract

Atmospheric nitrogen deposition is a major threat to biodiversity and ecosystem service provision around the globe. Through processes such as eutrophication and soil acidification, increased availability of reactive forms of nitrogen can lead to alterations of terrestrial nitrogen and carbon cycling. Research over the past couple of decades has enabled the establishment of thresholds for nitrogen impacts to different ecosystems. Although thresholds vary between systems, one common value used is 10 kg ha^{-1} yr^{-1}. Given that this value theoretically represents the rate at which nitrogen deposition starts to have an effect on ecosystem functioning, a common criticism of nitrogen addition experiments is that they often employ unrealistically high nitrogen addition rates (sometimes as high as 200 kg ha^{-1} yr^{-1}). Consequently, such large nitrogen applications have the potential for overestimating the effects of atmospheric nitrogen deposition. Long-term nitrogen addition experiments have shown that even low nitrogen applications (under 10 kg ha^{-1} yr^{-1}) can alter ecosystem processes. Atmospheric nitrogen deposition is predicted to continue increasing over the next few decades, meaning that systems currently under the threshold could be more severely impacted in the future. It is therefore imperative that more research is carried out at the lower ranges of nitrogen deposition in order to fully understand its consequences for ecosystems worldwide.

Using a nitrogen addition experiment in North Wales, UK, this study examines the effects of low levels of nitrogen (4.3, 4.8 ad 8.6 kg ha^{-1} yr^{-1}) on acid grassland nitrogen and carbon pools. Results show no significant effects of nitrogen treatments on any carbon and nitrogen pools after 6/7 years of applications, suggesting that nitrogen deposition under 10 kg ha^{-1} yr^{-1} is not enough to alter the nitrogen and carbon cycles at this site. However, it must be noted that as this experiment was only run for 7 years, this may not have been enough time for long-term effects of low levels of N addition to be observed.

5.2 Introduction

Anthropogenic activities such as fossil fuel burning, artificial nitrogen (N) fertiliser manufacturing, and animal husbandry produce over 180 Tg of reactive N (N_r) every year (Erisman et al., 2011). Consequently, humans now generate more N_r than is produced naturally in the whole world (Galloway and Cowling, 2002; Galloway et al., 1995). This huge influx of N_r has changed the global N cycle (Vitousek et al., 1997) and has led to the problem of atmospheric N deposition. Ecosystems around the globe that had previously adapted to low levels of N_r are now being exposed to regular inputs of this essential nutrient. Research has shown that atmospheric N deposition affects various ecological processes. Broadly, the main impacts of N deposition on terrestrial ecosystems are (1) eutrophication, (2) acidification, (3) direct foliar impacts, and (4) increased susceptibility to secondary stress factors (Bobbink et al., 1998).

In systems where N is a limiting factor, N addition often leads to increased primary productivity (Erisman et al., 2011; Gough et al., 2000; Morecroft et al., 1994; Shaver et al., 2001). In addition, higher plant biomass N content can lead to the production of higher quality litter, which is known to be more readily decomposable (Manning et al., 2008). N deposition is also known to reduce plant species richness (Stevens et al., 2010) and to affect plant community composition by favouring fast-growing plant species such as grasses (Stevens et al., 2009; Suding et al., 2005). N addition can also affect soil microbial activity and community composition. Higher N levels are known to promote bacterial activity and suppress fungi (Kirchmann et al., 2013), and dominance by fast-growing plant species may encourage changes to the soil microbial community via acidification, plant-specific differences in the release of root exudates and alterations in nutrient competition between plants and microorganisms (Bardgett et al., 1999). All these effects can lead to changes to the N and C cycles of the affected systems.

N deposition in the UK can range from around 5 kg ha^{-1} yr^{-1} in remote areas of Northern Ireland and Scotland, up to over 30 kg ha^{-1} yr^{-1} in areas with high levels of road traffic or intense agricultural activity (RoTAP, 2012; Stevens et al., 2004). In other European countries such as the Netherlands and Germany, N deposition can be even higher, with levels reaching over 40 kg ha^{-1} yr^{-1} (Stevens et al., 2010; Sutton, 2011). Although these levels are high, many experiments apply even larger rates of N addition (Carroll et al., 2000; Rousk et al., 2011; Tampere et al., 2015; Zeng et al., 2010). Such high N experiments are useful as they produce

results in relatively short amounts of time, however they also face criticism for not being representative of actual deposition levels and thus potentially overestimate the effects of N addition (Phoenix et al., 2012). A widely used threshold for N deposition impacts (or critical N load) is 10 kg ha^{-1} yr^{-1} (Sutton, 2011). Although in-depth research has shown this value does vary between ecosystems (Bobbink and Roelofs, 1995; Tipping et al., 2013), 10 kg N ha^{-1} yr^{-1} can sometimes be regarded as a rough general level above which changes in sensitive natural ecosystems may occur (Dentener et al., 2006).

Long-term N addition experiments have shown that even very low nitrogen applications (under 10 kg ha^{-1} yr^{-1}) can alter ecosystem processes. Phoenix *et al.* (2012) for example, show that long term N applications at levels as low as 7.7 kg ha^{-1} yr^{-1} (as ammonium sulphate) can alter plant productivity, lichen abundance and flowering patterns in a lowland heath. N_r production continues to increase every year (Galloway et al., 2008), meaning that systems currently under their N effects threshold could be more severely impacted in the future. It is therefore imperative that more research is carried out at the lower ranges of nitrogen deposition in order to fully understand its consequences for ecosystems worldwide. In addition, projections for future N deposition predict an increase in the proportion of reduced N pollution (Dennis et al., 2010), which is emitted largely due to agricultural practices. Enhancing current knowledge of the effects of low levels of reduced N on ecosystem functioning is therefore essential to our understanding of future effects of N deposition.

This study aimed to evaluate whether 6/7 years of low levels of N addition (4.3, 4.8 and 8.6 kg ha^{-1} yr^{-1}) have changed the C and N pools of a Welsh acid grassland. This ecosystem was chosen because grasslands are found across a wide range of temperate zones, are important for biodiversity and provide a number of ecosystem services, one of which is carbon (C) storage (Ward et al., 2016). In addition, the experiment investigated whether there were differences in the possible effects of low level N addition when NH_4Cl was applied compared with NH_4NO_3.

It was hypothesised that:

1. Overall changes to vegetation C and N pools would not be significant. However, N addition would lead to slight increases in plant N content and aboveground biomass, especially in the higher ammonium nitrate treatment.

2. No changes would occur in soil C and N pools, although N addition would slightly reduce soil pH in the ammonium chloride and the higher ammonium nitrate treatments.
3. N addition would lead to a small increase in the proportion of bacteria in the microbial community (lower fungal:bacterial ratio).
4. N addition would slightly increase whole system respiration rates and net primary productivity (NPP) in the higher ammonium nitrate treatment.

In order to test these hypotheses, CO_2 flux measurements and various soil and vegetation samples were collected from the experimental site and analysed in depth in the laboratory.

5.3 Results

5.3.1 Soil pH

Soil pH was not significantly different between treatments (p=0.22) (Figure 5.1a).

5.3.2 N pools

No significant differences between treatments were found for any above or belowground N pools. Total aboveground biomass N (as g m^{-2} and as mg g^{-1}) was not found to be significantly different between treatments (p=0.51 and 0.08 respectively). However, there is a trend for a slight increase in biomass N (as mg g^{-1}) with N addition (Figure 5.1b). Root biomass N (mg g^{-1}) was also not significantly different between treatments (p=0.70). Microbial biomass N (mg m^{-2} for 10 cm depth) was not significantly different between treatments (p=0.43), and neither was soil total N content (kg m^{-2}). This was true for soil total N (0-10 cm depth, p=0.97, Figure 5.1c) and for total N at different depths (0-5 cm, p=0.58, 5-10 cm, p=0.21, 10-15 cm, p=0.22). No significant differences between treatments were observed for the N content of the coarse, fine or very fine soil fractions (both as kg m^{-2} (p=0.64, 0.05 and 0.98 respectively) and as proportions of total N stocks (p=0.82, 1.00 and 0.98 respectively)). However, there was a trend for higher fine fraction N (kg m^{-2}) in the N2-C0 treatment (Figure 5.1d). Soil dissolved organic N (DON, mg m^{-2} for 10 cm depth) and available N (mg m^{-2} for 10

cm depth) were not significantly different between treatments (p=0.98 and 0.41 respectively).

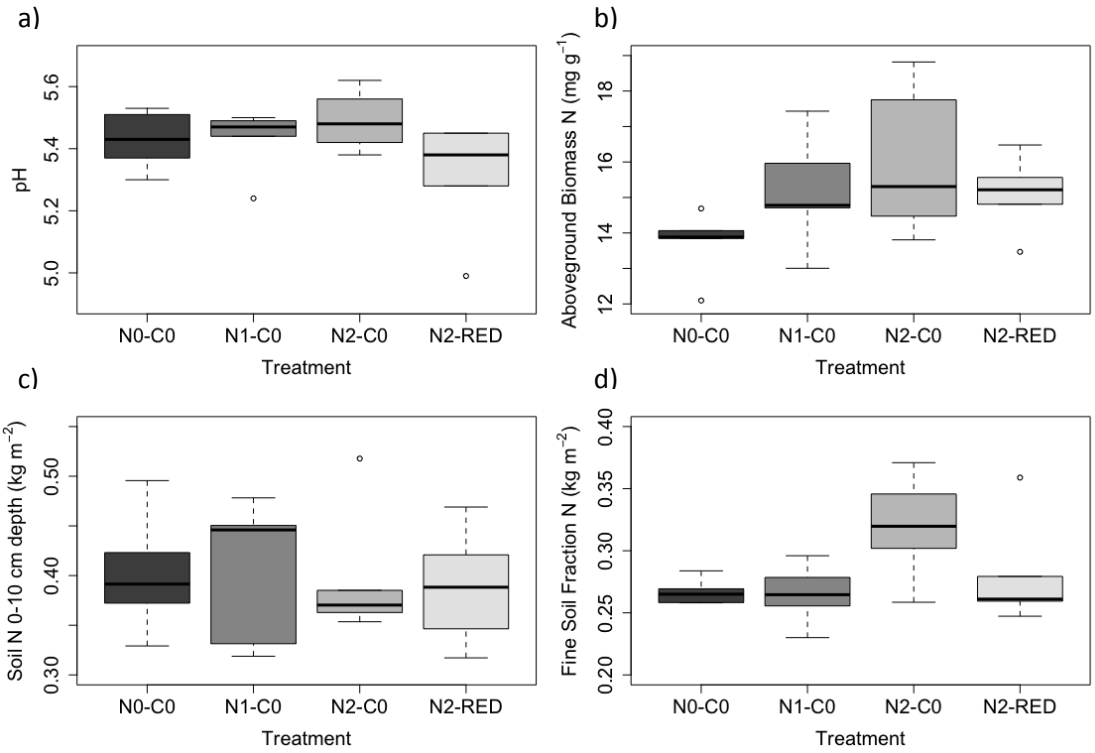

Figure 5.1: Soil pH (a), aboveground biomass N in mg g^{-1} (b), total soil N (c) and fine fraction soil N (d) for each treatment. N treatments consist of 0 kg N ha^{-1} yr^{-1} (N0-C0), 4.3 kg N ha^{-1} yr^{-1} as NH_4NO_3 (N1-C0), 8.6 kg N ha^{-1} yr^{-1} as NH_4NO_3 (N2-C0), and 4.8 kg N ha^{-1} yr^{-1} as NH_4Cl (N2-RED).

5.3.3 C pools

There were no significant differences found between treatments for any above or belowground C pools. Total aboveground biomass C (as g m^{-2}, p=0.20), rate of litter biomass loss (% $month^{-1}$, p=0.69), rate of root growth (g m^{-2} $month^{-1}$ for 15 cm depth, p=0.16) and root biomass C (mg g^{-1}, p=0.36) were not significantly different between treatments. However, there was a slight trend for lower root C in the N2-RED treatment (Figure 5.2a). Although microbial biomass C (mg m^{-2} for 10 cm depth, p=0.24) was not significantly different between treatments, there was a trend for increased microbial C in the N2-C0 and N2-RED treatments compared with control plots (Figure 5.2b). Soil C content (kg m^{-2}) was also not significantly different between treatments. This was true for total C (0-10 cm depth, p=0.98, Figure 5.2c) and for C at different depths (0-5 cm, p=0.51, 5-10 cm, p=0.44, 10-15 cm, p=0.28). Coarse (2mm-200 µm), fine (200-50 µm) and very fine (50-0.45 µm) soil fraction C contents (as kg m^{-2}, p=0.48, 0.08 and 0.77 respectively) and as proportions of total C stocks (p=0.72, 0.60 and 0.54 respectively) were not significantly different between treatments.

However, there was a trend for slightly higher fine fraction C (kg m^{-2}) in the N2-C0 treatment compared with control plots (Figure 5.2d). Soil dissolved organic C (DOC, mg m^{-2} for 10 cm depth) was not significantly different between treatments (p=0.97).

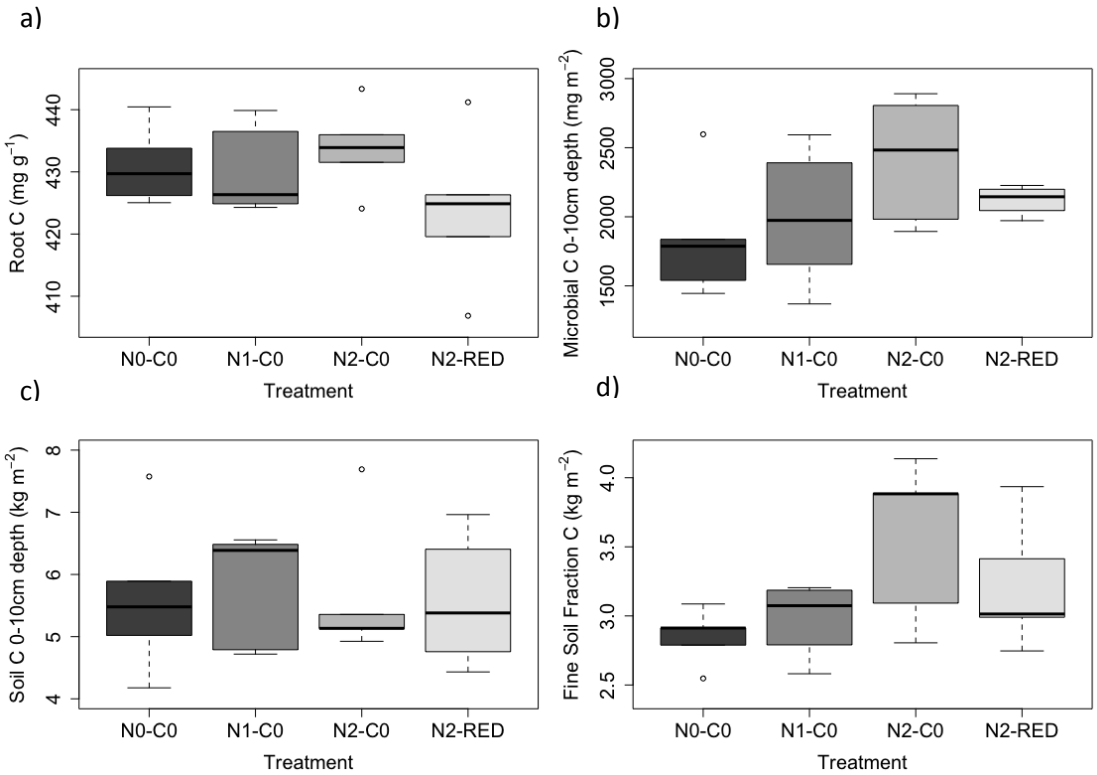

Figure 5.2: Root C (a), microbial biomass C (b), total soil C (c) and fine fraction soil N (d) for each treatment. N treatments consist of 0 kg N ha^{-1} yr^{-1} (N0-C0), 4.3 kg N ha^{-1} yr^{-1} as NH_4NO_3 (N1-C0), 8.6 kg N ha^{-1} yr^{-1} as NH_4NO_3 (N2-C0), and 4.8 kg N ha^{-1} yr^{-1} as NH_4Cl (N2-RED).

5.3.4 C:N ratios

No changes to C:N ratios were observed. Although total aboveground biomass C:N ratio (Figure 5.3a) was not significantly different between treatments (p=0.09), there was a slight trend for lower biomass C:N ratios with N addition. Root biomass C:N ratios (Figure 5.3b) were also not significantly different between treatments (p=0.77). Microbial C:N ratio (p=0.62), total soil C:N ratios (0-10 cm, p=0.75, 0-5 cm, p=0.19, 5-10 cm, p=0.50, and 10-15 cm depth, p=0.21) and soil fraction C:N ratios (coarse, p= 0.29, fine, p=0.44, and very fine, p=0.87) were not significantly different between treatments. However, there was a trend for slightly lower 10-15 cm depth soil C:N ratios in the N2-C0 and N2-RED treatments compared with control plots.

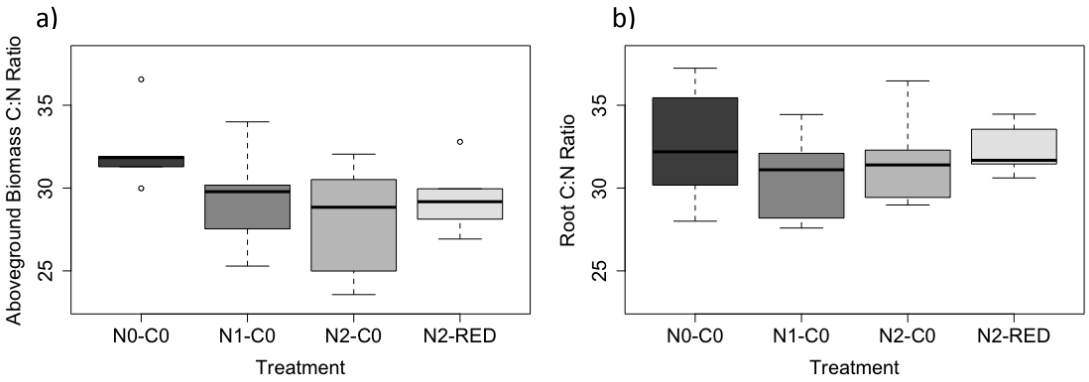

Figure 5.3: C:N ratios for aboveground (a) and root (b) biomass for each treatment. N treatments consist of 0 kg N ha^{-1} yr^{-1} (N0-C0), 4.3 kg N ha^{-1} yr^{-1} as NH_4NO_3 (N1-C0), 8.6 kg N ha^{-1} yr^{-1} as NH_4NO_3 (N2-C0), and 4.8 kg N ha^{-1} yr^{-1} as NH_4Cl (N2-RED).

5.3.5 Plant and microbial community structure

N addition did not lead to any significant changes to plant and microbial community structures. Total aboveground biomass (g m^{-2}) and species richness were not significantly different between treatments (p=0.20 and 0.67 respectively). Total PLFA biomass (µg g^{-1} dry soil, Figure 5.4a) was not significantly different between treatments (p=0.17). However, there was a trend for N2-C0 to have lower total PLFA biomass compared with control plots. Total fungal (p=0.32), total bacterial (p=0.30), Gram positive (Gram+, p=0.47) and Gram negative (Gram-, p=0.45) bacterial PLFAs were also not significantly different between treatments. Neither the soil fungal:bacterial ratio (Figure 5.4b), nor the Gram+:Gram- ratio were significantly different between treatments (p=0.92 and 0.72 respectively).

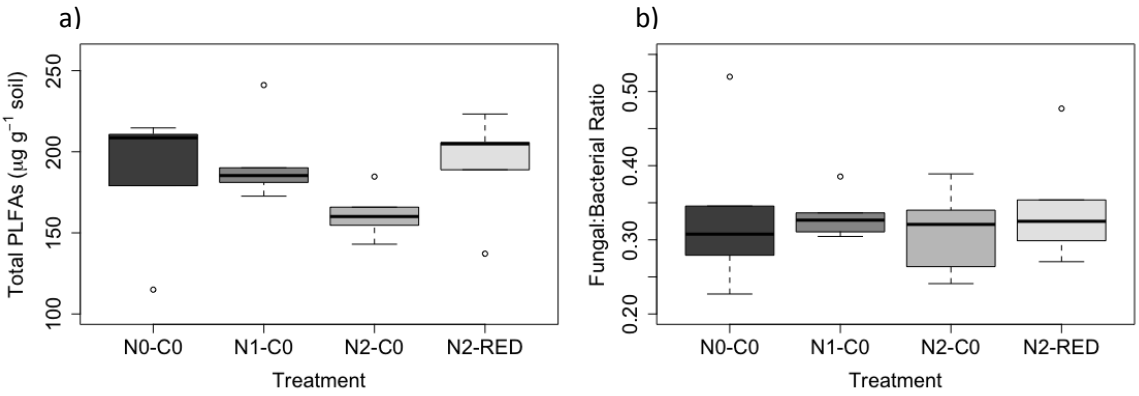

Figure 5.4: Total PLFA biomass (a) and fungal:bacterial ratio (b) for each treatment. N treatments consist of 0 kg N ha^{-1} yr^{-1} (N0-C0), 4.3 kg N ha^{-1} yr^{-1} as NH_4NO_3 (N1-C0), 8.6 kg N ha^{-1} yr^{-1} as NH_4NO_3 (N2-C0), and 4.8 kg N ha^{-1} yr^{-1} as NH_4Cl (N2-RED).

5.3.6 NPP and respiration

NPP values were highest in September and lowest in May, and respiration values were highest in June and lowest in October. However, neither NPP nor respiration were significantly different between treatments (p= 0.25 and 0.73 respectively, Figure 5.5).

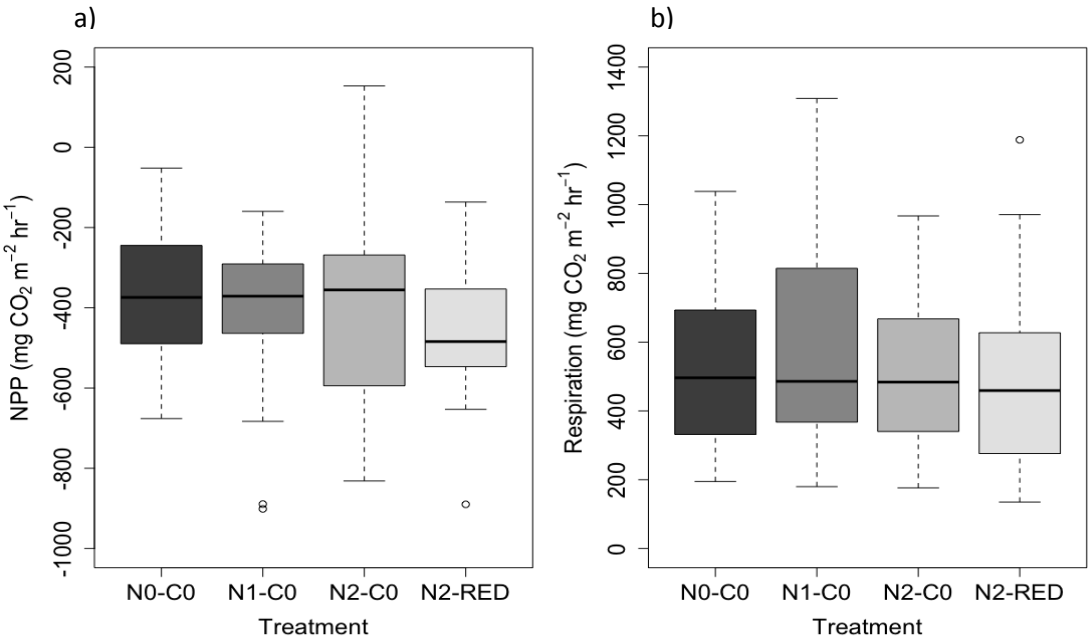

Figure 5.5: Net primary productivity (a) and respiration (b) for each treatment across all sampling days. N treatments consist of 0 kg N ha^{-1} yr^{-1} (N0-C0), 4.3 kg N ha^{-1} yr^{-1} as NH_4NO_3 (N1-C0), 8.6 kg N ha^{-1} yr^{-1} as NH_4NO_3 (N2-C0), and 4.8 kg N ha^{-1} yr^{-1} as NH_4Cl (N2-RED).

5.4 Discussion

5.4.1 Effects of N addition on N pools

None of the measures of N pools displayed any significant difference between treatments. This suggests that N addition in the range 4.3-8.6 kg ha^{-1} yr^{-1} was not high enough to have any observable effects on N cycling at this acid grassland, despite indications that this site is very N-limited (available N was found to be extremely low, with most plots displaying values of between 0 and 2 mg N m^{-2}). Background N deposition at Trefor is 9 kg ha^{-1} yr^{-1}, which is higher than the experimental N applications. This could mean that any changes observable at low N levels may already have occurred under regular N deposition at this site. Alternatively,

it is also possible that the experiment had not been running long enough for the effects of such low N doses to occur.

Despite the lack of significant effects, a few trends were observed. There was a trend for a slight increase in biomass N (as mg g^{-1}) with N addition, which could indicate that changes to vegetation chemistry were starting to occur. Pwllpeiran acid grassland in North Wales showed an increase in foliar %N after 15 years of N addition at a rate of 20 kg ha^{-1} yr^{-1}, but not at 10 kg ha^{-1} yr^{-1} (Phoenix et al., 2012). Foliar %N is known to respond relatively quickly to N addition in many systems, even at fairly low levels (UKREATE, 2008). However, these effects were found to be less pronounced in grassland experiments in a review by UKREATE (2008). In this experiment though, changes in foliar %N would be unlikely to have substantial effects on soil N (and C) pools, even in the long term. This is because biomass at this site was removed every year, and thus litter was not an important factor in this system. There was also a trend for slightly higher fine fraction N (kg m^{-2}) in the N2-C0 treatment, suggesting that N may be starting to accumulate in this soil fraction under 8.6 kg N ha^{-1} yr^{-1}. Indeed, N addition has been shown to increase the stability of recalcitrant organic matter by suppressing certain lignin-degrading enzymes (Peng et al., 2014). There was also a trend for slightly lower 10-15 cm depth soil C:N ratios in the N2-C0 and N2-RED treatments compared with control plots, which could suggest N accumulation further down the soil profile.

5.4.2 Effects of N addition on C pools

As with the N pools, none of the measures of C pools displayed any significant difference between treatments. A study by Wedin and Tilman (1996) found that total ecosystem C increased with low N addition rates (10 and 20 kg ha^{-1} yr^{-1}) in two C4 dominated fields, but not in a C3 dominated field (such as those common in the UK). Although the lack of changes to soil C pools had been predicted, it was thought that perhaps N addition would increase vegetation biomass (and thus the aboveground biomass C pool). However, this also did not occur. According to Phoenix *et al.* (2012), Pwllpeiran acid grassland also did not show any increases in productivity with N addition (10 and 20 kg ha^{-1} yr^{-1}).

At Trefor, there was a slight trend for lower aboveground biomass C:N ratios with N addition. This suggests that even N doses between 4.3 and 8.6 kg ha^{-1} yr^{-1} could potentially lead to an increase in the litter quality at this site. Nonetheless, due to the annual biomass harvest, this

is unlikely to have significant effects on soil C pools. Root growth rates and biomass C:N ratios also displayed no significant differences between treatments, and were therefore unlikely to have affected decomposition rates in the soil. However, there was a potential trend for lower root biomass C in the N2-RED treatment. N addition is known to reduce plant C allocation to roots due to a reduction in the need for nutrient mining (Zeng et al., 2010). Perhaps this effect might have begun to happen in the N2-RED treatment because ammonium uptake by plants is less energy intensive than oxidised N (which consists of 50% of the N addition in N1-C0 and N2-C0) and therefore easier for plants to absorb without investing in their root systems (Bloom et al., 1992). NPP and respiration were also not significantly different between treatments, which is probably due to the lack of change in plant biomass.

There was also a trend for increased microbial biomass C in the N2-C0 and N2-RED treatments compared with control plots. This could suggest that these treatments have somewhat stimulated labile organic matter decomposition. This is in line with other studies that suggest high N addition increases the decomposition of labile organic matter (Neff et al., 2002; Xu et al., 2004). However, a possible trend for slightly higher fine fraction C (kg m^{-2}) in the N2-C0 treatment compared with control plots was also observed. This might suggest an increase in recalcitrant organic matter C with N additions of 8.6 kg ha^{-1} yr^{-1}. At high N addition rates, N has been shown to produce recalcitrant compounds by reacting with litter components (Peng et al., 2014).

5.4.3 Effects of N addition on plant and microbial community structure

There were no effects of N addition on plant or microbial community structure, as results showed that N did not significantly affect species richness or the fungal:bactierial and Gram+:Gram- ratios between treatments. This is in contrast to a study by Clark and Tilman (2008), which showed that even N additions of 10 kg ha^{-1} yr^{-1} led to a 17% decrease in plant species richness in a heathland, though this was a long-term (23 year) experiment. Zong *et al.* (2016) on the other hand, found that species richness did not respond to N addition treatments (0, 10, 20, 40, and 80 kg ha^{-1} yr^{-1}) in an alpine meadow. However, they also found that changes to plant cover started to occur at the lowest rates of N addition.

There was a trend for N2-C0 to have lower total PLFA biomass compared with control plots. This suggests that 8.6 kg N ha^{-1} yr^{-1} may have been enough to begin reducing the active microbial biomass pool in these plots. This could potentially have repercussions for decomposition over longer time periods. At higher N addition rates, it has been shown that N can reduce total PLFA biomass, perhaps as a consequence of reduced soil organic carbon quality (Rousk et al., 2011).

5.5 Conclusions

Low levels (under 10 kg ha^{-1} yr^{-1}) of N addition did not have any significant effects on acidification, plant and microbial community compositions, or C and N pools at this acid grassland site. This suggests that a critical load for acid grasslands of 10 kg ha^{-1} yr^{-1} may be applicable at this site. In addition, there were no observable differences between the effects of N when applied as NH_4NO_3 or as NH_4Cl at these doses. Tipping *et al.* (2013) estimate that the threshold for acid grasslands is 7.9 kg N ha^{-1} yr^{-1}. Although the application of N at 8.6 kg ha^{-1} yr^{-1} did not produce any observable effects after 6/7 years, it is possible that this may not have been enough time for any long-term effects of N addition to occur.

Chapter 6: An in-depth assessment of direct and indirect effects of nitrogen deposition on carbon cycling and storage in grasslands

An in-depth assessment of direct and indirect effects of nitrogen deposition on carbon cycling and storage in grasslands

6.1 Abstract

In order to determine the effects of atmospheric nitrogen (N) deposition on carbon (C) cycling and storage in grasslands, it is necessary to differentiate between the direct effects of N fertilisation on soil and the indirect effects mediated by N-induced changes to plant community composition. By setting up a mesocosm experiment, we were able to manipulate species identity, number and functional group evenness in order to investigate the effects of community diversity on C cycling and storage in depth, and how this might be affected by N fertilisation. In addition, by using plant species identified as being associated with either high or low N environments, changes that might occur due to long term shifts in species composition caused by N deposition could also be assessed.

Community composition (especially the prevalence of species associated with either high or low N environments) significantly altered above and belowground biomass and chemistry, soil pH, microbial community composition, N mineralisation rate, ecosystem respiration and coarse soil fraction C storage. However, these effects were greatly affected by the presence of two dominant species, *Lotus corniculatus* and *Plantago lanceolata*. N addition altered ecosystem net primary productivity, microbial biomass N and C storage in the finer, mineral-associated soil fractions. Results suggest that while plant community composition had a much stronger influence on C storage in vegetation and microbal community structure, thus being the most important factor affecting fresh C input and decomposition in these systems, N addition may have had a more important role in the long-term storage of C in soil. In conclusion, both direct and indirect effects of N addition were important for C cycling and storage in these model grassland communities. This study further accentuates the need to take both effects into consideration when assessing the impact of N pollution on C stocks in grasslands.

6.2 Introduction

There is now compelling evidence that nitrogen (N) deposition affects the plant community composition of grassland environments (Bobbink et al., 1998; Dupre et al., 2010; Maskell et al., 2010; Stevens et al., 2011a; Stevens et al., 2010; van den Berg et al., 2011). N enrichment tends to favour fast growing, nitrophilous species such as grasses, often at the expense of slow growing species, leading to decreased plant species richness and diversity (Stevens et al., 2004). This evidence has led to a realisation among the scientific community that the impacts of N addition must be measured both in terms of its direct effects on soil chemistry and plant growth as well as on its indirect effects which are mediated by plant community change (Manning et al., 2006).

Although the effects of N on plant community composition have been clearly documented, what is less certain is how these altered plant communities will affect carbon (C) cycling and storage in soil. N-induced changes to diversity such as decreased species richness and functional evenness have been shown to alter aboveground productivity, root growth and turnover, and microbial diversity and biomass in grasslands (Cerabolini et al., 2010; Geisseler et al., 2016; Hector et al., 1999; Lamb et al., 2011; Lange et al., 2014; Steinbeiss et al., 2008; Tilman et al., 1996; Van der Krift and Berendse, 2002). Fast growing plant species such as grasses typically produce larger quantities of high quality litter, while slow growing species tend to be more conservative with nutrient use and thus have higher nutrient retention capabilities (Grime, 2001). By increasing the dominance of fast growing plant species, the indirect effects of N have the potential to speed up C and N cycling, which can lead to changes in the system's ability to store C. Therefore, in order to ascertain the effects of changes in plant community composition on C cycling and storage, it is necessary to differentiate between the direct and indirect effects of N deposition on soil.

A few studies have attempted to decouple the direct and indirect effects of N such as Manning *et al.* (2006; 2008), who developed plant communities under high and low N levels and then investigated what effects these different communities had on the soil. Another study by De Deyn *et al.* (2009) investigated the effects of N on different species mixtures by planting various combinations of three functional groups (grasses, forbs and legumes) into improved and unimproved soils. Although these studies yielded interesting results, the artificial nature of the experiments as well as the huge variability associated with this kind of research mean they must be built upon in order to determine what may be occurring in such

systems. Due to the complexity of the processes involved in plant-soil interactions, as well as the timescales involved, it is very difficult to determine whether any given process has an impact on soil C pools. Consequently, the use of highly controlled mesocosm experiments provides a relatively easy and cost-effective method of examining the processes involved in soil C storage in depth. Due to the issues surrounding the artificiality of these experiments, the extrapolation of results must be undertaken cautiously. However, useful information can be gathered about the minutiae of plant-soil interactions.

In order to obtain a clearer representation of the complex effects of N deposition on soil C, information must be gathered from research employing methods that attempt to replicate real life more closely by studying more sophisticated plant species mixtures. This mesocosm experiment seeks to build upon the foundations created by studies that have used experimental design focused on the importance of certain species identities and mixtures. However, rather than investigating systematic species mixtures such as those in De Deyn *et al.* (2009), it attempts to explore the interactions that occur within grassland plant communities when different aspects of plant community composition are altered. Rather than assessing species diversity as a whole, species identity, number and functional group evenness (in this case, dominance by grasses) are aspects of community diversity that have been manipulated in order to investigate diversity in depth. By examining these factors together, this experiment investigates how they interact, and whether any is more important with regards to C cycling and storage. In addition, by using plant species identified as being associated with either high or low N deposition (De Deyn et al., 2009; Payne et al., 2013; Stevens et al., 2011a; Stevens et al., 2004), changes that might occur due to long term shifts in species composition caused by N deposition can also be assessed.

Mesocosms were used to address the following hypotheses:

1. Species composition (indirect effects of N) would exert a stronger overall effect on C cycling and storage than N addition (direct effects of N), especially in soil.
2. Individual plant species would differ in their effects on C cycling and storage, and species associated with high N deposition would respond more efficiently to N addition, leading to stronger effects on C pools.
3. Plant communities dominated by species associated with either high or low N deposition would differ in their effects on C cycling and storage, and the effects of N

addition on C pools would be stronger for communities associated with high N deposition.

4. Communities containing species associated with both high and low N deposition (i.e. with a higher number of species) would be more beneficial for C storage and would be less affected by N addition than communities containing only one of these groups.

5. Communities with lower functional group evenness (i.e. dominated by grasses) would be less beneficial for C storage and would be more affected by N addition than communities with high evenness.

6.3 Results

6.3.1 Vegetation parameters

6.3.1.1 Monoculture treatments (Table 6.1)

Total aboveground biomass, and total aboveground biomass C (see Figure 6.1) and N were significantly different between plant species ($p<0.0001$ for all). However, these effects were highly dependent on N addition, as demonstrated by the significant interactions present between plant species and N addition for these variables ($p<0.001$ for all). N addition significantly increased the aboveground biomass, and aboveground biomass C and N of *A. capillaris, F. ovina, A. millefolium* and *A. odoratum.* Significant differences between species for aboveground biomass and aboveground biomass C were only found in the monocultures that did not receive N (with the exception of *C. rotundifolia*, which had significantly lower biomass and biomass C than other species even under N addition). The species with significantly higher biomass and biomass C without N addition were *L. corniculatus, P. lanceolata* and *T. repens*. The same three species also had the highest total aboveground biomass N without N addition. When N was added, the species with the highest biomass N were *L. corniculatus* and *T. repens*.

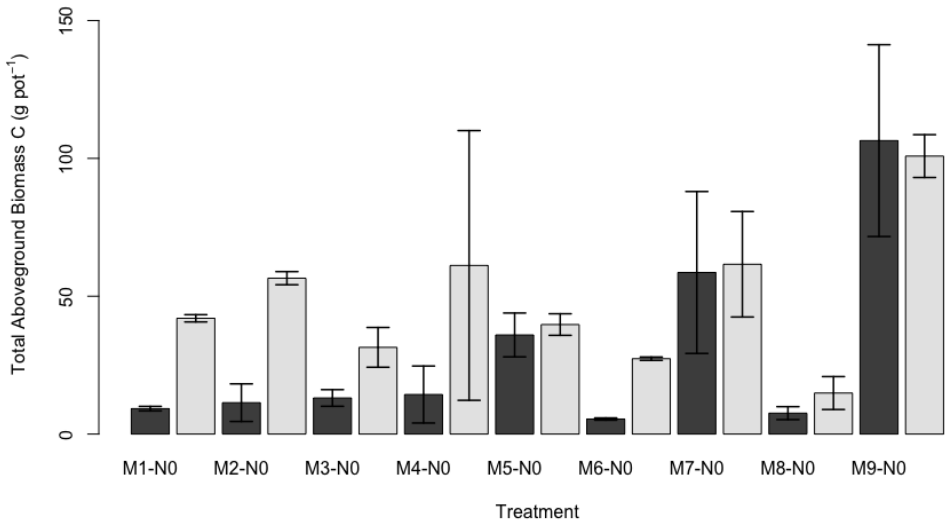

Figure 6.1: Total aboveground biomass C for monocultures with (light grey bars) and without (dark grey bars) N addition. Bars represent means, and error bars represent 1 standard deviation from the mean. Monoculture species are *A. capillaris* (M1), *F. ovina* (M2), *L. hispidus* (M3), *A. millefolium* (M4), *T. repens* (M5), *A. odoratum* (M6), *P. lanceolata* (M7), *C. rotundifolia* (M8), and *L. corniculatus* (M9).

Total aboveground biomass C:N ratio (see Figure 6.2) and N as mg per g (henceforth referred to as aboveground tissue N) were significantly different between species ($p<0.0001$ for both), but not between the levels of N addition. However, there were significant interactions between species and N addition for both these variables ($p<0.05$ for C:N and $p<0.01$ for aboveground tissue N). *F. ovina* had the highest C:N ratio and the lowest tissue N, but only when N was not added. The species with the lowest C:N ratios and the highest tissue N were *T. repens* and *L. corniculatus*, followed by *A. millefolium* with added N. Total aboveground biomass C as mg per g (henceforth referred to as aboveground tissue C) was significantly different between species ($p<0.0001$). There was also a significant interaction between species and N addition for this variable ($p<0.05$). The species with the highest tissue C were *L. corniculatus* and *C. rotundifolia*, followed by *F. ovina* with added N.

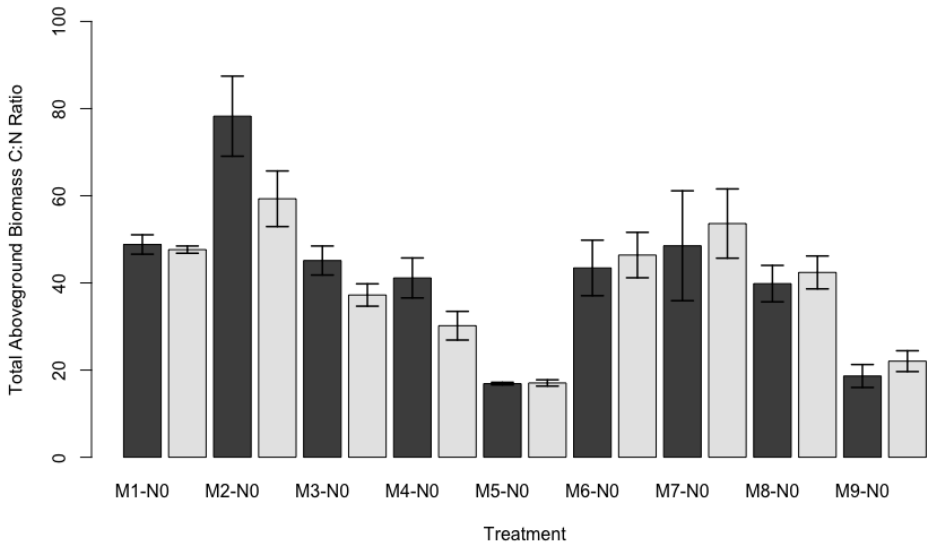

Figure 6.2: Total aboveground biomass C:N ratios for monocultures with (light grey bars) and without (dark grey bars) N addition. Bars represent means, and error bars represent 1 standard deviation from the mean. Monoculture species are *A. capillaris* (M1), *F. ovina* (M2), *L. hispidus* (M3), *A. millefolium* (M4), *T. repens* (M5), *A. odoratum* (M6), *P. lanceolata* (M7), *C. rotundifolia* (M8), and *L. corniculatus* (M9).

Total root biomass and root C (see Figure 6.3) and N were significantly different between species ($p<0.0001$ for all), but these effects were dependent on N addition, as demonstrated by the significant interactions present between plant species and N addition for these variables ($p<0.05$ for all). N addition led to a significant increase in root biomass and root C for *A. odoratum*, and to a significant increase in root N for *A. millefolium*. Under no N addition, the species with the highest root biomass and root C were *A. millefolium* and *L. corniculatus*, and the species with the highest root N was *L. corniculatus*. Under N addition, the species with the highest root biomass and root C was *A. millefolium*, the species with the lowest root biomass and root C was *T. repens*, and the species with the highest root N were *L. corniculatus* and *A. millefolium*.

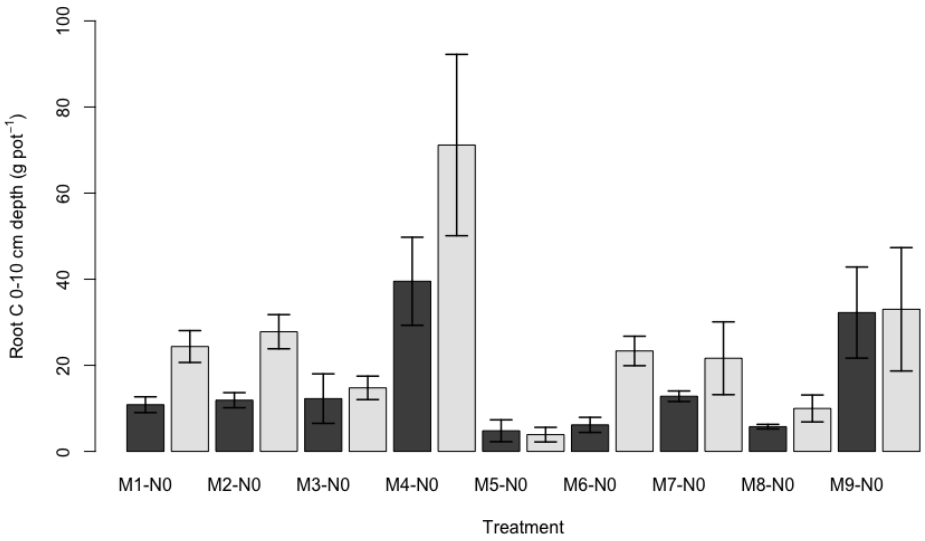

Figure 6.3: Total root C for monocultures with (light grey bars) and without (dark grey bars) N addition. Bars represent means, and error bars represent 1 standard deviation from the mean. Monoculture species are *A. capillaris* (M1), *F. ovina* (M2), *L. hispidus* (M3), *A. millefolium* (M4), *T. repens* (M5), *A. odoratum* (M6), *P. lanceolata* (M7), *C. rotundifolia* (M8), and *L. corniculatus* (M9).

Root C:N ratio (see Figure 6.4) and root N content as mg per g (henceforth referred to as root tissue N) were significantly different between species ($p<0.0001$ for both). However, these effects were dependent on N addition, as demonstrated by the significant interactions present between plant species and N addition for both variables ($p<0.001$ for the C:N ratio and <0.01 for root tissue N). N addition significantly decreased the root C:N ratio and significantly increased root tissue N in *A. millefolium*. The species with the highest root C:N ratio (and lowest root N content) were *A. capillaris, F. ovina* and also *A. millefolium* when it did not receive N. The species with the lowest root C:N ratio (and highest root N content) were *L. corniculatus* and *T. repens*, followed by *C. rotundifolia*. Root C as mg per g (henceforth referred to as root tissue C) was only significantly different between species ($p<0.0001$). The species with the highest root tissue C was *T. repens*, and the species with the lowest root tissue C was *A. millefolium*.

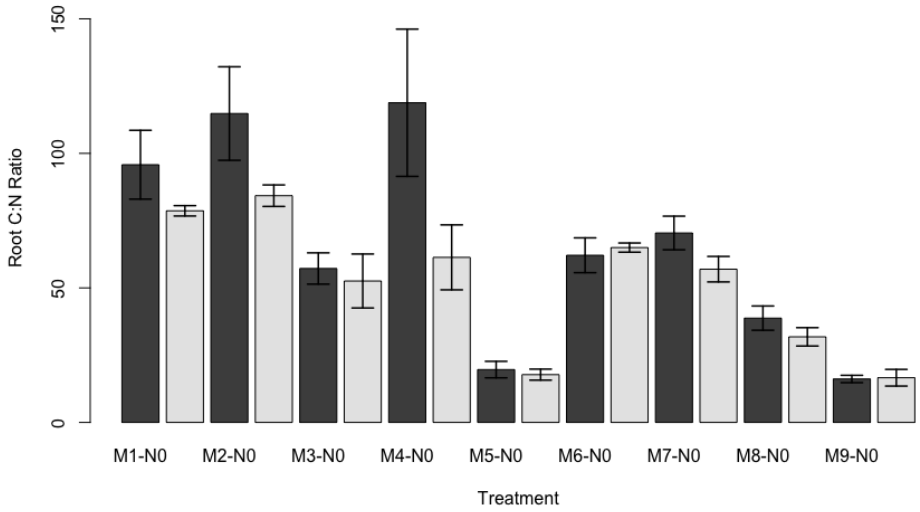

Figure 6.4: Root C:N ratios for monocultures with (light grey bars) and without (dark grey bars) N addition. Bars represent means, and error bars represent 1 standard deviation from the mean. Monoculture species are *A. capillaris* (M1), *F. ovina* (M2), *L. hispidus* (M3), *A. millefolium* (M4), *T. repens* (M5), *A. odoratum* (M6), *P. lanceolata* (M7), *C. rotundifolia* (M8), and *L. corniculatus* (M9).

Table 6.1: Plant species and fertiliser effects on vegetation parameters. p-values in bold indicate significant results.

Variable	Independent Variable	F-value	p-value
Total aboveground biomass (g pot-^{1})	Plant species	22.58	<0.0001
	N addition	70.91	<0.0001
	Plant species:N addition	4.89	<0.001
Total aboveground biomass C (g pot-^{1})	Plant species	23.01	<0.0001
	N addition	71.73	<0.0001
	Plant species:N addition	5.06	<0.001
Aboveground biomass tissue C (mg g^{-1})	Plant species	18.00	<0.0001
	N addition	6.00	<0.05
	Plant species:N addition	2.00	<0.05
Total aboveground biomass N (g pot-^{1})	Plant species	35.74	<0.0001
	N addition	64.61	<0.0001
	Plant species:N addition	5.71	<0.001
Aboveground biomass tissue N (mg g^{-1})	Plant species	76.21	<0.0001
	N addition	3.27	0.08
	Plant species:N addition	3.34	<0.01
Total aboveground biomass C:N Ratio	Plant species	76.59	<0.0001
	N addition	2.09	0.16
	Plant species:N addition	3.00	<0.05
Root biomass (g pot-^{1} for 10 cm depth)	Plant species	30.79	<0.0001
	N addition	29.10	<0.0001
	Plant species:N addition	2.38	<0.05
Total Root C (g pot-^{1} for 10 cm depth)	Plant species	30.39	<0.0001
	N addition	29.70	<0.0001
	Plant species:N addition	2.50	<0.05
Root tissue C (mg g^{-1})	Plant species	20.00	<0.0001
	N addition	0.00	0.65
	Plant species:N addition	1.00	0.21
Total Root N (g pot-^{1} for 10 cm depth)	Plant species	21.97	<0.0001
	N addition	40.61	<0.0001
	Plant species:N addition	2.37	<0.05
Root tissue N (mg g^{-1})	Plant species	146.34	<0.0001
	N addition	27.41	<0.0001
	Plant species:N addition	3.52	<0.01
Root C:N Ratio	Plant species	52.73	<0.0001
	N addition	28.02	<0.0001
	Plant species:N addition	5.27	<0.001

6.3.1.2 Species mixture treatments (Tables 6.2 and 6.3)

Total aboveground biomass was only significantly affected by N community prevalence, and not by species number, functional group evenness, or N addition. Total biomass was found to be significantly higher in 'low N' communities than in 'high N' communities ($p<0.0001$). However, aboveground biomass was strongly positively correlated with the percentage of

total biomass made up of the two 'low N' species *L. corniculatus* and *P. lanceolata* (R^2=0.73, p<0.0001). Together, these two species tended to make up a large percentage (often over 50%) of the aboveground biomass of all communities they were present in. *L. corniculatus* especially tended to dominate most of the pots where it was present.

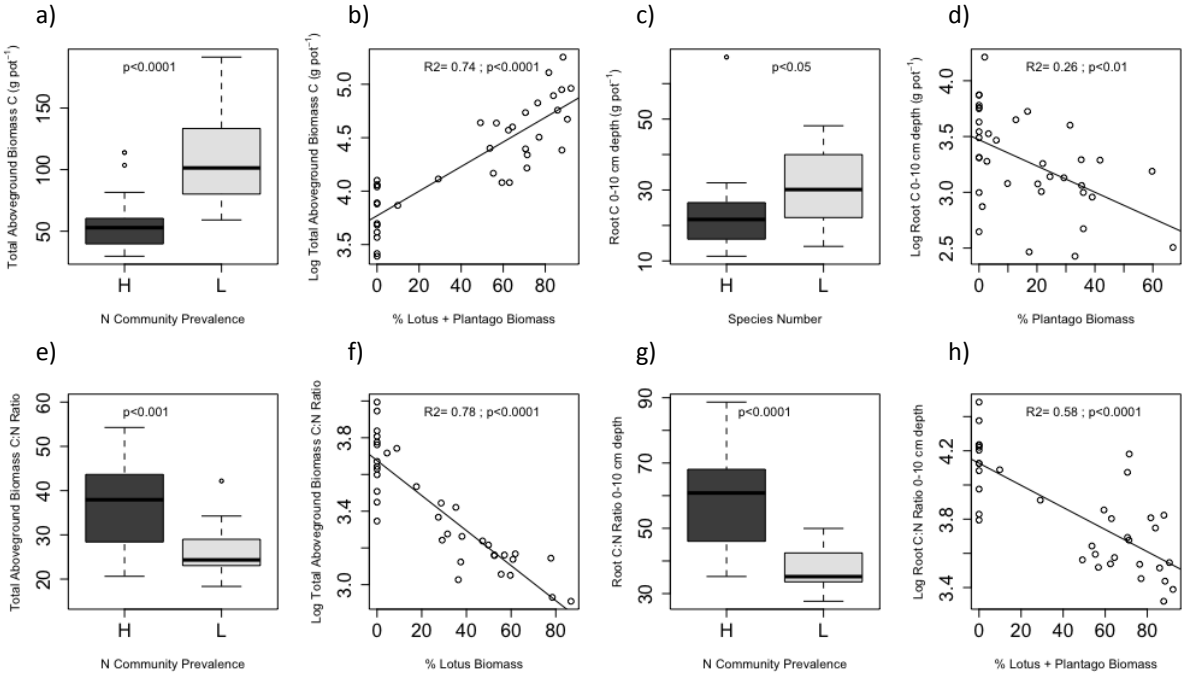

Figure 6.5: Total aboveground biomass C for high and low N communities (a) and its relationship with the percentage of total aboveground biomass made up of *L. corniculatus* and *P. lanceolata* biomass (b); Total root C for communities with high and low species numbers (c) and its relationship with the percentage of total aboveground biomass made up of *P. lanceolata* (d); Total aboveground biomass C:N ratios for high and low N communities (e) and its relationship with the percentage of total aboveground biomass made up of *L. corniculatus* (f); Total root C:N ratios for high and low N communities (g) and its relationship with the percentage of total aboveground biomass made up of *L. corniculatus* and *P. lanceolata* biomass (h).

Total aboveground biomass C (see Figure 6.5a) and N, aboveground tissue N, and biomass C:N ratio (see Figure 6.5e) were also only significantly affected by N community prevalence. Total aboveground biomass C and N and aboveground tissue N were significantly higher in 'low N' communities than in 'high N' communities (p<0.0001 for all), and biomass C:N ratio was significantly lower in the 'low N' communities (p<0.001). Total biomass C (see Figure 6.5b) and N were positively correlated with the percentage of total biomass made up of *L. corniculatus* and *P. lanceolata* (p<0.0001 for both, R^2=0.74 and 0.81 respectively), while aboveground tissue N was most strongly positively correlated with the percentage of total biomass made up of *L. corniculatus* only (p<0.0001, R^2=0.79). Biomass C:N ratio was most strongly negatively correlated with the percentage of total biomass made up of *L.*

corniculatus ($p<0.0001$, $R^2=0.78$; see Figure 6.5f). Aboveground tissue C was significantly affected by N community prevalence and by functional group evenness. Aboveground tissue C (see Figure 6.6) was significantly higher in both 'low N' communities ($p<0.0001$) and in mixtures with lower functional group evenness ($p<0.05$). There was also a significant interaction between N community prevalence and N addition for this variable ($p<0.05$). Aboveground tissue C was most strongly positively correlated with the percentage of total biomass made up of *L. corniculatus* ($p<0.0001$, $R^2=0.66$).

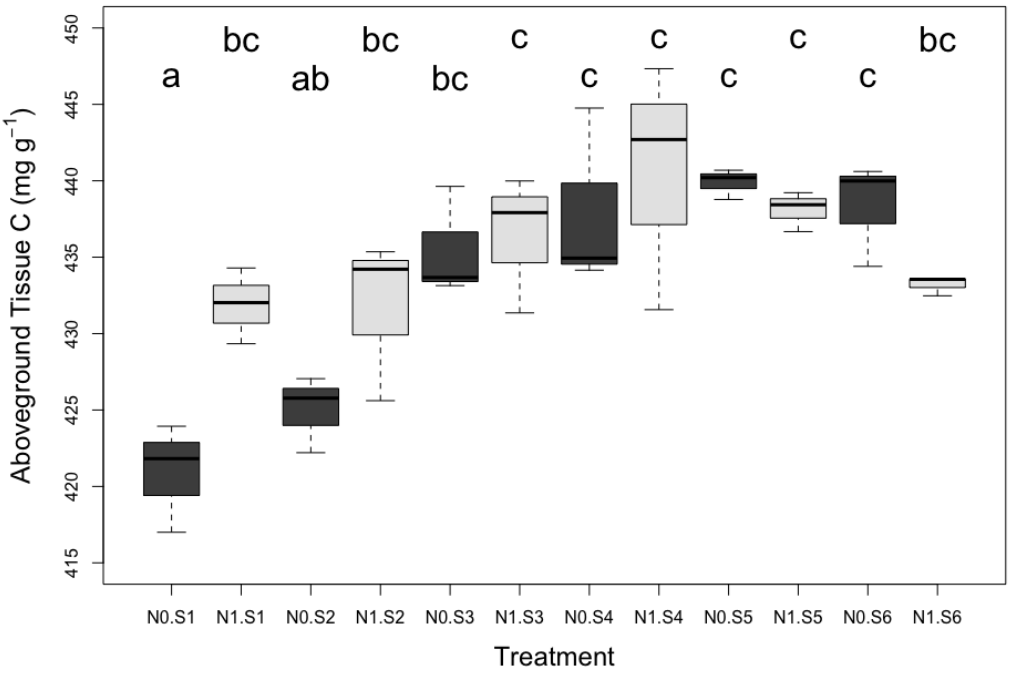

Figure 6.6: Aboveground tissue C for each species mixture treatment (S1-6). Dark grey boxes represent 0 kg N ha^{-1} yr^{-1}, light grey boxes represent 35 kg N ha^{-1} yr^{-1}. Letters above boxplots represent significant differences ($p<0.05$) between treatments as measured using a Tukey post-hoc test.

Total root biomass and root biomass C (see Figure 6.5c) were only significantly affected by species number. In addition, the effect of N addition on these variables was only marginally non-significant ($p=0.05$ for both). Root biomass and root C were significantly lower in mixtures with high species number ($p<0.05$ for both), and there was a trend for higher root biomass and root C with N addition. It was also found that root biomass and root C (see Figure 6.5d) were significantly negatively correlated with the percentage of total biomass made up of *P. lanceolata* ($p<0.01$, $R^2=0.26$ for both), and significantly positively correlated with the percentage of total biomass made up of *A. millefolium* ($p<0.05$, $R^2=0.16$ for both) and *A. capillaris* ($p<0.05$, $R^2=0.12$ for both).

There were trends for total root N to be higher in 'low N' communities as well as to increase with N addition. These trends were only marginally non-significant (p=0.06 and 0.07 respectively). Root tissue N and root C:N ratio (see Figure 6.5g) were only significantly affected by N community prevalence. Root tissue N was significantly higher and root C:N ratio was significantly lower in the 'low N' communities (p<0.0001 for both). Both root tissue N and root C:N ratio (see Figure 6.5h) were significantly correlated (positively and negatively respectively) with the percentage of total biomass made up of *L. corniculatus* and *P. lanceolata* (p<0.0001, R^2=0.58 for both). A significant positive correlation was also found between root tissue N and aboveground tissue N (p<0.0001, R^2=0.50). No such correlation was found between root tissue C and aboveground tissue C. No significant effects were found for root tissue C.

Table 6.2: Plant community treatment and fertiliser effects on aboveground vegetation parameters. p-values in bold indicate significant results.

Variable	Independent Variable	F-value	p-value
Total aboveground biomass (g pot-^{1})	Community prev. Species number Evenness N addition	29.44 2.21 1.62 0.20	<0.0001 0.15 0.21 0.66
Total aboveground biomass C (g pot-^{1})	Community prev. Species number Evenness N addition	30.09 2.28 1.74 0.24	<0.0001 0.14 0.20 0.63
Aboveground tissue C (mg g^{-1})	Community prev. Species number Evenness N addition Community : N addition	25.00 2.00 3.00 5.00 6.00	<0.0001 0.12 <0.05 0.13 <0.05
Total aboveground biomass N (g pot-^{1})	Community prev. Species number Evenness N addition	33.35 2.56 2.92 0.06	<0.0001 0.12 0.10 0.81
Aboveground tissue N (mg g^{-1})	Community prev. Species number Evenness N addition	17.98 1.43 2.95 1.25	<0.001 0.24 0.10 0.27
Total aboveground biomass C:N Ratio	Community prev. Species number Evenness N addition	17.05 1.34 2.77 1.48	<0.001 0.26 0.11 0.23

Table 6.3: Plant community treatment and fertiliser effects on belowground vegetation parameters. p-values in bold indicate significant results.

Variable	Independent Variable	F-value	p-value
Root biomass (g pot^{-1} for 10 cm depth)	Community prev. Species number Evenness N addition	0.99 4.21 0.64 4.05	0.33 <0.05 0.43 0.05
Total Root C (g pot^{-1} for 10 cm depth)	Community prev. Species number Evenness N addition	1.02 4.44 0.63 4.15	0.32 <0.05 0.43 0.05
Root tissue C (mg g^{-1})	Community prev. Species number Evenness N addition	0.10 0.00 0.30 0.30	0.79 0.97 0.62 0.60
Total Root N (g pot^{-1} for 10 cm depth)	Community prev. Species number Evenness N addition	3.91 1.40 0.00 3.66	0.06 0.25 0.99 0.07
Root tissue N (mg g^{-1})	Community prev. Species number Evenness N addition	44.09 1.70 2.68 0.24	<0.0001 0.20 0.11 0.63
Root C:N Ratio	Community prev. Species number Evenness N addition	42.56 1.67 2.44 0.29	<0.0001 0.21 0.13 0.59

6.3.2 Soil properties and microbial parameters

6.3.2.1 Monoculture treatments (Table 6.4)

Soil pH was only significantly affected by plant species ($p<0.0001$). The species with the highest soil pH was *F. ovina*, while the species with the lowest soil pH was *L. corniculatus*. However, the treatment with the lowest overall pH was the bare soil with added N. The soil fungal:bacterial ratio was significantly affected by both species and N addition ($p<0.05$ for both). When N was added to the legumes (i.e. *T. repens* and *L. corniculatus*), the fungal:bacterial ratio tended to decrease, whereas for other plant species the opposite tended to occur. Although individual species did not significantly differ from one another (as per the Tukey *post hoc* test) the lowest fungal:bacterial ratios tended to be in the bare soil treatments, as well as in the soil for *A. capillaris* and *F. ovina* (but only when N was not

added to these two species). No significant effects were found for soil Gram+:Gram- bacterial ratios, or total soil C and N. However, total soil C and N both tended to increase with N addition to legumes (and to the bare soil treatments), but decrease with N addition for all other species.

The total soil C:N ratio on the other hand, was significantly affected by plant species ($p<0.01$). The lowest C:N ratio was in the soil of the *T. repens* monocultures (especially when N was added). Microbial biomass C was significantly affected by plant species ($p<0.05$), whereas microbial biomass N was significantly affected by N addition ($p<0.01$). N addition significantly increased microbial biomass N. Although individual species did not significantly differ from one another (as per the Tukey *post hoc* test), the soil of the *A. millefolium, T. repens* and *L. corniculatus* monocultures tended to have lower microbial C, whereas the soil of the *C. rotundifolia* monocultures tended to have the highest microbial C. The microbial biomass C:N ratio was significantly affected by plant species ($p<0.01$). Once again, the individual species did not significantly differ from one another with regards to microbial C:N ratios. However, the lowest ratios tended to be in the soil of the *L. corniculatus* and *T. repens* monocultures.

Plant available N and N mineralisation rate were both significantly affected by plant species ($p<0.001$ for both). The highest levels of available N were present in the soil of the *T. repens* and the *L. corniculatus* (without N addition) monocultures, as well as in the bare soil treatment with N addition. N mineralisation rate was extremely low, with the exception of the soil from the *T. repens* and *L. corniculatus* monocultures. Soil DOC and DON were only significantly affected by plant species ($p<0.0001$ and <0.001 respectively). However, the effect of N addition on DOC was only marginally non-significant ($p=0.06$). The lowest DOC was in the soil of the *L. corniculatus* and *T. repens* monocultures, and although individual species did not significantly differ from one another (as per the Tukey *post hoc* test), the lowest DON tended to be in the soil of the *L. corniculatus, T. repens,* and *A. millefolium,* as well as the *P. lanceolata* (with added N) monocultures.

Table 6.4: Plant species and fertiliser effects on soil properties and microbial parameters. p-values in bold indicate significant results.

Variable	Independent Variable	F-value	p-value
pH	Plant species	8.81	<0.0001
	N addition	2.13	0.15
	Plant species:N addition	0.62	0.75
Fungal:Bacterial Ratio	Plant species	2.68	<0.05
	N addition	5.84	<0.05
	Plant species:N addition	1.49	0.21
Gram+:Gram- Bacterial Ratio	Plant species	1.60	0.17
	N addition	0.00	0.98
	Plant species:N addition	1.14	0.37
Microbial biomass C (mg pot^{-1} for 10 cm depth)	Plant species	2.42	<0.05
	N addition	1.25	0.27
	Plant species:N addition	0.11	1.00
Microbial biomass N (mg pot^{-1} for 10 cm depth)	Plant species	0.92	0.51
	N addition	11.28	<0.01
	Plant species:N addition	0.95	0.49
Microbial biomass C:N Ratio	Plant species	3.48	<0.01
	N addition	2.36	0.13
	Plant species:N addition	0.54	0.81
N mineralisation rate (mg pot^{-1} week^{-1} for 10 cm depth)	Plant species	18.07	<0.001
	N addition	1.52	0.23
	Plant species:N addition	1.20	0.33
Plant available N (mg pot^{-1} for 10 cm depth)	Plant species	26.67	<0.001
	N addition	0.60	0.44
	Plant species:N addition	1.96	0.09
Total soil C (g pot^{-1} for 10 cm depth)	Plant species	1.51	0.19
	N addition	0.22	0.64
	Plant species:N addition	1.35	0.26
Total soil N (g pot^{-1} for 10 cm depth)	Plant species	1.58	0.17
	N addition	0.01	0.90
	Plant species:N addition	1.50	0.20
Total soil C:N Ratio	Plant species	3.50	<0.01
	N addition	0.45	0.51
	Plant species:N addition	0.63	0.75
DOC (mg pot^{-1} for 10 cm depth)	Plant species	20.84	<0.0001
	N addition	3.68	0.06
	Plant species:N addition	1.64	0.15
DON (mg pot^{-1} for 10 cm depth)	Plant species	5.45	<0.001
	N addition	1.44	0.24
	Plant species:N addition	1.26	0.30

6.3.2.2 Species mixture treatments (Tables 6.5-6.8)

Soil pH was only significantly affected by N community prevalence. pH was significantly lower in 'low N' communities than in 'high N' communities ($p<0.01$). pH was also found to be significantly negatively correlated with the percentage of total biomass made up of *L. corniculatus* ($p<0.01$, $R^2=0.21$). The soil fungal:bacterial ratio was significantly affected by N community prevalence, with it being higher in the 'low N' communities than in the 'high N' communities ($p<0.01$; see Figure 6.7a). The fungal:bacterial ratio was also found to be significantly negatively correlated with soil DOC ($p<0.001$, $R^2=0.34$) and with soil DON ($p<0.05$, $R^2=0.18$), as well as significantly positively correlated with total root biomass N ($p<0.05$, $R^2=0.16$). Soil Gram+:Gram- bacterial ratio was significantly affected by species number, with the ratio being significantly higher for communities with high species number ($p<0.05$; see Figure 6.7b).

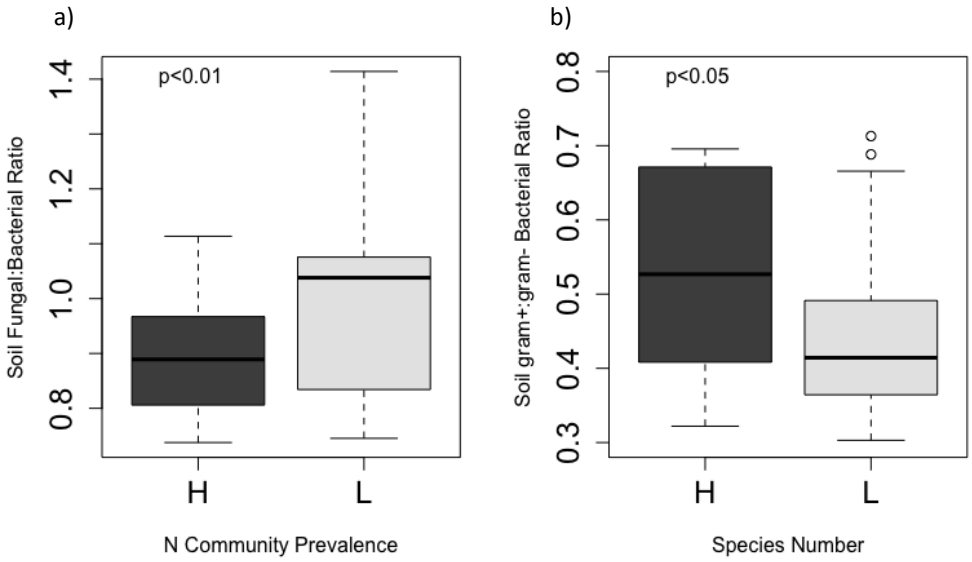

Figure 6.7: Soil fungal:bacterial ratios for high and low N communities (a), and soil Gram positive:Gram negative bacterial ratios for communities with high and low species numbers (b).

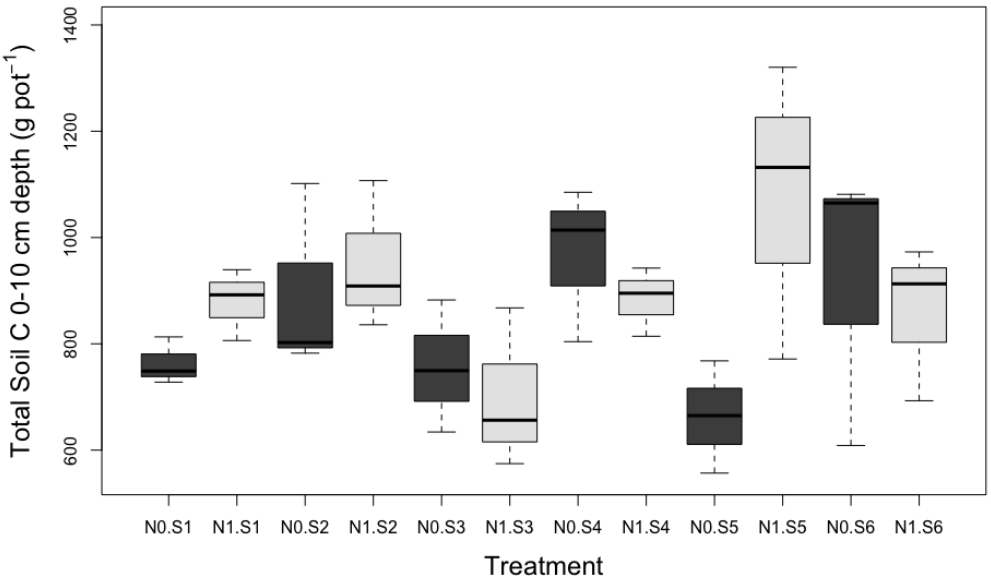

Figure 6.8: Total soil C (0-10 cm depth) for each species mixture treatment (S1-6). Dark grey boxes represent 0 kg N ha^{-1} yr^{-1}, light grey boxes represent 35 kg N ha^{-1} yr^{-1}.

Although N mineralisation rate was very low (between 0 and 0.8 mg per pot per week for 10 cm depth), it was also significantly affected by species number, with the rate being higher in communities with high species number ($p<0.05$). Microbial biomass N was significantly affected by N addition, with N addition significantly increasing this variable ($p<0.05$). No significant effects were found for microbial biomass C or C:N ratios, or for total soil C (see Figure 6.8), N or C:N ratios. However, there was a trend for soil C:N ratios to be higher for communities with high species number ($p=0.07$). No significant effects were found for plant available N, DOC or DON. However, there were trends for both DOC and DON to be lower for 'low N' communities than for 'high N' communities ($p=0.06$ and 0.07 respectively).

Table 6.5: Plant community treatment and fertiliser effects on soil properties and microbial parameters, PART 1. p-values in bold indicate significant results.

Variable	Independent Variable	F-value	p-value
pH	Community prev.	9.10	<0.01
	Species number	1.07	0.31
	Evenness	0.19	0.67
	N addition	0.15	0.70
Fungal:Bacterial Ratio	Community prev.	8.78	<0.01
	Species number	1.54	0.23
	Evenness	0.87	0.36
	N addition	0.15	0.70
Gram+:Gram- Bacterial Ratio	Community prev.	3.28	0.08
	Species number	5.02	<0.05
	Evenness	0.13	0.72
	N addition	0.24	0.63
Microbial biomass C (mg pot^{-1} for 10 cm depth)	Community prev.	1.57	0.22
	Species number	0.02	0.88
	Evenness	0.41	0.53
	N addition	0.53	0.47
Microbial biomass N (mg pot^{-1} for 10 cm depth)	Community prev.	0.32	0.58
	Species number	0.13	0.72
	Evenness	0.12	0.73
	N addition	7.44	<0.05
Microbial biomass C:N Ratio	Community prev.	1.33	0.26
	Species number	0.03	0.87
	Evenness	0.08	0.78
	N addition	1.91	0.18
N mineralisation rate (mg pot^{-1} week^{-1} for 10 cm depth)	Community prev.	0.01	0.93
	Species number	5.53	<0.05
	Evenness	0.00	0.98
	N addition	0.70	0.41
Plant available N (mg pot^{-1} for 10 cm depth)	Community prev.	0.08	0.78
	Species number	0.61	0.44
	Evenness	0.15	0.70
	N addition	0.01	0.92
Total soil C (g pot^{-1} for 10 cm depth)	Community prev.	1.47	0.24
	Species number	1.54	0.23
	Evenness	0.08	0.78
	N addition	1.21	0.28
Total soil N (g pot^{-1} for 10 cm depth)	Community prev.	1.03	0.32
	Species number	2.35	0.14
	Evenness	0.00	0.95
	N addition	0.47	0.50
Total soil C:N Ratio	Community prev.	0.01	0.92
	Species number	3.52	0.07
	Evenness	0.00	0.97
	N addition	0.09	0.77

Table 6.6: Plant community treatment and fertiliser effects on soil properties and microbial parameters, PART 2. p-values in bold indicate significant results.

Variable	Independent Variable	F-value	p-value
DOC (mg pot-^{1} for 10 cm depth)	Community prev. Species number Evenness N addition	3.99 0.81 0.06 1.12	0.06 0.38 0.81 0.30
DON (mg pot-^{1} for 10 cm depth)	Community prev. Species number Evenness N addition	3.48 0.00 0.55 0.08	0.07 0.96 0.46 0.78

Soils collected from the species mixture treatments were also separated into three size fractions (coarse, 2mm-200 µm; fine, 200-50 µm; very fine, 50-0.45 µm) in order to assess whether the species mixtures and the N addition had led to more subtle changes to soil C and N stocks. Coarse fraction C was significantly affected by N community prevalence, with C being higher in the 'low N' communities ($p<0.05$; see Figure 6.9a). Although no significant effects were found for coarse C as a proportion of total C stock, there was a trend for this variable to be higher in 'low N' communities ($p=0.06$; see Figure 6.9d). Fine fraction C on the other hand, was significantly affected by N addition, with C being higher with N addition than without ($p<0.05$; see Figure 6.9b). Fine C as a proportion of total C stock was also significantly affected by N addition, with this variable being higher with N addition as well ($p<0.01$; see Figure 6.9e). No significant effects were found for very fine fraction C (see Figure 6.9c), however, very fine C as a proportion of total C stock was significantly affected by N addition, with this proportion decreasing with N addition ($p<0.05$; see Figure 6.9f). DOC of the fractionation water and DOC as a proportion of total C stock were significantly affected by N addition, with both being lower with N addition than without ($p<0.001$ and <0.01 respectively).

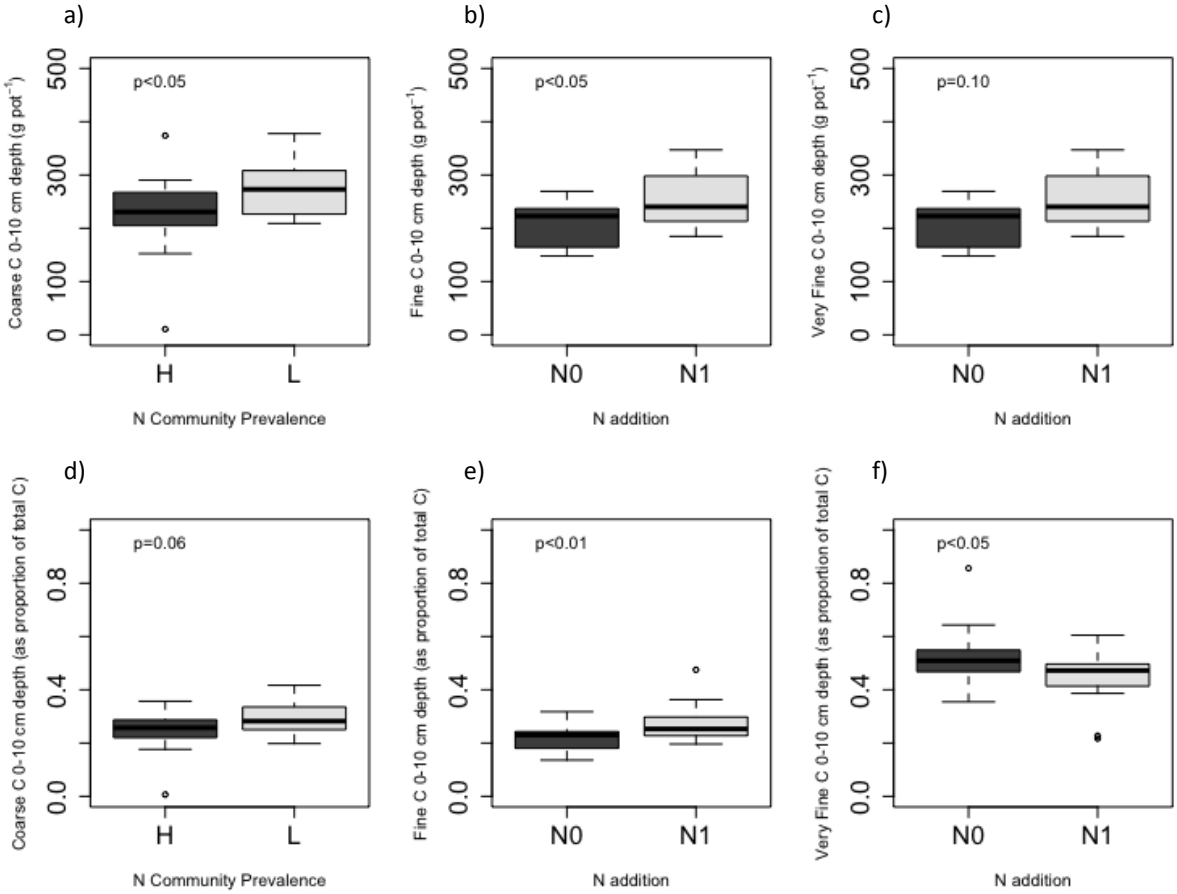

Figure 6.9: Coarse fraction C for high and low N communities (a); fine (b) and very fine (c) fraction C for 0 (N0) and 35 kg ha^{-1} yr^{-1} (N1) N addition; coarse C as a proportion of total C stocks for high and low N communities (d); fine (e) and very fine (f) fraction C as a proportion of total C stocks for 0 (N0) and 35 kg ha^{-1} yr^{-1} (N1) N addition.

No significant effects were found for both coarse fraction N and coarse N as a proportion of total C stock. However, there was a trend for higher coarse N in 'low N' communities ($p=0.06$). Fine fraction N was significantly affected by N addition, with fine N increasing with N addition ($p<0.01$). No significant effects were found for fine N as a fraction of total N stock, very fine fraction N, or very fine N as a proportion of N stock. No significant effects were found for the coarse fraction C:N ratio, or for the very fine fraction C:N ratio. However, fine fraction C:N ratio was significantly affected by N addition, with the ratio decreasing with N addition ($p<0.05$). Also, there was a trend for very fine fraction C:N ratio to decrease with N addition ($p<0.08$).

Table 6.7: Plant community treatment and fertiliser effects on soil physical fractionation results. All fractionation results were measured in g pot^{-1} to 10 cm depth, PART 1. p-values in bold indicate significant results.

Variable	Independent Variable	F-value	p-value
Coarse C (g pot^{-1} for 10 cm depth)	Community prev.	4.98	<0.05
	Species number	0.01	0.93
	Evenness	0.34	0.56
	N addition	0.61	0.44
Fine C (g pot^{-1} for 10 cm depth)	Community prev.	1.28	0.27
	Species number	0.38	0.54
	Evenness	0.03	0.87
	N addition	7.41	<0.05
Very fine C (g pot^{-1} for 10 cm depth)	Community prev.	0.97	0.33
	Species number	1.94	0.17
	Evenness	0.39	0.54
	N addition	2.90	0.10
DOC (g pot^{-1} for 10 cm depth)	Community prev.	0.06	0.82
	Species number	0.10	0.76
	Evenness	0.00	0.97
	N addition	15.16	<0.001
Proportion coarse C	Community prev.	3.80	0.06
	Species number	0.44	0.51
	Evenness	0.50	0.48
	N addition	0.78	0.39
Proportion fine C	Community prev.	1.04	0.32
	Species number	0.87	0.36
	Evenness	0.41	0.53
	N addition	8.93	<0.01
Proportion very fine C	Community prev.	2.41	0.13
	Species number	0.76	0.39
	Evenness	0.24	0.63
	N addition	4.54	<0.05
Proportion DOC	Community prev.	0.06	0.82
	Species number	0.78	0.39
	Evenness	0.14	0.71
	N addition	8.32	<0.01
Coarse N (g pot^{-1} for 10 cm depth)	Community prev.	3.98	0.06
	Species number	0.05	0.83
	Evenness	0.03	0.86
	N addition	0.24	0.63
Fine N (g pot^{-1} for 10 cm depth)	Community prev.	1.32	0.26
	Species number	0.13	0.73
	Evenness	0.00	0.98
	N addition	8.24	<0.01
Very fine N (g pot^{-1} for 10 cm depth)	Community prev.	0.07	0.79
	Species number	0.05	0.83
	Evenness	0.57	0.46
	N addition	0.18	0.68

Table 6.8: Plant community treatment and fertiliser effects on soil physical fractionation results. All fractionation results were measured in g pot^{-1} to 10 cm depth, PART 2. p-values in bold indicate significant results.

Variable	Independent Variable	F-value	p-value
Proportion coarse N	Community prev.	2.23	0.15
	Species number	0.00	0.96
	Evenness	0.24	0.63
	N addition	0.04	0.84
Proportion fine N	Community prev.	0.26	0.61
	Species number	0.02	0.89
	Evenness	0.43	0.52
	N addition	1.86	0.18
Proportion very fine N	Community prev.	0.63	0.44
	Species number	0.06	0.81
	Evenness	0.34	0.56
	N addition	1.30	0.26
Coarse C:N ratio	Community prev.	1.23	0.28
	Species number	0.19	0.67
	Evenness	0.36	0.55
	N addition	0.09	0.77
Fine C:N ratio	Community prev.	0.46	0.50
	Species number	0.04	0.84
	Evenness	0.26	0.62
	N addition	5.15	<0.05
Very fine C:N ratio	Community prev.	1.12	0.30
	Species number	1.08	0.31
	Evenness	0.18	0.67
	N addition	3.39	0.08

6.3.3 Ecosystem net primary productivity and respiration

6.3.3.1 Monoculture treatments (Table 6.9)

Ecosystem net primary productivity (NPP) was significantly affected by N addition ($p<0.01$). N addition significantly increased overall NPP (i.e. the amount of C sequestered by the monoculture treatments). Although not significantly different from the other species, there was a trend for higher NPP in the *A. millefolium* (with N addition) and *L. corniculatus* (without N addition) monocultures. Ecosystem respiration rates were significantly affected by plant species as well as N addition ($p<0.001$ for both). There was also a significant interaction between species and N addition for respiration rates ($p<0.01$). N addition led to a

significant overall increase in ecosystem respiration for the monoculture treatments. Without N addition, the species with the highest respiration were *L. corniculatus, P. lanceolata* and *T. repens*. With N addition the differences between species were smaller, but *A. millefolium,* followed by *L. corniculatus* and *T. repens* tended to display the highest respiration rates.

Table 6.9: Plant species and fertiliser effects on ecosystem net primary productivity (NPP) and respiration. p-values in bold indicate significant results.

Variable	Independent Variable	F-value	p-value
NPP (mg CO_2-C m^{-2} hr^{-1})	Plant species	0.98	0.47
	N addition	7.72	<0.01
	Plant species:N addition	1.13	0.37
	PAR	4.63	<0.05
Respiration (mg CO_2-C m^{-2} hr^{-1})	Plant species	8.93	<0.001
	N addition	43.92	<0.001
	Plant species:N addition	3.79	<0.01
	Soil moisture	0.65	0.43
	Soil temperature	0.01	0.95

6.3.3.2 Species mixture treatments (Table 6.10)

Ecosystem net primary productivity (NPP) was significantly affected by N addition ($p<0.05$; see Figure 6.10a). N addition significantly increased the amount of C sequestered by the species mixtures. There was also a significant correlation between NPP and total aboveground biomass ($p<0.05$, but only when PAR was used as a co-factor). Ecosystem respiration was significantly affected by N community prevalence ($p<0.001$; see Figure 6.10b). Respiration rates were significantly higher in the 'low N' communities. This effect was likely driven by total aboveground biomass (which is higher in 'low N' communities), as the two variables were significantly positively correlated ($p<0.0001$, $R^2=0.58$).

Table 6.10: Plant community treatment and fertiliser effects on ecosystem net primary productivity (NPP) and respiration. p-values in bold indicate significant results.

Variable	Independent Variable	F-value	p-value
NPP (mg CO_2-C m^{-2} hr^{-1})	Community prev.	2.80	0.11
	Species number	0.00	0.97
	Evenness	0.01	0.94
	N addition	5.72	<0.05
	PAR	16.14	<0.001
Respiration (mg CO_2-C m^{-2} hr^{-1})	Community prev.	21.48	<0.001
	Species number	0.14	0.71
	Evenness	0.55	0.47
	N addition	0.01	0.93
	Soil moisture	1.39	0.25
	Soil temperature	0.50	0.49

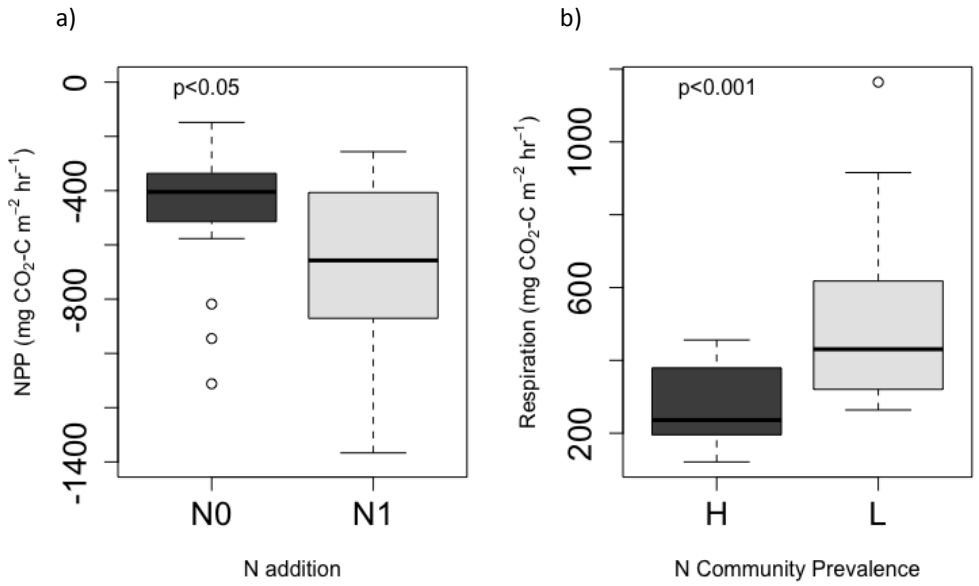

Figure 6.10: Net ecosystem primary productivity (NPP) for 0 (N0) and 35 kg ha^{-1} yr^{-1} (N1) N addition (a), and ecosystem respiration for high and low N communities (b).

6.4 Discussion

6.4.1 The direct effects of N

6.4.1.1 Monocultures

All above and belowground biomass variables with the exception of root tissue C showed significant interactions between plant species and N addition. Although N addition only significantly increased the biomass of *A. capillaris, F. ovina, A. millefolium* and *A. odoratum*, there was a trend for most species to gain biomass with N fertilisation. The only exceptions were the two legumes and *P. lanceolata*. The lack of effect of N on *L. corniculatus* and *T. repens* was likely due to these species' ability to fix N, therefore providing enough N for growth even without fertilisation. The ability of legumes to produce high levels of biomass via N_2 fixation is described by Craine *et al.* (2002). The lack of growth response to N addition from *P. lanceolata* contrasts with the findings of Berendse (1983), who found that N addition increased *P. lanceolata* biomass in monocultures. However, as *P. lanceolata* produced large quantities of aboveground biomass, one reason why N addition did not affect this species' biomass could have been due to a limitation by another nutrient. Nutrient co-limitation as a constraint on plant growth has been described by Harpole *et al.* (2011). This meant that without N addition, *L. corniculatus, T. repens* and *P. lanceolata* clearly had the largest aboveground biomass, but once N was applied this difference was no longer so apparent.

The positive impact of N addition on plant biomass is presented in many studies (Baer and Blair, 2008; LeBauer and Treseder, 2008; Semmartin and Oesterheld, 2001; Zhang et al., 2015). The results of this experiment suggest that N addition is most beneficial to the biomass of grasses, as well as the forb *A. millefolium*. This positive effect of N addition on grass biomass has been described in previous studies. In their meta-analysis, De Schrijver *et al.* found that N addition significantly increased the biomass of graminoid species, but did not elicit a response from forbs. Aboveground tissue chemistry of the different species was also dependent on N addition. *F. ovina* for example, had the highest tissue C:N ratio without N addition, but this was not the case when N was applied.

Root biomass of different species was also dependent on N addition. Although there was a trend for most species' root biomass to increase with N addition (the exceptions being the

legumes), the only significant increase was in the *A. odoratum* monocultures. This result is in line with the work of Manning *et al.* (2006), who found that N addition increased root biomass. There was also a trend for most species' root C:N ratios to decrease with N addition, however this decrease was only significant for *A. millefolium*. As the species with the largest root biomass, *A. millefolium*'s decrease in root C:N ratio with N addition could mean that N fertilisation may have a large effect on the quality of belowground organic matter input in communities where this species is abundant. This could have an impact on soil C storage and cycling, as lower root C:N ratios are known to increase root decomposition rates (Silver and Miya, 2001).

In the monoculture treatments, the only microbial parameters significantly affected by N addition were microbial biomass N and the fungal:bacterial ratio. In addition, there were no significant interactions between species and N addition for any soil or microbial variables measured. This suggests that N addition did not have strong direct effects on the soil or the microbial communities of the monoculture treatments.

N addition has been shown to increase both NPP and respiration rates in grasslands (Verburg et al., 2004; Zhou et al., 2014), and this is what occurred in the monoculture treatments. Furthermore, the effect of plant species on respiration was dependent on N addition. Without N addition, the highest respiration rates occurred in the *L. corniculatus*, *T. repens* and *P. lanceolata* monocultures. This was probably due to the higher biomass of these species. With N addition however, this difference became much less clear, and *A. millefolium* became the species with the highest respiration rates. By increasing respiration rates and NPP in the monocultures, N addition increased rates of C cycling in vegetation.

Overall, the productivity and chemistry of several grass and forb species depended on whether N was added or not. This suggests that the direct effects of N addition may have an important role in how species identity affects soil C storage and cycling via plant material input into the system. This result is in line with the work of Manning *et al.* (2006), who found that the direct effects of N on plant growth dominate ecosystem response to N addition. However, N addition had very little effect on how species identity affected any measured soil and microbial properties. This may have been because the experiment was running for too little time, or perhaps because the effects of species identity were strong enough to override the influence of N addition on soil biotic and abiotic factors, despite its effect on vegetation. It was expected that the species selected for being associated with high N environments

would have a larger effect on C cycling and storage with N addition due to higher aboveground productivity and increased tissue quality. However, this was not the case, as the effects of 'high N' and 'low N' species on C cycling and storage in monocultures were not clearly distinguishable.

6.4.1.2 Species mixtures

N addition did not significantly affect any above or belowground vegetation variables measured in the species mixture treatments. The lack of effect of N on the aboveground biomass of species mixture treatments was unexpected, as many studies have reported the positive effects of N fertilisation on aboveground plant biomass (Baer and Blair, 2008; LeBauer and Treseder, 2008; Semmartin and Oesterheld, 2001; Zhang et al., 2015). However, this effect was likely due to the fact that *L. corniculatus* and *P. lanceolata*, two species that were not affected by N addition in the monocultures, drove the biomass in these communities. There were nonetheless, marginally non-significant trends for root biomass, root C and root N to increase with N addition. Contrastingly, Bardgett *et al.* (1999) found that N addition (100 kg ha^{-1}) significantly reduced root biomass in temperate upland grassland species. Zeng *et al.* (2010) also found that N addition (200 kg ha^{-1} yr^{-1}) decreased the root C pool in a semi-arid grassland. Data suggests that the direct effects of N addition may have had a limited impact on C cycling and storage in the vegetation biomass of the species mixture treatments.

In the species mixture treatments the only microbial parameter measured that was significantly affected by N addition was microbial biomass N. These findings are in contrast with those of Bardgett *et al.* (1999), who found that N addition significantly altered the structure of the microbial community in favour of fungi in a temperate upland grassland. However, Bardgett *et al.* also concluded that overall, plant traits (and therefore plant identity and community composition) were more important than N fertilisation for determining microbial activity and possibly diversity. These results indicate that the direct effects of N only had a minor influence on the soil microbial community in the species mixture treatments. One reason for this could be that soil microorganisms are affected more by variations amongst plant species in root exudation patterns than by N addition (Bardgett et al., 1999).

Although N addition did not affect any overall soil C and N pools in the species mixture treatments, it was found to significantly affect the finer soil C and N fractions. These finer soil fractions typically contain older, more stable mineral-associated C, and are therefore arguably more important for long term C storage (De Deyn et al., 2011b; Puget et al., 2000). By increasing the amount and proportion of C stored in the fine soil fraction, N addition may have had a positive effect on soil C stocks. However, by decreasing the proportion of C stored in the very fine soil fraction, N addition may have also had a negative effect on long-term C storage. This result is in line with Manning *et al.* (2006), who found that mineral-associated C (defined as <53 μm) was lower under high N addition (44 kg N ha^{-1} yr^{-1}). Song *et al.* (2014) on the other hand, found that 9 years of N addition to a semiarid grassland increased C in the soil fraction associated with labile C, but did not affect the more stable, recalcitrant soil C.

In the species mixture treatments N addition positively affected ecosystem NPP. This is in line with the work of Manning *et al.* (2006), who found that N addition increased NEP in their experiment. NPP is known to increase with N addition in grasslands (Semmartin et al., 2007). By increasing NPP, N addition increased the amount of C entering the species mixture treatments via the vegetation. This suggests that N addition may play an important role in vegetation C uptake. In their terrestrial ecosystem model for example, McGuire *et al.* (1992) found that N-induced increases in NPP more than offset increased plant respiration in North America.

Overall, results suggest that unlike in the monoculture treatments, the direct effects of N did not affect vegetation productivity or chemistry in the species mixture treatments. They also had minor effects on soil microbial biomass and no effects on total soil C and N pools. However, N addition directly affected NPP and the finer soil C and N fractions, which could have implications for long-term C storage.

6.4.2 The indirect effects of N

6.4.2.1 Plant species identity (monocultures)

All above and belowground biomass variables measured were significantly different between plant species monocultures. However, as mentioned in section 5.5.1.1, all but root tissue C were also dependent on N addition. Nonetheless, the highest potential for C input from aboveground plant litter came from the legumes *L. corniculatus* and *T. repens*, and the forb *P. lanceolata*, and the highest quality litter came from the legumes. The results for *L. corniculatus* are in line with the findings of De Deyn *et al.* (2009), who found that *L. corniculatus* consistently stored the most C and N in vegetation compared with other grassland species monocultures.

However, it must be noted that because aboveground biomass was removed from the mesocosms, the most important plant input came from root turnover and exudation. Root biomass input is known to depend on species identity (Rees et al., 2005). The most productive species belowground was *A. millefolium*, and therefore this species could have had the highest belowground soil C inputs. However, the highest quality root tissue was found in the *L. corniculatus* and *T. repens* monocultures, so these species may have had a strong effect on the rate of soil C cycling. These findings are once again in accordance with the work of De Deyn *et al.* (2009), who found that *A. millefolium* produced the most root biomass and that *L. corniculatus* and *T. repens* had the lowest root C:N ratios.

Despite significant differences between species in both above and belowground growth and chemistry, total soil C and N stocks did not differ between monocultures. Large changes to total soil C and N pools were not expected due to the short-term nature of this experiment. However, the legume monocultures were expected to have some positive effects on total soil C and N as previous studies have shown that some legume species (including *L. corniculatus* and *T. repens*) can lead to increases in soil C and N stocks (De Deyn et al., 2009; De Deyn et al., 2011b). The lack of change in soil N may have occurred due to high uptake by plants, and the lack of change in soil C could have been because two growing seasons were not enough for changes to total soil C stocks to occur, especially given that plant aboveground biomass was harvested.

However, soil C:N ratios were significantly different between species, with the *T. repens* monoculture soil having the lowest ratio. This was likely due to N fixation by *T. repens*. Soil available N and N mineralisation rates were also different between plant species. Both variables were very low for all species monocultures except for those of *T. repens* and *L. corniculatus*, which was probably due to the legumes' ability to fix N. Craine *et al.* (2002) describe how non-legume species monocultures that can sustain large amounts of biomass tend to have low extractable inorganic nitrogen and N mineralization in their soils. Soil pH was also lower in the soils of the two legume monocultures, and this can probably be attributed to the higher levels of available N and N mineralisation in these soils (as nitrification releases H^+ ions (Bolan et al., 1991)).

Soil DOC and DON were found to be different between plant species. DOC in particular was noticeably lower in *L. corniculatus* and *T. repens* monocultures, which could be related to lower soil C:N ratios (van den Berg et al., 2012). Interestingly, microbial biomass C was found to be lowest in the soils of three of the most productive species (*L. corniculatus, T. repens* and *A. millefolium*). This suggests high levels of competition for C between plants and microbes. Indeed, it has been suggested that some microorganisms may be negatively affected by plants with a high N uptake rate (Moreau et al., 2015). The soil fungal:bacterial ratio was significantly affected by both species identity and N addition, with the lowest ratios occurring in the *A. capillaris* and *F. ovina* monocultures (with no N addition), and in the bare soil pots. These changes to the soil microbial community could have consequences for soil C storage in the long run. Bardgett *et al.* (1999) also found that soil fungal:bacterial ratios were significantly different between grassland plant species. They attributed these effects of species identity on soil biota to specific plant physiological traits, independent of environmental factors.

Overall, above and belowground vegetation was affected by species identity, although it must be noted that this often depended on N addition (as described in section 5.5.1.1). Therefore, the indirect effects of N mediated by species identity are likely to be less important than the direct effects of N on plant growth and chemistry. Soil and microbial variables on the other hand, were strongly affected by species identity, and apart from microbial biomass N and the fungal:bacterial ratio, were not affected by N addition. Therefore, the indirect effects of N mediated by species identity are likely to have an important effect on soil biotic and abiotic processes.

6.4.2.2 High vs. low N-associated communities

See Figure 6.11 for diagram of C fluxes and pools for these communities. N community prevalence was the most important aspect of community composition for aboveground biomass. However, the effects of N community prevalence on aboveground biomass were strongly driven by the influence of two 'low N' species (*L. corniculatus* and *P. lanceolata*), which dominated the biomass of the communities in which they were present. Dominant plant species are thought to have a disproportionate influence on ecosystem processes via the "mass ratio" effect, as they contribute most of the biomass that actively controls fluxes of energy and matter through the system (Cerabolini et al., 2010; Grime, 1998). In this experiment, total aboveground biomass, biomass C and biomass N were all higher in the 'low N' communities, but they were also highly positively correlated with the percentage of total biomass made up of *L. corniculatus* and *P. lanceolata*. In addition, the biomass C:N ratio was lower in the 'low N' communities, and this was strongly negatively correlated with the percentage of total biomass made up of *L. corniculatus*. A similar result was encountered by De Deyn *et al.* (2009) in their mesocosm experiment, whereby *L. corniculatus corniculatus* was the most productive species by far, consistently stored the most C and N in vegetation, and along with *T. repens*, also had the lowest biomass C:N ratio.

Unlike aboveground biomass, root biomass was not affected by N community prevalence. However, the root C:N ratio was lower in 'low N' communities, and this was negatively correlated with the percentage of total biomass made up of *L. corniculatus* and *P. lanceolata*. This indicates that root tissue quality in the 'low N' communities was higher than in the 'high N' communities. Studies have shown that better quality root litter has a higher rate of decomposition (Silver and Miya, 2001). Therefore, it is possible that the rate of root C turnover was greater in the 'low N' communities.

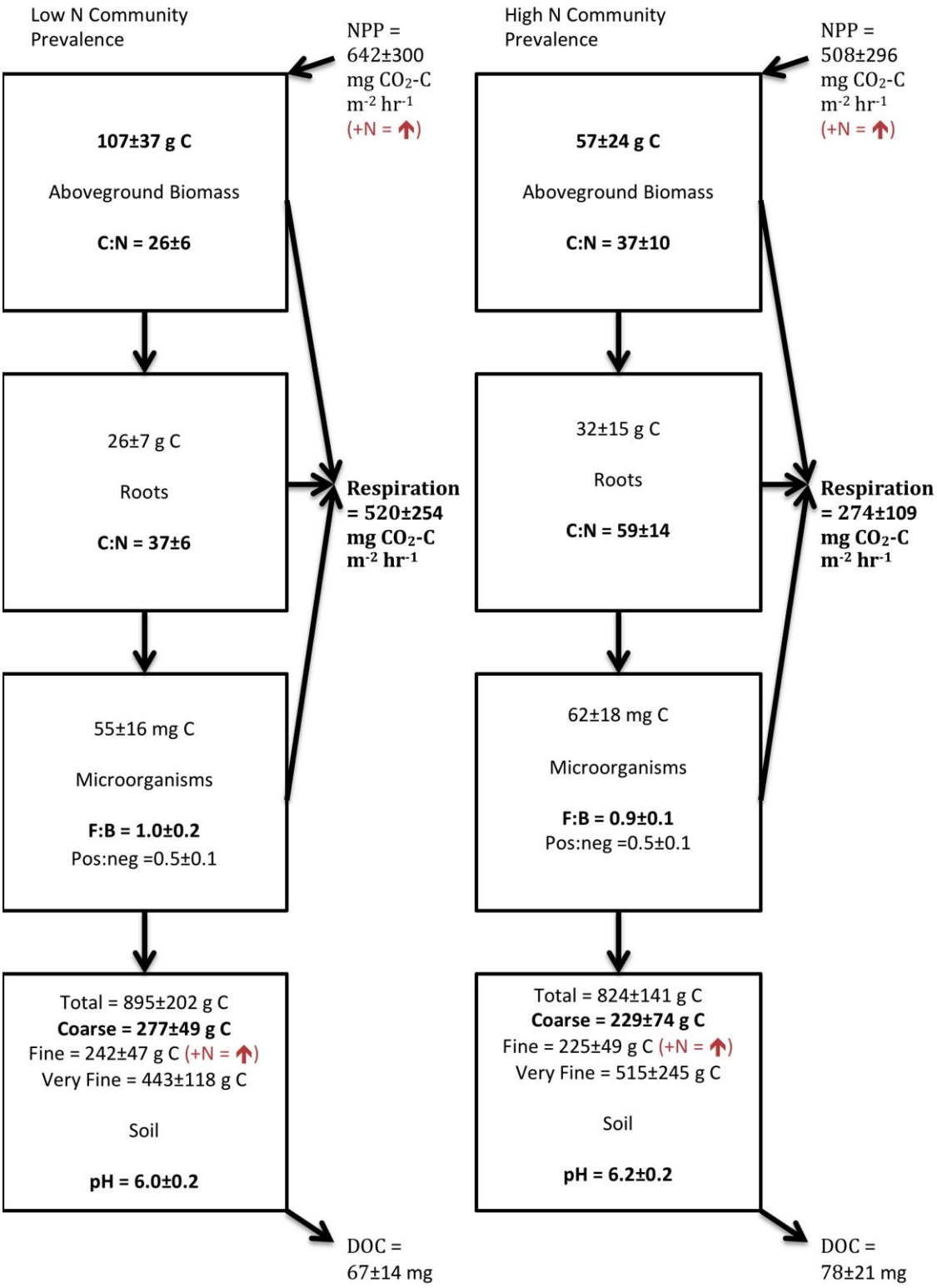

Figure 6.11: C pools and fluxes in the high and low N-associated communities. Writing in bold indicates significant differences ($p<0.05$) between communities. Writing in brackets indicates significant effects of N addition and direction of effect.

Despite changes in root tissue quality, microbial biomass C and N were not affected by N community prevalence, however, the fungal:bacterial ratio, a measure of microbial community structure, was higher in 'low N' than 'high N' communities. This result contrasts with the work of Habekost *et al.* (2008), who found that improved resource quality (defined as lower litter C:N ratios) enhanced bacterial growth at the expense of fungi in an experimental grassland. However, Manning *et al.* (2006) also found that fungi abundance was higher in 'low N' communities. The fungal:bacterial ratio was also significantly negatively correlated with DOC and DON, and positively correlated with total root N. This suggests that although root tissue quality did not affect microbial biomass, it may have affected microbial community structure by increasing the proportion of fungi relative to bacteria. This could have led to effects on soil C storage in the long run, as research shows that increased fungal activity is associated with increased soil C (Bailey et al., 2002). The correlations between the fungal:bacterial ratio and DOC and DON could indicate that a microbial community with a higher proportion of fungi may be better at mineralising organic forms of C and N. Research shows that fungi play an important role in gross N mineralisation (Balser and Firestone, 2005). The fungal:bacterial ratio was not significantly correlated with the proportions of *L. corniculatus* or *P. lanceolata* biomass, despite the fact that the functional characteristics of dominant plant species are known to be important determinants of soil biological properties (Bardgett et al., 1999).

pH was found to be significantly lower in the 'low N' communities. This was probably due to the presence of *L. corniculatus*, as legumes are known to decrease soil pH (Dakora and Phillips, 2002). There were also marginally non-significant trends for DOC and DON to be lower in the 'low N' communities, likely due to higher uptake caused by greater plant biomass. Orwin *et al.* (2014) also found that communities dominated by *L. corniculatus* lost the least DOC and DON in leachates. Although total soil C and N were not affected by N community prevalence, coarse fraction C was found to be significantly higher in 'low N' communities. Coarse C is known to be comprised of young, plant matter-derived C that typically has a relatively short turnaround period of a few years (Puget et al., 2000). It is possible that this increase in coarse fraction C may have been affected by the dominance of *L. corniculatus* in the 'low N' communities, as increasing the proportion of legume species in a grassland community has been shown to lead to increases in soil C storage (Li et al., 2016a).

Ecosystem respiration rates were found to be significantly higher in the 'low N' communities, and highly positively correlated with total aboveground biomass. Research shows that ecosystem respiration is highly linked to plant aboveground biomass (Flanagan and Johnson, 2005). This suggests a higher rate of C loss from vegetation in 'low N' communities as a consequence of increased C stored as aboveground biomass.

The prevalence of 'low N' species (driven by *L. corniculatus*, and to a lesser extent, *P. lanceolata*) led to higher C and N stocks in aboveground biomass, but it also increased the quality of both the above and belowground plant tissue in the system as well as the rate of ecosystem respiration. These changes to biomass decomposability and the increase in C loss via respiration could have led to an acceleration of the C cycle in the 'low N' communities. In addition, the prevalence of 'low N' species lowered soil pH, increased the proportion of fungi in the microbial community, and increased coarse fraction C, meaning that community composition also altered soil biotic and abiotic properties. Therefore, it is possible that community composition may have had an impact on C storage via altered inputs from vegetation. Changes to microbial community composition could also have had larger implications for C storage over time.

It was hypothesised that the 'high N' communities would cause greater changes to the C cycle when exposed to N than the 'low N' communities. However, only one significant interaction was found between an aspect of community composition and N addition: an interaction between N community prevalence and N addition for aboveground tissue C (see Figure 6.6). This variable only increased with N addition in treatments that did not include *L. corniculatus* (species mixture treatments 1 and 2). Although this effect was not present in the other species mixture treatments, the 'low N' communities displayed higher overall aboveground tissue C content regardless of N addition. This suggests that N addition did have a greater effect on 'high N' communities for aboveground tissue C content, but that 'low N' communities, driven by *L. corniculatus* (as confirmed by the significant correlation between aboveground tissue C content and the percentage of total biomass made up of *L. corniculatus*), still had a larger impact on C regardless.

In this experiment, *L. corniculatus* and *P. lanceolata* drove vegetation aboveground biomass, and vegetation aboveground chemistry was driven by the N-fixing ability of *L. corniculatus*. This dominance affected C cycling via input from vegetation, and may even have had a part in altering soil biotic and abiotic properties (such as microbial community structure, soil pH,

and coarse soil fraction C storage). A previous study by Bezemer *et al.* (2006) reveals that it is possible for species identity to affect both abiotic and biotic soil properties within a short period of time (two growing seasons). Real grasslands are not dominated by species such as *L. corniculatus* and *P. lanceolata*, and these species only became dominant due to the artificial nature of this experiment. Although the dominance of *L. corniculatus* and *P. lanceolata* illustrates how far removed from reality these communities were, their effects demonstrated the impact of dominant species on ecosystem processes, particularly when one of these species is a legume. Research has shown that N_2 fixation by legumes may be a major driver of soil C (and N) storage (Cong et al., 2014).

6.4.2.3 High vs. low species number

Many studies report positive correlations between aboveground productivity and species richness in grasslands (Cardinale et al., 2007; Cong et al., 2014; Hector et al., 1999; Roscher et al., 2005; Spehn et al., 2005; Tilman et al., 1996), which are frequently attributed to increased trait variability, leading to greater niche differentiation and superior exploitation of resources. However, in this study species number did not have a significant effect on aboveground biomass (nor did it affect ecosystem NPP or respiration), as biomass was strongly determined by two dominant species (*L. corniculatus* and *P. lanceolata*).

Belowground biomass on the other hand, was negatively affected by species number. This result is contrary to the works of Mommer *et al.* (2010) and of Mulder *et al.* (2002), who report positive effects of species richness on belowground biomass. For Mommer *et al.* the increase in root biomass with richness was driven by increased root biomass of *A. odoratum*. Mulder *et al.* suggest that increased root biomass was a consequence of greater growth rates in communities with high species richness. They also add that although the percentage of legume biomass aboveground had a very strong effect on aboveground biomass (as occurred with *L. corniculatus* in the species mixture treatments), it did not affect root biomass.

As previously mentioned however, species number did not affect the aboveground productivity of the species mixture treatments. In addition, neither *A. odoratum*, nor *L. corniculatus* or *T. repens* percentage aboveground biomass were significantly correlated with root biomass or root C. However, both root biomass and root biomass C of the species

mixture treatments were significantly negatively correlated with the percentage of total aboveground biomass made up of *P. lanceolata*. Also, there was a marginally non-significant (p=0.06) trend for species mixtures with higher species number to have a larger percentage of their aboveground biomass made up of *P. lanceolata*. This could therefore suggest that the decline in root biomass and root C with increased species number could at least partly be due to the increased presence of *P. lanceolata* in the high species number mixtures. A possible reason for this could be that by dominating aboveground biomass, and therefore probably restricting the growth of other species, *P. lanceolata* may have limited the belowground productivity of the other species, including that of *A. millefolium*, which had the highest belowground productivity.

Species number did not significantly affect any measured soil properties. However, species number did significantly affect two microbial parameters. Higher plant species number was expected to benefit the diversity of soil biota as it included more plants differing in root morphology and root chemical composition (Habekost et al., 2008). Although fungal:bacterial ratios were not affected by species number, mixtures with high species number had significantly higher Gram+:Gram- bacterial ratios. Studies have indicated that Gram-positive bacteria use more soil organic matter-derived C sources, while Gram-negative bacteria prefer plant biomass and fresh organic material inputs (Bird et al., 2011; Habekost et al., 2008; Kramer and Gleixner, 2008; Reinsch et al., 2014). As aboveground biomass was removed at the end of both growing seasons in this experiment, the impact of litter inputs to the soil was minimised and belowground organic matter inputs became even more important for plant-soil feedbacks. Therefore, the decrease in the proportion of Gram-negative bacteria with higher species number could be associated with the aforementioned decrease in root biomass and root biomass C in mixtures with a higher species number. However, it must be noted that no significant correlation was found between the Gram+:Gram- bacterial ratio and root biomass (p=0.28) or root C (p=0.25).

N mineralisation rate was also significantly affected by species number, with the rate being higher in communities with high species number. This finding is in line with the work of Zak *et al.* (2003), who reported greater N mineralisation rates with increasing plant species richness. They proposed that one mechanism behind this effect was that increasing plant richness also led to greater microbial activity. Mueller *et al.* (2013) also found that plant species richness increased N mineralisation, but they suggest that the strongest driver of N mineralisation in their long-term experiment was root N concentration. Neither microbial

biomass C, nor root tissue N were found to be significantly correlated with N mineralisation rate (p=0.52 and 0.19 respectively). In addition, although the monoculture treatments showed that the highest N mineralisation rates by far were in the soils of the legume species (which also had the highest root N concentrations), no significant correlation was found between N mineralisation and the proportion of total biomass made up of *L. corniculatus* and *T. repens* in the species mixture treatments (p=0.44).

Due to the relatively high turnover rates of roots (Van der Krift and Berendse, 2002), decreasing root biomass and total root C in the high species number treatments could have led to decreased organic matter input into the soil. The decrease in the proportion of Gram-bacteria found in high species number mixtures also suggests a decrease in fresh plant matter inputs to the soil. This may have had long-term negative consequences for soil C stocks for these communities, especially given that a large proportion (up to 80%) of total plant biomass in temperate grasslands can be attributed to belowground biomass (Jackson et al., 1996). In the work of Steinbeiss *et al.* (2008) for example, higher species richness significantly increased both root biomass and C stocks after four years. Although the authors state that greater plant material input was not a simple cause of increased soil organic C, the implication is that it is still likely to play an important role in this process. Increased N mineralisation rates with higher species number on the other hand, could have led to greater soil available N stocks, which could have a positive effect on aboveground productivity (and thus aboveground C stocks) in the long-term.

6.4.2.4 High vs. low functional group evenness

Functional evenness and its opposite, functional dominance, are increasingly recognised as important factors in the regulation of ecosystem processes which include C storage (Hillebrand et al., 2008). In this experiment it was expected that lower functional group evenness would lead to increased aboveground productivity, and decreased root growth and microbial diversity. However, functional group evenness had the least effect on C cycling and storage out of all three measures of diversity (functional evenness, species number and N community prevalence). In fact, the only variable that was significantly affected by functional group evenness was total aboveground tissue C. The reason why evenness did not affect C cycling and storage in this experiment was probably the fact that by the final harvest, the percentage of total biomass made up of grasses did not significantly differ between high and

low evenness mixtures (p=0.85). This means that the experimental design was ultimately unsuccessful in its attempt to simulate differences in functional group evenness.

6.5 Conclusions

Species identity affected several processes involved in C storage and cycling in the monoculture treatments due to differences in above and belowground biomass and chemistry, which led to changes in microbial activity and community composition. However, the effect of species identity on most vegetation variables measured depended on whether N was added or not (i.e. the direct effects of N). In addition, the effects of 'high N' and 'low N' species on C cycling and storage in monocultures were not clearly distinguishable.

Of the community composition aspects manipulated in the species mixture treatments, the most important was N community prevalence. Many above and belowground vegetation variables, as well as soil biotic and abiotic properties were affected by N community prevalence. This led to changes in C cycling and storage that tended to be stronger in the 'low N' communities. However, this effect was overwhelmingly driven by the presence of two dominant 'low N' species, *L. corniculatus* and *P. lanceolata*. Nonetheless, a significant increase in coarse fraction C in the 'low N' communities indicates that community composition had the greatest effect on fresh C storage in these systems. These results contrast with Manning *et al.* (2006), who found that direct effects on plant growth dominate ecosystem response to N deposition. Aside from having a negative effect on root biomass, species number had a limited effect on C cycling and storage. The functional group evenness treatments in this experiment were unsuccessful.

Due to the short-term nature of this experiment, it was expected that species composition would exert a stronger overall effect on C cycling and storage than N addition, especially in the soil. However, results suggest N addition played a more important role in C cycling and storage in the species mixture treatments than was anticipated. Despite community composition exerting a much stronger effect on above and belowground vegetation in species mixture treatments, N addition increased ecosystem NPP. In addition, the direct effects of N may have been more important for older soil C storage, as N addition significantly altered finer, mineral-associated fraction C.

In conclusion, both direct and indirect (via plant community change) effects of N addition were important for C cycling and storage in these model grassland communities. This study further accentuates the need to take both effects into consideration when assessing the impact of N pollution on C stocks in grasslands.

Chapter 7: General Discussion

General Discussion

Terrestrial ecosystems are important C sinks, thus making them indispensible for climate change mitigation. Increasing levels of atmospheric N deposition are known to affect various chemical and biological processes that contribute to C cycling. However, there is still much uncertainty regarding the true impact of N enrichment on terrestrial C storage. The overarching aim of this thesis was, with a focus on acidic grasslands, to investigate how N addition affects ecosystem C cycling and storage. In order to address this aim, above and belowground C and N pools and gas emissions were measured in a series of field, mesocosm and microcosm studies. This chapter discusses the key findings of this thesis, their implications, and the potential for future work in this field of study.

7.1 Reduced N is likely to have the strongest long-term effects on C storage in acid grasslands (Chapter 3)

Soil and vegetation were sampled from a seven-year N addition experiment in Revna, Norway. N was added to experimental plots as NH_4NO_3 (35 and 70 kg ha^{-1} yr^{-1}), $NaNO_3$ (70 kg ha^{-1} yr^{-1}) and as NH_4Cl (70 kg ha^{-1} yr^{-1}).

Reduced N as NH_4Cl led to the largest decreases in soil pH and aboveground biomass C:N ratios compared with control plots. In addition, only the reduced N treatment significantly lowered the soil fungal:bacterial ratio compared with control plots, and there was a non-significant trend for this treatment to decrease total aboveground biomass. These findings support the work of van den Berg *et al.* (2016), who found that acid grasslands appear to be more sensitive to reduced rather than oxidised N deposition.

Although no changes to the total soil C pool were found, these results indicate that reduced N addition could lead to changes in C storage over time, potentially by changing rates of organic matter decomposition via increased litter quality (Nicolardot et al., 2001) and increased proportion of bacteria in the microbial community (Six et al., 2006). In addition, lower soil pH (and perhaps toxic effects of ammonium) may eventually lead to changes in plant growth and community composition (de Graaf et al., 1998; Kleijn et al., 2008; Lucassen et al., 2003), and therefore to indirect effects of N on C.

These findings are especially significant as forecasts for future atmospheric N deposition predict an increase in the proportion of reduced N (Engardt and Langner, 2013). Emissions of NH_3 from agriculture have been the least affected by environmental policies (Sutton, 2011), and must therefore be targeted by current and future policy-makers in order to reduce the effects of N deposition on terrestrial ecosystems. Results from Chapter 3 highlight the importance of incorporating the differential effects of N form in future research into the effects of N addition on C storage.

7.2 N addition has had a negative effect on microbial respiration in an acid grassland (Chapter 4)

Intact soil cores taken from a seven-year N addition experiment in Norway were incubated at 10, 16 and 22°C for two weeks. A set of cores was also spiked with P before the start of the incubation. Gas samples were collected and analysed for CO_2 and N_2O content.

N addition had a significant negative impact on soil respiration rates in this acidic grassland. This finding is consistent with several studies such as Ramirez *et al.* (2010), Treseder (2008) and Liu and Greaver (2010). Decreases in microbial respiration indicate that N addition had a negative impact on microbial activity at this site. We propose that this may have been due to a combination of N-enhanced C and P limitations (Harpole et al., 2011; Penuelas et al., 2012; Rousk et al., 2011), as well as possible effects of N-induced acidification (Chen et al., 2016). Given that the NH_4Cl treatment produced the largest reductions in CO_2 emissions, these findings also strengthen the idea put forth in Chapter 3 that reduced N may play an especially important role in soil C storage.

As microbial respiration is a major pathway for C loss from a system (Raich and Schlesinger, 1992; Ryan and Law, 2005), our results suggest that N addition may have a long-term beneficial effect on soil C storage. However, this might not be the case in systems where P is not a limiting nutrient/is also added. Adding P to cores led to an increase in respiration compared with cores incubated at the same temperature without P. This suggests that the negative effects of N on respiration could be dependent on whether P is a limiting nutrient or not. Other studies have also noted the effect of N addition on P limitation. Elser *et al.* (2007) for example, found widespread evidence that fertilisation with only N or P quickly

induced limitation by the other nutrient. Future research and C models should consider the effects of multiple nutrients on terrestrial C storage.

7.3 Effects of low levels (under 10 kg ha^{-1} yr^{-1}) of N addition on C cycling and storage in an acid grassland were not observable after seven years (Chapter 5)

Soil and vegetation samples were collected from a seven-year low N addition experiment in Trefor, Wales. N was added to experimental plots as NH_4NO_3 (4.3 and 8.6 kg ha^{-1} yr^{-1}) and as NH_4Cl (4.8 kg ha^{-1} yr^{-1}). No significant effects of low doses of N addition were found for this acid grassland site. This could indicate that a critical load of 10 kg ha^{-1} yr^{-1} may be applicable for this site.

However, long-term N applications have been shown to alter vegetation growth and phenology at levels as low as 7.7 kg ha^{-1} yr^{-1} in a lowland heath for example (Phoenix et al., 2012). In fact, Tipping *et al.* (2013) estimate that the critical load for acid grasslands is 7.9 kg ha^{-1} yr^{-1}. Tipping *et al.* used plant species richness data from an N deposition gradient across the UK to calculate this critical load, and therefore the sites had likely been exposed to N deposition for many years. This suggests that the effects of low levels of N addition take a long time to be observed, and therefore data from short-term (under 10-year) N addition experiments should be used with caution when determining critical loads. In addition, the field site in Wales had a background N concentration of 9 kg ha^{-1} yr^{-1}. This means that the effects of very low levels of N addition may have already occurred at this site.

Overall, results suggest that short-term effects of low level N addition on C cycling and storage cannot be observed, and therefore that long-term N addition experiments are crucial in order to understand the real consequences of chronic N addition, even at low levels. Furthermore, additional research is needed in areas with the lowest levels of background N deposition in order to investigate the effects of low levels of N addition on 'pristine' environments.

7.4 Direct and indirect effects of N addition may differ for young and old C storage (Chapter 6)

Aspects of plant community diversity were manipulated and subjected to N addition (35 kg ha^{-1} yr^{-1} as NH_4NO_3) in a mesocosm experiment set up at Lancaster University Field Station.

Plant community composition (i.e. the indirect effects of N addition) had the strongest influence on C storage in vegetation and on microbial community structure. This result contrasts with the work of Manning *et al.* (2006), who found that direct effects on plant growth dominate ecosystem response to N deposition. Community composition also significantly altered coarse soil fraction C, which is known to be comprised of young, plant matter-derived C that typically has a relatively short turnaround period of a few years (Puget et al., 2000). Therefore, plant community composition was the most important factor affecting fresh C input, decomposition and storage in the mesocosms. However, it must be noted that many of these effects were driven by two dominant 'low N' species (*L. corniculatus* and *P. lanceolata*). Although this highlights the artificial nature of this experiment, it also demonstrates the impact of dominant species on ecosystem processes.

The direct effects of N addition on the other hand, may have had a more important role for the storage of older C in soil. N addition led to an increase in fine fraction C (and in the proportion of total C stored in this fraction). N also significantly decreased the proportion of total soil C stored in the very fine soil fraction, and it must be noted that very fine soil fraction C was also the only soil C pool significantly affected by one of the N treatments (70 kg ha^{-1} yr^{-1} NH_4NO_3) in Chapter 3. Research suggests N addition accelerates the degradation of easily degradable litter and light soil C fractions, but may slow the decomposition of recalcitrant litter and stabilise C compounds in mineral-associated soil fractions (Lu et al., 2011a; Xu et al., 2004). Although the observed effects of N on fine C were positive, the negative effects on the proportion of total C stored in very fine soil fraction suggest that C storage could be negatively affected in the long term.

These results suggest that both direct and indirect effects of N are important for C cycling and storage in acid grasslands, and that both effects have to be taken into consideration in further research.

7.5 Summary diagram

Figure 7.1 illustrates the overall effects (both direct and indirect) of N addition on C cycling and storage as reported in this thesis.

Reduced N addition (70 kg ha^{-1} yr^{-1} NH_4Cl) decreased pH, which in turn may have contributed to the trend for lower aboveground biomass in this treatment (Chen et al., 2013) (Chapter 3). All forms of N addition had inhibitory effects on microbial activity (Chapter 4), potentially via the effects of reduced pH (Chen et al., 2016), N-enhanced C and P limitation (Harpole et al., 2011; Penuelas et al., 2012; Rousk et al., 2011) or even via direct inhibitory effects of N (Ramirez et al., 2010).

N addition reduced aboveground biomass C:N ratios (Chapter 3), and although there were no observed effects of N on aboveground biomass, there was a trend for a decline in biomass with reduced N addition (Chapter 3). Decreased biomass C:N ratios would have led to lower C:N ratios in the plant litter.

The 'high N' communities had higher aboveground and root C:N ratios than the 'low N' communities (Chapter 6), although this was due to dominance by *L. corniculatus*. There was also a marginally non-significant trend for N addition to increase root biomass in the mesocosms (Chapter 6).

N addition increased NPP, but did not affect ecosystem respiration (Chapter 6). Plant community composition did not affect NPP, but the 'high N' communities had lower rates of ecosystem respiration than the 'low N' communities (Chapter 6). This was due to increased biomass in the 'low N' treatments, which was driven by two dominant species (*L. corniculatus* and *P. lanceolata*).

No effects (direct or indirect) were observed for soil microbial biomass C. However, soil fungal:bacterial ratios were lower in 'high N' communities than in 'low N' communities (Chapter 6). In addition, lower fungal:bacterial ratios observed in the 70 kg ha^{-1} yr^{-1} NH_4Cl treatment in Norway may have been mediated by increased litter quality (Lange et al., 2014) (Chapter 3). Lower species number led to a decrease in soil Gram+:Gram- bacterial ratios (Chapter 6). N addition had a negative effect on microbial respiration (Chapter 4). Soil respiration was not measured in the mesocosms.

Neither direct nor indirect effects of N addition affected total soil C. However, N addition to the mesocosms led to an increase in fine fraction C (and in the proportion of total C stored in the fine fraction) and to a decrease in the proportion of total C stored in the very fine soil fraction (Chapter 6). The 70 kg ha^{-1} yr^{-1} NH_4NO_3 treatment in Norway also led to a decrease in very fine fraction C (and in the proportion of total C stored in the very fine fraction) compared with control plots (Chapter 3). Coarse fraction C was lower in 'high N' communities than in 'low N' communities (Chapter 6). DOC was found to be lower in all 70 kg ha^{-1} yr^{-1} treatments in Norway (Chapter 3). There were marginally non-significant trends for DOC to be higher in 'high N' communities than in 'low N' communities (Chapter 6).

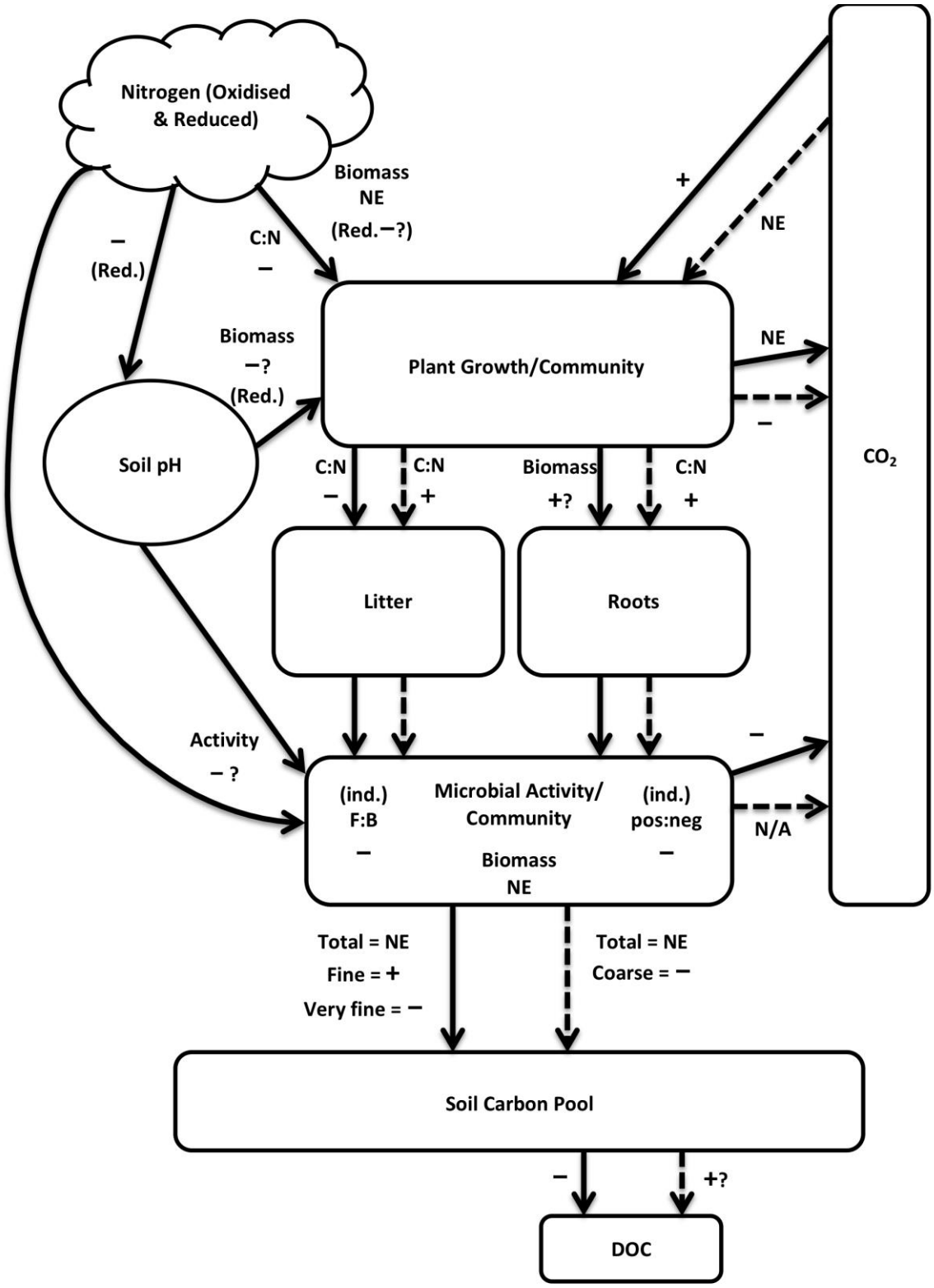

Figure 7.1: The effects of N addition on C cycling and storage in acid grasslands. Solid arrows represent direct effects of N, dashed arrows represent indirect effects of N. 'NE' stands for 'no effect', '+' stands for positive effect and '-' stands for negative effect. Question marks are used where effects were not clear/significant.

7.6 Future Work

While the information obtained from this thesis will help to understand the mechanisms by which N affects C storage in terrestrial ecosystems, many challenges still remain.

Firstly, there is a great need for more long-term N addition experiments such as those described by Phoenix *et al.* (2012). Due to the long turnover times associated with C, experiments are likely to need decades in order to provide reliable long-term data for the effects of N on C. Therefore, more studies of the effects of N on C storage should be carried out on already existing long-term N addition experiments.

Secondly, isotopic labelling studies would enable an in-depth understanding of C allocation to different soil C pools, and would be especially useful for differentiating between the direct and indirect effects of N on C storage. An example of how isotopic labelling can be used to track soil C allocation under N addition can be found in Finn *et al.* (2016).

Thirdly, more N addition experiments should take into account both the effects of different N forms, and the effects of other limiting nutrients. A good example of an experimental design that incorporates different nutrients is the 'Nutrient Network', or 'NutNet', which incorporates nitrogen, phosphorous, potassium and other micronutrients in a fully factorial design (Hautier et al., 2014).

Finally, it must be noted that grasslands are by no means the largest soil C sinks. Ecosystems such as peatlands and bogs hold huge quantities of C, and are often at most risk from the effects of climate change. Research into the effects of N addition on C storage, how this might change with warming, and how direct and indirect effects might differ in these systems is essential.

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