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Using Stable Isotopes To Investigate Interactions Between The Forest Carbon and Nitrogen Cycles

Richard Nair

THE UNIVERSITY
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Richard Nair
August 2014

A paper based on first research chapter of this thesis (Chapter 3) is published as a jointly authored paper (Tree Physiology (2014) 34 (10): 1130-1140 (doi:10.1093/treephys/tpu084)). Richard Nair was the primary author of the manuscript, performed all fieldwork and statistical analyses. Manuscript feedback, logistical support, and some field assistance were provided by all other authors.

As the publication is very similar to Chapter 3 a copy of this is located within "TP-2014-157.zip", submitted electronically along with this thesis.

Abstract

Nitrogen (N) fertilization due to atmospheric deposition (N_{DEP}) may explain some of the net carbon (C) sink (0.6-0.7 Pg y-1) in temperate forests, but estimates of the additional C uptake due to atmospheric N additions ($\Delta C/\Delta N$) can vary by over an order of magnitude (5 to 200 $\Delta C/\Delta N$). High estimates from several recent studies [e.g. Magnani (2007), Nature 447 848-850], deriving $\Delta C/\Delta N$ from regional correlations between N_{DEP} and measures of C uptake (such as eddy covariance -derived net ecosystem production, or forest inventory data) contradict estimates from other studies, particularly those involving ^{15}N tracer applications added as fertilizer to the forest floor. A strong $\Delta C/\Delta N$ effect requires nitrogen to be efficiently acquired by trees and allocated to high C:N, long-lived woody tissues, but these isotope experiments typically report relatively little ($\sim$ 20 %) of ^{15}N added is found above-ground, with less than 5 % of the total ^{15}N applied found in wood. Consequently the high correlation-derived $\Delta C/\Delta N$ estimates are often attributed to co-variation with other factors across the range of sites investigated.

However, ^{15}N-fertilization treatments often impose considerably higher total N loads than ambient N_{DEP}, while almost all exclusively only apply mineral ^{15}N treatments to the soil, often in a limited number of treatment events over relatively short periods of time. Excessive N deposition loads can induce negative physiological effects and limit the resulting $\Delta C/\Delta N$ observed, and applying treatments to the soil ignores canopy nitrogen uptake, which has been demonstrated in numerous

studies. As canopies can directly take up nitrogen, the chronic, (relatively) low levels of ambient N_{DEP} inputs from pollution may be acquired without some of the effects of heavy N loads, with trees obtaining this N before it reaches the soil, allowing canopies to substitute for, or supplement, edaphic N nutrition. The strength of this effect depends on how much N uptake can occur across the canopy under field conditions, and if this extra N supplies growth in woody tissues such as the stem, as well as the canopy. Similarly, such mineral fertilizer isotope trace experiments are also unable to trace N in the decomposing litter and humus layers of the soil, which even under heavy N_{DEP} loading contribute most of the N utilised for forest growth. Recent literature suggests that some organic (early decomposition) forms of N may be taken up by roots. If this litter N is not retained or distributed in the same way as mineral fertilizers, its contribution to plant nutrition and $\Delta C/\Delta N$ may need to be reassessed under nitrogen deposition.

We tested some of these assumptions in the nursery and the field. In order to facilitate litter ^{15}N tracing, we conducted an experiment injecting large trees with $^{15}N\text{-}NH_4NO_3$ to create ^{15}N-labelled litter, tracing the applied isotope into a full harvest of the canopy. Such labelled litter substitute was used to replace the litter layer in a Sitka Spruce plantation (*Picea sitchensis* L. (Bong.)), where the fate of this ^{15}N from litter decomposition in the soil system was compared against the fate of ^{15}N in deposition. Similarly, in potted Sitka Spruce saplings, we used combination treatments of ^{15}N-labelled litter, soil-targeted ^{15}N-deposition, and canopy targeted ^{15}N-deposition, investigating ^{15}N return in different age classes of above and below ground biomass.

We found that i) ^{15}N recovery in canopies (needles and branches) in our injected trees was almost all of the injected ^{15}N five months after injection, ii) canopy application of N_{DEP} led to 60 % ^{15}N return in above-ground parts of saplings compared to 21 % in soil applications and iii) a litter-derived ^{15}N source was

retained 55 % more in topsoil, and 36 % more in roots, than a similar deposition ^{15}N source applied as mineral fertilizer.

We discuss the implications of such findings in the context of ^{15}N return in different plant organs and ecosystem pools, seasonal variation in N content, and overall inferences of a forest $\Delta C/\Delta N$ effect. Our results suggest that the total $\Delta C/\Delta N$ effect driven by a high N sequestration from canopy uptake in wood is $\sim$ 114:1, more than double that of ^{15}N tracer experiments but not as high as upper estimates from correlative studies, and that litter-derived organic N is better retained in trees and soils in excess of similar amounts of mineral ^{15}N from deposition. Existing forest ^{15}N-fertilization experiments could under-estimate the overall $\Delta C/\Delta N$ effect of atmospheric N deposition.

Acknowledgements

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Contents

Chapter 1

Introduction

1.1 Uncertainties in Forest Responses to Global Change

The biggest uncertainties in future earth system models are derived from the biosphere, and how the 2000 gigatonne (gt) terrestrial carbon sink in plants and soil will respond in the future to anthropogenic global change (Friedlingstein *et al.*, 2006; Schaphoff *et al.*, 2006; IPCC AR4 WG1, 2007). Most of the variation in interannual atmospheric CO_2 concentrations are driven by changes in the amount of carbon (C) held in this pool, as CO_2 is taken up by green plants in photosynthesis and released in aerobic respiration, with the factors driving these two opposing fluxes varying in strength with the seasons. As well as these interannual changes in CO_2 uptake and release, the terrestrial biosphere is a net sink for CO_2, as more C is annually fixed than respired ((1.4 Pg C y^{-1} in 1990-1999, Prentice *et al.*, 2001)). This uptake is not the equilibrium (no net exchange of C with atmospheric CO_2 in either direction) which would be expected if, at a global scale, forest ecosystems are not changing in function or scale. As increasing atmospheric CO_2 concentrations are expected to cause major perturbations to the earth's climate (Solomon *et al.*, 2009), understanding the causes for this increasing sink are important to assess the future strength and longevity of this sink for some of the increasing amounts of atmospheric CO_2

Temperate and boreal forests make up a particularly large fraction of the overall terrestrial CO_2 sink, taking up 0.6-0.7 Pg C y^{-1} (Goodale *et al.*, 2002), holding large C stocks in high C/N woody biomass and slow-decomposing soil pools, with a large potential to gain or lose C due to relatively small changes in the factors which govern CO_2 uptake and release (Reay *et al.*, 2008). The overall driver for this sink in these regions is human activity, but disentangling numerous effects is difficult (Law *et al.*, 2002); the net uptake may include the effects of rising concentrations of CO_2 on photosynthesis rates ('CO_2 fertilization', Norby, 1999), a feedback from temperature changes (Saxe *et al.*, 2002), ongoing changes in land use and management such as forest regrowth (Sedjo, 1992), as well as the effects of nitrogen deposition from the atmosphere (N_{DEP}, Prentice *et al.*, 2001; Ciais *et al.*, 2008).

The nitrogen cycle is not as well understood as the C cycle, and until recently, N_{DEP} was thought to be settled as a fairly minor driver of this C sink (Nadelhoffer *et al.*, 1999c; de Vries *et al.*, 2006), taking up around 50 kg C kg N $^{-1}$. Trees are relatively poor competitors for N additions (Nadelhoffer *et al.*, 1999c) when compared to other ecosystem sinks, and a higher proportion of N than observed in field manipulative studies (for a recent summary, Templer *et al.*, 2012) needs to be acquired by high C/N wood for a strong overall effect of N_{DEP} on C uptake ($\Delta C/\Delta N$; the change in C due to a change in N inputs).

Observations indicating a low $\Delta C/\Delta N$ effect were primarily drawn from experimental studies where fertilizers were applied to forest soils, but subsequent work which correlated wet N_{DEP} against forest NEP (net ecosystem production) across multiple sites in Europe (Magnani *et al.*, 2007) found an apparent N_{DEP} effect of several hundred kg C kg N $^{-1}$. While this finding was controversial (de Vries *et al.*, 2008; De Schrijver *et al.*, 2008; Magnani *et al.*, 2008), it spurred continued interest in the effects of N_{DEP} in forests, including a questioning of the assumptions of previous ecosystem N budgets and N fertilization . These past manipulations were suggested to poorly represent canopy (Sievering *et al.*, 2007) N retention, and longer term edaphic (Jenkinson *et al.*, 1999; Högberg, 2012) interactions with N_{DEP}, which could lead to underestimations of the overall forest carbon response.

1.2 Nitrogen Limitation Across the Globe

In order to discuss nitrogen effects on forests, it is first necessary to briefly discuss the nitrogen cycle.

Nitrogen is one of the most important plant nutrients, as it is a constituent of amino acids (and thus proteins), and DNA, and is a critical control for primary production of the biosphere (Gruber and Galloway, 2008). However, while N_2 gas is very abundant, making up 78 % of the atmosphere, only a few families of microorganisms can fix (reduce) N_2 into ammonium (NH_4^+), making it available for biological utilisation as NH_4^+, or, following nitrification, nitrate (NO_3^-). Various constraints prevent N fixing organisms from proliferating (Vitousek and Howarth, 1991) in many ecosystems, in particular a positive feedback of increasing ecosystem N availability which excludes N fixers due to fitness costs of fixation in late-successional, relatively N-abundant ecosystems (Vitousek *et al.*, 2002; Menge *et al.*, 2010), so 'fresh' fixed atmospheric N in reactive forms is often in short supply. Most plant-acquired N is therefore acquired by recycling of dead organic matter from the soil (Figure 1.1) having been released from decomposing biomass by chemical and microbial processes (Marschner and Rengel, 2007). In terrestrial ecosystems where rates of litter recycling (N mineralization and nitrification, usually by soil microbes) are low, N availability usually limits growth and productivity (C uptake)

(Vitousek and Howarth, 1991). Although overall nitrogen stocks can be high in many regions (the majority of N in the global biological system is held in upper 100 cm of soils (Batjes, 1996) and only small amounts are typically found in plants ($>$ 10 %) and microbes (1.5 %)), low rates of remobilisation of N from soil stocks mean little is available for plant uptake. In some forests with large amounts of standing biomass, this difference may be smaller (c. 30-50 % in plants Butterbach-Bahl *et al.*, 2011), but decomposition rates still typically limit the availability of new N for growth.

Decomposition rates increase with temperature (Moore, 1986) and soil water content (Stark and Firestone, 1995), as long as the soils do not become aerobic, so high latitude ecosystems, such as such as temperate and boreal forests, are particularly likely to be N limited (Vitousek and Howarth, 1991), with N availability (Hobbie *et al.*, 2002; Seitzinger *et al.*, 2006) the major restriction on growth (Vitousek and Howarth, 1991; LeBauer and Treseder, 2008; Reich *et al.*, 2006; Butterbach-Bahl *et al.*, 2011) as it occurs before other factors (e.g. phosphorus (P), light intensity, water) become limiting. Even when temperature or water constraints on decomposition rate are not present in warmer ecosystems, slow N cycling and subsequent N limitation can also occur due to soil properties such as pH, soil texture and ion exchange capacity (Reich *et al.*, 1997; Côté *et al.*, 2000), SOC content (Côté *et al.*, 2000), organic matter inputs and soil C:N ratios (Côté *et al.*, 2000; Adams *et al.*, 2004). Controls on N uptake therefore differ considerably to those controlling C, where availability is largely uniform around the globe and uptake is driven by capacity for primary production, or potassium (K), which is derived from weathering of rocks, independent of limits on biological activity (Butterbach-Bahl *et al.*, 2011).

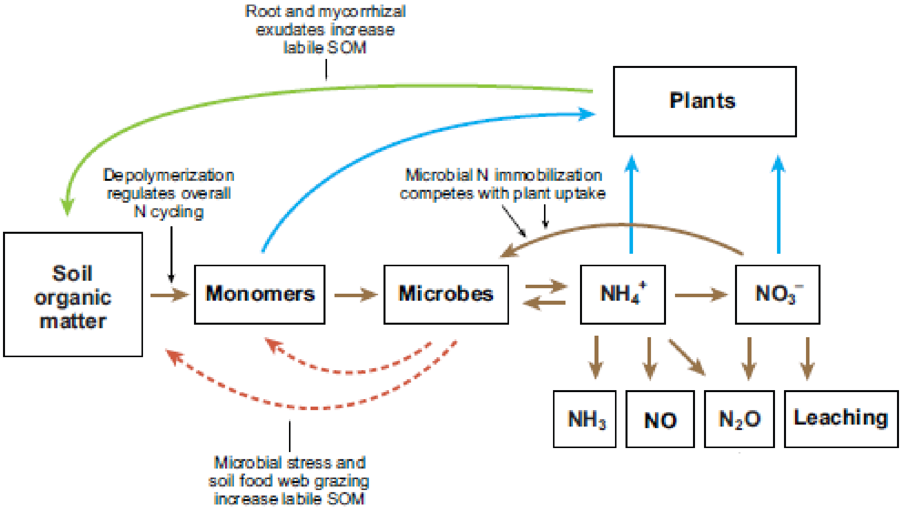

Figure 1.1: The soil nitrogen cycle (Jackson *et al.*, 2008), adapted from Schimel and Bennett (2004). N uptake of mineral N depends on mineralization of organic matter by microbes (Section 1.2), although there is also evidence that plants may be able to acquire organic N (Section 1.6).

1.3 Anthropogenic Modification of the N cycle

An industrial method to fix N into reactive, plant-available forms (NH_3 from N_2 and H_2 in the Haber-Bosch processes) was developed in the early 20th century to meet N demand for weapons manufacture, but also allowed much greater application of fertilizers for N-limited agricultural production which had previously been limited to guano and evaporite N deposits. These pre-industrial inputs were only capable of mobilising around 0.2 Tg N y^{-1} (Smil, 2001), and at this time were the main anthropogeneic peturbation of the N cycle alongside limited cultivation of leguminous species (e.g. rice, alfalfa, clover) either for their own purpose or to increase background soil fertility for other crops (Galloway *et al.*, 2004). The Haber-Bosch process enabled huge increases in food production, quadrupling the output of agricultural lands (Smil, 2001), and feeding the huge increases in human population in the 20th century (Smil, 1999; Butterbach-Bahl *et al.*, 2011).

As an N source, this new Haber-Bosch derived N was substantially different than previous fertilizer (manure and crop residue) as it represented a new source of N fixation, rather than recycling of already-fixed nitrogen. It also soon provided orders of magnitude more reactive N than background N fixation; while the total N fixation by terrestrial ecosystems is 110 Tg N y^{-1} (Gruber and Galloway, 2008), in 1860, around 10 Tg reactive N y^{-1} was derived from anthropogenic activity, but this fraction had increased to 187 Tg N y^{-1} by 2005 (Galloway *et al.*, 2008). In the modern world, human activity is the major form of N fixation on Earth.

1.4 Nitrogen Deposition

Unsurprisingly, the huge increases in availability of use of reactive N resulted in emissions of both oxidised (NO_x) and reduced (NH_y) forms of nitrogen to the environment (Galloway *et al.*, 2004), affecting ecosystems aside from the intended recipients of N fertilizers, and far from the sites of industrial activity. Reactive N emissions are also derived from cultivation of species with N-fixing symbioses, transport and industrial processes, which burn fossil fuels and produce reactive N by oxidizing N_2 or releasing sequestered N in fuels (Galloway *et al.*, 2004). Of these activities, agriculture is the largest total source of N (Galloway *et al.*, 2008), as only around 10 % of applied N is retained in food (Galloway and Cowling, 2002) but NO_x emissions to the atmosphere are greatest from industrial sources (van Aardenne *et al.*, 2001).

Rising levels of reactive N via nitrogen pollution has obvious influences on N-limited ecosystems as they are released from growth constraints, resulting in changes to ecosystem structure, function, and composition (Vitousek *et al.*, 1997; Bobbink *et al.*, 2010), while trophic changes occur as more N-limited species are able to proliferate and N-sensitive species decline (Fenn *et al.*, 2003). Many studies have been performed on the effects of N pollution, particularly the toxic and succession effects of high N runoff from agricultural fields (Butterbach-Bahl *et al.*, 2011), and the phytotoxic effects of high NO_x in combination with the heavy sulphur deposition in North America and Europe prior to emissions control legislation in the 1980s. While many ecosystems now have recovered from high acid loads following these political changes (e.g. Likens *et al.*, 1996), and eutrophication effects, while strong, are local and well-studied (Smith and Schindler, 2009)), emissions of NH_3, NO_x, and various organic forms of N continue to enter the atmosphere unabated and are transported great distances (Schlesinger, 2009), readily redepositing as wet (in rain) or dry (directly from the atmosphere) deposition, in timescales of minutes to days (Aneja *et al.*, 2008). This results in chronic N inputs to many ecosystems with total N magnitudes much smaller than the local N inputs from eutrophication, but spread over a much larger area (Figure 1.2). Currently, N_{DEP} in Europe may be declining slightly (Waldner *et al.*, 2014) although this effect is frequently masked by high variability across sites. Even with a longer term decreasing trend, N_{DEP} will remain a major component of many ecosystem total available N into the future.

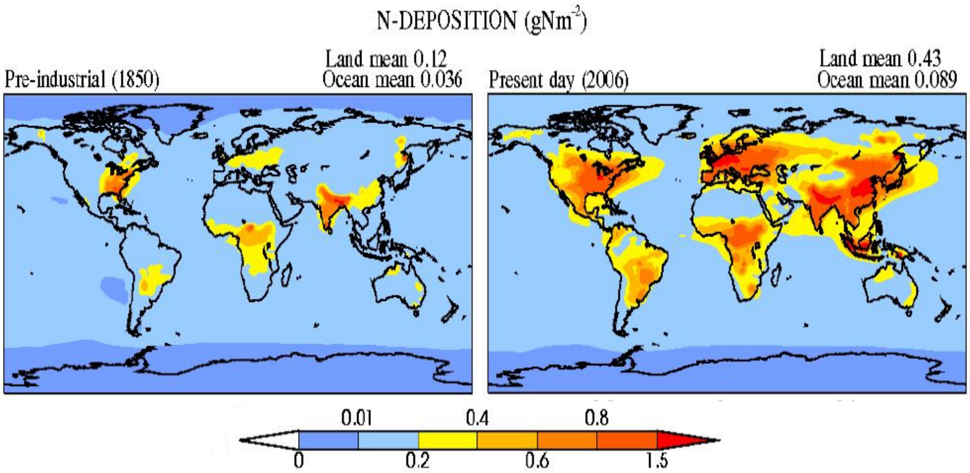

Figure 1.2: Global patterns of nitrogen deposition in pre-industrial (1850,left panel), and present day (2006, right panel). Adapted from Bala *et al.* (2013).

In general, N_{DEP} is greatest in regions of high fertilizer use and industrial activity (compare in Figure 1.2, North America, Europe, India, China) but it is not deposited uniformly across ecosystems in these regions. Of the total ~ 46 Tg N y^{-1} deposited on land (Galloway *et al.*, 2004), around 1/3 is deposited onto forests (Schlesinger, 2009) as their high canopy surface area and elevation of biomass increase overall N interception (Houle *et al.*, 1999). Forests are 2-3 times more susceptible to N_{DEP} than open land (Fowler *et al.*, 2004).

As temperate and boreal forests are N-limited (Vitousek and Howarth, 1991), susceptible to high N inputs (Fowler *et al.*, 2004), and are also very C efficient (having high C/N ratios due to large amounts of woody biomass Reay *et al.*, 2008), such ecosystems are good candidates for an increase in C uptake by nitrogen deposition. The scale of this uptake has been examined in depth in the literature on both a local and regional scale and is summarised in the next section to explain why estimates of the total effect of N on C uptake ($\Delta C/\Delta N$) can differ so greatly.

1.5 Uncertainty in Changes in Forest C Uptake due to N deposition

The first suggestions of an anthropogenic nitrogen effect on C uptake were from models in the 1980s, and predicted relatively small C uptakes (0.2 Pg globally) as a consequence of N and P pollution combined (Peterson and Melillo, 1985). As more global data became available, modelled estimates gradually increased to 1.0 - 2.3 Pg y^{-1} (Schindler and Bayley, 1993), or a terrestrial 0.3 to 1.3 Pg y^{-1} (Townsend *et al.*, 1996), while at the same time as these models were developed, N deposition effects were also being studied on smaller scale in N budget manipulations such as the European NITREX project (Wright *et al.*, 1995). Modelled nitrogen effect on C uptake largely depended on the C/N (Carbon to Nitrogen) ratios of the N sinks (Levy *et al.*, 2005; de Vries *et al.*, 2008), the C effect maximised if N within plants was allocated to woody biomass with high C/N ratios and long lifetimes, and lowered if the main plant sink was low C/N tissues such as leaves, twigs, or fine roots. Globally, this means that responses are strongest in forests, as they contain far more woody biomass than other ecosystems (Townsend *et al.*, 1996).

Many experimental studies (for a summary up to 1993 see Schindler and Bayley (1993)) showed that even at high extra inputs, most additional N could be retained within ecosystems (Goodale *et al.*, 2002). However, the potential C response was constrained by results from ^{15}N-isotope tracer fertilizer experiments (summarized in a meta-analysis of NITREX and North American nitrogen addition experiments using isotope tracers by Nadelhoffer *et al.* (1999c)), which found that from a synthesis of experiments, the major forest sink for N from N_{DEP} is the soil, with soil microbes (SMB), and aggregations with mineral and organic soil accounting for around 70 % of isotope tracers added to these systems. Trees obtained only about 20 % of the N applied in these experiments and only 5% of the total (1/4 of N acquired by trees) was found in woody biomass.

As the C/N ratio of the soil sink (30C:1N) and non-woody tree sink (25C:1N) are much lower than wood (500C:1N), the potential gain in carbon uptake as a result of N_{DEP} was limited to about 20 % of terrestrial carbon uptake, or 0.30 - 0.38 Pg y^{-1}, at the lower end of modelled scale estimates of carbon uptake enhancements (Högberg, 2007) and corresponding to a $\Delta C/\Delta N$ effect of 50 kg C kg N^{-1}. Other methodologies estimating an N effect also tended to be low and agree with previous predictions; using calculations based on ecosystem N budgets, de Vries *et al.* (2006) also found 46 kg C kg N^{-1} (or 10 % of C sequestration) in Europe could be attributed to N deposition, while field manipulations also found low effects, while only 5 kg C kg N^{-1} was calculated from results of a ^{15}N-tracer experiment at Harvard Forest, USA (Currie *et al.*, 2004), where most of the added N was retained in mineral soil. N saturation also limited an N effect in many systems - at very high levels of N_{DEP}, other factors become limiting (Aber *et al.*, 1989, 1998, 2003; Fenn *et al.*, 1998), leading to high N losses from these systems so all excess N would not apply to a total $\Delta C/\Delta N$ effect.

This low response to N due to low competitiveness of trees for soil N from N_{DEP} seemed settled, until Magnani *et al.* (2007) found a strong correlation of wet N_{DEP} with net ecosystem productivity (NEP). This implied a fertilization effect of N_{DEP} of several hundred kg C kg N $^{-1}$ (Magnani *et al.*, 2008), several orders of magnitude greater than was expected based on previous work. Unsurprisingly, these findings generated considerable debate (Högberg, 2007; de Vries *et al.*, 2008; De Schrijver *et al.*, 2008; Sutton *et al.*, 2008; Magnani *et al.*, 2008), with the Magnani *et al.* (2007) approach criticised for ignoring differences in local climate (Sutton *et al.*, 2008), GPP, or soil N mineralization capacity (de Vries *et al.*, 2008), but even under subsequent revision to 175-225 kg C kg N $^{-1}$ (Magnani *et al.*, 2008), this estimate was still considerably in excess of the apparent strength N_{DEP} on C sequestration in other studies. Later, Högberg (2012) also suggested that high rates of N deposition may also correlate with high edaphic (internal) N , which may be caused with historical N_{DEP} and eutrophication, or underlying ecosystem fertility and explain much of the correlation in Magnani *et al.* (2007). Across a range of European forest sites, N_{DEP} only contributed an average of 13.5 % of total N supply (Högberg, 2012).

Despite these criticisms, there are several reasons why Magnani *et al.* (2007) could have had such different results compared to other studies. As this was a correlative study, it is possible that the manipulative experiments from which other conclusions of $\Delta C/\Delta N$ and N partitioning were drawn did not sufficiently represent the real world. In particular, N-budget based calculations typically calculate N_{DEP} as throughfall plus stemflow, leaving nitrogen retained in the bark and canopy unaccounted for (Lovett, 1994; Kreutzer *et al.*, 2009), while ^{15}N-fertilizer traces (c.f. Nadelhoffer *et al.*, 1999b) typically apply ‘atmospheric’ N deposition directly to the soil, rather than to the canopy. A substantial retention of N in the canopy or direct foliar uptake of nitrogen (Sievering *et al.*, 2007), could both reduce total amounts and change the fashion by which N_{DEP} reaches the soil system, and even allow some N to bypass soil sinks if taken up directly by the canopy (Sievering, 1999).

The methodology of experiments summarised in Nadelhoffer *et al.* (1999b) was also not suited to trace changes in the forest floor N supply under N deposition treatments (Jenkinson *et al.*, 1999). The majority of N for plant growth is supplied from litter mineralization, being N previously held in biomass and recycled through the soil, even under heavy N_{DEP} (Högberg, 2012). A longer term response of forests to N_{DEP} may therefore also occur via changes in specific leaf area and litterfall rates (Reich *et al.*, 1999), belowground C (Burton *et al.*, 2000), and C allocation patterns (Palmroth *et al.*, 2006) as a response to tissue N concentrations and N availability. In general, the fate of litter N is difficult to trace experimentally and conclusions about an overall $\Delta C/\Delta N$ effect hinge on this N mineralised from litter being partitioned between pools of variable C/N ratios in the same manner as labelled N_{DEP} treatments. While C/N ratios of litter pools are often related to N losses, this may be driven more by tree species than nitrogen deposition (Cools *et al.*, 2014), and total N budget estimates relying on unlabelled methods may conflate C/N ratio differences with a N_{DEP} effect.

Incorporating canopy uptake and changes in C allocation due to increasing N into a model (Dezi *et al.*, 2010) which had produced a $\Delta C/\Delta N$ of 27.8 (McMurtrie *et al.*, 2001), alongside forest management practices common in North America and Europe found a relatively strong response (up to 121 kg C kg N^{-1}), although improving estimates of N losses in soil water in the model and changes in litter N only had small effects. While forest management was the most important factor in increasing this estimate, this study suggested that with model modifications the full range of low responses, e.g. Nadelhoffer *et al.* (1999c); de Vries *et al.* (2006),or high responses e.g. Magnani *et al.* (2008), could be represented using reasonable assumptions and that low estimates of an N_{DEP} effect on forest C could also be questioned regarding their validity in describing the forest nitrogen cycle under N_{DEP}.

Subsequent work using a similar methodology to Magnani *et al.* (2007) in North America (Thomas *et al.*, 2009) also found a relatively greater effect on above-ground C (73 kg C kg N $^{-1}$), which, while not as strong as Magnani *et al.* (2008), supports the argument that lower estimates from other methods may be considered to be overly conservative. Similarly, more localised studies across Switzerland and Italy using correlative methods Ferretti *et al.* (2014) suggest that $\Delta C/\Delta N$ response in these regions could be in the higher range of estimates. A summary of major studies with comparisons of $\Delta C/\Delta N$ is presented in Table 1.1.

1.6 Root Uptake of Nitrogen

The most basic understanding of plant nutrition is that all nutrients aside from CO_2 and oxygen are acquired from the soil by roots. Plants acquire can also acquire N by exchanging C with mycorrhizal fungi symbioses (which increase the available surface area for nutrient acquisition (Simard *et al.*, 2003), while beneficial symbioses can also occur with rhizosphere micro-organisms (Walker *et al.*, 2003). From the plant's perspective, this interface with the soil biota is adapted to maximise potential nutrient acquisition from the soil, exploring the belowground environment to exploit patches of high resource availability (Hodge, 2004). For reviews of methods of nutrient acquisition by roots, see Maathuis (2009); Jackson *et al.* (2008).

Because concentrations of $NH_4{}^+$ and $NO_3{}^-$ (the predominant mineral N forms in the soil) are higher inside than outside plant cells, plants must actively pump these by expending energy from photosynthesis. Transport is performed by specialised

Study	ΔC/ΔN			Methodology	Region
	Total	Soils	Trees		
Nadelhoffer *et al.* (1999c)	50	21	29	meta-analysis of ^{15}N amendments	North America and Europe
McMurtrie *et al.* (2001)	27.8[1]			GDAY model	modelled
de Vries *et al.* (2006)	46	21	25	N and C budgets	Europe
Hyvönen *et al.* (2008)	36[2]	11	25	long term N addition experiments	Fennoscandia
Hyvönen *et al.* (2008)	71	21	50	modified de Vries *et al.* (2006)[3]	Europe
Magnani *et al.* (2007)	400			NEP against wet N_{DEP}	Europe
Magnani *et al.* (2008)	200			revised estimates [4] Magnani *et al.* (2007)	Europe
Thomas *et al.* (2009)	100[5]		73	Forest inventory data against N_{DEP}	North America
Dezi *et al.* (2010)	121			modified GDAY model	modelled
Ferretti *et al.* (2014)	159			Forest inventory data against N_{DEP}	Italy

Table 1.1: Estimates of N use efficiency (ΔC/ΔN) of forests between different studies. Estimates are averages where a range of values is often reported and do not always explicitly separate soil tree ΔC/ΔN. [1]: Output as reported by Dezi *et al.* (2010) in comparison to modified version of model. [2]: Response was strongly dependent on C/N of humus layer, results presented are average but could be as low as 0 in low C/N humus ecosystems. [3]: Recalculated Europe-wide measurements using method of de Vries *et al.* (2006) but scaling factors from Hyvönen *et al.* (2008). [4]: Revised estimates incorporating dry N_{DEP}. [5]: Presents only above-ground estimates, total calculated by assumed similarity to other soil ΔC/ΔN results.

enzymes, divided into High and Low-Affinity Transport Systems (HATS and LATS), the activity of which are affected by factors such as pH, temperature, and soil nitrogen ion concentrations, and depend on maintenance of a proton gradient (Jackson *et al.*, 2008). Plants both impact the N cycle by taking up this nitrogen and by supplying carbon belowground which is utilised by microbes which are often the dominant sink for mineral N (Knops *et al.*, 2002). Between species, uptake in roots is adapted to suit soil properties (Falkengren-Grerup, 1995), species in reduced and low pH soils tending to prefer reduced NH_4^+, and those in high pH, oxidised soils NO_3^- (Maathuis, 2009). This difference allows plants to extract as much N as possible from their environment, and likely reflecting the typical availability of ions rather than a physiological preference for one form over another as, once acquired by the cell, plants must reduce NO_3^- to NH_4^+ to incorporate it into organic compounds, another step which requires energy expenditure.

Until the 1990s, it was widely perceived that this model of mineral N uptake was universal and as roots could only take up mineral N forms (Pate, 1973) and competed so poorly against soil microbes, plant N uptake in most soils was from N remaining in the NO_3^- pool after microbial utilization (Schimel and Bennett, 2004) (due to the typical negative charge in many soils, most NH_4^+ is often immobilised in cation exchange leaving NO_3^- the dominant available pool).

This 'microbial bottleneck' of competition (Knops *et al.*, 2002) was once thought to limit plant responses to N, but since this time, considerable evidence has built up (Chapin *et al.*, 1993; Lipson and Näsholm, 2001; Näsholm *et al.*, 1998, 2009; Rennenberg *et al.*, 2009) that although mineral uptake usually predominates (Harrison *et al.*, 2007; Jackson *et al.*, 2008), the root system and its symbioses can access organic N (such as amino acids) in the soil which are released from the decomposition of N-containing polymers during the decomposition process. Plants may also be able to actively control the N cycle (Chapman *et al.*, 2006) through feedbacks in mycorrizal symbioses (both in competition with saprotrophs

(Leake *et al.*, 2003), and via mycorrizal uptake of organic N (Hodge *et al.*, 2001; Hodge and Storer, in press)) allowing direct access to organic N without having to directly compete against soil microbes. Traditionally such associations were thought to have no N benefit (Read and Perez-Moreno, 2003), and globally, there are still questions about how important mycorrizal fungi are for N uptake on the ecosystem scale (Meyer *et al.*, 2009). While obtaining organic N from the soil is more energetically costly than ion uptake (Jackson *et al.*, 2008), this process may be especially prevalent in heavily N limited ecosystems (Rennenberg *et al.*, 2009), where a greater carbon investment in this process may allow plants to bypass limits in growth imposed by their poor ability to compete directly with soil microbes (Schimel and Bennett, 2004). This may offer a net energy benefit as organic N does not need to be reduced to usable NH_4^+ like NO_3^-. A lab study has also suggested that organic N sources may be partitioned differently within plants to mineral N (Cambui *et al.*, 2011), which may lead to differences in overall $\Delta C/\Delta N$, even if similar amounts of mineral and organic N were obtained.

Either way, plants allocate large amounts of fixed C belowground (Högberg *et al.*, 2001; Warren *et al.*, 2012) for building and maintaining the root architecture for nutrient uptake as well as the active transport of N compounds themselves (Cannell and Thornley, 2000). C assignment to different organs and processes may change under N_{DEP} if N is more abundant or differently available to the established soil sink. In particular, if substantial amounts of N can also be accessed by the canopy under N deposition (Sievering, 1999), both the C cost of N acquisition and the total magnitude of N fluxes into the plant may be reduced allowing an increased $\Delta C/\Delta N$ effect.

1.7 Canopy Uptake of Nitrogen

A high retention of N_{DEP} by the canopy has been suggested by numerous studies (e.g. 40-65 % (Schulze, 2000), 70 % (Gaige *et al.*, 2007), 80 % (Sievering *et al.*, 2007)). If N remains on the branches and foliage before being washed off by subsequent rain events, this both changes the rate and magnitude of N_{DEP} reaching the soil, and potentially makes this N directly available for nutrition by processes occurring across the canopy (Sievering, 1999). Canopy uptake of N is widely acknowledge in the literature, but estimates of its importance vary. As aside from human activity, sources of atmospheric deposition of N are minimal, there may seem to be few ecological reasons why trees have evolved to acquire N across the canopy, and as this canopy-acquired N can be assumed to be largely acquired by foliage, a strong $\Delta C/\Delta N$ effect depends on this N being assigned to higher C/N pools within the tree. Without stable isotopes and rigorous separation of biomass between different pools, it is also difficult to distinguish between actual uptake in canopies and retention by epiphytes, ion exchange in the canopy, or adsorption to bark surfaces (Dail *et al.*, 2009). Where ^{15}N isotope tracer tracers have been used to try and account for this uptake rather than using estimates based on throughfall and stemflow N concentrations, uptake by the canopy is 5-30 % (Friedland *et al.*, 1991; Wilson and Tiley, 1998; Ammann *et al.*, 1999) of total N inputs. However these experiments are rare and expensive, and other estimates of total nutritional value of canopy interactions can be higher, varying from very little importance (1.7 % total nutrition, (Schulze, 1989)), to 15-30 % (Tomaszewski, 2003), or 50 % of total nutrition (Sievering *et al.*, 2007). Even with these unlabelled N calculations, canopy uptake information at an ecologically relevant scale is still rather sparse and there is likely to be a strong effect of methodology, site and species (Templer *et al.*, 2005) which makes it difficult to interpret the overall importance of canopy uptake.

The mechanism of nitrogen uptake by the canopy is complicated and poorly understood, and possibly involves multiple processes. A review of nitrogen uptake mechanisms in foliage is given in Sparks (2009) and will only be discussed briefly here. In summary, NO, NO_2, or NH_3 dissolve as nitrous and nitric acid into the apoplast, and dissociate, into ${NH_4}^+$, ${NO_2}^-$ and ${NO_3}^-$, and are taken up into the cytoplasm and assimilated as ${NH_4}^+$ (Sparks, 2009). ${NH_4}^+$ and ${NO_3}^-$, deposited directly onto the leaf surface (primarily by dry deposition) may be taken up by another pathway which is not as well understood but may involve cation exchange (Sparks, 2009) and thus favour ${NH_4}^+$, or diffuse through the stomata before subsequent cuticular transport (Bowden *et al.*, 1989; Boyce *et al.*, 1996). Uptake could also occur across twigs by simple diffusion (Klemm *et al.*, 1989), which may cause high observed bark ^{15}N Wilson and Tiley (1998), while under very high N loads nitrifying chemolithoautotrophs may colonise the stomatal cavity (Papen *et al.*, 2002), and affect the potential for foliar nitrogen utilization by converting NH_x to NO_x, which may affect N uptake if the capacity for acquisition of cations and anions by the foliage differs.

1.8 Effect of Canopy Uptake On Plant Structure and Growth

Information on whole-tree exposure to reactive nitrogen has been available since the 1980s, when high levels of acid rain and artificial misting experiments allowed researches to examine the effects of high S (sulphur) and N loads from pollution. Physical damage to foliar tissues were commonly observed (Wellburn, 1990; Maurice and Crang, 1986) due to high N loads and ammonia concentrations (Krupa, 2003) although this may have been due to excessively high N concentrations, and high occurrences of S and ozone pollutants in tandem with N inputs (Harisson *et al.*, 2000). From more recent experiments using lower N_{DEP} magnitudes, usually using wet N_{DEP}, observational estimates of canopy uptake of nitrogen are around 2-10 kg N ha^{-1} y^{-1} (Kreutzer *et al.*, 2009; Kristensen *et al.*, 2004), and changes in canopy functioning such as photosynthetic efficiency and carboxylation rates have been observed (Sievering *et al.*, 2007; Tomaszewski and Sievering, 2007; Wortman *et al.*, 2012) when leaves are exposed to excess N.

As well as a canopy response, a change in whole-tree C uptake and growth can also depend on how N is assigned within the tree, as retention in the needles or the canopy would not have as large C effect as assignment to stemwood due to twentyfold differences in C/N ratios (Nadelhoffer *et al.*, 1999c). Movement of canopy-acquired N in trees is particularly important, as trees do not have the same seasonal growth constraints as annual plants, can store nitrogen between growing seasons and utilise it in periods of high C and N demand where N requirements can be met by remobilisation, and alleviate the carbon cost and rate limitations of root N uptake.

The location of this storage differs between species (Millard and Grelet, 2010), and many evergreen species are able to exploit their long-lasting foliage in a manner that deciduous species cannot, mobilising N from these foliar storage in the next growing season (Millard and Proe, 1992). A similar mechanism operates during leaf senescence (Näsholm, 1994; Norby *et al.*, 2000; Hörtensteiner and Feller, 2002) which may also allow deciduous species to make use of this uptake (Tagliavini *et al.*, 1998) and mobilise excess N from the leaves. However, in the only field-scale canopy-application of ^{15}N tracers in the literature, Dail *et al.* (2009) found a low 1-3 % N retention in high C/N bolewood from the experiment at Harvard Forest where 70 % of N amendments were retained in the canopy (Gaige *et al.*, 2007). Large amounts of extra retention being in bark, the measurement of which included epiphytic mosses, lichens, and cyanobacteria with low C/N ratios, and little capacity for a large $\Delta C/\Delta N$ response.

Larger trees may also take long periods to respond to moderate increases in N_{DEP} due to additional nitrogen first saturating storage compartments before it is assigned elsewhere in tree (Sheppard *et al.*, 2004), while trees also have relatively plastic nitrogen pools and do not sequester N in the same way as carbon (Millard and Grelet, 2010), so even if N is initially assigned to storage pools it may subsequently be translocated to other organs with a different C/N ratio. When seasonal changes or age cause senescence, limiting nutrients such as N are usually translocated to other parts of the tree and even in living foliage (Nussbaum *et al.*, 1993), some N from foliage is incorporated into amino acids and moved elsewhere in the plant via the vascular system. Schulze (1989) estimated that only 1.7 % of plant total N nutrition is likely to come from re-use of N from the foliage, but if N uptake potential of the canopy is high, under N deposition, these mechanisms may be able to operate on far larger amounts of 'excess' canopy N. Nitrogen additions are commonly used to encourage forest growth (often in mixed NPK fertilizers), establishing a competitive advantage for young trees over heather and bracken. However, overall growth responses tend to be lower under N deposition than a combination of all three macronutrients (Hyvönen *et al.*, 2008), especially on more fertile soils as P and K become limiting.

The effects of N on trees can involve numerous physiological differences as additive nitrogen manipulations are well documented to have effects on foliar structure, size, and nutrient contents (e.g. (Schaberg *et al.*, 1997; Elvir *et al.*, 2006), increasing foliar N, decreasing other foliar nutrient concentrations, and increasing needle size, while similar effects can be seen across natural nitrogen deposition gradients (e.g. Rueth and Baron, 2002). Reductions in overall root system size may also occur under N deposition (although these experiments tend to be direct applications to the soil), as N becomes more abundant belowground (Nadelhoffer, 2000), but higher turnover and reduced mycorrhizal symbioses (Egerton-Warburton and Allen, 2000; Lee and Jose, 2003) may also be a consequence. Overall, growth responses to N appear to be highly dependent on methodologies used (Nadelhoffer, 2000) and far more studies may be required for improved elucidation of such processes and their outcomes.

Under canopy nitrogen uptake there may be additional pathways to affect the roots and soil, as glutamine is synthesised in leaves taking up nitrogen (Nussbaum *et al.*, 1993; Sparks, 2009). This amino acid may play a role in downregulating belowground N acquisition as it is both rapidly transported by the phloem (Högberg *et al.*, 2008; Nussbaum *et al.*, 1993), and high concentrations in roots correlate with reduced N uptake (Geßler *et al.*, 1998) under both nitrate (Nussbaum *et al.*, 1993) and ammonium (Geßler *et al.*, 1998) deposition manipulations. Reductions in root exudates (Yano *et al.*, 2000) in response to N_{DEP} may also occur as less C is invested belowground, affecting the abundance and community structure of associated microorganisms, although in ectomycorrhizal species, this suppression may be relatively weak (Wallenda and Kottke, 1998) and it may be difficult to disentangle a specific canopy uptake effect from an overall higher soil N concentration. The degree to which belowground C-assignment is reduced may also depend on the availability of other nutrients (phosphorus or trace nutrients such as potassium or magnesium) or water for uptake, which may be under heavier demand due to N fertilization, but if less C is assigned to roots and spent to acquire nitrogen from the soil, more should be available to sequester elsewhere.

1.9 Effect of Nitrogen Deposition on the Soil System

While more studied, the effects of nitrogen deposition on the soil system are also relatively poorly understood, but are examined thoroughly in a meta-analysis by Knorr *et al.* (2005).These are complicated and depend on magnitudes of inputs, ambient N regimes, litter quality, and may change over time as litter decomposes. In an unperturbed system, roots and mycorrhizal fungi are responsible for decomposition, reduction, and assimilation of N from dead organic matter, the effect of N_{DEP} on decay rates being just as important as direct growth responses and responses to available N as, even under high rates of N_{DEP}, the majority of ecosystem N is still derived from edaphic sources (Schulze, 2000; Högberg, 2012). Nonetheless, there are several potential effects of N_{DEP} on this process: direct effects of nitrogen availability on litter decomposition (Knorr *et al.*, 2005), reductions of C inputs to the soil due to higher N availability (Burton *et al.*, 2000) (either in the soil or by the canopy), and changes in litter input quality or magnitude under nitrogen deposition (Reich *et al.*, 1999), as well as interactions between all these effects.

1.9.1 Direct Effects on Decomposition Rates

Early in the decomposition process, N inputs to the soil may enhance litter decomposition as decomposers such as saprotrophic fungi must gain N to colonise decomposing litter (Swift *et al.*, 1979; Parton *et al.*, 2007). However, sustained low levels of N deposition may also have a direct effect on soil community structure (Lilleskov *et al.*, 2001; Frey *et al.*, 2004) and promote overall reduced decomposition (Janssens *et al.*, 2010) as increased soil N availability may cause shifts in microbial populations towards more N limited, C efficient species (Ågren *et al.*, 2001), reducing overall rates of decomposition. This relationship follows the dynamics of decomposing litter and changes in internal quality; initial positive responses to N are due to release of water soluble, non-lignified carbohydrates and protein being decomposed (Melillo *et al.*, 1982; Berg *et al.*, 1993; Zeller *et al.*, 2000), while older litter fractions are made up of high C/N, recalcitrant lignin, which decomposes slowly and is inhibited by excess N content (Berg and Matzner, 1997; Carreiro *et al.*, 2000). Overall, Knorr *et al.* (2005) found no effect of the amount of N additions averaged over 500 studies, but strong interactive effects; high quality litters decomposing faster at sites with low ambient N deposition, presumably because these sites were highly N limited. In none of the 500 studies in Knorr *et al.* (2005) was N_{DEP} less than twice the ambient level and the authors identified a need for more studies at lower, more realistic levels.

1.9.2 Changes in C Assignment

As previously discussed, N availability, either purely in the soil or from the canopy, may reduce C inputs by trees to the soil if less energy is required to acquire sufficient N for growth (Nadelhoffer, 2000; Egerton-Warburton and Allen, 2000; Lee and Jose, 2003). This C may be used for other purposes above-ground, resulting in changes in the overall $\Delta C/\Delta N$ effect. Below-ground, plant-derived C is important in determining C losses from soil respiration (Högberg *et al.*, 2008), as well as below-ground community structure (Högberg, 2007; Yarwood *et al.*, 2009), which may affect rates of decomposition and N recycling as, like N, different C availability may drive changes in the viability of different ecological strategies.

1.9.3 Changes in Litter Quality

Finally, an important and long-term effect on soil N cycling is changes in litter inputs both in terms of tissue C/N concentrations, and leaf size and lifetimes (and thus total litter inputs to the soil). Reich *et al.* (1999) found these traits tended to increase with leaf N across different biomes, and changes in these trends have been confirmed multiple times under chronic N deposition experiments (e.g Magill and Aber, 1997; Schaberg *et al.*, 1997; Schoettle, 1999; Bauer *et al.*, 2004). As litter quality is an important determinant of decomposition rates on its own and with N_{DEP} (Knorr *et al.*, 2005), changes in this due to nitrogen deposition may also be important over the long term, and particularly absent in short-term N fertilization studies.

1.10 Stable Isotopes in Ecology

Isotopes of an element such as carbon (^{12}C and ^{13}C), nitrogen (^{14}N and ^{15}N) or oxygen (^{16}O, ^{17}O, and ^{18}O) have different quantities of neutrons in the atomic nucleus but otherwise undergo near-identical chemical reactions. Minor differences in preference for one form of an isotope occur in chemical reactions (a process known as fractionation). This means that the ratio of heavier (^{H}X) and common (^{L}X) forms of biologically common isotopes (e.g. ^{H}X/^{L}X) can be used by ecologists to distinguish between i) changes in processes at natural abundance where rates of a fractionating reaction my change and thus differences in the measured ratio of isotopes can infer changes in the rates of different reactions and ii) introductions of highly enriched or depleted ^{H}X where the isotope abundance of a source pool is so distinct from natural variation that subsequent changes in other pools can be attributed to this source, allowing the tracking of this tracer through a system of interest. In particular, the stable forms of these isotopes (e.g.^{13}C, ^{15}N, ^{17}O, ^{18}O) do not undergo radioactive decay, are particularly well suited to use as tracers where radioactive half-lives and/or safety concerns may limit application. The heavier isotopes of elements are typically much rarer than their light counterparts, and are commonly specifically named in scientific texts (e.g.^{13}C, ^{15}N, ^{17}O, ^{18}O), while the common, lighter form (^{12}C, ^{14}N, ^{16}O) is not always directly specified and often depends on context.

As the heavier forms of the elements are comparatively rare, being able to identify them using analytical techniques (Mass Spectrometry, Tunable Diode Laser Spectroscopy, Nuclear Magnetic Resonance Spectroscopy) allows them to be used as tracers in scientific studies. Applications of isotopes in ecology are as diverse as tracking animal migrations (e.g Rubenstein and Hobson, 2004) and food sources (e.g Szepanski *et al.*, 1999) to measuring water use efficiency in trees (e.g Guerrieri *et al.*, 2010) and quantifying C movement between soil and plants (Ineson *et al.*, 1996). In the context of the N cycle, the ^{15}N tracer is useful for measuring uptake and partitioning of ^{15}N sources (e.g. Jackson *et al.*, 1989; Nadelhoffer *et al.*, 1999c) and is a very useful tool for investigating changes in the ^{15}N cycle. Unlike ^{13}C, which is both more common (1.109 % of all carbon) and variable within and between ecosystems (especially between C3 and C4 plants (Ineson *et al.*, 1996)), ^{15}N is both rarer (0.3663 % of all nitrogen) and shows sustained variation and high heterogeneity at natural abundance (Figure 1.3) especially in soils (Högberg, 1997; Weber and Bol, 2008). Within the terrestrial biosphere, ^{15}N has no major natural differences to exploit, while a large number of different pools of variable size mean it is difficult to use this natural abundance variation to trace N dynamics (Robinson, 2001). In this case, highly ^{15}N-enriched material must be introduced and used to follow ^{15}N enrichment in different pools from this artificial source.

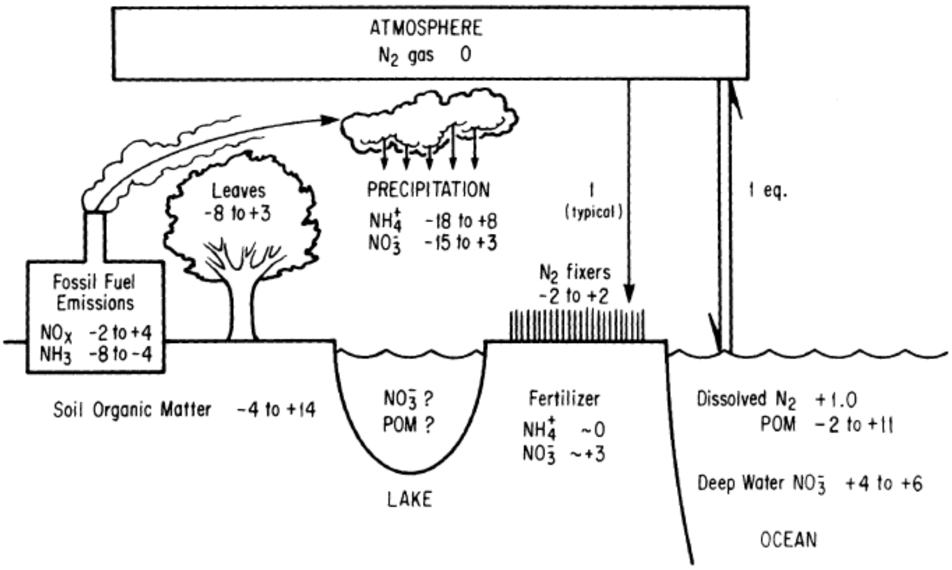

Figure 1.3: ^{15}N isotope natural abundance (δ^{15}N ‰ in different pools (Peterson and Fry, 1987). δ^{15}N is often near the 0 ‰ of atmospheric N_2 and does not vary substantially, especially in terrestrial systems.

1.11 Objectives of Thesis

The main objective of this thesis was to investigate some of the remaining questions in the effect of N_{DEP} on forests, particularly to investigate two potential ways in which N_{DEP} may not be fully represented in manipulative studies using ^{15}N-isotope tracers.

We aimed to conduct experiments using different sources of nitrogen which may affect the assignment of N within the tree and soil pool, and compare these with an experimental model of nitrogen deposition in a similar manner to ^{15}N inputs summarised in Nadelhoffer *et al.* (1999c).

Due to the relatively short duration of a PhD project compared to the typical time which a large N dose may take to saturate storage compartments in trees (Sheppard *et al.*, 2004), or affect foliar N concentrations feeding back into litter quality and N recycling (Magill and Aber, 1997), a series of experiments were designed and implemented, which traced the relatively short-term fate of a ^{15}N label from fertilizer, applied to foliage or to soil, or released from decomposition of ^{15}N-enriched litter.

If N is acquired by the canopy it may reduce tree C assignment to below-ground pools due to lower demand for N from this source, and subsequently change overall $\Delta C/\Delta N$ if C can be assigned to more C efficient biomass, or if more N in total can be acquired by the plant, allowing it to overcome a N limited state.

Similarly, if N mobilised from litter decomposition is more available to trees due to organic uptake, or differently partitioned (Cambui *et al.*, 2011) within trees than mineral N, the overall $\Delta C/\Delta N$ effect may be inferred to be greater than the effect of N deposition to the soil plus N decomposition effects on litter, as this litter-derived organic N can be acquired before passing through heavy competition with microbes in the NH_4^+ and NO_3^- pools.

Using the stable isotope ^{15}N also allowed experiments at very low N_{DEP} magnitude close to ambient conditions. This methodology, used in the second and third research chapters, allowed a focus on ^{15}N partitioning effects, independent of changes in soil and plant processes related to the magnitude effects of additional N added (for example, changes in litter decomposition under different nitrogen deposition magnitude (Knorr *et al.*, 2005)). This low N_{DEP} magnitude also meant that the total N input to these experiments was close to real world deposition and without the cumulative effect from adding a large N dose on top of a system already receiving atmospheric deposition, thus measuring a response from total N in excess of realistic N_{DEP} to the system.

1.11.1 Hypotheses

In Chapters 4 and 5, the following broad hypotheses were tested to compare the fate of ‘external’ mineral fertilizer additions of ^{15}N when applied to the soil surface, with similar treatments applied to the canopy, as a more realistic model of deposition (Chapter 4), and with ‘internal’ ^{15}N from decomposing litter (Chapter 5). The third hypothesis arises as a conseqeuence of the first two:

1. Recovery of N in fertilization treatments (used to simulate N_{DEP}) is greater in canopy- than soil-targeted fertilization treatment over the same magnitude due to exposure to, and uptake of, N by the canopy (Chapter 4).
2. Recovery of ^{15}N in trees from ‘internal’ litter pools under N deposition treatments is greater than the recovery of ^{15}N added ^{15}N-labelled mineral fertilizer additions to the soil, due direct plant uptake of organic forms of N which allows them to bypass microbial competition (Chapter 4, 5)

3. Recovery of ^{15}N in trees from ‘internal’ litter pools under N deposition treatments is lower under canopy- than soil- targeted fertilization treatments in biomass pools which receive proportionally more ^{15}N from canopy-targeted treatments (Chapter 4)

While similar treatments had been performed independently at different sites (c.f. Nadelhoffer, 2000; Zeller and Colin-Belgrand, 2001; Dail *et al.*, 2009), they had never been directly compared against each other in a single study. As site-specific responses are likely to be an important part of ^{15}N retention (see comparisons of multiple ^{15}N tracer studies, (e.g. Templer *et al.*, 2012)), understanding the quantitative differences between such treatments has never been directly possible.

The major difference between these two studies was that Chapter 4 was a pot study where canopy treatments could be applied, while Chapter 5 was a field study where the soil system could be studied in depth. For the field experiment, I also had to produce a large quantity of ^{15}N-labelled litter, as at natural abundance, ^{15}N content is highly conserved (Högberg, 1997; Callesen *et al.*, 2012), and there is no natural variation which can be used provide a source of ^{15}N-labelled litter without its artificial creation. This is described in a separate research chapter, Chapter 3, where effects of tree size, canopy position, and phenology on canopy ^{15}N return from a stem-injected treatment are also discussed.

1.12 Summary of Research Chapters

This thesis is written as a series of three research chapters which are intended for publication independently as scientific papers. Each uses ^{15}N-tracers in experiments on Sitka Spruce (the most common forestry species in Britain). A short supplementary description of stable isotope tracer theory is described in

Chapter 2, which follows this introduction. The contents and purposes of each research chapter (Chapters 3,4,5) are as follows:

3. **Stem Injection of ^{15}N-NH$_4$NO$_3$ into mature Sitka Spruce (*Picea sitchensis*)**

   This chapter describes a field experiment where 98 % ^{15}N-labelled ammonium nitrate was introduced via stem injection to the vascular system of adult *Picea sichensis*, where it was translocated into the canopy, and the relative expression of the label in the canopy was assessed. Previously, stem injection of ^{15}N has primarily been performed on small trees or without replication, and but here we were able to assess ^{15}N return and recovery in adult *Picea sichensis*is analysed statistically and discussed within the context of tree N storage phenology.

4. **Differences in ^{15}N-Return in Sitka Spruce between Canopy and Soil ^{15}N Treatments**

   This chapter describes a pot-based experiment addressing all three main hypotheses in a relatively controlled mesocosm experiment.

   Six replicated treatments are described where ^{15}N fate from litter and deposition could be compared when N deposition treatments were applied to the soil or to the canopy and an artificial litter source was available to 4-year old trees. We recorded a time-series of needle ^{15}N expression for 14 months after treatments started, and an endpoint recovery of the ^{15}N-label within the stems, branches, and roots of the whole plants, in both the current year cohort (which grew under the treatment regimes) and older biomass. From ^{15}N expression in individuals with labelled litter, and individuals experiencing labelled deposition, a synthesis of the fate of N from both of these pools was obtained.

5. **A Field Study Comparing the Fate of ^{15}N from Sitka Spruce Litter Mineralization with ^{15}N from Nitrogen Deposition**

The third research chapter describes a field experiment similar to the soil ^{15}N-deposition and labelled litter where a more realistic, field-scale soil profile was available, but a canopy treatment was not possible. ^{15}N was used as a tracer both from direct soil deposition (which could be assumed to amend throughfall and stemflow N_{DEP}) and from the ^{15}N-labelled litter, to soil pools (litter, roots and bulk soil) over a timeseries. After 16 months, at the end of the experiment these pools were assessed in a mass balance, along with K_2SO_4-extractable N and soil microbial biomass. ^{15}N-recovery was examined from deposition or litter sources of the ^{15}N isotope between these alternative pools.

Chapter 2

Stable Isotopes

2.1 Stable Isotope Notation

We have used stable isotopes in all 3 chapters of this thesis so briefly describe isotope notation in this section. Stable isotope abundance (how much of a particular isotope is present) is typically recorded in one of two fashions, δ (delta), in units of ‰ (permille), or atom %, in percentage units, both of which have been used in different contexts in the following research chapters.

2.1.1 $\delta\ ^{15}$N

The δ-abundance of a heavy isotope (expressed in units per mille, ‰) is calculated in equation 2.1, R$_{sample}$ and R$_{standard}$ being the ratio of the heavy (^{H}X, e.g. ^{15}N) to light (^{L}X, e.g. ^{14}N) isotope (^{H}X / ^{L}X) in the sample in question (R$_{sample}$), and R$_{standard}$ that of a standard reference compound (which is N_2 in air for nitrogen, and Pee-Dee Belemnite, a fossil, for carbon).

$$\delta^H X = [R_{sample}/R_{standard} - 1] * 1000 \tag{2.1}$$

As this expression of abundance includes two ratios, it can be a negative value, *(*i.e. less ^{H}X relative to ^{L}X than in the standard). This is commonly the case for ^{13}C in natural systems as the reference compound for ^{13}C is unusually highly ^{13}C enriched.

2.1.2 Atom % ^{15}N

The amount of isotope ^{H}X can also be expressed as atom percent (equation 2.2), where the amount of ^{H}X is compared to the total amount of the element. This is always positive and cannot exceed 100 %.

$$atom\%^H X =^H X/X \tag{2.2}$$

2.1.3 Conversion between δ ^{15}N and Atom % ^{15}N

δ X and atom % X are not linearly related to each other, but at natural abundance values tend to be very close to this relationship. δ^{15}N is commonly used to express ^{15}N contents at close to natural abundance ^{15}N and at this level atom % notation will differ only in the third or fourth decimal place. Atom % is typically used for artificially enriched material, although it is fairly trivial to convert between the two (equation 2.3).

$$atom\% = \frac{100 * R_{standard} * (\delta/1000 + 1)}{1 + R_{standard} * (\delta/1000 + 1)} \tag{2.3}$$

In this thesis, atom % is used in Chapters 3 and 4, as ^{15}N contents were high in the artificial N injection and in some of the treatments in the potted experiment. In the field experiment (Chapter 5), many of the differences were much closer to natural abundance, so δ^{15}N is used as the primary notation. Where comparisons between the two are made, values from different experiments are always compared in the same units.

Chapter 3

Stem Injection of ^{15}N-NH$_4$NO$_3$ into Mature Sitka Spruce

3.1 Introduction

Interest in the role of the nitrogen cycle in ongoing global change has driven a large number of studies into the effects of N deposition and the dynamics of N pools within ecosystems (e.g. Nadelhoffer *et al.* (1999c); Magill *et al.* (2004); Magnani *et al.* (2007)). Nitrogen's stable isotope, ^{15}N, is often used as an enriched tracer in spikes of mineral ^{15}N additions (e.g, Högberg (1997); Nadelhoffer *et al.* (1999b); Mulholland and Tank (2000); Templer *et al.* (2012)), or at natural abundance (e.g,

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Högberg (1990); Dijkstra *et al.* (2008)), to investigate N dynamics beyond that which can be measured in bulk changes in pools and fluxes. However, as ecosystem $\delta\ ^{15}$N is typically highly conserved (Robinson, 2001), soil δ^{15}N is spatially variable (Högberg, 1997) and temperate decomposition rates are relatively slow (Vitousek and Howarth, 1991), it is consequently very difficult to trace ^{15}N from litter pools, without a source of biomass with a δ^{15}N high enough to allow detection. Labelled biomass must be even more enriched if short-term recovery of the label is desired, or if one intends to trace the label into relatively uncompetitive pools, with high dilution, such as trees (Nadelhoffer *et al.*, 1999c).

Biomass enriched in ^{15}N can be produced by application of labelled fertilizer (Weatherall *et al.*, 2006b; Langenbruch *et al.*, 2013), foliar sprays (Zeller and Colin-Belgrand, 1998), or by direct injections into the plant vascular system (Swanston and Myrold, 1998). This latter methodology is potentially most efficient as valuable ^{15}N-labelled material is not lost via misting (Bowden *et al.*, 1989), exposed to soil sinks (Nadelhoffer *et al.*, 1999c), or exported from the immediate area by soil hydrology. Injection techniques (Roach, 1939) were first utilised to apply enriched ^{15}N compounds by Horwath *et al.* (1992) and consist of a reservoir of injection substrate introduced to the tree either passively (Proe *et al.*, 2000; Christenson *et al.*, 2002; Garten and Brice, 2009; Churchland *et al.*, 2012), or under pressure (Horwath *et al.*, 1992; Swanston and Myrold, 1998), via a purpose-drilled hole accessing the cambium and plant vascular system where the solution is taken up via a Venturi effect. This method can be used to trace the fate of injected elements either within the trees (Horwath *et al.*, 1992; Swanston and Myrold, 1998; Augusto *et al.*, 2011), or into the soil system (Garten and Brice, 2009; Churchland *et al.*, 2012) but it has rarely been used (Christenson *et al.*, 2002; Weatherall, 2005) as a method with the primary purpose of creating labelled biomass, typically targeting relatively young and small trees, where total biomass is low, and the canopy both open and easily sampled.

It is difficult to draw conclusions about the overall effectiveness of this method because of the large variety in the species employed (Table 3.1), but generally, it appears that in conifers, injected N is heterogenously distributed within tree crowns both in the short term (Augusto *et al.*, 2011), and even more so as N is translocated throughout the canopy by the tree. These differences may be caused by within canopy variation in N demand due to exposure and related photosynthetic activity (Ellsworth and Reich, 1993), or variations in needle age and N storage potential (Proe and Millard, 1994), which may vary in larger trees, both due to allometric scaling of tree proportions (Niklas, 1995), and the effects of canopy closure on crown size. Both of these changes also reduce the absolute amounts of foliage to woody biomass within the tree (Ritson and Sochacki, 2003) which may also affect the fate of injected ^{15}N between foliar and woody pools. Evergreen species also retain needles for several years (6-8 in Sitka spruce (*Picea sitchensis* (Bong.) Carr.) (Norman and Jarvis, 1974)), so younger trees may not represent the full range of needle age classes present in older individuals.

As well as tree biomass size and proportions, the size of N pools within the tree and their sink strengths change over the growing season, both due to phenological variation in nutrient assignment (Weinstein *et al.*, 1991), and overwinter storage of ^{15}N in current year needles and roots (*e.g.* Millard and Proe (1992) for Sitka spruce). In a study on 4-year *Pinus radiata* (D.Don), Proe *et al.* (2000) initially recovered 45 % of the injected ^{15}N in the canopy one week after injection, rising to 83 % at the end of the growing season (eight months after injection), with a bias in ^{15}N recovery away from the upper canopy, while in Sitka spruce saplings, the majority of an injected ^{15}N-NH$_4$NO$_3$ solution was found in the above-ground biomass of the harvested trees (Weatherall, 2005).

The aim of this study was to produce a quantity of ^{15}N enriched Sitka spruce (*Picea sitchensis*) biomass suitable for a subsequent field study, requiring hundreds of kgs of dry, isotope-labelled foliage for replacement of litter layers. As the intention was

Table 3.1: Summary of selected stem injection experiments using a ^{15}N label. Studies have been included to demonstrate the variety of purposes, species, and tree sizes employed.

Reference	Species	Habit	Purpose	Height (m)	n
Horwath *et al.* (1992)	*Populus* clone	Deciduous	Trace to roots / soil system	not given	8 (2×4)
Swanston and Myrold (1998)	*Alnus rubra*	Deciduous	Partitioning within crown	5	10
Proe *et al.* (2000)	*Pinus radiata*	Evergreen	Partitioning within crown	5-6	8 (2×4)
Christenson *et al.* (2002)	*Quercus velutina*	Deciduous	Herbivorous moth frass	9	1
Weatherall (2005)	*Picea sitchensis*	Evergreen	Labelled biomass	3.2	7
Garten and Brice (2009)	*Liquidambar styraciflua*	Deciduous	Partitioning belowground	17	24 (2 ×3×2)
Augusto *et al.* (2011)	*Pinus pinaster*	Evergreen	Foliar labelling	4.8	3

to produce as much labelled foliar biomass as possible, it was planned to inject trees on the edge of our target stand, because they were expected to have relatively more foliage than inside the closed canopy (Zavitkovski, 1981). A potential consequence of this approach is that edge trees may display spatial variability in ^{15}N recovery because of factors that affect intra-canopy ^{15}N distribution. The trees ranged in heights from 9 to 13 m, and we investigated differences in ^{15}N recovery and distribution in the canopy due to variations in tree and crown morphology.

3.2 Methods

3.2.1 Site and Stand Characteristics

A 20-year old stand comprising 90 % Sitka spruce and 10% *Larix decidua* (European larch) was selected in Cardrona Forest, a mixed conifer plantation forest in the Scottish Borders (55°61’50" N -3°12’87" E) , about 38 km south of Edinburgh. The site was a hillside with well draining, brown forest soil (annual rainfall of 887 mm, mean monthly temperatures between 0° C and 18° C). The stand was selected as it fulfilled the criteria of having a long, accessible stand edge (0.6 km) of (predicted by forest inventory GIS) 10-12 m tall Sitka spruce, close to a forest road, while not being located on any major recreational routes through the forest.

3.2.2 Injection Method

Stem injections took place in July and August 2011 with the trees remaining in the field until December 2011. Twenty-one trees with Diameter at Breast Height (DBH) between 12 and 25 cm and no visible wounds or deformities at breast height (1.3 m) were prepared for the ^{15}N injection along the stand edge.

Our injection apparatus (Figure 3.1) was based on a passive uptake design (Proe *et al.*, 2000). The apparatus consisted of a reservoir (an inverted 1L bottle with two 10 mm holes in the raised base), affixed to the tree and connected by a 3 mm diameter tube to a 20 mm diameter, double-holed bung. A second 3 mm tube from the bung was closed with an adjustable plastic tap. The trees were prepared by removing an area of bark around 1 m from the ground on the inside of the stand with sandpaper, and drilling a 35 (depth) x 20 (radius) mm hole using a hand drill with a wood auger bit. Once drilled, the hole was immediately plugged with the bung and coated on its sides and surface with a commercially available waterproof silicon sealant. For each tree, the reservoir was pre-filled with DI (deionised) water and allowed to flow through the apparatus by operating the tap, flooding the wound site and draining out, to refill the wound as quickly as possible and limit cavitation. Once air bubbles had been flushed from the system, the tap was closed, leaving 1 L empty volume in the reservoir, which was then filled with dilute red Safranin dye, and the tap adjusted to bring the coloured solution to the injection site. The next day, trees without obvious uptake or with evidence of leaks (8 of the 21 trees prepared for ^{15}N injections) were eliminated from the experiment. For the remaining trees, the apparatus was partially drained using the tap to leave 1 L of empty capacity, and filled with 1 L of the injection solution.

The injection was 1 L of 21 g L^{-1} double labelled 98 atom % $^{15}NH_4{}^{15}NO_3$ (CK Gas Products, Hampshire, UK), delivering approximately 7.53 g ^{15}N or 0.3 to 0.8 % of the total tree N pool, depending on the size of the tree. NH_4NO_3 was used for the injections as both of its constituent ions are transported in the xylem stream (Marschner and Marschner, 2012), with a label equally distributed between the anion and cation in case of differential assignment within the tree. After the introduction of the solution, the uptake (in ml) from the bottles was recorded from every reservoir every 1-2 days, and at each occasion the reservoir refilled to 1 L by addition of further DI water to prevent the equipment running dry between refills, while steadily diluting the solution. A linear rate of uptake from the bottles was assumed and the bottles were topped up until the estimated NH_4NO_3 concentration in each bottle was below 1 g L^{-1} in all bottles. The bottles were then allowed to run dry and stand for several days before deconstruction.

3.2.3 Sampling Strategy and Analysis

All 13 trees were felled in December 2011, 4.5 months after the injection, along with an unlabelled tree as a control. All branches were immediately cut away from the main stem and bundled into six categories per tree ((Figure 3.2), representing the specific location of removal along the main stem, in combination of three vertical sections: Canopy$_{BOT}$ (from the base of the tree to 3.5 m up the trunk), Canopy$_{MID}$ (from 3.5 to 7.5 m up the trunk), and Canopy$_{TOP}$ (from 7.5 m to the top of the tree), and two radial sections: Canopy$_{IN}$ (comprising 120° inside the stand), and Canopy$_{OUT}$ (comprising 240° facing out of the stand), with each of the six spatial positions having both a vertical (Canopy$_{TOP}$, Canopy$_{MID}$, or Canopy$_{BOT}$) and radial (Canopy$_{IN}$ or Canopy$_{OUT}$) identifier. The bundles were either removed from site immediately and transported to the location of further processing, 28 km away, or, due to the large volume of biomass, left on site for three weeks, until early January 2012. Both sets of branches were stored outside away from sites where water would accumulate, under tarpaulin, until all had been collected in early January. During this period most precipitation at both sites was snow which had not substantially melted at the time of collection of the remaining biomass.

Once all branches were collected, all the bundled sections were moved inside a dry polytunnel and chopped into small sections using a chainsaw and manual loppers. This material was then dried in batches in a timber kiln for up to two weeks at 70°C, but, due to the time required per batch, around 3/4 of the material was found to be sufficiently dry to cause needle shedding after temperatures in the polytunnel reached 40°C in spring 2012. Moisture content of these samples was compared to the kiln-dried samples to make sure they were similarly dry.

For ^{15}N analysis, three subsamples of 30 needles per section were drawn from the total needle harvest, after the dried needles had been well mixed, resulting in a composite sample of the total needle pool of each section. These were gently washed in distilled water to remove surface residues and any residual wood dust from the processing, then redried in an 80°C oven until mass loss had ceased (usually 24h, although some samples remained in the oven for up to 48h) and milled inside plastic micro test tubes in a Retsch MM400 ball mill (Retsch Ltd UK), for 20-30 minutes until the sample was homogenised into a fine powder. In addition to the thirteen trees sampled for ^{15}N recovery within the complete needle biomass, sub-samples of three branches from each of the six vertical/horizontal combination sections for five trees were taken to separate the 2011 cohort of needles from those produced in previous years. These sections were identified by growth beyond the most recent branch whorl, and separated from the main biomass of the branch before drying. The whole yield of needles harvested from the branch for both the current year biomass and the older biomass was weighed and dried independently to allow a calculation of the proportion of current year needles in the section.

Sampling of the woody biomass component was performed on five trees after the needles had been removed. Cuttings were taken from the branches in each section and replicated by sampling from three entire harvested branches, distinct from the tree stem at their base, in each section. The entire branches were not homogenised for sampling but sections for analysis were taken from a range of distances along the branch to attempt to sample a representative range of tissues, taking three branch ‘cookies’ per branch per sample, containing the entire radial section 1 cm in length. These samples were washed and redried like the needles, then milled in large metal cups with two large ball bearings in the MM400 ball mill although some larger sections were split and only a radial fraction of the disc analysed. Care was taken to clean the cups thoroughly with distilled water and 100% ethanol between successive measurements. For both the needles and the branches, 2.5 - 3.5 mg of the milled powder per sample was weighed into a 8.5 mm ultra-clean tin capsule and analysed for [N] and $\delta\ ^{15}$ N on a SerCon Callisto CF-IRMS Isotope Ratio Mass Spectrometer (School of Biological Sciences, University of Aberdeen, UK), along with standards of known isotopic abundance every 10 samples to allow the entire run to be corrected for drift. A small number (5%) of less enriched samples were analysed at the School of Geosciences, University of Edinburgh on a VG PrismIII dual inlet Isotope Ratio Mass Spectometer with CE Instruments NA2500 Elemental Analyser, with some samples run on both devices to ensure comparability. When analysing particularly highly enriched samples (with δ^{15}N in the 1000s), a minor enhancement of the ^{15}N ratio of subsequent samples is observed (A Midwood, pers. comm.). In order to reduce the effect of this artefact, samples of suspected high enrichment were run on the mass spectrometer in order of expected increasing enrichment.

3.2.4 Simple Predictive Model

A simple allometric model was used to calculate the expected ^{15}N abundance based on tree and canopy size if the ^{15}N injected was evenly distributed throughout the tree. We used measurements of total dry needle biomass made at felling, as well as DBH and measured tree height (length of intact stem + stump after felling), and used allometric equations to predict the ^{15}N recovery within the tree. To calculate the branch biomass of the trees we used equations for foliar and crown biomass, but, as our trees had comparatively more lateral biomass than typical due to their edge profile, we used the actual needle biomass to derive crown and branch biomass by rearranging the standard equations:

$$DBH = \sqrt[p]{\frac{log_{\beta}(1 - NeedleBiomass/\alpha_{needles}) - \alpha_{branches}}{\gamma}} \tag{3.1}$$

as given in McKay *et al.* (2003), where $\alpha_{needles}$ and β are constants for leaf biomass models for spruces and firs, and $\alpha_{branches}$, p and γ are species-specific constants for a crown biomass model for Sitka spruce. Branch biomass was then calculated as the difference between the crown biomass model ($\alpha + \gamma$.DBH.p (McKay *et al.*, 2003)) and the measured needle biomass.

Predicted N recovery was based on biomass and measured average N %, assuming that all N in the canopy was a valid sink for the injected N, with no losses such as gaseous N emissions or leakages from the apparatus. ^{15}N was allocated evenly based on the calculated size of N pool the canopy, divided into separate branch and needle pools. No spatial variation in allocation due to radial or vertical components was included in this null model, and no enrichment was allocated to the roots, but this was assumed to be minimal ($<$ 5%) based on earlier work on Sitka spruce saplings (Weatherall *et al.*, 2006b), nor was any ^{15}N allocated to stemwood, where C/N ratios are higher (Gundersen, 1998), and a greater proportion of the total biomass is not growing. This assumed no net growth over the injection period (*i.e.*, that the size of the N pools within the tree were the same at the time injected as when felled) and no losses of ^{15}N due to senescence or shedding of needles from the oldest age classes of needles. While both growth and litterfall would have been ongoing in the trees, the end of the growing season is usually a period of fine root growth and starch production, rather than stem elongation (Ford and Deans, 1977; Weinstein *et al.*, 1991), and litterfall does not appear to have a seasonal component in Sitka spruce (Hansen *et al.*, 2009).

Predicted ^{15}N recovery in each section was therefore calculated as follows:

$$^{15}N_{predicted} =^{15} N_{initial} +^{15} N_{injected}.(\frac{N_{section}}{N_{crown}}) \tag{3.2}$$

where $^{15}N_{initial}$ is the initial total ^{15}N content of the section in question, $N_{section}$ the total N of the section (determined post-harvest based on per-section average [N]), N_{crown} the total tree level N specific to each individual tree, and $^{15}N_{injected}$ the (constant) total ^{15}N of the injection.

3.2.5 Expressions of ^{15}N recovery

The predicted and observed ^{15}N atom % (referred to as ^{15}N enrichment), were expected to vary among trees because of variable dilution due to tree size. Therefore we also calculated a percentage recovery (referred to as ^{15}N recovery, equation (3.3)), assuming an even distribution of all injected ^{15}N throughout the canopy (equation (3.2)) which allowed comparison of relative ^{15}N recovery between different sections of the canopy while accounting for an expected lower ^{15}N enrichment in larger trees due to dilution.

$$Rec_{section} = Rec_{crown} \cdot {}^{15}N_{injected} \cdot \frac{N_{section}}{N_{crown}} \tag{3.3}$$

Rec_{crown} being the total recovery of the injection, in % units, specific to each tree.

3.2.6 Statistical Analyses

All statistical analyses were conducted in R (R Core Team, 2013) v3.1.0.

We used analysis of variance (ANOVA) to compare ^{15}N recovery and amount of label among the trees, and examine the relationships between both of these measures of ^{15}N distribution and tree-level variables such as uptake rate or tree biomass.

Among canopy sections, we constructed linear mixed effect models to predict needle ^{15}N atom %, ^{15}N recovery, distribution of needle biomass and proportion of current year needles. The triplicate samples from each section were averaged to give a single ^{15}N value for each metric per section. The models all used tree as a random (block) factor and vertical and horizontal section positions and average section-level needle biomass and N content and as fixed factors. We also included two tree-level metrics as fixed factors; the ratio of canopy (needle and branch) biomass to total biomass (referred to as canopy ratio), and the total biomass of the tree. These were transformed for normal distribution if appropriate and spatial autocorrelation of ^{15}N recovery among sections based on proximity within the canopy was accounted for by including a correlation matrix based on Manhattan distance between the average modelled distal end position of all branches within each section, using tree height, dbh, and standard geometry, assuming tree stems were extended cones.

We compared models with up to five-way interactions using ΔAICc (small-sample corrected Akaike Information Criterion) and dropped terms stepwise to minimise AICc until the model with the lowest AICc was found. R^2 values are reported as marginal ($R^2_{(m)}$), indicating the proportion of the variance accounted for by the fixed factors using the methodology for pseudo-R^2 for mixed effect models detailed by Nakagawa and Schielzeth (2013). Models for tree-level responses were linear regressions without the tree-level random effect, and presented as adjusted R^2. Branch ^{15}N was compared in the same manner, but separately, due to the limited number of replicated trees. Likewise, as we only measured the proportion of current year cohorts across six of the 13 trees, these were not included in the overall model and analysed separately. For each model, residuals were assessed visually and also tested for conformity to expected (gaussian) distribution.

3.2.7 Error Propagation

When calculating ^{15}N recovery in different tree pools a large number of different parameters with associated uncertainty were included (e.g. biomass, N content, atom % ^{15}N). We propagated errors using standard methodologies (based on

Pythagoras' theorem) where two measured quantities with error, e.g. x ± Δx and y ± Δy, have a sum z with error $\Delta z = \sqrt{\Delta x^2 + \Delta y^2}$.

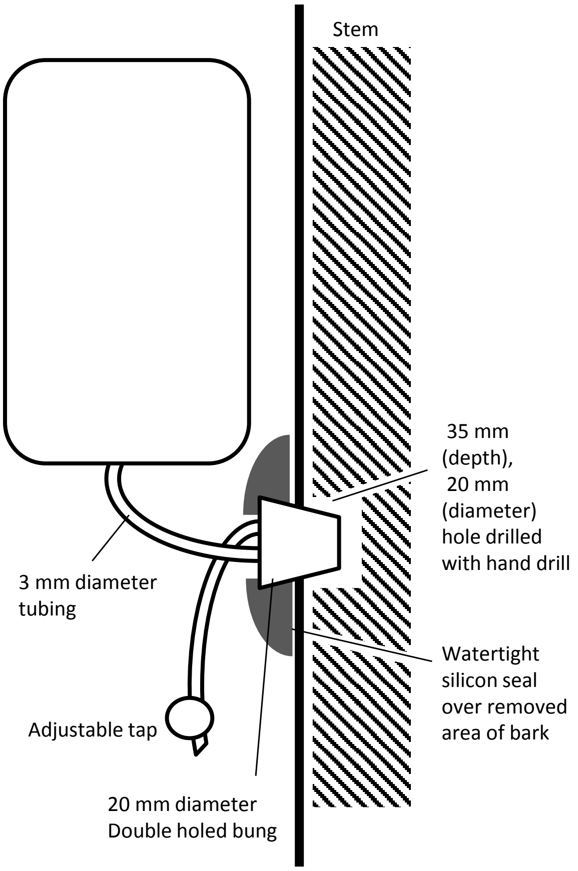

Figure 3.1: Diagram of the stem injection apparatus. The reservoir bottle is filled via two holes (not shown) in the top surface of the bottle and flow of labelling solution proceeds down the 3 mm pile to the bung, into the small reservoir created by the drilled hole in the stem. This flow is regulated via the outflow on the second 3 mm tube controlled by the adjustable tap. Not shown are the securing of the reservoir to the tree, nor the vascular tissues within the stem which transport the labelling solution away from the injection site.

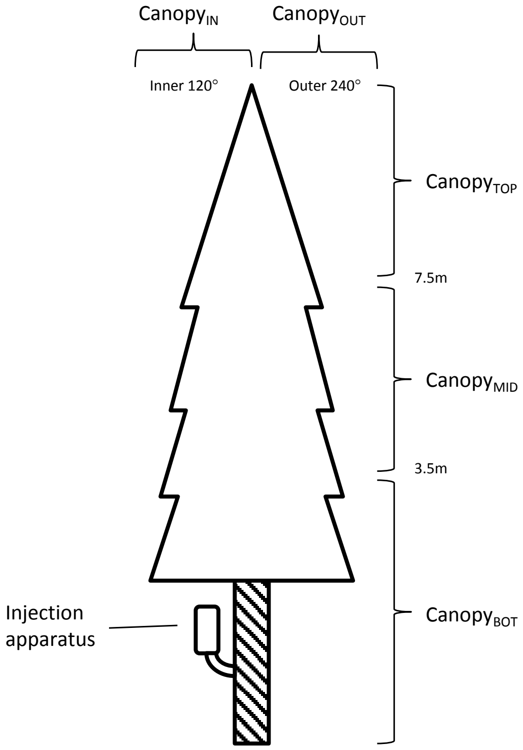

Figure 3.2: Diagram of within-tree sampling strategy. Trees were harvested in six sections per tree, split into three vertical (Canopy$_{TOP}$, Canopy$_{MID}$, Canopy$_{BOT}$) and two lateral (Canopy$_{IN}$, Canopy$_{OUT}$) classes.

3.3 Results

3.3.1 Solution Uptake

No damage or phytotoxic foliar ‘burns’ were observed in preliminary unlabelled tests. The 21 g L^{-1} solution took between 2 and 10 days to reach the endpoint estimated concentration of 1 g L^{-1}, and uptake times (mean 6.4 ± 2.3 (s.d.) days) displayed by individual trees were not related to total tree mass ($P > 0.05$), needle mass ($P > 0.05$), or canopy ratio ($P > 0.05$).

3.3.2 Biomass harvest

At harvest in December 2011, 22.6 ± 7.3 (s.d.) kg needle litter was rendered per tree (293.6 kg in total). The harvested needle biomass decreased up the tree as successive sections were smaller, and was broadly evenly distributed laterally (67.3 % of the mass of harvested needles were from Canopy_{OUT}, 2/3 of the total circumference of the stem). When harvests were standardised using the total circumference of the tree (Figure 3.3a) to compare yields from an equal area, the significant variables affecting section level needle biomass were vertical position ($P < 0.0001$), the interaction between vertical and horizontal positions and total tree height ($P < 0.0001$, $R^2_{(m)} = 0.53$) but not horizontal position ($P > 0.05$)

The fraction of needle biomass harvested in the current year cohort (Figure 3.3b, Table 3.2) increased vertically (Canopy_{TOP} 17.8 % (CV = 4.7 %); Canopy_{MID} 7 % (CV = 7 %); Canopy_{BOT} 2.9 % (CV = 0.03 %), $P < 0.001$, $R^2_{(m)} = 0.96$) across the subsample (n = 6 trees), but this did not change significantly between horizontal sections ($P > 0.05$), nor was there an interaction ($P > 0.05$) between the sections.

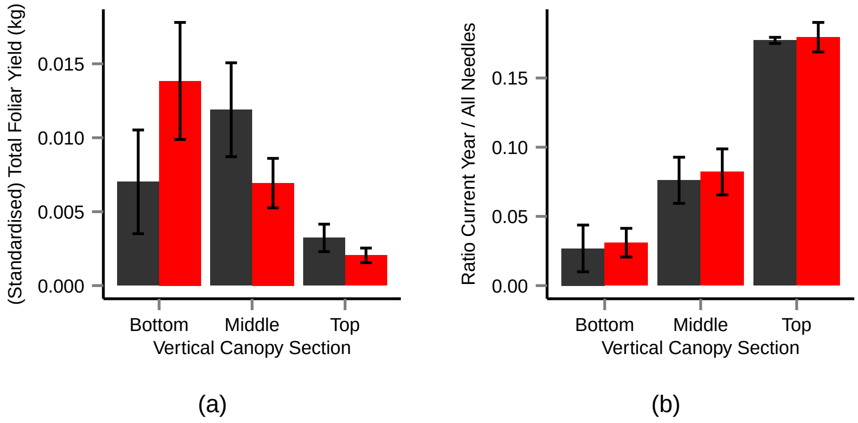

Figure 3.3: Comparison across vertical canopy sections of standardised needle biomass yield (a) and proportion of current cohort needles (b). Shading indicates lateral sections; inside the stand (dark grey) and outside the stand (red). Error bars show 95% CI for both figures.

3.3.3 ^{15}N abundance and label recovery

Average per-tree needle nitrogen content was 1.18 % (CV = 11 %), and the average abundance of ^{15}N was 1.89 atom % (CV = 30 %). Baseline values of atom % ^{15}N in the control was about 0.38 atom % ^{15}Nin all sections. ^{15}N abundance in the branches was 2.35 atom % (CV = 99 %), while N content of the branch sections analysed was 0.6 % (CV = 44 %). The observed needle ^{15}N atom % when considered on the level of individual tree crowns, correlated with the prediction of equation (3.2) ($P < 0.001$, $R^2_{adj} = 0.651$, Figure 3.4a) and was not significantly different than a 1:1 relationship ($P = 0.161$). This ^{15}N expression decreased with increasing canopy biomass ($P = 0.003$, Figure 3.4b), and with canopy ratio ($P = 0.025$) ($R^2_{adj} = 0.571$), but was not related to total biomass ($P > 0.05$), N contents of needles ($P > 0.05$), or ranked uptake rate of solution ($P > 0.05$). Branch ^{15}N abundance also broadly correlated with predicted ^{15}N recovery, based on the estimated branch biomass (Equation (3.1), $P = 0.039$, $R^2_{adj} = 0.587$).

This ^{15}N abundance meant that an average of 53.1 % (CV = 29 %) of the total ^{15}N

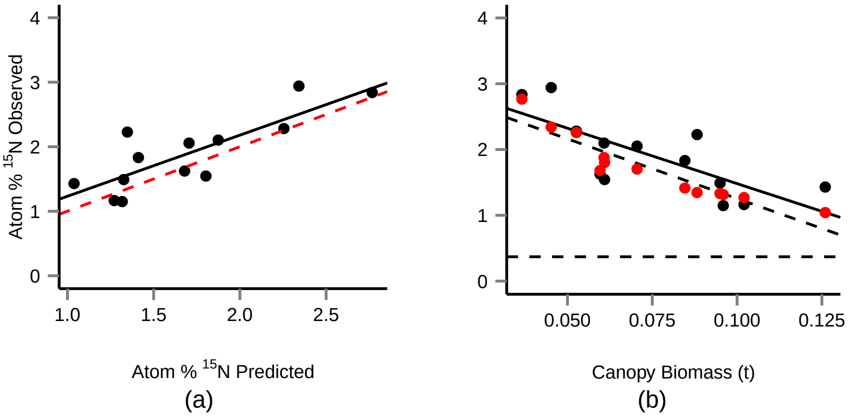

Figure 3.4: Mean measured ^{15}N abundance per-tree was closely correlated with predicted values based on allometric biomass. Panel (a) shows linear relationship between predicted, and observed mean per-tree needle atom % from data (points, black line, adjusted $R^2 = 0.651$), and statistically similar 1:1 relationship (red dashed line). In (b), observed (black) and predicted (red) per-tree needle atom % show the predicted dilution effect caused by increasing canopy biomass. Best fit lines indicate linear relationships for observed atom % (solid) and predicted atom % (dashed), while horizontal line indicates natural abundance.

injected into the stem was accountable in the needles, and an average of 68.5 % (CV = 81 %) was accountable in the branches, totalling 118.4 % (CV = 43 %) of the total ^{15}N injected. In the needles, 112.9 % (CV = 20 %) of the predicted ^{15}N recovery was found, while 89 % (CV = 73.7 %) was found in the branches. There was no effect of canopy ($P > 0.05$) or tree size, ranked uptake rate ($P > 0.05$), or average needle % N content ($P > 0.05$) on the recovery of the total injection in the needles ($P > 0.05$) or branches ($P > 0.05$) when totalled for the tree. ^{15}N recovery was highly variable among trees with a minimum of 33.5 % of the injection returned in foliage, a maximum of 88.9 %, and a mean of 53.1 (CV = 28 %).

^{15}N enrichment varied among the six canopy sections. Despite the lower average ^{15}N abundance, the bottom sections of the canopy were very highly variable, displaying both the highest individual needle enrichment (4.39 atom %), as well

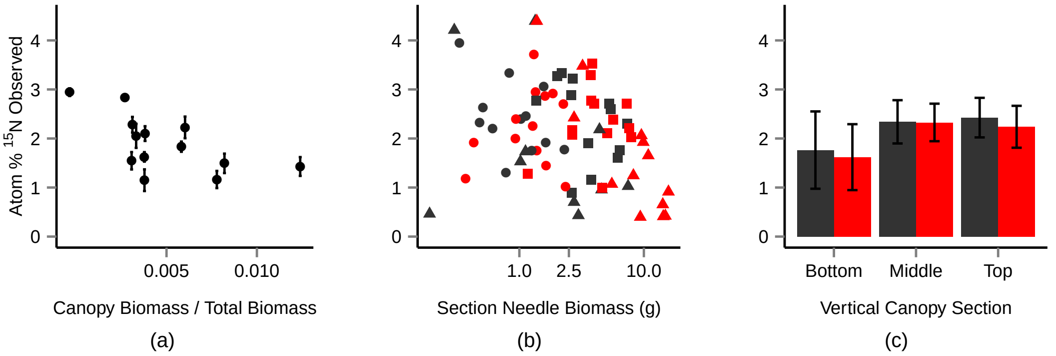

Figure 3.5: Relationship between atom % ^{15}N measured in needle biomass of (a) the entire trees and (b and c) of individual tree sections , compared with (a) the ratio of whole canopy / tree biomass, (b) the harvested needle biomass, (c) canopy section. In (b) and (c), lateral canopy sections are shaded grey (inside the stand) and red (outside the stand), and in (b), canopy sections are divided into as Canopy$_{BOT}$ (triangle), Canopy$_{MID}$(diamond) and Canopy$_{TOP}$ (circle). Error bars show 95% CI.

as the lowest enrichments (0.399 atom %). ^{15}N abundance in the needles was driven by vertical position (P = 0.016), canopy ratio (P = 0.004), and needle biomass (P = 0.0305), (Figure 3.5, Table 3.3), with a greater ^{15}N enrichment displayed in smaller sections, smaller canopies, and higher up the tree; Canopy$_{TOP}$ (2.33 atom %, CV = 25 %) and Canopy$_{MID}$ (2.33 atom %, CV = 24 %), were significantly ($P < 0.05$) greater than Canopy$_{BOT}$ (1.68 atom %, CV = 101 %), but not significantly different from each other (Tukey HSD, P = 0.451). Neither total biomass or any interaction terms remained in the most parsimonious (AICc) model when reduced by stepwise regression, which had a $R^2_{(m)}$ of 0.28.

This difference led to Canopy$_{BOT}$ accounting for considerably less ^{15}N (88.3 ± 61.8 %) than Canopy$_{MID}$ (163.8 ± 69.9 %) and Canopy$_{TOP}$ (158.4 ± 44.2 %). The canopy ^{15}N allocation (Table 3.3) was significantly related only with vertical section (P = 0.0005, Figure 3.6), although normalised needle biomass remained in the most parsimonious model (P = 0.0707). The $R^2_{(m)}$ for this model was 0.23. Among the vertical sections of the canopy (Tukey HSD), there was a significant

Table 3.2: Average biomass, ^{15}N abundance, and proportion current year needles among canopy sections. Shown as mean ± S.E.

Canopy Position		Outside Stand ($2\pi/3$ rad)	Inside Stand ($4\pi/3$ rad)
Canopy$_{BOT}$ ($<$ 3.5 m)	Needle Biomass (kg)	119.92 ± 4.85	30.40 ± 2.15
	Atom % ^{15}N	1.15 ± 1.24	1.42 ± 1.45
	Current Year Needles (%)	2.59 ± 1.8	2.99 ± 1.1
Canopy$_{MID}$ (3.5 - 7.5m)	Needle Biomass (kg)	60.07 ± 2.94	51.53 ± 1.95
	Atom % ^{15}N	2.35 ± 0.70	2.23 ± 0.81
	Current Year Needles (%)	7.04 ± 1.6	7.57 ± 1.6
Canopy$_{TOP}$ ($>$ 7.5m)	Needle Biomass (kg)	17.71 ± 6.05	13.98 ± 5.69
	Atom % ^{15}N	2.29 ± 0.79	2.28 ± 0.74
	Current Year Needles (%)	15.0 ± 0.01	15.2 ± 0.01

Table 3.3: Summary of most parsimonious linear models for needle ^{15}N atom % and needle ^{15}N recovery. numDF is the degrees of freedom in the numerator, denDF the degrees of freedom in the denominator of the F-value.

^{15}N atom percent model

Variable	numDF	denDF	F-value	P-value
Intercept	1	58	449.6300	$< .0001$
Vertical section	2	58	4.4740	0.0156
Ratio canopy / whole tree biomass	1	11	13.1145	0.0040
Needle biomass in section	1	58	4.9162	0.0305

^{15}N recovery (as % predicted in section) model

Variable	numDF	denDF	F-value	P-value
Intercept	1	58	254.63674	< 0.001
Vertical section	2	58	8.74377	0.0005
Needle biomass in section	1	58	3.39084	0.0707

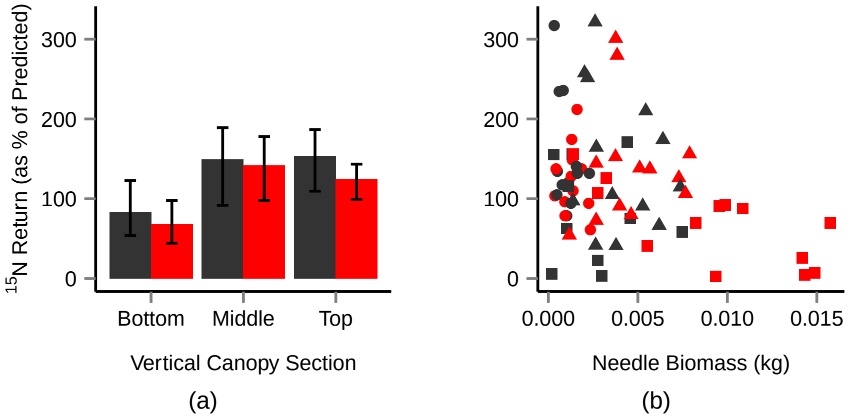

Figure 3.6: Relationship between recovery of predicted ^{15}N (%) with respect to (a) vertical sections and (b) needle biomass. Lateral canopy sections are shaded dark grey (inside the stand) and red (outside the stand), and in (b), sections are represented by as Canopy$_{BOT}$ (triangle), Canopy$_{MID}$ (diamond) and Canopy$_{TOP}$ (circle). Bars in (a) show are 95 % CI.

difference in ^{15}N allocation between Canopy$_{MID}$ and Canopy$_{BOT}$ ($P < 0.001$), but no significant differences in recovery in Canopy$_{TOP}$ against recovery to the Canopy$_{MID}$, or between Canopy$_{TOP}$ and Canopy$_{BOT}$, were found.

Within individual trees, observed ^{15}N abundance in branches was much more variable than in needles (Canopy$_{BOT}$ 2.69 atom % (CV = 137 %); Canopy$_{MID}$ 3.45 atom % (CV = 89 %); Canopy$_{TOP}$ 2.11 atom % (CV = 69 %)), driven by an apparent heterogeneity of recovery, particularly in Canopy$_{BOT}$ where some samples displayed atom % at natural abundance while others were as high as 10.4 atom % (the highest recorded). Average atom % varied among trees and with biomass (Figure 3.7). When the observed atom percent was expressed as a recovery of the expected label, this varied among sections but there was no significant statistical relationship was found with the measured variables.

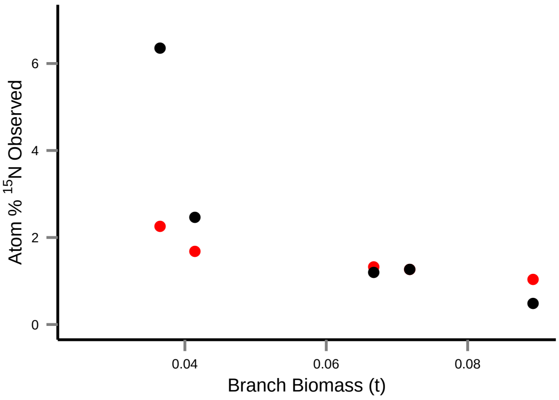

Figure 3.7: Mean observed (atom %) ^{15}N label in branch sections, compared to total branch biomass for each tree. Red is predicted ^{15}N abundance, black observed ^{15}N abundance.

3.4 Discussion

The variability among species investigated, concentrations of solution, objectives, and methods reported in the literature makes it difficult to compare results from stem injection experiments. Given the presence of multiple age cohorts of needles in evergreen conifers variation in the expression of an isotope label would also be expected. We found a high variability in the atom % ^{15}N abundance, and therefore limit discussion as far as possible to injections of conifers where the intra-canopy N dynamics are expected to be as comparable as possible to our trees.

3.4.1 Overall ^{15}N Recovery

The harvested biomass of every injected tree was ^{15}N enriched, in both the needles and the branches, with an average recovery greater than 121 % of the applied label when the measured ^{15}N excess over the control was scaled to the entire canopy. This was fairly variable (CV = 22.8 %), predominantly due to the high and variable label recovery in the branches (CV = 99.7 %) which contributed

46.9 % of the calculated total label recovery, with the average branch atom % matching or exceeding the prediction in 4 of the 5 trees analysed despite the prediction assuming all N was assigned to branches or needles. The predictive model assumed a similar turnover rate (and therefore a similar proportion of N replaced) between foliar and branch pools based on observed branch and needle N content, but real differences in this rate may have driven the discrepancies from predictions. As there are no alternative explanations for an additional enriched ^{15}N source for the trees, the high total recovery for the ^{15}N label in the canopy from these measurements contrasts with Seiter and Horwath (1999) and Garten and Brice (2009), who inferred a large allocation of injected N to belowground processes from low accountancies in aboveground tissues. While the greatest foliar ^{15}N recovery may be expected when injections are timed with periods of peak foliar growth, refilling of N storage pools in conifer foliage may also account for a strong ^{15}N recovery in needles. This experiment took place late in the growing season, when most growth is in roots and structural tissues (Weinstein *et al.*, 1991), and when root N uptake is greater than plant N demand (Millard and Grelet, 2010). The high canopy ^{15}N content observed is consistent with sequestration of this additional N in overwinter storage pools in the needles, while belowground demand for N may be fully satisfied by ongoing root uptake.

3.4.2 Needle ^{15}N Distribution

Overall, measured needle biomass for each section varied considerably beyond the expected allometric distribution based on tree size, presumably because of the edge nature of the trees. This accounted for 53.1 % of the overall canopy ^{15}N in excess of the natural abundance control, considerably greater than other studies using smaller trees (e.g. Horwath *et al.* (1992); Augusto *et al.* (2011)), although Proe *et al.* (2000) found a similar recovery (45 %) in the foliage of 5-8 m conifers, one week after injection. While it is difficult to compare label recovery between studies, our canopy estimates of ^{15}N recovery are substantially greater than Augusto *et al.* (2011) (42-62 %), which suggested that their recovery may be due to lower canopy:biomass ratios (Ritson and Sochacki, 2003) in their larger trees compared to Proe *et al.* (2000), while we used edge profile trees with relatively large canopies which may have contributed to their relatively greater short-term allocation to a relatively larger crown.

Our samples were all collected 4.5 months after the injection, in winter, and were from the entire foliar biomass (including both the 2011 needle cohort and older needles). At this time, needle ^{15}N abundance was expected to be biased towards current year needles (Augusto *et al.*, 2011) as most conifers store nitrogen in roots and one-year-old needles, in photosynthetic proteins such as RuBisCo (Millard *et al.*, 2007), remobilising this N in the next growing season (Millard and Proe, 1992). Foliar ^{15}N abundance was biased towards the upper canopy, consistent with Proe *et al.* (2000), where crown zones were assigned based on age of the relevant stem section. Our results are however in contrast to Augusto *et al.* (2011) who reported no difference between upper and lower canopies. Canopy nitrogen storage is thought to be more important in larger trees (Miller, 1986), as they have larger pools of current year needles available, and greater N requirements in the spring. N storage pools can be rapidly mobilised to overcome limited uptake from the soil, providing a resource ready for the development of new foliage the next growing season (Augusto *et al.*, 2011). Current year needles, expected to be the store for this excess ^{15}N, were approximately 2.8 %, 7.3 %, and 15.1 % of the total foliar yield for Canopy_{BOT}, Canopy_{MID}, and Canopy_{TOP} respectively, significantly biased towards the upper canopy sections.

However, ^{15}N atom % abundance and recovery did not exactly follow this distribution, with equal apparent total allocation of injected ^{15}N to the middle and upper foliage regardless of the difference in the total mass of new cohort needles. ^{15}N labels absorbed from the soil are typically found in regions of high metabolic rates (Mead and Pritchett, 1975), which are usually located within the canopy in regions of greater exposure to sunlight and more photosynthetic potential (Ellsworth and Reich, 1993; Hollinger, 1996), and the apparent inconsistency in N allocation compared to new needles may be due to different spatial demands for N for photosynthetic function.

Dilution (Swanston and Myrold (1998)) explained much of the variation in ^{15}N atom % in the most parsimonious model, both at the level of individual trees (canopy ratio) and in individual sections within the trees (needle section biomass), but when this effect was removed by the ^{15}N recovery model, only vertical position of the section remained significant, average recovery in the upper canopy being greater than in the lower canopy. Respectively, the ^{15}N atom % and recovery models explained 28 % and 23 % of the variability in the amount of ^{15}N label, calculated from 30 needles per individual sample with considerable variation typically found among replicates from the same section. The within-section variability was not explainable by the measured variables as, aside from N content of samples, no explanatory variables were measurable to the individual sample level. Exposure (Kohyama, 1980; Zavitkovski, 1981) and competition (Vanninen and Mäkelä, 2000) would have varied within each canopy section due to individual needles positions, as well as variable amounts of different age classes (Norman and Jarvis, 1974). Alternatively, uneven allocation may have been due to the heterogeneous distribution of the label within the tree over the time period of the study.

3.4.3 Branch ^{15}N Distribution and contrast with foliar ^{15}N

Branch atom % ^{15}N was even more varied than in needles, with recorded atom % as high as 10.66 % but often with measured ^{15}N at natural abundance levels, especially so in Canopy$_{BOT}$, where the coefficient of variation was 137 %. This variance was mostly due to one of the five trees analysed for branch ^{15}N having a consistently very high ^{15}N enrichment in the branches (resulting in an average branch atom % in the whole tree of over 6 %), with it having the third highest needle ^{15}N content of the 13 trees, and highest from the five trees where branches were also analysed.

Wood contained a much larger range of ages and potentially a greater range of living tissues within individual branches, especially in larger trees where needle lifespan is much shorter than the age of the tree. Depending on the position of the branch, there was also potentially variability in growth and metabolic rate among branches due to environmental factors. We also used the needle biomass to predict branch biomass in the allometric model, rather than direct measurement, expecting it to be more accurate than DBH for these trees where release from competition would increase branchiness (Mäkinen and Colin, 1998; Ackerman *et al.*, 2013). If, in this case, we substantially overestimated the branch masses, this would also have caused an overestimation in the label recovery in the branches. Alternatively, this high variation in both ^{15}N recovery and ^{15}N atom % (which we measured directly and is not dependant on branch biomass estimates) may also have been due to variation in N allocation. Sap flow in many trees is sectorial (Larson *et al.*, 1994; Orians *et al.*, 2004; Gloser *et al.*, 2008), and the injection in summer 2011 may have initially reached specific regions of the canopy in the same sector as the injection site. As foliar N pools are dynamic and N is assigned both to maximise photosynthetic capacity across the canopy and for storage (independent of plant N (Fife *et al.*, 2008; Millard and Grelet, 2010)), variation in needle and branch ^{15}N may have been due to a more gradual movement of ^{15}N to some parts of the canopy.

In the autumn, N-uptake is typically greater than total tree N demand, as shoot extension and foliar production have ceased (Weinstein *et al.*, 1991), but production of storage proteins continues. In contrast, no major N storage in conifers occurs in bark and wood (Millard and Grelet, 2010) during this period, and radial wood production in branches, stem and roots is ongoing in early autumn (Weinstein *et al.*, 1991). New wood laid down following the August injections may be a more continuous structural sink for ^{15}N while foliar sinks may be more transient as the N moves around the canopy in order to maximise ^{15}N storage in foliage at the end of the year. The high branch ^{15}N in some branches may reflect the branches first reached by the ^{15}N label and the highest ^{15}N abundances in branches at the base of the canopy may be structural sinks closest to the injection site where the additional ^{15}N is least diluted by N already in the sap, translocated from foliage throughout the upper canopy.

Rates of uptake of the solution from the injection site varied among trees but did not correlate with ^{15}N abundance or recovery of the expected label, and there were no relationships between the uptake rates and measured total biomass, canopy size or ratio, needle biomass or total N % (all $P > 0.05$). In non-labelled experiments, these rates are highly variable (Sanchez-Zamora and Fernandez-Escobar, 2004) among tree species and seasons of injection. While we expected uptake to be rapid due to movement of the xylem stream (Meinzer *et al.*, 2001), this variation may have been due to the difficulty of standardising stem wounding, accessing different depths of the stem with different flow rates (Delzon *et al.*, 2004). Variations in canopy morphology (Fiora and Cescatti, 2008) within individual trees may also have driven differences in relative flow rate experienced by a single location radially, or around the circumference (Čermák *et al.*, 2007) of the stem.

There was not a significant difference in total needle biomass between the inside and outside of the stem, once this was adjusted to compare identical proportions of the circumference despite expectations due to the trees' position on the edge of the stand and a well known release from competition on the exposed size. As branch biomass was calculated using an assumed linear relationship with measured needle biomass, the very high recovery in some sections may be a result of this relationship varying throughout the canopy.

3.5 Conclusion

We were able to successfully label the entire canopy with an apparent total recovery of the label in both the needles and branches based on scaling the ^{15}N recovery through the canopy biomass. The entire needle biomass was the main sink for the injected ^{15}N, accounting for over 50 % of the total injection, allocation being greatest towards the upper canopy, which contains a greater proportion of young needles. ^{15}N recovery in branch biomass was considerably more varied, particularly at the bottom of the tree, likely due to the distribution of sap flow and demand for N for growth in wood, but not foliage, during the autumn. The overall high recovery can partly be attributed to the habit of the trees and the method of injection, which is well established to allow higher recovery of applied ^{15}N than soil applications, but it is likely that the seasonality of the injection also played a part in the variation observed as at other times of the year N may be assigned in different proportions to above and below-ground pools due to phenological growth patterns. These differences highlight the importance of considering seasonal N dynamics and partitioning of the ^{15}N label among biomass age classes in stem injection studies, particularly in conifers, while overall it is clear that the technique is a viable and efficient method for creating ^{15}N biomass labelled in a cheaper, and larger scale than using labelled fertilizer on saplings.

Chapter 4

Differences in ^{15}N-Return in Sitka Spruce between Canopy and Soil ^{15}N Treatments

4.1 Introduction

Forests in the northern hemisphere are carbon (C) sinks of a net 0.6-0.7 Pg C y^{-1} (Goodale *et al.*, 2002) and are also typically nitrogen (N) limited (Vitousek *et al.*, 2002; LeBauer and Treseder, 2008) with intense competition for ecosystem N among plant, soil microbial and mineral sinks (Kaye and Hart, 1997). As these forests currently receive extra inputs of N transported by the atmosphere (Galloway *et al.*, 2004) from human activity (Vitousek *et al.*, 1997; Holland *et al.*, 1999), nitrogen deposition (N_{DEP}) has been suggested as one potential driver for this net forest growth.

Estimates of the effects of nitrogen deposition (N_{DEP}) on forest C uptake (henceforth referred to as $\Delta C/\Delta N$) can vary substantially with one estimate of C accumulation as great as 120-150 kg C kg N $^{-1}$ (Magnani *et al.*, 2008), based on a comparison of European net ecosystem productivity (NEP) against N deposition (Magnani *et al.*, 2007). However, for a sink of this magnitude, N_{DEP} needs to be accumulated in trees and sequestered in high C/N, long-lived bolewood (Townsend *et al.*, 1996), which contradicts the results of manipulative experiments and N budgets. Isotope tracer experiments where ^{15}N-distinct N is directly applied to the forest floor find that the forest floor (soils and microbial biomass (SMB)) are the major sinks (70 %) for ^{15}N tracers, while only around 20 % can be traced into trees and even less (5 %) into wood (Nadelhoffer *et al.*, 1999c). Estimates from studies based on N budgets also tend to be low (e.g. 46 kg C kg N $^{-1}$ in Europe; (c.f de Vries *et al.*, 2006)) and one particular ^{15}N amendment and modelling synthesis, from Harvard Forest (Currie *et al.*, 2004), found a ΔC/ΔN effect as small as $>$ 5 kg C kg N $^{-1}$, with most N for forest growth derived from mineral soil. Consequently, the high estimates from Magnani *et al.* (2007) have been suggested to be due to covariance with factors such as edaphic N and site history (de Vries *et al.*, 2009; Högberg, 2012), climate, GPP, and dry N_{DEP} contributions (de Vries *et al.*, 2008; Sutton *et al.*, 2008).

However, ^{15}N amendment experiments (e.g. Nadelhoffer *et al.*, 1999c) typically apply ^{15}N directly to the soil and cannot include interactions and uptake across the canopy (Sievering, 1999), nor interactions with unlabelled edaphic N pools (Jenkinson *et al.*, 1999). Estimates from these studies could therefore underestimate the total ΔC/ΔN effect. A recent modelling study (Dezi *et al.*, 2010) suggested a strong ΔC/ΔN effect (up to 121 kg C kg N $^{-1}$) when management, foliar uptake, changes in litter quality, and soil processes were taken into account, and canopy uptake of N may raise allocation to wood to 10 or 15 % of total N_{DEP} (Sievering, 1999), doubling the effect assumed from soil ^{15}N traces, while a study in North America from forest inventory data used in a similar manner to Magnani *et al.* (2007) found a strong wood effect, which, if combined with estimates from ^{15}N studies for soil ΔC/ΔN, yields a total effect of around 100 kg C kg N $^{-1}$.

While more difficult to obtain a carbon effect from, estimates of ecosystem N retention can also be obtained from N budgets, which incorporate canopy uptake as a difference between the sum of measured throughfall and stemflow and total N_{DEP} inputs onto the forest (Friedland *et al.*, 1991; Sievering *et al.*, 2007). The difference between these values can sometimes result in high estimates of canopy N retention (e.g. Sievering *et al.*, 2007, 80 % of N_{DEP}) and consequently correlate with relatively small changes in measured C stocks from plant growth. However, these methods typically do not include losses of N back to the atmosphere as trace gases, which may occur alongside uptake and utilization of N via the canopy.

Nitrogen uptake can occur across both foliage and twigs, via mechanisms of ion exchange (Bowden *et al.*, 1989; Boyce *et al.*, 1996; Sparks, 2009) and simple diffusion (Klemm *et al.*, 1989) across twig surfaces. These different methods may vary in their ability to take up specific nitrogen species (Wilson and Tiley, 1998) due to transport and reduction costs, as well as internal cell N concentrations. Overall, estimates of the total canopy uptake effect vary considerably, from around 3/4 of N_{DEP} retained or taken up (Gaige *et al.*, 2007; Sievering *et al.*, 2007), to lower estimates when directly measured of between 25 % (Friedland *et al.*, 1991) and 30 % (Ammann *et al.*, 1999) of dry ^{15}N load, or as little as 5 % of ^{15}N mists recoverable in foliage and branches one day after application (Wilson and Tiley, 1998). These estimates are often difficult to interpret as varying methods, N_{DEP} magnitudes (Chiwa *et al.*, 2004), and timescales interact with species and site specific effects, as well as both positive (Sievering *et al.*, 2007; Wortman *et al.*, 2012) and negative (Maurice and Crang, 1986; L'Hirondelle *et al.*, 1992; Wellburn, 1990) impacts on canopy physiology such as changes in photosynthetic performance or N-related phytotoxic effects. Uptake estimates from individual branches and leaves must also be carefully interpreted as N_{DEP} interacts with multiple overlapping branches and leaves as it passes through the canopy (Boyce *et al.*, 1996). In the only available ^{15}N-tracer study carried out at the forest catchment scale, where ^{15}N was applied to the canopy, twigs, branches, and bark were found to be a major sink (25 - 50 % recovery) of helicopter-applied ^{15}N three years after application of a ^{15}N treatment (Dail *et al.*, 2009), but it was unclear whether the bark retention was due to uptake by trees or canopy mosses and lichens as this relatively high ^{15}N return was not in the bolewood, which is necessary for a strong $\Delta C/\Delta N$ effect.

Mineral fertilizer N applications onto the soil are also unable to directly trace the fate of N from soil and litter pools, and litter recycling is usually the major source of N nutrition (Schulze, 2000; Högberg, 2012), even under high N_{DEP} regimes. N_{DEP} applications may release N mineralization from N limitations on decomposers (e.g. Manning *et al.*, 2008), but also may have toxic and suppressive effects on litter decomposition. A meta-analysis (Knorr *et al.*, 2005) of the effects of N addition on litter decomposition revealed complex effects of N additions on mass loss of litter quality, magnitude of N_{DEP} relative to ambient deposition, and the length of the study period. Rates of decomposition are also tied to litter decomposition stage (Berg and Matzner, 1997; Carreiro *et al.*, 2000), environmental conditions (Manzoni *et al.*, 2008), and background levels of N availability (Knorr *et al.*, 2005). In the 500 sites synthesised by Knorr *et al.* (2005), N additions generally had an overall inhibitory effect on litter mass loss, but not at sites where N addition rates were less than double the ambient N deposition level.

Changes in litter decomposition affect overall availability of N, and subsequent changes in aboveground C if more is available to trees. Canopy uptake of N may also affect decomposition as this may change demand for below-ground edaphic-derived N and assignment of C to root systems and symbioses, in the same way as increased N concentrations in the soil (*c.f.* Nadelhoffer, 2000). Finally, changes in the soil system can occur concurrently with processes in the canopy and interactions between plant C assignment and removal of N from throughfall by canopy retention may impact soil processes in addition to the effects of nitrogen deposition to soil processes alone. However, tracing the N released from decomposition is difficult and requires ^{15}N-enriched biomass to determine the magnitude of N allocation to different ecosystem pools. These experiments are rare and expensive as ecosystem N is spatially variable (especially in the soil) (Högberg, 1997), and litter decomposition is typically slow, necessitating high enrichment of ^{15}N-labelled biomass.

Up to now, there have been no published studies where the allocation of N from canopy ^{15}N deposition can be directly compared to the assignment of N within trees under ^{15}N deposition to the soil over sustained time periods. Canopy deposition studies also typically do not address the potential for changes in a third factor, i.e. soil N recycling via litter decomposition, and in general, knowledge of the response of edaphic N to N_{DEP} is sparse and difficult to predict. We designed an experiment utilising saplings where N from these three sources could be traced via combinations of treatments with a ^{15}N label using one enriched ^{15}N source per treatment. Bulk N_{DEP} was kept unaltered from ambient deposition and at the same magnitude in all N-amendment treatments to allow a direct comparison between the foliar-applied ^{15}N label and the soil wet ^{15}N deposition treatment with minimal effect of total N abundance relative to controls. We also applied ^{15}N-labelled needle litter to investigate uptake and partitioning of available N coming from litter recycling We aimed to trace the ^{15}N label over time in the foliage, and sample total ^{15}N abundance in twigs, wood, roots, soil, and soil microbial biomass extracts. By harvesting our saplings at the end of the experiment, we were able to scale these ^{15}N abundances to a mass balance ^{15}N return, allowing comparison of N partitioning between the treatments.

4.2 Methods

4.2.1 Study Site

Our study consisted of 3-year old *Picea sichensis* (L. Bong.) saplings, located outdoors at Forest Research Northern Research Station, Scotland (55°86'N, 3°20'W). Thirty selected individuals from a cold-stored (4 °C, lifted January 2011) batch of 2 year old saplings were potted in 60 L pots on a mix of 90% homogenised stagnohumic gley topsoil (Clement, 2004), harvested from from Griffin Forest, central Scotland (56°37'N, 3°47'W), and 10% low N/P/K compost. In June 2011, the seedlings were randomly arranged in a 0.5 m spaced grid of 5 by 6 trees, surrounded in an overall 9 by 6 grid by an additional 60 trees, potted on 100% low N/P/K compost, and left to establish until summer 2012. Annual precipitation at the research site was 704 mm, while annual temperatures varied between 1 - 19 °C. The trees remained in the initial configuration for the duration of the experiment and remained healthy, with the exception of two individuals from the treatment group suffering major needle discolouration and loss by the end of the experiment. These were removed from the data collected once it became apparent that these individuals were unhealthy, reducing the sample size of the two affected treatments by one.

4.2.2 Experimental Treatments

Individual trees were assigned to six treatments based on current basal diameter and height as well as a series of soil CO_2 efflux measurements made over spring and summer 2012 with a EGM-4 CO_2 IRGA (PPsystems USA), as proxy for differences in below-ground N cycling potential. Due to an aphid infestation in spring 2012, six trees had lost some of the 2011 cohort of needles and were each

Table 4.1: Treatment descriptions for the six experimental treatments. Total N_{DEP} for all deposition treatments was 54 g N ha^{-1} y^{-1} applied as $NH_4NO_3$and ^{15}N-enriched treatments were 98% double-labelled ^{15}N-NH_4NO_3. Deposition target indicates the method of application of the treatment, whether to the canopy or the soil.

Treatment ID	Litter	Deposition	Target
NA_{litter}-Water (control)	Natural abundance	Water	Soil
$^{15}N_{litter}$-Water	^{15}N-enriched	Water	Soil
NA_{litter}-$^{15}N_{soil}$	Natural abundance	^{15}N-enriched N_{DEP}	Soil
NA_{litter}-$^{15}N_{canopy}$	Natural abundance	^{15}N-enriched N_{DEP}	Canopy
$^{15}N_{litter}$-NA_{soil}	^{15}N-enriched	Natural abundance N_{DEP}	Soil
$^{15}N_{litter}$-NA_{canopy}	^{15}N-enriched	Natural abundance N_{DEP}	Canopy

assigned to different treatments to avoid systematic biases

The six treatments (Table 4.1) were designed to test a unique combination ^{15}N source to the trees; a) litter type (either natural abundance NA_{litter}, or ^{15}N-enriched, $^{15}N_{litter}$, b) deposition type, either , N_{DEP} to the soil, ($_{soil}$) or $N_{DEP}$to the canopy, ($_{canopy}$), and c) ^{15}N enrichment of the treatment; either water control applied directly to the soil, (*Water*), natural abundance nitrogen, (NA), or ^{15}N nitrogen (^{15}N). All treatments, except the water control on unlabelled litter, had a single enriched ^{15}N source (for example, the NA_{litter} litter treatment was paired with a ^{15}N-enriched N_{DEP} treatment).

4.2.3 Artificial Litter Layer

Litter with a ^{15}N-label was obtained from three 6 - 7 m tall *Picea sichensis* trees, stem-injected with ^{13}C and ^{15}N double-labelled aspartic acid (see Churchland *et al.*, 2012) at Gisburn Forest, UK (54°01'N, 2°22'W). These were felled in November

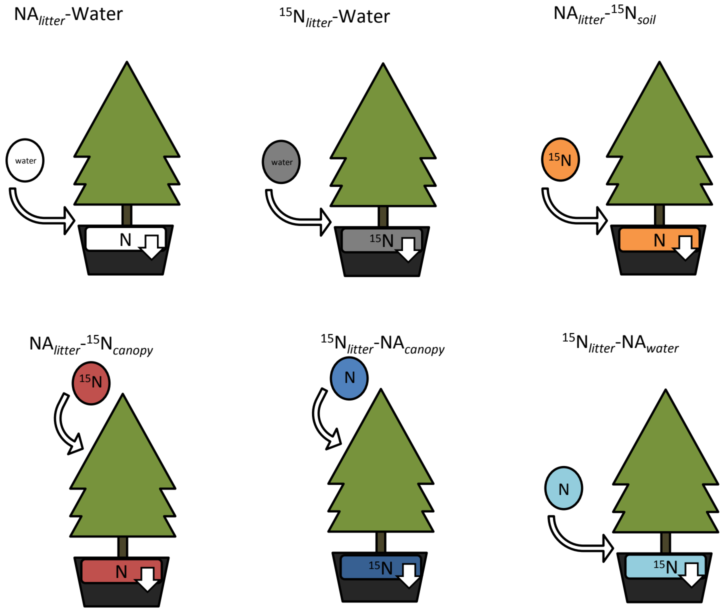

Figure 4.1: Experimental set up of the six treatments. Each treatment (Table 4.1) received either a canopy or soil targeted deposition treatment (circles) and a litter layer (rectangle in pot). In all treatments except the control there was a single source of ^{15}N enrichment, either in the deposition or the litter and a total N application of 54 g ha^{-1} y^{-1} N deposition. Colours correspond to treatments on later graphs (Figures 4.2, 4.3, 4.4).

2010, and left on site until January 2011, when three additional trees were harvested as a source for 'control' litter without ^{15}N enrichment. The entire branch biomass of each tree was dried in a 70 °C oven until needles were easily separated from the branches, then stored in paper sacks in dry polytunnel until deployment. A random sample of 100 needles from each of the trees was measured for N and C isotope content 1 month before deployment, using the same methodology as later samples. In the control trees, atom % ^{15}N was at natural abundance (0.366 atom %), while the labelled trees had a foliar ^{15}N abundance of 0.629, 1.216, and 1.597 atom %. All trees had a foliar ^{13}C content at natural abundance (despite the ^{13}C injection), and N content (1.118 % (s.d. = 0.06)) of the needle harvest from each tree was not significantly different (ANOVA, $P > 0.05$) between the sources.

Each of the 30 trees in the experiment received 0.8 kg of this litter in August 2012, spread in an even layer (approximately 4-5 cm depth) across the soil surface, with the three labelled and three unlabelled tree sources distributed at random between the appropriate recipients. Each individual only received litter from a single tree, to reduce the potential for interactive effects of litter mixing (e.g. Gartner and Cardon, 2004; Smith and Bradford, 2003).

4.2.4 Deposition Treatments

The simulated nitrogen deposition applied to 4 of the 6 treatments was equivalent to approximately 54 g ha^{-1} y^{-1} N in excess of background N_{DEP}, as of either 98% ^{15}N double labelled NH_4NO_3, or unlabelled NH_4NO_3. Ammonium nitrate was chosen since it contains both mineral ions typically found in nitrogen deposition. This treatment was applied in DI water solution every 3-6 weeks from February 2013 until March 2014 as either a 0.5 L (soil applications), or a solution of 10-15 ml (canopy applications), the canopy solution being increased in volume (but not in N content) in summer 2013 to match increases in the canopy biomass of the trees. Soil treatments were sprayed onto the litter surface using a pressurised hand sprayer, while foliar applications were applied directly onto needles and twigs using a brush pre-soaked with treatment solution. A brush was used for this application, rather than a spray (c.f Bowden *et al.*, 1989; Boyce *et al.*, 1996; Dail *et al.*, 2009) as we wanted to ensure that we knew how much N was available for uptake by each tree without needing to measure and consistently recalibrate models for canopy surface area with each successive treatment application over the 13 months of the experiment. These treatments began at the top of the tree and were continued on each branch and stem section in turn down through the canopy until the solution was exhausted. The bottle was then washed out with an additional 10 ml rainwater and poured directly down the stem of the tree. Control water treatments were applied directly to the litter surface in the same manner as the soil applications.

4.2.5 Routine Biomass Measurements and maintenance

Routine measurements of tree growth were made every two months during the growing season and every three months outside the growing season. At each occasion, tree height was measured with a plumbline marked in centimetres, and basal diameter was measured as the mean of two calliper measurements at across the stem at the litter surface. At each of these instances, leaf litter not derived from the experiment was removed and weeds growing in the pot were uprooted, manually shredded, and left on the soil surface, so none of the ^{15}N label in their foliage was removed from the potted system. The pots were free-draining and ^{15}N exported in flow through the pot was not retained.

4.2.6 Time Series Measurements

Twenty-five needles per tree were collected from the entire canopy on 13 occasions between August 2012 and May 2014, 10 of these being after the deposition treatments began in February 2013. This number was chosen to obtain as representative a sample as possible without detrimental effects of cumulative defoliation on the trees. After bud burst in May 2013, a harvest of the 2013 needle cohort was made alongside the general harvest, which was then specifically targeted on the 2011-2012 cohorts of needles. In the first instance (May 2013), the 2013 cohort sample was a single, entire bud, but subsequent harvests were taken from the entire current cohort of biomass in the same manner as the general needle harvest. For the May 2014 measurement, this sample was taken from the entire harvested needle biomass and contained more ($\sim$ 100) needles. All samples were collected immediately before application of the regular deposition treatments, to allow as much time as possible for movement of the assimilation products within the tree (in contrast to the short-term foliar ^{15}N return, e.g. Wilson and Tiley (1998)), and to allow as much retention of ^{15}N remaining on leaf surfaces in the soil as possible by natural washing by rain events. The harvested needles were immediately transported to the lab and either immediately processed (see below), or frozen at -4°C until it was convenient to dry them (usually within 7 days).

To avoid extensive damage from repeat sampling of the same trees, branch and twig samples were only collected in October 2013 and at the termination of the experiment in March 2014. In October, one random branch per tree was removed with secateurs from both the current year cohort and one from the oldest age class of branches (which contained biomass from the 2011, 2012, 2013 and 2014 growing seasons). Three $\sim$ 0. 75 cm ‘cookies’ containing the entire radial section (bark included) were cut from the entire length of each branch and used for isotope analysis, while the whole branch was dried to obtain dry weight. In March 2014, a similar method was used, but radial cookies were collected from three different branches. Likewise, litter samples were only collected every three months to avoid depleting the litter pools in the pots and were a small fraction (< 5 %) of the total litter in the pots.

4.2.7 Destructive Measurements

The experiment was terminated in March 2014. After recording of basal diameter and height, the stem was cut at the base to kill the tree and the branches immediately removed with clippers at their junction with the stem, then separated into the two age classes (2013 and older cohorts) used in the sequential needle samples. The stem was also separated into these two sections by cutting at the divide between annual growth stages. This resulted in sections which contained the vertical growth achieved during each year but did not separate the radial growth occurring across the whole stem length.

All biomass was dried in paper sacks inside a 80 °C timber oven until mass loss had ceased (3 days). After drying, the needles were separated from the dry branches and each section was weighed.
The litter layer on the surface of each pot was removed using a trowel and a 7 cm diameter, 20 cm (to the base of the pot) soil core were taken from each pot at a random location between the main stem and the edge of the pot. The soil cores were separated into root and soil components while moist and the soil homogenised. 15 g of homogenised soil was dried in an 80 °C oven to prepare the soil for total ^{15}N/N and ^{13}C/C measurement as well as water content calculations based on mass loss. A further 15 g dry weight equivalent subsample of field-moist soil was fumigated for 3 days with chloroform in a vacuum oven, then extracted in 45 ml 0.05 M K_2SO_4 for 3 hours on a 220 rpm shaker along with an unfumigated control. The extract solution was freeze-dried and subsamples of the salt were analysed on a CN analyser for C and N content. The remaining salt was rehydrated,

adjusted in volume to deliver an appropriate amount of N for mass spectrometry, and concentrated via diffusion onto PTFE-enclosed acidified paper disks using the method of Stark and Hart (1996). These discs were analysed on the mass spectrometer for ^{15}N content measurement only to improve the accuracy of the result.

Soil microbial biomass N was calculated using N content and a K_{EN} (conversion factor to equate extractable N with actual soil microbial N) of 0.54 (Brookes and Landman, 1985), where SMB N was (total N extracted from fumigated soil / total N extracted)*K_{EN}. Microbial ^{15}N was calculated from these measurements along with ^{15}N abundance of the control and fumigated pools by equation 4.1:

$$\frac{\delta^{15}N_{SMB} = \delta^{15}N_{fumigated} * N_{fumigated} - \delta^{15}N_{unfumigated} * N_{unfumigated}}{N_{fumigated} - N_{unfumigated}} \tag{4.1}$$

4.2.8 Sample Processing

All biomass samples were washed in distilled water to remove remaining surface residues and dried in a 80 °C oven until mass loss had ceased (usually 1 - 2 days). Needles on the branches and twigs were removed after drying and before milling. The samples were milled on a Retch MM-200 ball mill, in metal capsules with a single ball, until a fine powder was produced, except for the needle samples between August 2012 and February 2014, which, due to their small volume, were milled in plastic micro-test tubes with two small ball bearings. A subsample of this powder (~3 mg) was analysed for [N], ^{15}N, [C] and ^{13}C on a SerCon Callisto CF-IRMS Isotope Ratio Spectrometer (School of Biological Sciences, University of Aberdeen, UK), along with standards of known isotope abundance.

4.2.9 Final Experiment Calculations and Statistical Analyses

The dry masses of the whole tree sections from March 2014 were used to calculate growth metrics to compare trees and to calculate an above-ground mass balance at the termination of the experiment. Rather than compare raw mass of the tree compartments we calculated three metrics, bulking the ^{15}N/N deposition type combinations together to produce three treatments for bulk nitrogen regime with n = 10 (water control, foliar deposition, soil deposition) to test these growth effects. The metrics calculated were Above Ground Biomass (AGB, the sum of both the 2011-2012 and 2013 needle, branch, and stem cohorts), AGB % Canopy (the percentage of the total biomass made up by the canopy), Canopy % Foliage (the percentage of the canopy biomass that was foliage) and height increment (length of leader at the end of the 2013 growing season as a percentage of height of tree at the end of the 2012 growing season).

The isotope analyses used all treatments where the source of ^{15}N enrichment differed, giving six treatments with n=5 each. Time series data were analysed as linear mixed effect models where the fixed effects were time and treatment while individuals were random effects. In addition, we fitted an autoregressive moving average correlation structure to account for autocorrelation of individuals through time, using REML method due to small sample sizes. We also allowed for a greater variation in ^{15}N content later in the experiment by implementing a variance structure, as later in the experiment there was greater potential for variation with a greater cumulative application of N. Comparisons between treatments in these models were performed by the Tukey HSD post-hoc test.

The mass balance was calculated using the March 2014 biomass, [N], and ^{15}N measurements, assuming that all enrichment above natural abundance was derived from the experimental treatments. Below-ground compartments (soil, roots, microbial extracts) were included in the mass balance but as uncertainty in ^{15}N-label distribution was much greater between these treatments, the total recovery is combined for a single below-ground ^{15}N return.

The mass balance calculation was made by subtracting the atom % ^{15}N in the control NA$_{litter}$-water treatment from observed atom percent in the five ^{15}N-enriched treatments, to calculate $^{15}N_{excess}$, using the total mass of ^{15}N added in the deposition treatments, or estimated to be released from the litter based on a separate litterbag experiment (see Chapter 5), ($^{15}N_{added}$), and the average N mass of the pool in question (N), in equation 4.2 to work out the total ^{15}N return, $^{15}N\ return(\%)$.

$$^{15}N\ return(\%) = \frac{^{15}N_{excess} * N}{^{15}N_{added}} * 100 \tag{4.2}$$

Uncertainty in the mass balance calculations were propagated fully using standard methodology (as described in Chapter 3.2.7) to take into account uncertainty in original measurements and averages. All statistical analyses were performed in R (R Core Team, 2013) v3.1.0, using the package 'nlme' (Pinheiro *et al.*, 2013) and the glht function in the 'multcomp' package (Hothorn *et al.*, 2008).

4.3 Results

4.3.1 Needle Time Series

Differences in ^{15}N content of the ^{15}N-labelled N_{DEP} treatments (NA_{litter}-$^{15}N_{soil}$ and NA_{litter}-$^{15}N_{canopy}$), and the water control were apparent within a month of the deposition treatments beginning (Figure 4.2). The ^{15}N content of needles in cohorts older than the experiment (2011-2012) in the ^{15}N deposition treatments increased over time, to about 0.41 atom % (NA_{litter}-$^{15}N_{canopy}$) or 0.38 atom % (NA_{litter}-$^{15}N_{soil}$) by April 2014, while the control NA_{litter}-water treatment remained consistently at natural abundance (0.365 %). Over this period the corresponding needles in ^{15}N litter treatments ($^{15}N_{litter}$-Water,$^{15}N_{litter}$-NA_{canopy}, and $^{15}N_{litter}$-NA_{soil}) did not display a consistent trend in enrichment, although variance was very high in these treatments, especially early in the growing season. The explanatory factors of treatment, date, and the treatment:date interaction were all significant ($P < 0.001$) in explaining changes in ^{15}N abundance, but only the NA_{litter}-$^{15}N_{canopy}$ (post-hoc TukeyHSD, $P < 0.001$ with all other treatments) and NA_{litter}-$^{15}N_{soil}$ treatment ($P = 0.03$ against NA_{litter}-Water) were significantly different from the unlabelled control, NA_{litter}-Water (Table 4.2). In the most parsimonious model, the correlation structure did not improve parsimony due to the high variance early in the growing season.

N content of the needles fluctuated with an overwinter peak in N content in both 2013 and 2014, although peak [N] was not as great in the second year. This periodicity was not observed in the 2013 cohort of needles, which had a high N (∼7.5 %) soon after budburst, but did not peak over the winter (Figure 4.3)

In the 2013 cohort of needles, the ^{15}N enrichment was greater than the water control in both the NA_{litter}-$^{15}N_{canopy}$ and NA_{litter}-$^{15}N_{soil}$ treatments (Figure 4.4, top), and also in $^{15}N_{litter}$-NA_{canopy}, although in this treatment, this was due to a single individual which consistently displayed a high needle ^{15}N abundance on all assessment dates for this cohort. While this difference between treatments in the 2013 cohort of needles was significant ($P < 0.0176$) in determining the ^{15}N abundance along with time ($P < 0.0001$), there was no interaction term or correlation structure in the best AIC model, and the high variation in ^{15}N expression meant that only the NA_{litter}-$^{15}N_{soil}$ — NA_{litter}-Water comparison was significant ($P = 0.008$) in the TukeyHSD test, although the NA_{litter}-$^{15}N_{soil}$ comparison with the $^{15}N_{litter}$-Water and $^{15}N_{litter}$-NA_{soil} treatments also had borderline P values (Table 4.3).

4.3.2 Destructive Harvest

The two trees which died were in the NA_{litter}-Water, and NA_{litter}-$^{15}N_{soil}$ treatments which reduced their sample size to 4 for the ^{15}N-enriched treatment groupings at the destructive harvest.

At this time, there were no significant differences among bulk N treatment groupings in any of the biomass variables measured (all $P > 0.05$, Table 4.4) . Across the whole experiment, above ground biomass was 370 ± 119 g per tree, 74 ± 5 % (s.d.) of this being canopy, while canopy mass was 38 ± 2 % needles. The trees gained on average 12.4 ± 5.8 % (s.d.) of their height over the 2013 growing season, whist litter mass when removed from the pots at the end of the experiment averaged 65.5 ± 33.3 % (s.d.) of the original dry mass applied.

The ^{15}N content of the major biomass components (Table 4.5 and Table 4.6) varied among treatments. There were significant effects of the treatment in both 2013 ($P = 0.003$) and 2011-2012 ($P < 0.001$) needle cohorts, both stem cohorts ($P < 0.001$, $P < 0.001$), and the 2013 branch cohort (0.009), but not the 2011-2012 branch cohort ($P = 0.39$). Within the treatments , it was only NA_{litter}-$^{15}N_{canopy}$

	NA_{litter}-Water	$^{15}N_{litter}$-Water	$^{15}N_{litter}$-NA_{soil}	$^{15}N_{litter}$-NA_{canopy}	NA_{litter}-$^{15}N_{soil}$
$^{15}N_{litter}$-Water	0.06	/	/	/	/
$^{15}N_{litter}$-NA_{soil}	0.16	-0.11	/	/	/
$^{15}N_{litter}$-NA_{canopy}	0.08	0.02	0.08	/	/
NA_{litter}-$^{15}N_{soil}$	0.42 *	0.36	0.25	0.33	/
NA_{litter}-$^{15}N_{canopy}$	1.08 ***	1.02 ***	0.91 ***	0.99 ***	0.66 ***

Table 4.2: Tukey HSD comparisions between treatments in most parsimonious mixed effect ^{15}N abundance model for the 2011-2012 cohort needles timeseries, mean difference (columns-rows) and significance at $P < 0.05$ level indicated by *, at $P < 0.001$ level by ***.

	NA_{litter}-Water	$^{15}\mathrm{N}_{litter}$-Water	$^{15}\mathrm{N}_{litter}$-NA_{soil}	$^{15}\mathrm{N}_{litter}$-NA_{canopy}	NA_{litter}-$^{15}\mathrm{N}_{soil}$
$^{15}\mathrm{N}_{litter}$-Water	0.003	/	/	/	/
$^{15}\mathrm{N}_{litter}$-NA_{soil}	0.004	0.001	/	/	/
$^{15}\mathrm{N}_{litter}$-NA_{canopy}	0.013	0.009	0.009	/	/
NA_{litter}-$^{15}\mathrm{N}_{soil}$	0.023 ***	0.02^{a}	0.020^{b}	0.011	/
NA_{litter}-$^{15}\mathrm{N}_{canopy}$	0.017	0.014	0.019	0.014	- 0.006

Table 4.3: Tukey HSD comparisions between treatments in most parsimonious mixed effect ^{15}N abundance model for 2013 cohort needle timeseries, mean difference (columns-rows) and significance at $P < 0.01$ level indicated by **. Borderline significant differences are represented by *a*, indicating $P = 0.086$, and *b*, $P = 0.072$.

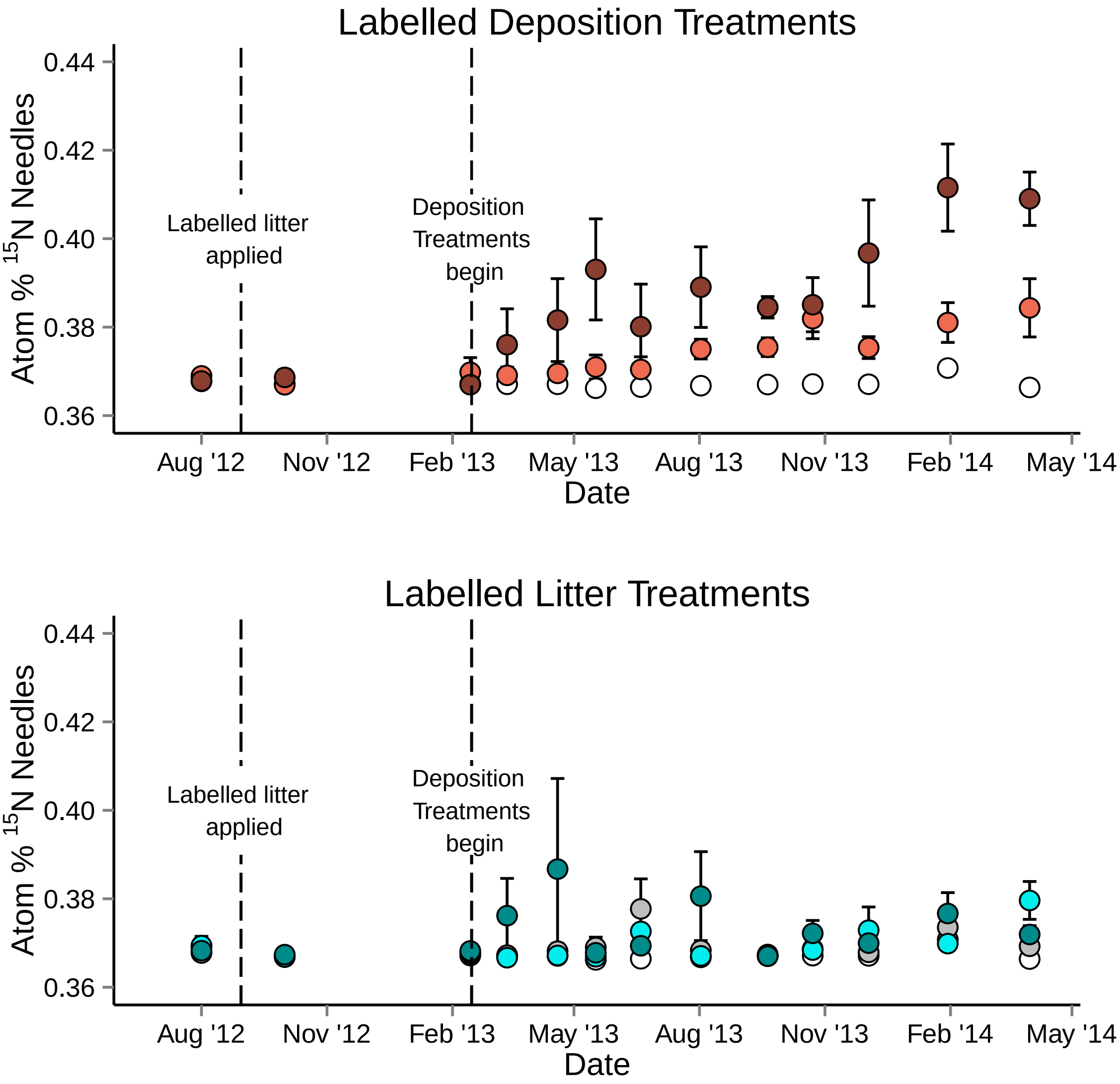

Figure 4.2: ^{15}N content (atom %) of needles older than the 2013 cohort from ^{15}N-labelled deposition treatments (above) and ^{15}N-labelled litter treatments (below). NA$_{litter}$-Water deposition is shown on both plots (white); on the upper plot treatments are NA$_{litter}$-^{15}N$_{canopy}$ (red), NA$_{litter}$-^{15}N$_{soil}$ (orange), and on the lower plot treatments are ^{15}N$_{litter}$-NA$_{canopy}$ (dark blue) ^{15}N$_{litter}$-NA$_{soil}$ (light blue) ^{15}N$_{litter}$-Water (grey). Error bars show standard error of the mean.

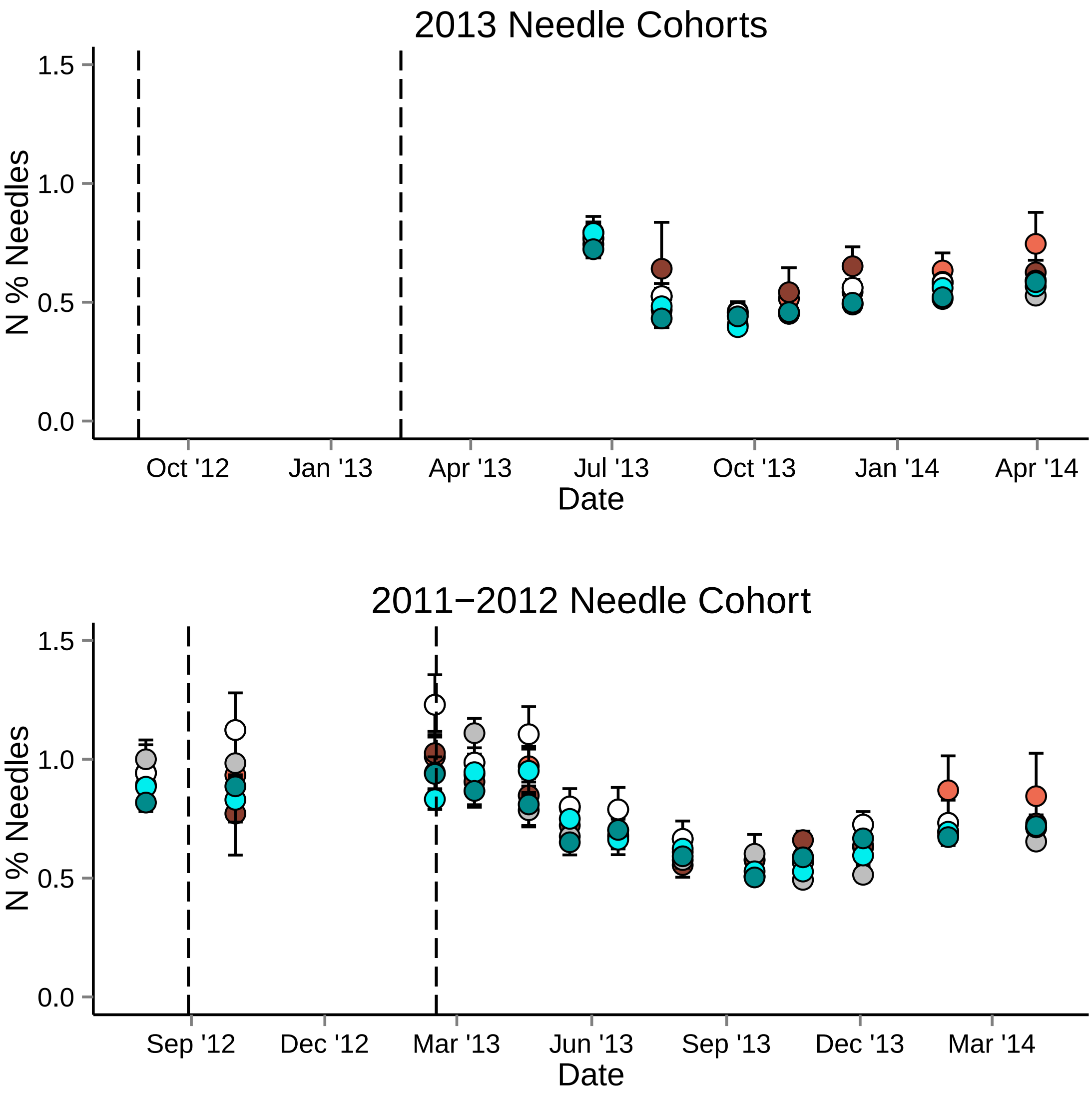

Figure 4.3: N content of needles in both measured cohorts, 2013 (top) and 2011-2012 (bottom). While a yearly cycle is observed this does not differ between treatments NA$_{litter}$-^{15}N$_{canopy}$ (red), NA$_{litter}$-^{15}N$_{soil}$ (orange), ^{15}N$_{litter}$-NA$_{canopy}$ (dark blue) ^{15}N$_{litter}$-NA$_{soil}$ (light blue) ^{15}N$_{litter}$-Water (grey), and NA$_{litter}$-Water (white). Error bars show standard error of the mean.

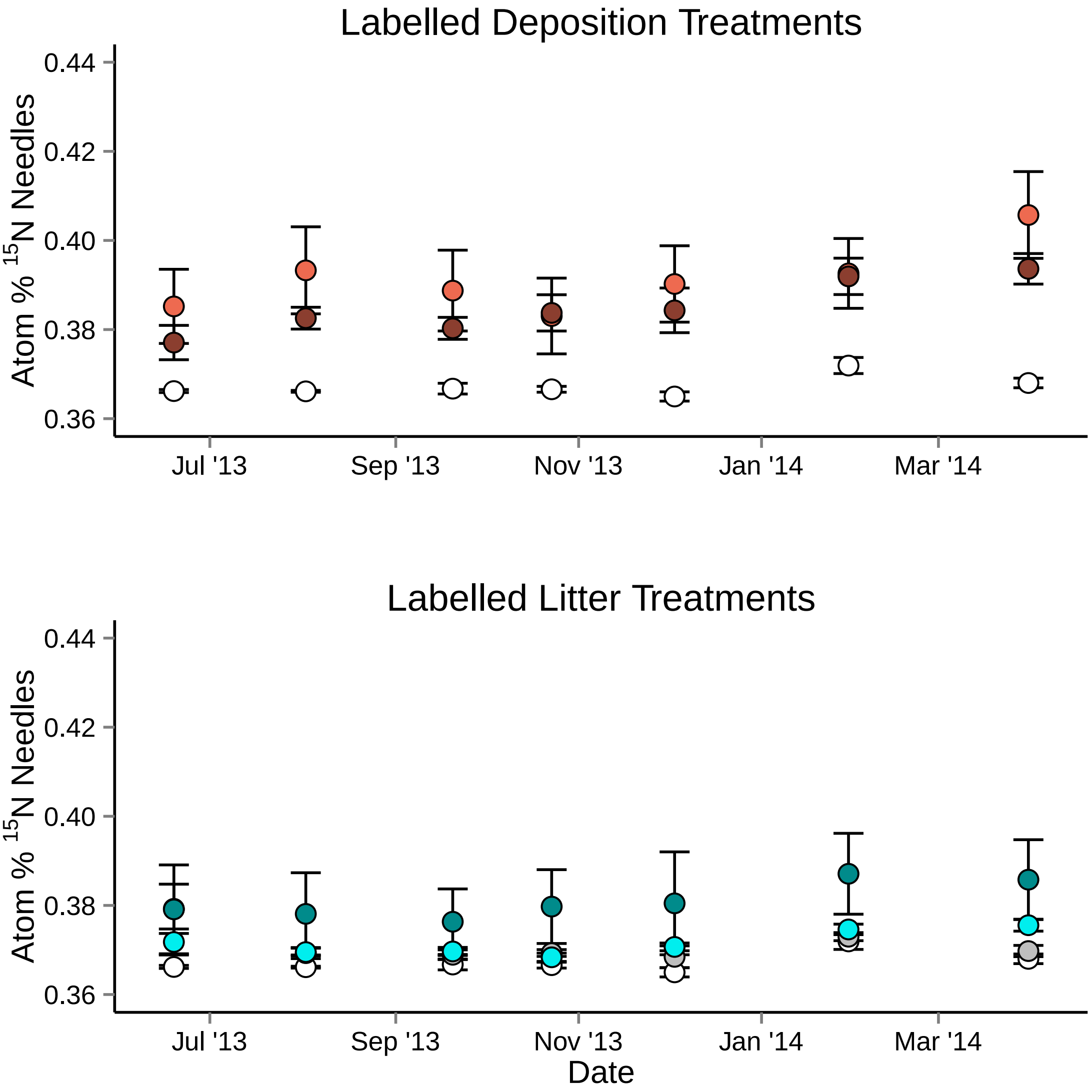

Figure 4.4: ^{15}N content (atom %) of 2013 needle cohort from ^{15}N-labelled deposition treatments (above) and ^{15}N-labelled litter treatments (below). NA$_{litter}$-Water deposition is shown on both plots (white); on the upper plot treatments are NA$_{litter}$-^{15}N$_{canopy}$ (red), NA$_{litter}$-^{15}N$_{soil}$ (orange), and on the lower plot treatments are ^{15}N$_{litter}$-NA$_{canopy}$ (dark blue) ^{15}N$_{litter}$-NA$_{soil}$ (light blue) ^{15}N$_{litter}$-Water (grey). Error bars show standard error of the mean.

	n	AGB (g)	AGB % Canopy	Canopy % Foliage	Height increment (% of initial height)
Water Control	9	357 (± 42)	69.9 (± 3)	34 (± 3)	9.4 (± 2)
Soil N Deposition	9	309 (± 60)	74 (± 5)	38 (± 4)	7.0 (± 3)
Canopy N Deposition	10	391 (± 60)	71 (± 1)	36 (± 4)	7.9 (± 3)

Table 4.4: Total above ground dry biomass (AGB), proportion of dry biomass which is 'canopy' (branches and needles), and proportion of canopy which is foliage between nitrogen deposition treatments. Uncertainty displayed is standard error of the mean of n individuals obtained by combining complementary ^{15}N labelled treatments to form three bulk N deposition treatments. There were no significant differences in biomass among treatments at the end of the experiment.

which significantly differed from the other treatments in the stem sections P $<$ 0.001, P $<$ 0.001), as well as the 2011-2012 needles (P $<$ 0.001), while, in the 2013 needles, when considering the measurement for this time only, all five ^{15}N labelled treatments were significantly different from the NA_{litter}-Water control (p $<$ 0.001).

Root atom % N was not statistically different between treatments, but the lowest mean atom % was found in the NA_{litter}-Water treatment, and the highest ^{15}N atom % in the NA_{litter}-$^{15}N_{soil}$, NA_{litter}-$^{15}N_{canopy}$, $^{15}N_{litter}$-NA_{soil}, and $^{15}N_{litter}$-NA_{canopy} treatments (Table 4.5). Soil ^{15}N also did not differ between treatments. There were no statistical differences in total N extractable from 0.05M K_2SO_4 unfumigated extracts between pots (P $>$ 0.05, mean = 0.010 mg g^{-1} dry soil), nor between the N content of microbial biomass (P $>$ 0.05), which was fairly variable and estimated at 0.041 ± 0.03 mg g^{-1} soil. Microbial atom % calculated from the fumigations was very variable and was not related to treatment, and there was also no statistical difference in bulk soil ^{15}N. As there was more uncertainty in the soil system results as they were not measured over time, they were combined for the mass balance.

4.3.3 Mass Balance Estimates of Above-Ground ^{15}N Return

Using the mass balance approach detailed above, the total estimated ^{15}N recovery in the above-ground parts of the tree was calculated. The highest returns of the ^{15}N label were calculated as 63.67 ± 6.05 % (st.dev) in the NA_{litter}-$^{15}N_{canopy}$ treatment and 20.58 ± 6.31 % in the NA_{litter}-$^{15}N_{soil}$. These two treatments were significantly (P $<$ 0.001, P $<$ 0.01) different than the NA_{litter}-Water control and are shown in Table 4.7. ^{15}N recovery was highest in the 2013 and 2011-2012 needles, and 2011-2012 stem in these treatments, while in the NA_{litter}-$^{15}N_{canopy}$ treatments there was also a high recovery in the 2013 branches. The labelled litter treatments displayed a low overall N return based on our initial field-based mass loss estimate, which remained non-significant even when we revised these based on conservative literature values (Titus and Malcolm, 1999; van Huysen *et al.*, 2013) which better fit the overall measured mass loss. These treatments displayed a lower total ^{15}N return and high standard deviation; $^{15}N_{litter}$-Water 9.623 ± 6.54 (s.d.), $^{15}N_{litter}$-NA_{soil} 12.59 ± 6.38, and $^{15}N_{litter}$-NA_{canopy} 11.52 ± 6.05, and were not significantly different than the water control (all $P > 0.05$).

4.4 Discussion

4.4.1 Overall ^{15}N Return

Conventional forest nitrogen deposition experiments (e.g. Nadelhoffer *et al.*, 1999c) apply tracers directly to the soil in a similar manner to the NA_{litter}-$^{15}N_{soil}$ treatment in this study. Estimates of N recovery in plant biomass in such experiments is around 20 %, partitioned between woody (5 %) and non-woody (15 %) biomass (Nadelhoffer *et al.*, 1999c) while in our NA_{litter}-$^{15}N_{soil}$ treatment, total ^{15}N recovery was very similar to this, at 20.5 ± 5.5 %. In this treatment, total woody recovery of the ^{15}N label (in 2011-2012 branches, and both stem sections, and excluding the 2013 branches, which were primarily non-woody twigs) was 3 %, although standard deviation was high (4 %).

In contrast, the highest recovery of the ^{15}N label above ground in all sections except the 2013 needles (where NA_{litter}-$^{15}N_{soil}$ had the highest return), was in NA_{litter}-$^{15}N_{canopy}$ treatment, with a total ^{15}N return above ground of 64 % of that applied. Total woody recovery in this treatment was primarily located in the 2011-2012 stem section (14.6 % ± 3). This was two to three times as much of the total above-ground ^{15}N return within the treatment than in the NA_{litter}-$^{15}N_{soil}$

	Needles	Branches	Stem	Roots
NA_{litter}-Water (control)	$0.3664 \pm 0.002\ ^{a}$	$0.3664 \pm 0.017\ ^{a}$	$0.3783 \pm 0.006\ ^{a}$	0.3670 ± 0.045
$^{15}N_{litter}$-Water	0.3693 ± 0.002^{b}	$0.3693 \pm 0.002\ ^{a}$	$0.3831 \pm 0.008\ ^{a}$	0.3763 ± 0.002
$^{15}N_{litter}$-NA_{soil}	$0.3796 \pm 0.010\ ^{b}$	$0.3796 \pm 0.010\ ^{a}$	$0.4378 \pm 0.085\ ^{a}$	0.3946 ± 0.009
$^{15}N_{litter}$-NA_{canopy}	$0.3719 \pm 0.004\ ^{b}$	$0.3719 \pm 0.004\ ^{a}$	$0.3787 \pm 0.006\ ^{a}$	0.3813 ± 0.017
NA_{litter}-$^{15}N_{soil}$	$0.3789 \pm 0.010\ ^{b}$	$0.3664 \pm 0.000\ ^{a}$	$0.4063 \pm 0.003\ ^{a}$	0.3909 ± 0.015
NA_{litter}-$^{15}N_{canopy}$	$0.4090 \pm 0.013\ ^{b}$	$0.3729 \pm 0.011\ ^{b}$	$0.4953 \pm 0.062\ ^{b}$	0.3945 ± 0.045

Table 4.5: Mean ^{15}N abundance in 2011-2012 cohort tree compartments. Values shown ± standard deviation. Lowercase letters indicate significant differences among treatments for the same pool at the $P < 0.05$ level or higher.

	Needles	Branches	Stem
NA_{litter}-Water (control)	$0.3680 \pm 0.002\ ^{a}$	$0.3678 \pm 0.002\ ^{a}$	$0.3673 \pm 0.001\ ^{a}$
$^{15}N_{litter}$-Water	$0.3698 \pm 0.003\ ^{b}$	$0.3838 \pm 0.026\ ^{a}$	$0.3735 \pm 0.015\ ^{a}$
$^{15}N_{litter}$-NA_{soil}	$0.3756 \pm 0.003\ ^{b}$	$0.3840 \pm 0.021\ ^{a}$	$0.4879 \pm 0.032\ ^{a}$
$^{15}N_{litter}$-NA_{canopy}	$0.3757 \pm 0.013\ ^{b}$	$0.3750 \pm 0.008\ ^{a}$	$0.3858 \pm 0.008\ ^{a}$
NA_{litter}-$^{15}N_{soil}$	$0.4019 \pm 0.023\ ^{b}$	$0.3811 \pm 0.029\ ^{a}$	$0.4097 \pm 0.057\ ^{a}$
NA_{litter}-$^{15}N_{canopy}$	$0.3936 \pm 0.008\ ^{b}$	$0.4469 \pm 0.030\ ^{b}$	$0.5734 \pm 0.135\ ^{b}$

Table 4.6: Mean^{15}N abundance in 2013 cohort tree compartments. Values shown ± standard deviation. Lowercase letters indicate significant differences among treatments for the same pool at the $P < 0.05$ level or higher.

treatment. As the method of ^{15}N application was the only difference between these two treatments, differences in ^{15}N recovery can be attributed to N partitioning differences due to the mode of ^{15}N uptake, and while the overall treatment effects of canopy versus soil labelling were clear, these differences must be considered in the context of seasonality (N content in the needles varied throughout the year (figure 4.3)), as the harvest in March was during the period when N is remobilised from storage to satisfy the demands of the new growing season (Millard and Grelet, 2010).

In evergreen conifers such as Sitka Spruce, N is stored overwinter in the youngest age class of needles (Millard and Proe, 1992), in contrast to deciduous species which store N in stems and roots (Millard and Grelet, 2010). This N can contribute 9 - 46 % of N for new shoot growth (Millard and Proe, 1992), independent of soil N supply (Millard and Proe, 1993; Weatherall *et al.*, 2006b). Mobilization of these reserves to new shoot growth can occur both by direct remobilization of needle N (Millard, 1996), and xylem-phloem-xylem recyling via the roots (Marschnert *et al.*, 1997), although it is not known if the latter process operates in conifers (Weatherall *et al.*, 2006b). We did not find a clear pattern in atom % ^{15}N of the roots in our March 2014 sample which could confirm that recycling was occurring

	Canopy ^{15}N Deposition	Soil ^{15}N Deposition
2013 Needles	7.17 ± 3.04 %	9.50 ± 3.40 %
2011-2012 Needles	13.41 ± 2.40 %	3.94 ± 2.66 %
2013 Branches	20.77 ± 2.86 %	4.82± 3.46 %
2011-2012 Branches	3.12 ± 0.14 %	0.02 ± 0.07 %
2013 Stem	1.04 ± 0.04 %	0.21 ± 0.02 %
2011-2012 Stem	14.64 ± 3.15 %	2,78 ± 4.04 %
Total Woody Biomass	18.80 ± 3.23 %	3.01 ± 4.04 %
Total Above Ground	60.14 ± 5.75 %	21.28 ± 6.85 %
Below-Ground[1]	7.28 ± 3.55 %	12.13 ± 3.57 %
Total ^{15}N Recovery	67.42 ± 6.76 %	33.41 ± 7.72 %

Table 4.7: ^{15}N Return as % of total applied ^{15}N label in in the above- and below-ground sections of the two labelled deposition treatments. Also presented are total percentage accountancy in woody sections (stem and 2011-2012 branches), total ^{15}N return above-, and below-ground and in total. [1] indicates combined soil and root recovery as total root mass was very variable. Errors terms are standard deviation obtained by propagating the error in measurements of different pools while total recovery and error are obtained by summing the recovery and propagating the error of individual pools making up the total.

via this pathway as no significant differences were found among the treatments in this pool. A slightly elevated ^{15}N content over the water control was evident from all ^{15}N-enriched treatments but the variation within these treatments was not great enough for detection of a difference, nor were there detectable differences in soil bulk ^{15}N or K_2SO_4-extractable N at this time. Using *Picea sichensis* litter with a greater N enrichment in this study, Weatherall *et al.* (2006a) in a similar potted experiment on younger seedlings found only (> 2.5 %) of N released from litter was retained in seedlings. The ^{15}N enrichment of the biomass and potential N uptake from the litter used in this study may have been too low for a detectable trace to tree or microbial pools on the mass spectrometer. Zeller and Colin-Belgrand (2001) found 0.2-0.7 % of litter ^{15}N to be retained in roots after 3 years. While such recoveries are likely to depend on species and nutrient supply, if similar levels were found in this experiment this may have influenced the low return despite our higher estimated ^{15}N release.

4.4.2 ^{15}N Return in Needles Over Time

Over the the 2013-2014 winter season, the N content of all needle treatments in the 2011-2012 cohort was relatively conserved (Figure 4.3) with only a relatively small reduction in N content over this period early in the growing season. Since most of the decline in needle N content over the previous year occurred after April, the proportionately higher ^{15}N return in wood in the NA_{litter}-$^{15}N_{canopy}$ treatment is unlikely to be explained by high concentrations of mobile labelled N in the phloem derived from the needles as opposed to permanent pools within the stem.

4.4.3 ^{15}N Return in Wood

In a canopy-level ^{15}N-fertilization experiment, Dail *et al.* (2009) addressed the stratification of the bole by separating tree cores into three stem sections (bark, recent wood, sapwood) and did not sample the heartwood, and analysed branches by homogenising wood ‘cookies’, bark included, a methodology we adopted for both branch and stem measurements which meant stem sections comprised bark, recent wood and sapwood (heartwood was not present due to the small size of the saplings). This study found total separate recoveries of labelled $^{15}NH_4^+$ and $^{15}NO_3^-$ were 31.4 and 61.4 % respectively, with bark sections retaining 45 % amounts of $^{15}NO_3^-$ and otherwise a low recovery of ^{15}N in the bole (1.5 % of both ions) . In our experiment, we observed a high recovery of N label in the stem (14.6 %) in the canopy fertilization treatment (NA_{litter}-$^{15}N_{canopy}$), where direct bark adsorption or uptake by lichens or cyanobacteria (c.f Reiners and Olson, 1984; Dail *et al.*, 2009) were unlikely as the stem bark surface was directly exposed to very little ^{15}N in the canopy treatment, as this was primarily applied to the branches. While we did not separate this pool from the higher ^{15}N stemwood, if the high stem ^{15}N recovery was within bark rather than stemwood as a whole (we were not able to quantify this), then the high abundances we observed would have been due to transport within the stem and preferential assignment rather than uptake due to location of this organ.

4.4.4 Potential $\Delta C/\Delta N$ effect of Canopy Fertilization Treatments

We deliberately used a low N_{DEP} concentration, designed to avoid a treatment effect of N deposition to allow the ^{15}N effects of the isotope to be studied without complications resulting from the physiological effects of large amounts of additional N (e.g. Schaberg *et al.*, 1997; Elvir *et al.*, 2006). Our deposition treatment, 54 g

N ha^{-1} y^{-1} was several orders of magnitude less than N amendments typically used in experimental N addition experiments (e.g Wallenstein *et al.*, 2006; Gaige *et al.*, 2007; Metcalfe *et al.*, 2013) a small fraction of ambient nitrogen deposition in southern Scotland (13-16 kg ha^{-1} y^{-1}), which our plants could be expected to be receiving. Total N deposition recieve by the pots was therefore in the range of 13-16 kg ha^{-1} y^{-1}). While retention of the ^{15}N-label in the soil may have meant that the NA$_{litter}$-^{15}N$_{soil}$ and NA$_{litter}$-^{15}N$_{canopy}$ treatments were able to take up slightly different total amounts of ^{15}N (NA$_{litter}$-^{15}N$_{soil}$ ^{15}N having more exposure to sinks within the soil), this difference was a minor proportion of the unlabelled background N expected to be available from the soil due to the low dosage of our amendments.

While we worked on saplings rather than mature forests where estimates of N$_{DEP}$ (e.g. Nadelhoffer *et al.*, 1999c) effects on forest C have been drawn, our high ^{15}N return in this section is likely to represent a stronger allocation to stemwood under canopy nitrogen deposition, as well as overall differences partitioning of applied ^{15}N within the tree (e.g. 30 % of total ^{15}N return was in needles in our NA$_{litter}$-^{15}N$_{canopy}$ treatment, compared to 60 % when equivalent ^{15}N deposition was applied to the soil) in the short timeframe of our study, as well as the overall greater retention of N$_{DEP}$ by trees under this pathway. These differences may have major effects on tree ΔC/ΔN and contribute to an overall stronger effect than calculated from traditional isotope studies. In this chapter we refrain from making direct inferences to the scale of this difference from soil-targeted ^{15}N-N$_{DEP}$ studies, but overall estimates can be found in the discussion section of the thesis, section 6.6.1.

4.4.5 Litter-Derived ^{15}N

As well as avoiding fertilization effects, the low N_{DEP} magnitude used in this study was also chosen to allow a reasonable comparison in return of this label with ^{15}N release from the labelled litter in treatments $^{15}N_{litter}$-Water, $^{15}N_{litter}$-NA_{soil}, and $^{15}N_{litter}$-NA_{canopy}. Experiments using labelled litter are sparse (Hatton *et al.*, 2012) and have found only small returns of N aboveground following application (2 % of litter ^{15}N over 4 years (Zeller and Colin-Belgrand, 1998; Zeller *et al.*, 2000; Zeller and Colin-Belgrand, 2001)). In our experiment, ^{15}N recovery from the litter was limited, with these treatments indistinguishable from the natural abundance control in the ^{15}N timeseries for the 2011-2012 needles, and the 2013 needles (high averages for $^{15}N_{litter}$-NA_{canopy} being driven by a single enriched individual). When the entire trees were sampled at the end of the experiment, total above-ground ^{15}N recovery was $\sim$ 10 % of estimated mineralized N in all three ^{15}N-litter treatments, and statistically indistinguishable from the NA_{litter}-Water treatments due to high variances. This recovery was mainly due to the needles and branches, and may have been detectable due to the larger sample size of this final sampling, against a background of high ^{15}N variation in the trees.

We initially estimated litter ^{15}N release based on a separate experiment (Chapter 5) but from litter mass remaining at the end of this nursery experiment thought that using these litterbag-derived values was unrealistic. When this rate was revised to literature values for early-stage decomposition of Sitka Spruce litter of around 5% N, (Titus and Malcolm, 1999; van Huysen *et al.*, 2013), measured mass loss and estimated N release based on N content of the litter at the end of the experiment fit these overall trends. While in the early stages of decomposition the colonisation of litter by decomposers often raises internal N concentrations (Parton *et al.*, 2007), rapid mass loss early in decomposition counteracts the per-mass increase in [N] and results in net N mineralization from the litter. Even after a decade large amounts of ^{15}N from labelled litter may remain associated with soil aggregates or remain in undecomposed litter (Zeller and Colin-Belgrand, 1998; Zeller *et al.*, 2000; Zeller and Colin-Belgrand, 2001; Hatton *et al.*, 2012) and our total N return and inability to distinguish between these treatments and the NA$_{litter}$-Water control over the needle time series is similar to the low short-term recovery of ^{15}N from *Fagus sylvatica* litter in above-ground biomass in these studies (e.g. Zeller and Colin-Belgrand (2001)).

The high variance in these treatments early in the experiment (Figure 4.3) may indicate acquisition of the litter-derived ^{15}N by the trees but replication was insufficient to explain this frequent high ^{15}N content. Towards the end of the time series, needle ^{15}N may have also began to diverge from NA$_{litter}$-Water (Figure 4.2). When we sampled from the entire needle biomass at the end of the experiment, with the largest cumulative ^{15}N release by this time, ^{15}N abundance for the 2013 needles was significantly different than the NA$_{litter}$-Water control in all three ^{15}N-litter treatments as well as the ^{15}N-N$_{DEP}$treatments (although not significantly different on the whole-tree level). While sample size was the same at this date as previously (5 trees), the needle sample was from the harvest of the entire canopy which could also indicate a heterogeneity in ^{15}N partitioning from the litter-derived ^{15}N not accommodated by the sampling strategy. Similarly, the high ^{15}N enrichment in 2013 needles from a single replicate of the ^{15}N$_{litter}$-NA$_{canopy}$ treatment was consistent throughout the time series. This individual received the highest ^{15}N litter of the labelled sources, although this was with three other individuals which did not also display a high ^{15}N atom %.

While we were unable to distinguish the fate of litter ^{15}N under nitrogen deposition, this is clearly an area where more research is required. This may be addressable experimentally with ^{15}N-labelled litter over longer timescales, greater enrichment, and greater replication.

4.5 Conclusion

Magnani *et al.* (2007) found from a correlation of NEP against N_{DEP}, that nitrogen deposition has a strong effect on forest C sequestration, but this contradicts by an order of magnitude estimates by stable isotope traces (Nadelhoffer *et al.*, 1999c) where a ^{15}N label has been largely retained in the soil, and not recovered in trees (Högberg, 2007). While our experiment employed saplings, we were able to demonstrate that both the magnitude and proportional recovery of canopy-applied ^{15}N across different biomass classes uptake differed by a factor of three when compared to soil-applied ^{15}N deposition treatments, thereby going some way to explain the discrepancies across different studies. Both an absolute and relative greater recovery of applied ^{15}N was observed in stems under canopy N_{DEP}, which may have major implications for the overall $\Delta C/\Delta N$ effect due to the high C/N of this pool. This area remains largely unexplored, and appropriately designed stable isotope experiments may provide a useful tool to resolving the still unanswered questions regarding the N deposition effect on the forest carbon sink.

Chapter 5

A Field Study Comparing the Fate of ^{15}N from Sitka Spruce Litter Mineralization with ^{15}N from Nitrogen Deposition

5.1 Introduction

Quantitative estimates of an effect of anthropogenic nitrogen (N) deposition (N_{DEP}) on forest growth can vary by an order of magnitude (c.f. Nadelhoffer *et al.* (1999c); Currie *et al.* (2004); de Vries *et al.* (2006); Magnani *et al.* (2008)), and since N-limited (Vitousek and Howarth, 1991) northern and boreal forests are a substantial C sink (0.6 - 0.7 Pg y^{-1}, Goodale *et al.* (2002)), understanding why estimates of the effect of N addition on C sequestration ($\Delta C/\Delta N$) vary so greatly is important to separate N_{DEP} from the many other anthropogenic factors (see Sedjo (1992); Norby (1999); Prentice *et al.* (2001); Saxe *et al.* (2002)) which could be driving this change. As ecosystem N is derived from both N deposition and internal N (from litter recycling), the ability for different studies to account for the fate of internal recycled ecosystem N may have an effect on the magnitude of a ΔC/ΔN effect calculated. If this recycled N is partitioned differently, understanding overall effects of N_{DEP} on ecosystem ΔC/ΔN from ^{15}N-isotope studies would also require understanding of movement of N from litter under the N_{DEP} treatment and not a simple partitioning of N_{DEP} N plus litter-derived N between ecosystem pools of different C/N ratios.

When calculated from N budgets and ^{15}N labelled deposition experiments, ΔC/ΔN is usually low (e.g. 46-50 kg C kg N $^{-1}$ (Nadelhoffer *et al.*, 1999c; de Vries *et al.*, 2006), with the effect split evenly between soils (21 kg C kg N $^{-1}$) and trees (25-29 kg C kg N $^{-1}$). Typically, the majority of ^{15}N tracers added to forests are retained in the forest floor and soil (Templer *et al.*, 2012), and in an influential meta-analysis, Nadelhoffer *et al.* (1999c) found that 70 % of ^{15}N tracers added in N_{DEP} experiments are typically retained in soil mineral and microbial (SMB) pools, while only about 5 % of tracers are found in high C/N woody biomass necessary for a stronger C effect. However, a correlation across many locations of N_{DEP} with NEP (net ecosystem productivity) (Magnani *et al.*, 2007, 2008) found a very large effect of N_{DEP} on the whole ecosystem C sink (trees and soil, 175 to 225 kg kg C kg N $^{-1}$), which implies more N than indicated by deposition experiments is sequestered in high C/N pools. Another study using similar method (Thomas *et al.*, 2009) also found a relatively high ΔC/ΔN (73 kg C kg N $^{-1}$) in trees alone, which is likely to be around 100 kg C kg N $^{-1}$ if similar, conservative effects on soil ΔC/ΔN as Nadelhoffer *et al.* (1999c) or de Vries *et al.* (2006) are assumed. It has been suggested that the Magnani *et al.* (2007) findings may have confounded variability in wet N_{DEP} with several other factors (dry N_{DEP} (de Vries *et al.*, 2008); climate (Sutton *et al.*, 2008); edaphic N mineralization (de Vries *et al.*, 2008); anthropogenic N history (Högberg, 2012)), but ^{15}N traces and ecosystem N manipulations may also contain inaccuracies derived from a failure to account for interactive effects of N_{DEP} on canopies (Sievering, 1999) and soils (Jenkinson *et al.*, 1999). When these factors were included in a model, along with common forestry practice (Dezi *et al.*, 2010; McMurtrie *et al.*, 2001), they raised the C effect to 121 kg C kg N $^{-1}$ from 28 kg C kg N $^{-1}$.

Net nitrogen mineralization in the soil is frequently in the range of 30-100 kg $ha^{-1}y^{-1}$ (Persson and Wirén, 1995; Scott and Binkley, 1997; Reich *et al.*, 1997) in temperate regions, while atmospheric inputs without human inputs are < 0.5 kg $ha^{-1}y^{-1}$. Average anthropogenic deposition rates are 10 kg $ha^{-1}y^{-1}$ in some regions (Galloway *et al.*, 2008) and may reach ~ 50 kg $ha^{-1}y^{-1}$ by 2050 (Galloway *et al.*, 2004). Therefore, even under relatively heavy N_{DEP} regimes, the majority of ecosystem N is still derived from edaphic sources (Schulze, 2000; Högberg, 2012) and the forest response to N deposition could be as dependent on changes in belowground N cycling under N_{DEP} as it is on the long-term retention of N inputs from deposition.

NH_4^+ and NO_3^- ions were once thought to be the sole source of N nutrition for plants (e.g. Pate (1973)), and as plants are poor competitors for this mineral N in soils, microbial utilization of this pool was understood to limit N supply (Schimel and Bennett, 2004). Soil organic matter (SOM) and abiotic mineral fractions in the soil also tend to be strong sinks for these forms of N, and frequently account for > 50 % of mineral ^{15}N additions (Templer *et al.*, 2012). Under this understanding, recovery of mineral N_{DEP} would reasonably represent the background plant-available N pool as all other N would pass through a bottleneck of microbial decomposition (Knops *et al.*, 2002) into NH_4^+ and NO_3^- pools before acquisition. Changes in litter decomposition rates affecting overall differences in total net N mineralization due to N_{DEP} (see Knorr *et al.*, 2005, for a review)) would therefore be the major controls on changes in plant N availability and combine additively N_{DEP} inputs to a total N availability under N_{DEP} which could ultimately affect ecosystem $\Delta C/\Delta N$.

However, it is now widely accepted that plants can acquire and utilize organic N (Näsholm *et al.*, 2009) as amino acids (Chapin *et al.*, 1993; Näsholm *et al.*, 1998), peptides (Paungfoo-Lonhienne *et al.*, 2008), and even proteins (Paungfoo-Lonhienne *et al.*, 2008) which may provide a pathway for extra N uptake in N-limited (Rennenberg *et al.*, 2009) ecosystems (e.g. Chapin *et al.* (1993); Näsholm *et al.* (1998); Schiller *et al.* (1998)) as N can be acquired by plants as organic products of depoloymerisation by extracelluar enzymes (Schimel and Bennett, 2004), before competition for mineral N. As well as increasing the available N pool organic N uptake may offer energetic advantages to plants due to a lower C cost of uptake (Zerihun *et al.*, 1998; Gruffman *et al.*, 2013), and a lack of a need to reduce NO_3^- to usable NH_4^+, as amino acid N is already in this form (Zerihun *et al.*, 1998). While lab (Paungfoo-Lonhienne *et al.*, 2008) and hydroponic (Gruffman *et al.*, 2013) experiments have measured uptake of amino acids and proteins, in field studies, N is available to plants, microbes, and soil sinks simultaneously from all states of decomposition from large organic molecules to mineral ions. Experiments where ^{15}N is traced from decomposing biomass in the field (e.g. Zeller *et al.*, 2000) are limited as labelled litter needs to be of the appropriate species and provenance to provide useful ecological insight, as well as having sufficient ^{15}N content to overcome low decomposition rates, high long-term retention of tracers in undecomposed litter fractions (Hatton *et al.*, 2012), and heterogeneous background soil (Högberg, 1997) and litter (Weber and Bol, 2008; Callesen *et al.*, 2012) $\delta^{15}N$. In the short term, small amounts of ^{15}N tracer can be recovered in above- and below-ground biomass of trees growing on ^{15}N-isotope distinct litter (Zeller and Colin-Belgrand, 2001; Weatherall *et al.*, 2006a).

As litter-derived N may be acquired by different mechanisms to the mineral N from deposition, this study investigates if movement of ^{15}N from litter sources, which could contain a mix of organic and inorganic N available to soil sinks, is comparable to fertilization treatments to the soil, which only apply mineral ^{15}N and are relatively well understood (see Nadelhoffer *et al.*, 1999c). To do this, we combine a litter replacement experiment, where we removed and replaced the litter layer with ^{15}N-labelled litter, with a ^{15}N fertilization experiment, where we applied a solution of ^{15}N-labelled NH_4NO_3 to the soil surface over the litter layer, in plots in a Sitka Spruce (*Picea sitchensis* (L. Bong.)) plantation. We aimed to avoid introducing an artificial treatment effect caused by large magnitude N additions (which would increase soil fertility), but retain the ability to track similar amounts of highly enriched ^{15}N label from both sources. Over 15 months, we traced the isotope from the two sources into roots and soil fractions from two soil layers, as well as making measurements of soil microbial and soil extractable N isotope ratios at the end of the experiment to quantify longer-term N retention in these pools.

5.2 Methods

5.2.1 Study Site

Our study was located in an experimental plot at Cloich Forest, a managed Sitka Spruce plantation 34 km outside of Edinburgh, UK (55°42’ N, 03°16’ W). The plot is approximately 400m above sea level and was established in 1970, with Sitka spruce (*Picea sitchensis* (Bong.) Carr), planted at 3000 stems per hectare (2 m inter-tree spacing). The soil at the site is a shallow peat overlaying Silurian Ordovacian greywacke (Sheppard *et al.*, 1995). As there is no significant understory and the litter at the site is entirely derived from the Sitka spruce canopy, the forest floor is composed of slow-decomposing acidic needles with a relatively thick layer of partially decomposed litter (O_h Layer) above a thicker, dark-coloured (A_h Layer) of organic dominated peaty topsoil with a sharp divide before an orange-brown B horizon. In this study we were only concerned with the organic horizons, the depths of which varied over a scale of around 2 m due to the impact of a ploughing regime at establishment. Soils were approximately 30 cm deep on furrows and 45 cm deep on ridges across the site. Layer depths varied with microsite topography; litter layers were considerably deeper in furrows (max 7 cm) than on ridges (min 1 cm), while the O_h layer varied from 3 cm (ridges) to 11 cm (furrows). The A_h horizon began by 16 cm deep at all sites we sampled across all plots. Local climate is typical of southern Scotland with annual minimum temperatures of -0.2 °C in December and maxima of 18.8 °C in July. Annual rainfall is 980 mm, which frequently falls as snow in the winter. In 1986, sections of the site were selectively thinned to 4 m and 6 m spacing (Greens *et al.*, 1995). Our experiment took place entirely in the unthinned 2 m spacing section, where average dbh was 21.5 ± 5.70 (s.d.) cm. Previous work at the site (Greens *et al.*, 1995) had removed low level branches from the trees to improve access, so we repeated this procedure where necessary, removing all branches from trees up to 1.5 m.

5.2.2 Artificial Litter Layer

In the selected area, we established 12 rectangular plots each containing a single central tree and up to eight peripheral trees (a single tree was missing from the corner of some plots), with an edge of c.4 m on each side. These were randomly assigned to one of four treatments, in a crossed design of two factors (N_{DEP}-^{15}N and litter decomposition ^{15}N) . In three replicate plots (treatment for litter decomposition ^{15}N), the entire litter layer was removed with a shovel and replaced with artificially produced Sitka Spruce litter with a distinct ^{15}N signature. These treatments received ^{15}N labelled litter (at 1.53 ± 0.4 (s.e.) atom % ^{15}N (3227 δ^{15}N ‰), 1.87 ± 0.1 atom % ^{15}N (4184 δ^{15}N ‰), and 2.09 ± 0.2 atom % ^{15}N (4807 δ^{15}N ‰). A second treatment received unlabelled litter, at natural abundance(0.366 % (1 δ^{15}N ‰) for all plots). As the labelled litter was derived from multiple trees (three per plot), injected with ^{15}N (Chapter 3), we minimised the within-treatment variance in the ^{15}N content by mixing the litter for each plot well before application. Mean N content by dry weight in artificial litter at application was 1.2 %, while C % was 51.0 % (C/N ratio 34). The fresh litterfall at the site had an average N content of 1.1 % and C content of 47.1 % (C/N ratio 47.5). Total dry masses of ^{15}N-labelled litter deployed were 23.0 kg, 22.2 kg, and 21.7 kg, and unlabelled litter 29.81 kg 29.52 kg and 27.07 kg. The remaining six plots were left undisturbed as controls against the litter replacement.

5.2.3 N_{DEP} Regime and Experimental Treatments

On the three plots where litter had been removed and replaced with natural abundance litter, and three replicate undisturbed plots, we applied the nitrogen deposition treatments as a 1 L spray direct to the soil (litter) surface with a pressurised hand sprayer, adding 98% ^{15}N, double-labelled NH_4NO_3 in 4-5 week intervals from February 2013 until April 2014 (in June/July 2013, November/December 2013 and January/February 2014 the treatment was applied bimonthly with no reduction in the total application dose per month). In total, the deposition treatments added 0.62 g of ^{15}N to each plot over the whole experiment. The three plots where the litter layer was removed and replaced with ^{15}N-labelled litter plots received unlabelled deposition (^{15}N 0.367 atom %) as this deposition treatment, and the remaining three non-litter swapped plots received DI water in place of a nitrogen additions. This resulted in four treatments: two treatments of swapped litter, $^{15}N_{Dep}$s and $^{15}N_{Litter}$s where the ^{15}N-label was either available in deposition ($^{15}N_{Dep}$s) or the litter ($^{15}N_{Litter}$s), and two treatments of unswapped litter, $^{15}N_{Dep}$u, which received ^{15}N deposition as a control against changes in soil processes as a result of the litter swap, and Water Control, which received de-ionised water without an enriched ^{15}N source. The treatments are summarised in Table 5.1.

Table 5.1: Summary of combination treatments for the field experiment.

Treatment ID	Litter Layer	Deposition
$^{15}N_{Litter}$s	Swapped, ^{15}N-enriched	natural abundance NH_4NO_3
$^{15}N_{Dep}$s	Swapped, natural abundance	98 %^{15}N- NH_4NO_3
$^{15}N_{Dep}$u	Unswapped, natural abundance	98 %^{15}N- NH_4NO_3
Water Control	Unswapped, natural abundance	Water

5.2.4 Litterbags

We estimated rates of ^{15}N loss from the litter by use of a separate litterbag experiment established in April 2013, three months after deposition treatments started in the main experiment. These were not established with the main experiment to avoid effects of disturbance caused by litterbag removal. 60 litterbags (20 ^{15}N-labelled litter, 40 unlabelled litter) were constructed from 1.1 mm aperture polypropylene mesh and were filled with 2 g of litter, either derived from labelled, or unlabelled trees. These were sealed with a hot glue gun and buried in the litter layer on site in three additional plots which had been established at the same time as the main experiment. These plots included labelled litter (1.23 atom %), where we buried 20 natural abundance litterbags, unlabelled litter, where we buried 20 ^{15}N-labelled, 3.71 atom % ^{15}N litterbags, and an unlabelled litter control, where we buried natural abundance litterbags.

These treatments allowed us to quantify mass loss and changes in N content of the litterbag, as well as to estimate the transfer of litter-derived ^{15}N to other litter within the litter pool, via the spatial separation of unlabelled litter within a ^{15}N-labelled plot). Three litterbags were retrieved per plot on nine occasions between April 2013 and May 2014. The litter from these pots was processed in the same way as sequential litter samples from the main experiment.

5.2.5 Sampling Strategy

We took soil samples from the plots using an 5.5 cm diameter, 20 cm deep soil auger on 8 occasions over the 15 month period of the experiment, from January 2013 (immediately before the first deposition treatment), until May 2014 (one month after the last deposition treatment). On three occasions, a larger corer (6.5 cm diameter) was used and masses were adjusted appropriately when calculating total N contents. At each sampling, cores were removed at three random locations per plot (36 cores in total) by removing the surface litter layer, which was bagged and removed separately, and driving the auger directly into the soil. The locations were chosen randomly based a coordinate system within the plot and a random number table, but reselected if the core location was within 5 cm of a previous core, or if the auger encountered an irremovable stone or other obstacle. The soil from the cores were separated on-site into the O_h (organic partially decomposed humus) and A_h (organic-influenced topsoil) soil horizons and bulked together to give one composite sample per plot per date for each of the two soil horizons,

except for the first three dates when the A_h horizon was not sampled. If the B horizon was encountered by the corer, this portion of the soil was discarded.
The soil samples were stored in a coolbox and transported back to the lab (approximately 2 hours from sampling time) and either held overnight at 4°C, or processed immediately.

5.2.6 Processing and Measurement

We immediately weighed all soil cores to establish field wet weight and then allowed the soil to equilibrate to ambient humidity at room temperature (rewetting if necessary to prevent drying), before sieving it to pass through a 2 mm mesh. From this $<$ 2mm soil fraction, small needle and root debris was removed from this subsample with tweezers, and subsamples (15- 20 g) were weighed into stainless steel trays and dried in a 80°C oven overnight, until a stable mass was reached. After drying the soil was reweighed and used to calculate the dry mass of the whole core, then milled in a stainless steel capsule on a Retsch MM400 ball mill (Retsch Ltd UK), until a fine powder was achieved, suitable for mass spectrometry. The material that did not pass through the sieve was washed in DI water and then gently dried and sorted to separate larger roots from stones and other debris. The total mass of dry roots from each set of 3 composite cores was recorded, then washed again and redried at 80 °C, before milling on the ball mill in the same manner as soil samples. Litter samples were washed in DI water to remove surface residues and dried overnight in the 80 °C oven. These were then milled on the ball-mill like all other samples. At the end of the experiment, we made a single point assessement of soil microbial biomass N and ^{15}N content. Samples for fumigation were obtained from the $<$ 2mm soil fraction with small debris removed, and 10 g (dry weight) of wet soil was weighed into glass jars for fumigation, and into identical jars as an unfumigated control. The fumigation samples were exposed to chloroform in a dark vacuum oven for three days. Both fumigated and unfumigated samples were shaken for three hours with 50 ml 0.05M K_2SO_4 and filtered through pre-leached Whatman no.1 filter paper to extract the available N. The filtrate was freeze dried for two days to remove all water and a small subsample ($\sim$ 10 mg) was analysed for C and N content on a Carlo Erba NA 2500 Elemental Analyser. The remaining filtrate was re-hydrated with DI water and prepared for ^{15}N analysis by the N diffusion technique of Stark and Hart (1996) by adjusting the pH of the solutions with conc. NaOH, adding 0.4 g of Devarda's alloy, and trapping the solution N on a pre-prepared PTFE-enclosed $KHSO_4$-infused paper disk.

All samples were analysed on a SerCon Callisto CF-IRMS Isotope Ratio Mass Spectrometer at the University of Aberdeen, UK, along with samples of known isotope abundance and method blanks for the N diffusion disks. To calculate N and ^{15}N in these samples, the method blank discs were subtracted from the sample N contents so that N in the filters was not attributed to the extractable pool.

5.2.7 Statistical Analyses and Mass Balance

We modelled the change in δ^{15}N content in O$_h$ and A$_h$ horizon roots and soil separately, with linear mixed effects models and treatment and date as fixed factors and plot as a random factor. A correlation structure was used to control for the effect of psuedo-replication within successive measurements of the same plots over time and a weighting structure to allow the residuals to increase later in the experiment when measured ^{15}N inputs and δ^{15}N ‰ were greater. All statistics were performed in R v 3.01 (R Core Team, 2013), linear mixed effect models were run with the nlme package (Pinheiro *et al.*, 2013), and post-hoc TukeyHSD tests were performed with the general linear hypothesis (glht) in the multcomp package (Hothorn *et al.*, 2008). We also calculated R^2_m (Nakagawa and Schielzeth, 2013), in order to break down linear model R^2 into a component relating to the fixed effects we were interested in.

As dry masses of soil horizons and roots were highly variable and did not differ statistically between treatment plots, we used their average masses and [N] contents of these values to calculate N pool sizes in the bulk soil, roots, litter, and microbial biomass as enrichment over the natural abundance, water control treatment. As the experiment was designed to be maintained in the long term, plot litter masses were intended to be based on the dry masses of litter removed at the start of the experiment, and tracer return was expressed as a % of total applied N_{DEP} treatments (for the deposition treatments), or total N calculated to have been released from the ^{15}N-labelled litter. The net N loss calculated from the litterbags was applied to the litter layer of the whole plots, based on a best fit model of (exponential) mass loss fit to the litterbag data, where litter mass was measured over time and all litter within the bags was known to be derived from the litter replacement. Net N release was calculated based on changes in this mass and N content over time, assumed to be equal to the decrease in this pool since the beginning of the experiment.

5.3 Results

5.3.1 N Content of Soil, Root and Litter

There were no differences over time in our soil system pools (Figure 5.1). In most pools, N content remained constant except for the litter, where average N content started below the control ($^{15}N_{Dep}$u and Water Control) in the two swapped treatments ($^{15}N_{Litter}$s and $^{15}N_{Dep}$s) but increased over time until all treatments were similar. There were no statistically significant differences between treatment in N content in any of the five pools (soil in the O_h and A_h horizons, roots in the O_h and A_h horizons, and litter) over the treatment period.

5.3.2 δ^{15}N Changes Over Time in Soil and Litter

Changes in the δ^{15}N of the O_h horizon were clearly detectable over the course of the 1.5 year experiment (Figure 5.2), with a variable increase in ^{15}N content from the ^{15}N-litter treatment ($^{15}N_{Litter}$s) developing in late 2013, in contrast to the two ^{15}N-deposition treatments ($^{15}N_{Dep}$s and $^{15}N_{Dep}$u), which had mean δ^{15}N slightly above natural abundance but did not differ significantly from the water control (Table 5.2).

By May 2014, the O_h soil had a δ^{15}N of 65.9 ± 13.6 ‰ (s.d.) in $^{15}N_{Litter}$s, 29.5 ± 14.5 ‰ in $^{15}N_{Dep}$s, 26.0 ± 6.9 ‰ in the N_{DEP}-control, and 2.2 ± 0.4 ‰ in the water control without an additional ^{15}N source. Although in the $^{15}N_{Litter}$s treatment, a higher mean δ^{15}N was observed in March 2014 than May 2014, the standard deviation on this was very large as our sample size was small.

We fit a linear relationship to these data, which had a significant effect of both treatment ($P = 0.002$) and date ($P < 0.001$) on δ^{15}N content in this horizon.

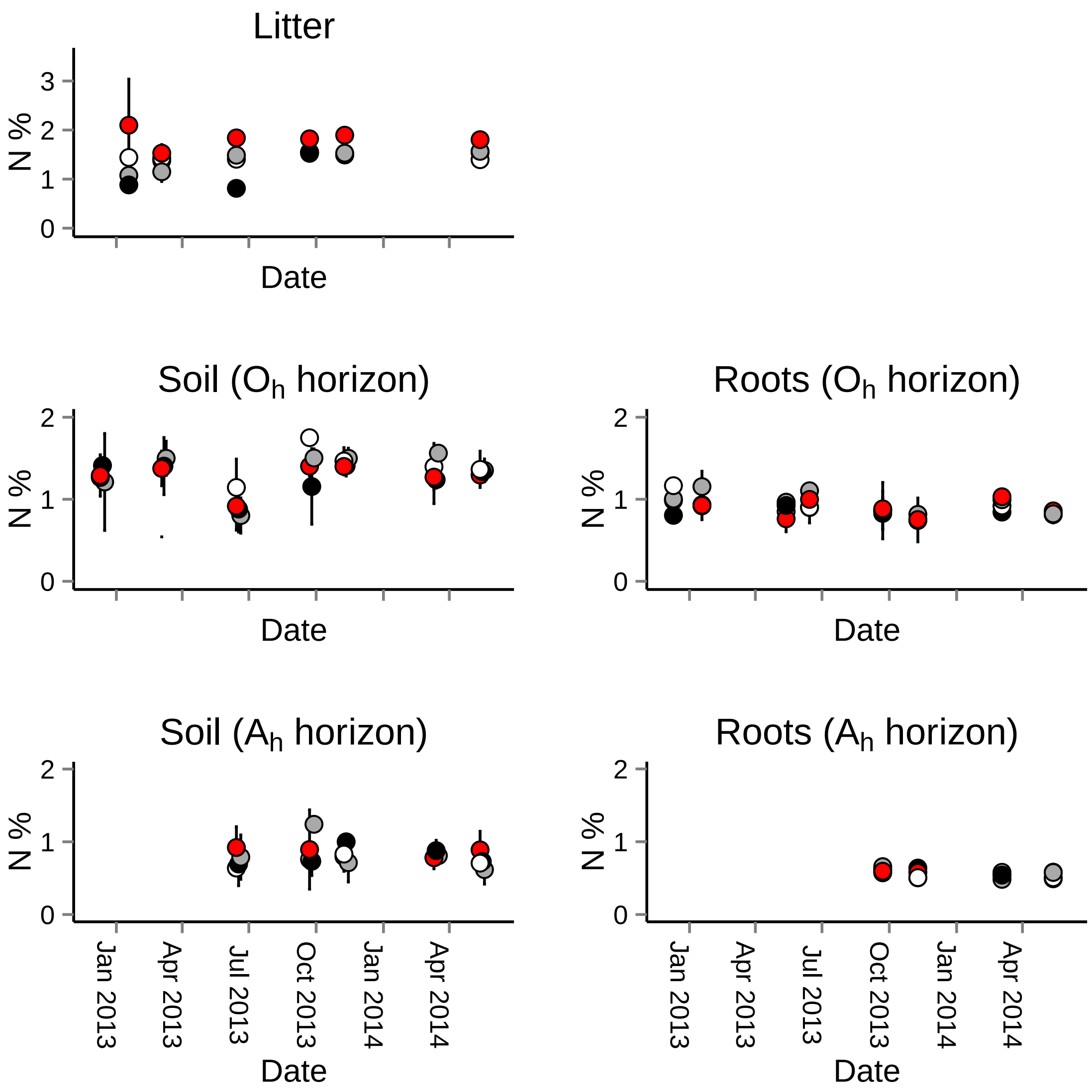

Figure 5.1: N (% mass) of forest floor pools over time. Treatments are: Water Control (White), ^{15}N$_{Dep}$u (Grey), ^{15}N$_{Dep}$s (Black) ^{15}N$_{Litter}$s (Red). A slight jitter has been applied to the x axis to distinguish treatments.

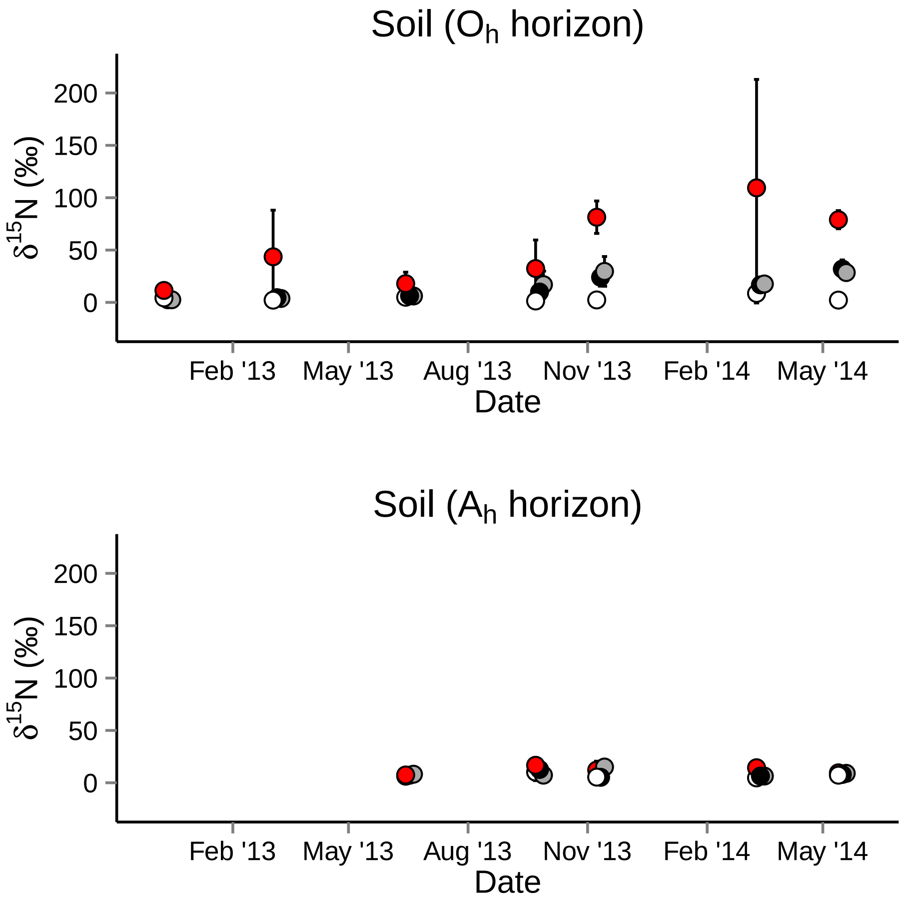

Figure 5.2: ^{15}N content (δ^{15}N ± standard deviation) of soil layers over time. Treatments are: Water Control (White), ^{15}N$_{Dep}$u (Grey), ^{15}N$_{Dep}$s (Black) ^{15}N$_{Litter}$s (Red). A slight jitter has been applied to the x axis to distinguish treatments.

The R^2_m indicated that fixed effects (treatment and date) accounted for 49 % of the variation. In contrast, there was no significant return between treatments (P = 0.065) or over time (P = 0.758) of the ^{15}N label in A_h horizon soil (Figure 5.2). δ^{15}N measured in the water control, A_h horizon was 6.5 ± 0.8 ‰, similar to unlabelled control treatments in other ^{15}N-N$_{DEP}$ experiments (*c.f.* Nadelhoffer *et al.*, 1995), and slightly more enriched than O_h fractions (3.6 ± 1.0 ‰), at natural abundance.

Table 5.2: Tukey HSD comparisons for treatments in the O_h horizon soil most parsimonious model. Asterisks indicate significance at the $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) level.

O_h HORIZON	$^{15}N_{Dep}u$	$^{15}N_{Dep}s$	$^{15}N_{Litter}s$
Water Control	0.540	0.426	<0.001 ***
$^{15}N_{Dep}u$		0.997	0.004 **
$^{15}N_{Dep}s$			0.027 *

Table 5.3: Tukey HSD comparisons for treatments in the litter layer most parsimonious model. Asterisks indicate significance at the $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) level.

LITTER	$^{15}N_{Dep}u$	$^{15}N_{Dep}s$	$^{15}N_{Litter}s$
Water Control	0.103	0.042 *	<0.001 ***
$^{15}N_{Dep}u$		0.996	0.001 ***
$^{15}N_{Dep}s$			0.001 ***

This change in the O_h horizon in ($^{15}N_{Litter}s$) was derived from the high $\delta^{15}N$ of the litter layer (around 2500 ‰), although measured $\delta^{15}N$ fluctuated (Figure 5.3). Variance was very high in this treatment, which was expected as the litter mixes used for the swap were not completely homogeneous, being derived from several trees.

Otherwise, a consistent increase was visible in $\delta^{15}N$ in the two labelled N_{DEP} treatments over the experiment (Figure 5.3) reaching a $\delta^{15}N$ in May 2014 of 670 ± 70 ‰ in the $^{15}N_{Dep}s$ treatment, and 600 ± 90 ‰ in the $^{15}N_{Dep}u$ treatment. When the high ^{15}N labelled litter treatment was removed from the dataset to facilitate comparisons between the other 3 treatments (which could be expected to have the same mean $\delta^{15}N$ if there was no effect of ^{15}N treatments), TukeyHSD comparisons (Table 5.3) indicated that the two ^{15}N-N_{DEP} treatments were significantly ($P = 0.004$) different from the control, but not from each other ($P = 0.654$).

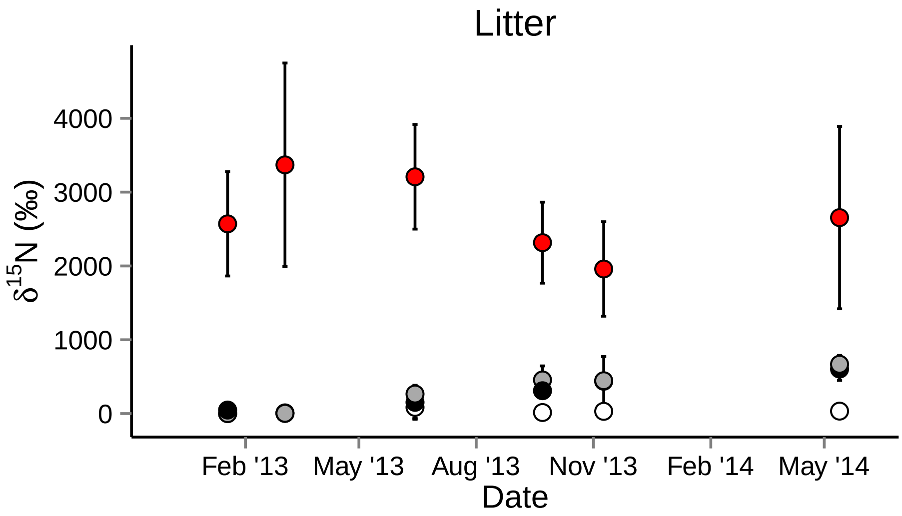

Figure 5.3: ^{15}N content (δ^{15}N ± standard deviation) of the litter layer over time. Treatments are: Water Control (White), ^{15}N$_{Dep}$u (Grey), ^{15}N$_{Dep}$s (Black) ^{15}N$_{Litter}$s (Red).

5.3.3 δ^{15}N Changes Over Time in Roots

δ^{15}N also increased over natural abundance in the roots over time, beyond the values measured in the control, although variance was fairly large, especially in ^{15}N$_{Litter}$s (Figure 5.4). The treatments in the O$_h$ horizon followed a broadly linear increase, reaching maxima of 149.7 ± 29 (s.d.) (^{15}N$_{Litter}$s), 79.7 ± 18 (^{15}N$_{Dep}$s), and 65.9 ± 26 ‰ (^{15}N$_{Dep}$u). The mixed effect model for this treatment had a significant effect of date ($P = 0.036$), treatment ($P < 0.001$) and their interaction ($P < 0.001$) which overall explained 69 % (R^2_m) of the variation (Table 5.4).

In the A$_h$ layer, all treatments were significantly different from the water control but not each other (Table 5.4) over time ($P < 0.001$) and treatment ($P < 0.01$) but there was no significant interaction between date and treatment in the most parsimonious model. R^2_m for this was lower, explaining only 25 % of the variation.

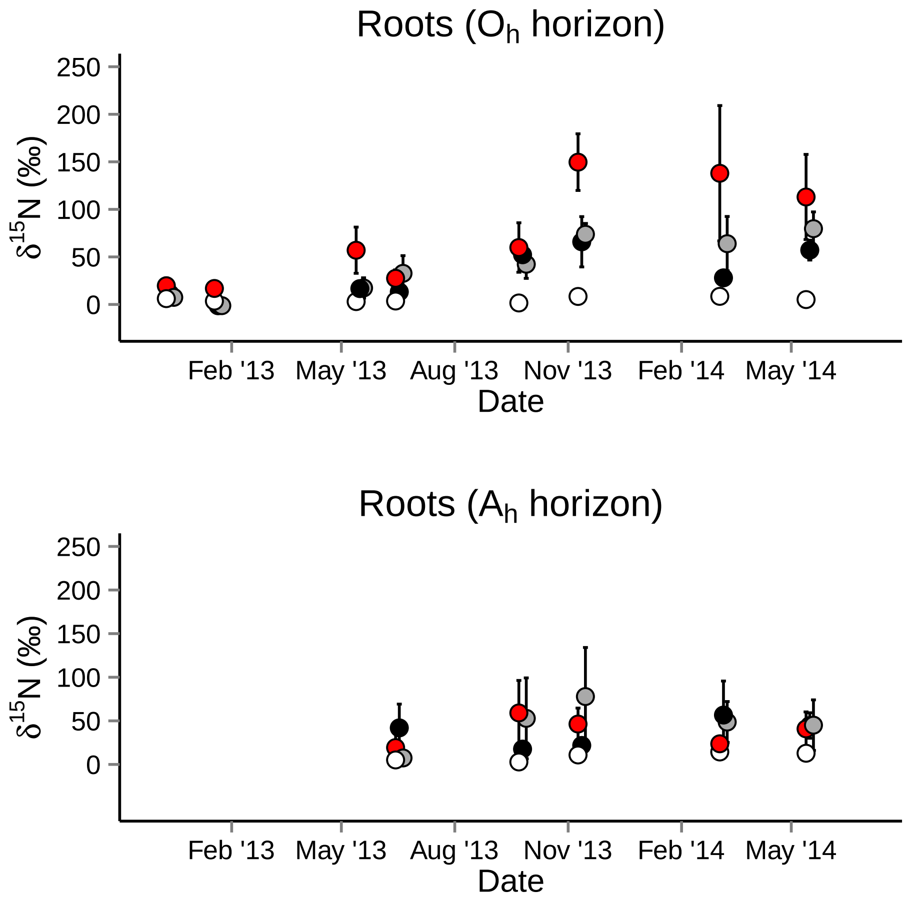

Figure 5.4: ^{15}N content (δ^{15}N ± standard deviation) of roots over time. Treatments are: Water Control (White), ^{15}N$_{Dep}$u (Grey), ^{15}N$_{Dep}$s (Black) ^{15}N$_{Litter}$s (Red). A slight jitter has been applied to the x axis to distinguish treatments.

5.3.4 K_2SO_4 extractable ^{15}N and Microbial ^{15}N Return

In May 2014, there were no significant differences in 0.05 M K_2SO_4 extractable N between the four treatments and two soil layers ($P > 0.05$). The mean N extractable was 0.024 ± 0.03 (s.d.) mg g^{-1} in the O$_h$ horizon, and 0.010 ± 0.01 mg g^{-1} in the A$_h$. δ^{15}N of the extract was significantly greater in all three N$_{DEP}$ treatments (mean = 171.2 ± 40 ‰) than the water control in the O$_h$ horizon (mean 66.3 ± 8 ‰,$P = 0.004$)) but were not significantly different from each other. There were no significant differences in the A$_h$ layer extractable N δ^{15}N.

Table 5.4: Tukey HSD comparisons for treatments in the roots most parsimonious models. Asterisks indicate significance at the $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) level.

O_h HORIZON	$^{15}N_{Dep}u$	$^{15}N_{Dep}s$	$^{15}N_{Litter}s$
Water Control	< 0.001 ***	< 0.001 ***	<0.001 ***
$^{15}N_{Dep}u$		0.977	0.007 **
$^{15}N_{Dep}s$			0.018 *
A_h HORIZON	$^{15}N_{Dep}u$	$^{15}N_{Dep}s$	$^{15}N_{Litter}s$
Water Control	0.033 *	< 0.001 ***	0.015 *
$^{15}N_{Dep}u$		0.581	0.998
$^{15}N_{Dep}s$			0.756

We did not apply a correction factor for microbial nitrogen and the unaltered difference in N extracted between fumigated and unfumigated samples was 0.092 ± 0.06 (s.d.) mg g^{-1} in the O_h horizon and 0.043 ± 0.04 (s.d.) mg g^{-1} in the A_h. δ^{15}N of theO_h microbial biomass was significantly higher in all ^{15}N treatments (mean $\delta^{15}N = 171.8 \pm 140$ ‰) than the water control ($\delta^{15}N = 29.2 \pm 9$, $P = 0.02$) but were not different from each other. There were no significant differences in δ^{15}N of this pool in the A_h horizon, where the control δ^{15}N was 47.4 ± 13 ‰.

5.3.5 Litterbag Mass Loss

Mass loss from our litterbags was almost 50 % of their mass over a year of decomposition (Figure 5.5), which fit a logarithmic curve ($R^2 = 0.92$) but it was noticeable that the initial mass was above this best fit line, indicating a rapid mass loss at the start of the experiment. Over this time, the N content rose from 1.5 to ∼ 2.25 % in all bags, although all measurements were fairly variable due to the small sample size. ^{15}N content (presented here as atom % rather than δ^{15}N to ease comparisons on a log scale) stayed relatively constant in the labelled litter treatment, while the unlabelled litter decomposing in the labelled litter displayed some variance in δ^{15}N over time but did not significantly differ from the control unlabelled litter without a ^{15}N source ($P > 0.05$).

5.3.6 Mass Balance Estimates of Soil System ^{15}N Return

Based on change in litter mass and N content from the litterbags, our labelled litter released 25.5 ± 4 (s.e.) kg N ha^{-1} y^{-1}. At the ^{15}N content of our litter, this was equivalent to a mean N release of 0.604 ± 0.09 g per plot for the ^{15}N-labelled litter over the time of the whole experiment, and comparable to the 0.622 ± 0.03 g ^{15}N added in deposition over the same time period.

The mass balance calculated using these rates of release is presented in Table 5.5. Most of the soil ^{15}N return was in the O_h horizon in which the highest recovery was from the $^{15}N_{Litter}$s treatment ($\sim$ 50 % of ^{15}N release), compared to means of 25-30 % in the ^{15}N-N_{DEP} treatments $^{15}N_{Dep}$s and $^{15}N_{Dep}$u. ^{15}N recovery in the A_h horizon soil was low in all treatments, accounting for 1-3 % of ^{15}N available. Similarly root ^{15}N return was higher in the O_h horizon and greater in the $^{15}N_{Litter}$s treatment, although as this pool was relatively smaller, the high δ^{15}N observed accounted for only about 8 % of the ^{15}N decomposed from litter, or 4-5 % of that added in deposition. ^{15}N return in roots in the A_h horizon was also small, but proportionally larger when compared to O_h horizon at 1-2 %, between all three ^{15}N-enriched treatments.

Only small proportions of the soil ^{15}N return were derived from microbial biomass which accounted for 0.59 ± 0.17 % (se) for $^{15}N_{Dep}$u, 0.96 ± 0.23 % for $^{15}N_{Dep}$s and 1.48 ± 0.48 % for the $^{15}N_{Litter}$s treatment in the O_h horizon. Total microbial expression of this label in the A_h horizon had a mean of 0, similar to the low return in the soil in this horizon, so there was little movement of the ^{15}N label applied this deep in the course of the experiment. As this total amount of extracted N from the soils was low and already included in total soil ^{15}N return, we did not include extractable K_2SO_4 N in our mass balance calculations.

Litter was the biggest sink for ^{15}N additions in the two N_{DEP} treatments. When we used the litter layer masses from litter removed at the start of the experiment and assumed this whole pool was a ^{15}N sink with the measured δ^{15}N (~ 600 ‰), we calculated very high estimates of ^{15}N recovery which in this pool alone was almost 100 % of ^{15}N additions. The litter mass (8000 kg ha^{-1}) was considerably higher than values from other studies in temperate conifer forests (e.g. 1210 kg ha^{-1}, Micks *et al.* (2004), 3800 kg ha^{-1}, Morison *et al.* (2011)), which likely arose from taking parts of the fermentation (O_f) layer along with the L horizon at the start of the experiment. As this seemed unrealistic, we used GGBiosoil conifer plot averages (Morison *et al.*, 2011) and reduced litter mass to ~ 3800 kg ha^{-1}. As a result of these calculations we present in Table 5.5 the litter ^{15}N sink without confidence intervals. From these revised values, we estimated litter assimilated ~ 33 % of our ^{15}N-N_{DEP} treatments. Similar values for litter ^{15}N recovery in litter could not be calculated from the main experiment and we found no significant differences in unlabelled litterbag treatment in labelled litter, indicating no litter-litter transfer. As discussed later, we felt that no litter to litter transfer was unlikely and have omitted this pool from Table 5.5.

In total, we could account for 29.96 ± 5.2 (s.e.) % of the applied ^{15}N in the soil and roots $^{15}N_{Dep}$u (62.72 % of the applied ^{15}N when litter was included), 35.23 ± 6.9 % $^{15}N_{Dep}$s (78.57 % with litter), or 63.94 ± 14.0 % ^{15}N in $^{15}N_{Litter}$s, where an estimate of litter ^{15}N retention was not available.

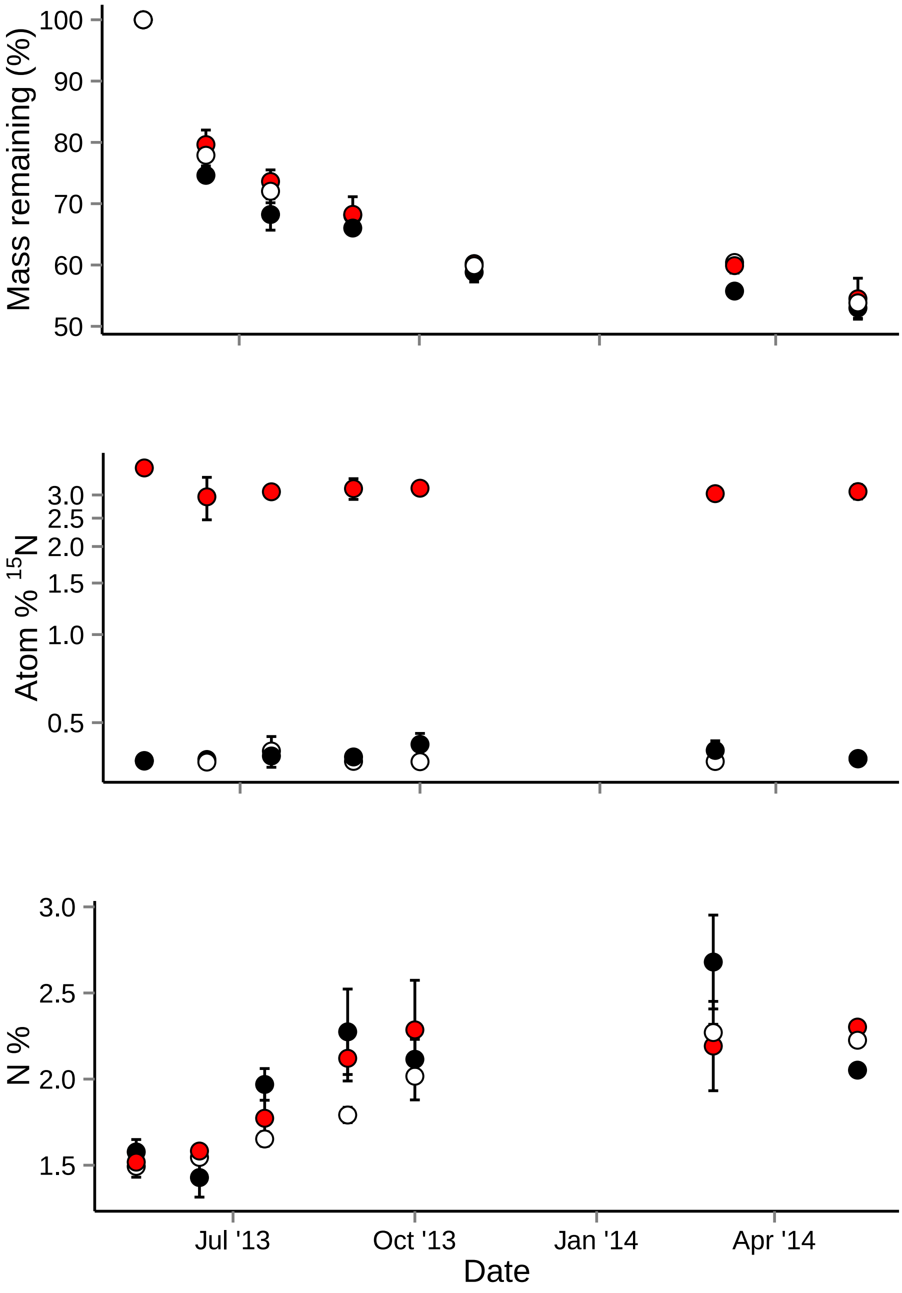

Figure 5.5: Decomposition plots for the litterbag experiment. Figures show (top) mass loss (middle) changes in ^{15}N content, and (bottom) changes in N content over time. Treatments are: Unlabelled litterbag in unlabelled litter (White), unlabelled litterbag in ^{15}N-litter (Black), and ^{15}N-litterbag in unlabelled litter (Red). Error bars show standard deviation.

Table 5.5: ^{15}N Mass balance for the ^{15}N-enriched treatments (May 2014)

	LITTER	O_h HORIZON			A_h HORIZON		
	Litter	Roots	Soil	Microbes	Roots	Soil	Microbes
Mass (kg ha^{-1})	3800_a	8100	61600	5.70_b	7800	174800	7.70_b
		(3000)	(1400)	(1.0)	(2000)	(31000)	(1.1)
N (%)	1.58	0.89	1.35		0.66	0.81	
	(0.3)	(0.1)	0.4		(0.1)	(0.3)	
A1) $^{15}N_{Dep}u$	32.76_a	3.66	23.73	0.59	1.58	0.99	0.13_c
		(1.4)	(5.7)	(0.17)	(0.5)	(0.4)	(0.11)
A2) $^{15}N_{Dep}s$	36.49_a	5.25	32.83	0.96	1.60	2.40	-0.04_c
		(2.1)	(8.5)	(0.23)	(0.6)	(0.5)	(0.07)
B) $^{15}N_{Litter}s$	NA_a	8.00	51.89	1.48	1.32	2.73	-0.10_c
		(3.4)	(13.5)	(0.48)	(0.4)	(0.9)	(0.06)

Table shows mean percent recoveries of ^{15}N tracer, applied either as deposition treatments (A1,A2), or released from the ^{15}N labelled litter (B) by May 2014. Values in parentheses are standard error of the mean. Subscripted values indicate: (a) Estimates are obtained using GGBiosol conifer plot estimates (Morison *et al.*, 2011) rather than measured masses and hence standard errors derived from masses are omitted for this pool. Litter-to-litter ^{15}N retention in $^{15}N_{Litter}s$ was not quantifiable. (b) Microbial N is a proportion of the measured soil pool and should not be included as a separate component of the total. This figure is not adjusted by a correction factor for total microbial biomass and thus N % is also not presented. (c) In some cases the A layer microbial biomass was on average ^{15}N depleted relative to the control, hence a negative accountancy.

5.4 Discussion

In this study, around 50 % of litter mass (from litterbags) was lost over the course of the experiment. When changes in N concentration were taken into account, this indicated a net loss of 20 % N and ^{15}N. We did not use this decomposition when calculating the final litter pools in the main experiment (as we replaced our litter masses with literature estimates), but did assume our $^{15}\text{N}_{Litter}$s litter in the treatment had released this amount of ^{15}N over the whole experiment (0.604 ± 0.09 g per plot), which was comparable to the total ^{15}N added in deposition treatments (0.607 ± 0.03 g). In this discussion we assume that a) ^{15}N return was not an effect of differing magnitudes of ^{15}N availability and b) differences between treatments were due to the different ^{15}N sources rather than litter swap effects. This latter assumption depended on ^{15}N retention not being affected by the different litter provenances in swapped ($^{15}\text{N}_{Litter}$s and $^{15}\text{N}_{Dep}$s) and unswapped ($^{15}\text{N}_{Dep}$u and Water Control) treatments. We therefore address differences in litter decomposition and ^{15}N return before considering the results in detail.

5.4.1 Litter ^{15}N release and retention

Rates of litter decomposition are related to many abiotic and biotic factors (Berg and McClaugherty, 2008) and vary with soil depth (Wang *et al.*, 2013), as decomposer activity changes through the litter profile (Lindahl *et al.*, 2007). The mass loss from our litterbags was similar to the decomposition rate of ^{15}N enriched *Picea sitchensis* litter from a harvest of seedlings in van Huysen *et al.* (2013). We applied these rates to the entire replaced litter pool, including the relatively exposed surface where a great deal of needles were still relatively intact, and deeper portions where litter was dark and humified by the end of the experiment, assuming that this N release was representative of the average of the whole layer. However, as litter quality is an important determinant of decomposition rates (Knorr *et al.*, 2005; Ge *et al.*, 2013), we were concerned that differences between our high-quality swapped litter (which was harvested before senescence) and litter already at the site, as well as effects on decomposition and ^{15}N retention due to the removal and replacement of the litter layer in $^{15}N_{Litter}$s and $^{15}N_{Dep}$s treatments, may have affected ^{15}N release and recovery, a potential effect of litter quality was particularly evident as our litterbags rapidly lost mass during the first month of decomposition, reflecting the early loss of nonstructural C and acid-hydrolisable materials (Berg, 2000; Hobbie *et al.*, 2010). These fractions may have been more abundant in our swapped litter as it was entirely derived from trees harvested before senescence (Chapin, 1980; Chapin *et al.*, 1993), but as we found no significant differences in ^{15}N recovery between the swapped ($^{15}N_{Dep}$s) and unswapped ($^{15}N_{Dep}$u) litter, ^{15}N-deposition treatments in any of the pools measured, this initial difference in litter quality and application disturbance between treatments did not appear to have an effect on ^{15}N return. By definition, it was not possible for a similar unswapped control using labelled litter.

Our estimates of N release from litter also assumed that much of the ^{15}N lost in terms of mass was reincorporated into litter and expressed as the increasing N content. Litter δ^{15}N increased over time under the two ^{15}N-N_{DEP} treatments, indicating a similar incorporation of mineral N from decomposition into the litter as observed in many other studies (Nadelhoffer *et al.*, 1995; Koopmans *et al.*, 1996; Downs *et al.*, 1996). The increase was fairly variable which, as spatial variation in δ^{15}N due to inputs of ^{15}N-depleted litter from the canopy only amounted to a few permil (Weber and Bol, 2008), may have been due to insufficiently even applications of the ^{15}N-deposition treatments or differences in rates of decomposition and colonisation on the microsite scale (Wang *et al.*, 2013) due to local litter layer depth and associated thermal (Ogée and Brunet, 2002) and water retention (Putuhena and Cordery, 1996) properties. Variation in δ^{15}N in the $^{15}N_{Litter}$s treatment was also large, and may have been derived from a heterogenous ^{15}N signal within the canopies harvested (Chapter 3, Nair *et al.* (2014)), despite our mixing of the litter.

There was also considerable heterogeneity in litter δ^{15}N in the unlabelled litterbag treatment decomposing within a ^{15}N-labelled litter layer which we intended to use to quantify litter to litter N transfer but overall, there were no statistical differences in ^{15}N between this and an unlabelled control. Leaf-to-leaf (Schimel and Hättenschwiler, 2007) and litter-to-litter exchanges of N(Berglund *et al.*, 2013) can be demonstrated in ^{15}N tracer studies, but this may require direct physical contact (unlike our mesh litterbags) between different sources and is most easily performed in litter mixes where distinct components can be identified (e.g. Berglund *et al.*, 2013). We have assumed this statistically indistinct ^{15}N return in litter from litter was an artefact of the experimental design and as δ^{15}N in the labelled litter treatments in both the litterbag experiment and from our main plots did not decrease over time, a similar proportion of the N released from labelled litter was reincorporated locally into the litter layer, resulting in the net 20 % N loss we used to calculate ^{15}N release.

5.4.2 Overall differences in ^{15}N return in the Forest Floor

If there were differences in soil depths, we expected that these would be a higher ^{15}N return in the O$_h$ horizon as ^{15}N could be assimilated in these sinks before reaching the A$_h$ horizon. While some ^{15}N could move lower in the soil due to progressive pool turnover and infiltration of this N lower into the soil horizons (as observed in long-term trace experiments, such as Eickenscheidt and Brumme, 2012), we continued to add ^{15}N over time to the forest floor, continually renewing availability of ^{15}N to the pools closest to the soil surface. In the A$_h$ horizon, microbial ^{15}N return accounted for none of the applied ^{15}N (return in this horizon was often slightly negative and had a mean of 0 %), otherwise soil and root ^{15}N return were low (1-3 %), and in terms of δ^{15}N changes over time, no significant changes in N content were observed between any treatments in the A_h soil. In other studies small changes (a few ‰) in lower regions of organic-dominated soil have been found over several years (e.g. Nadelhoffer *et al.*, 1999a, 2.9 % of ^{15}N in 0-10 cm mineral soil, and 1.1 % in 10-20 cm depth), although some returns in this layer can be higher (*c.f.* Tietema *et al.*, 1998, 20-30 %), and may reflect differential sink strength in the upper layers of the forest floor or differential rates of turnover of N pools. Across the ^{15}N-tracer studies used in Nadelhoffer *et al.* (1999c)'s meta-analysis, taking in 18 sites over two years, only in two experiments were higher ^{15}N returns found in A rather than O soil layers.

Overall, our ^{15}N recovered from the forest floor was concentrated in litter and O_h soil; the $\sim$ 35 % of ^{15}N additions found in litter were the largest overrall sink in the soil system for ^{15}N-N_{DEP} treatments, and larger than ^{15}N return found in studies at low levels of N_{DEP} for example, 9% (Micks *et al.*, 2004), or 20% (Koopmans *et al.*, 1996), while comparable to ^{15}N recovery at higher deposition magnitudes ^{15}N (e.g. Downs *et al.*, 1996, $\sim$ 50 %). The ^{15}N return in the O_h horizon agreed with the 20-50 % estimates in the literature for 'low deposition' experiments (e.g. Tietema and Emmett, 1998; Nadelhoffer *et al.*, 1999b), while microbes contained little of this O_h layer N in our study, some 2-3 % of O_h soil ^{15}N in all 3 treatments. When ^{15}N is applied in an enriched pulse, microbes typically assimilate 30-50 % of ^{15}N-NH_4NO_3 in the short term (Jackson *et al.*, 1989; Zak *et al.*, 1990; Zogg *et al.*, 2000; Morier *et al.*, 2012) but this rapidly falls off over the longer term (Zogg *et al.*, 2000; Providoli *et al.*, 2006; Templer *et al.*, 2012) as these pools turn over quickly, and N released can be re-immobilised, incorporated into other sinks, such as organic matter (Hatton *et al.*, 2012) or obtained by fungal hyphae and moved into other decomposing biomass (Hart *et al.*, 1993). Over time, the sinks with the longest turnover time should incorporate more ^{15}N at the expense of microbes, even though the latter may be better short-term competitors. There were no significant differences in the microbial biomass ^{15}N content, but we did not apply a correction factor for extraction efficiency as there is little literature information for appropriate values for forest soils at 0.05 M K_2SO_4. Applying a similar 0.54 K_{EN} as in Brookes and Landman (1985) would indicate microbial ^{15}N return almost two times larger and suggest a larger absolute difference between treatments. In the $^{15}N_{Litter}$s treatment, which had the highest ^{15}N recovery, relatively more freshly mineralised ^{15}N may have been available shortly before sampling progressively decomposing litter compared to infrequent ^{15}N applications as litter decomposition occurred continuously up to the point of final sampling while the last mineral additions were more than a month prior.

Our root masses were larger than similar pools in other studies (c.f Nadelhoffer *et al.*, 1999b; Templer *et al.*, 2005) as we included all coarse and fine roots in the cores in our measurements, which may explain a slightly higher total ^{15}N return in this pool across all treatments of this study than found elsewhere. The differences between O_h and A_h horizons were less pronounced than the soil, as roots returned 4 - 8 % ^{15}N in the O_h horizon, and 1-2 % in the A_h. Root ^{15}N return is not always stratified between soil layers (e.g Zeller and Colin-Belgrand, 2001; Templer *et al.*, 2005), but is typically a fraction of soil ^{15}N as other soil sinks are stronger competitors for N (Nadelhoffer *et al.*, 1999c) and much of the ^{15}N acquired by roots is incorporated into biomass in the above-ground portion of the tree. Movement of root-acquired ^{15}N within the tree may have been observed in our experiment, as in the O_h horizon root ^{15}N return was consistently $\sim$ 15 % of the soil ^{15}N return, while the relative fraction of ^{15}N return was much greater (50-160 % of soil ^{15}N return) in the A_h horizon in all treatments. As apart from this root signal, A_h horizon ^{15}N return was very low, the relatively high root expression in the A_h horizon may be the result of movement of N within the plant with N assimilated in the ^{15}N-abundant O_h horizon subsequently expressed elsewhere. However to assess the potential for this overall effect on N nutrition, we also need to consider potential losses of ^{15}N to soil water and gaseous pools between the treatments.

5.4.3 Possible ^{15}N losses and Differences in ^{15}N Retention in the Soil

The results of this study suggest that over 15 months, a greater amount of litter nitrogen than deposition nitrogen was retained in the upper portions of the soil but the relative partitioning of this nitrogen only changed in roots between the two soil horizons. Total ^{15}N return (as a percentage of total N availability) was 60-80 % in the soil N_{DEP} and was probably higher in the labelled litter, where we were not able to successfully measure litter ^{15}N retention but recovered 64 % even without this pool. In the soils and roots, where we had measurements for ^{15}N recovery in all treatments, total ^{15}N returns were in total 50 to 100 % greater in the $^{15}N_{Litter}$s treatment than the labelled N_{DEP} treatment ($^{15}N_{Dep}$s and $^{15}N_{Dep}$u), with this difference primarily located within the soil pools. These differences may have been due to differences in ^{15}N losses between the treatments resulting in an overall greater ^{15}N availability for soil sinks.

Studies which quantify ^{15}N losses via leachate at ambient low deposition levels find $<$ 10 % loss in this pathway (Tietema and Emmett, 1998; Zak *et al.*, 2004; Providoli *et al.*, 2005), and ^{15}N losses as gases (such as NO_x) from N_{DEP} are rarely quantified (Templer *et al.*, 2012) but likely to be low (Tietema and Emmett, 1998; Christenson *et al.*, 2002). Assuming overall 10 % losses from leaching and gas (c.f. Nadelhoffer *et al.*, 1999c), the two N_{DEP} treatments accounted for a total of $\sim$ 70-80 % of ^{15}N, without including the above-ground ^{15}N sink in plant woody and foliar tissues, consistent with meta-analyses of ^{15}N tracer studies (Nadelhoffer *et al.*, 1999c; Templer *et al.*, 2012) without an above-ground ^{15}N recovery component. However, if similar retentions in the labelled litter treatment are assumed to have occurred in the litter as the other treatments, and similar proportions of N obtained by trees as in the literature, this treatment accounts for most of the ^{15}N without losses. Eickenscheidt and Brumme (2012) found around 1 % of ^{15}N from labelled Beech litter to have been lost as N_2O over 10 years, but leachate ^{15}N has not been measured in other labelled litter studies (Zeller and Colin-Belgrand, 2001; Blumfield and Xu, 2004; Weatherall *et al.*, 2006b).

N released from litter is available in multiple forms from proteins to mineral ions unlike our applied ^{15}N-N$_{DEP}$, which was only NH_4NO_3. Larger N-containing organic molecules may be not as vulnearable to gas loses via nitrification, nor as mobile in soil as mineral ions, particularly NO_3^- (Butterbach-Bahl *et al.*, 2011). A wide variety of forms of organic N are detectable in the soil from ^{15}N-NMR spectroscopy, including c. 40 % of this N as proteins (Schulten and Schnitzer, 1997). Different forms of ^{15}N retention from labelled litter is reported in other studies (e.g. Hatton *et al.*, 2012), while in this study we were only measuring bulk ^{15}N recovery in homogenised soil and were not able to separate different forms of ^{15}N retention and organic ^{15}N availability. Comparisons of amino acid ^{15}N recovery in soils do not find a difference in soil ^{15}N retention (unlike plant ^{15}N uptake) when compared to mineral ^{15}N additions (Näsholm *et al.*, 1998; McFarland *et al.*, 2010), but we could find no studies in the literature investigating direct retention of additions of larger ^{15}N-labelled polymers in the field, nor in other monomer products litter-derived polymer cleavage, such as amino sugars or nucleic acids, which are both obtainable by plants but may also be released from litter and make up a large proportion of soil N (Schulten and Schnitzer, 1997). The monthly incidence of excess N$_{DEP}$ events in our study may also have meant that more of the N$_{DEP}$-^{15}N than decomposition-derived ^{15}N was lost from the system due the slow, constant release of N from mineralization being acquired by the most competitive soil sinks. We could identify no studies where mineral N$_{DEP}$ addition rates were explicitly compared to quantify whether differences between chronic N$_{DEP}$ or spikes of application delivering similar magnitudes over time could effect ^{15}N recovery as more competitive sinks become saturated, but as we applied ^{15}N in N$_{DEP}$ in a very dilute dose, consider effects of this type unlikely in our study.

5.4.4 Inferences of Plant ^{15}N Uptake from Differences in Total ^{15}N Return

^{15}N tracer from polymers such as peptides and proteins are known to be taken up by mycorrhizal plants (Bajwa and Read, 1985; Abuzinadah *et al.*, 1986; Kerley *et al.*, 1995; Paungfoo-Lonhienne *et al.*, 2008), but these studies are not performed in the field, and it is difficult to separate the fate of these molecules using ^{15}N rather than products of breakdown of complex polymers under realistic field conditions where an abundance of different forms of ^{15}N are available. Tracer techniques are not able to conclusively identify the form of uptake from a simple ^{15}N pool mass balance, as ^{15}N tracers cannot distinguish between ^{15}N-labels from organic forms, and their mineralization products. In this experiment, relatively more total ^{15}N (9.32 ± 3.4 (s.e.) %) was found overall in the roots under the ^{15}N$_{Litter}$s treatment than under the labelled deposition treatments (5.24 ± 1.5 and 6.85 ± 2.2). It was unclear whether this difference was due to a preferential uptake of litter-derived N by the roots, or a different total ^{15}N availability in root-available pools to ^{15}N-N$_{DEP}$ additions as, while in absolute terms more ^{15}N was expressed in roots in the ^{15}N$_{Litter}$s plots, ^{15}N return in roots in the O$_h$ horizon in each treatment was a similar proportion of the total ^{15}N recovery in the same soil layer. As above-ground ^{15}N return, ^{15}N losses, and ^{15}N content of roots leading out of the plots were not measured in this study and the direct comparison of absolute ^{15}N content of the roots between treatments is difficult, we are cautious in assigning the missing N from our overall mass balance to above-ground N uptake. We note, however, that the previously mentioned differences in ^{15}N return in roots occupying different soil horizons (in section 5.4.2) may also reflect a difference in whole-plant ^{15}N acquisition. As the difference in total ^{15}N return between roots and soil was much smaller in the A$_h$ horizon than the O$_h$, it appeared from our ^{15}N return that ^{15}N from the O$_h$ horizon was being assigned to roots deeper in the soil. Where measured, root ^{15}N from labelled litter is generally less than above-ground ^{15}N recovery (Zeller and Colin-Belgrand, 2001; Weatherall, 2005). We suggest that this differential recovery between labelled litter and labelled deposition may reflect similar differences between aerial biomass pools, a different effect of litter and deposition-derived N on whole-tree nutrition and thus differences in total tree $\Delta C/\Delta N$ effect between these sources.

5.5 Conclusion

The fate of ^{15}N from organic sources in the soil is poorly understood, but in this study, with N_{DEP} at close to ambient levels, it appeared that litter-derived ^{15}N was better retained in soil and roots than N_{DEP}-^{15}N of a similar magnitude. The most important implication of this difference in contribution of total nitrogen to plant nutrition between litter and N_{DEP} sources is that mineral N availability from N_{DEP} and N release from litter as a result of associated changes in decomposer community structure (Frey *et al.*, 2004), litter C/N ratios (McNulty *et al.*, 1991) and ultimately, decomposition rates (Knorr *et al.*, 2005)) are not simply additive (N_{DEP} plus recycled N), as different amounts of ^{15}N released from litter may be obtained by soils, roots and microbial biomass. As C/N ratio varies between these pools, this differential partitioning of litter-derived nitrogen would affect overall estimates of $\Delta C/\Delta N$. From the higher root return in this study, high C/N trees may be more responsive to increases in availability of litter-recycled N than low C/N soils. However, our study was limited to a single site and small sample sizes, and better estimates of the difference in ^{15}N retention could be obtained with more available ^{15}N enriched material (allowing greater replication), a full destructive removal of litter at the end of the experiment (allowing a more accurate ^{15}N mass budget to be produced), and measurement losses of ^{15}N via trace gas emissions. Currently, we refrain from making numerical estimates to an overall importance of litter-derived ^{15}N to an overall $\Delta C/\Delta N$ effect (this is covered further in the thesis discussion, section 6.6.2), but ^{15}N recoveries in this experiment suggest that the fates of decomposition N and deposition N in ecosystems are not the same, and long-term changes in litter N release may explain some of the difference between estimates of an overall $\Delta C/\Delta N$ effect between different studies.

Chapter 6

Discussion

This thesis has described three field experiments which were designed to test some commonly held assumptions in isotope tracer experiments simulating nitrogen deposition in forests.

Chapter 3 did not address this problem directly but provided a large source of ^{15}N-distinct litter for use in later experiments, as well as providing insight into distribution of N within the canopy in large trees. **Chapter 4** was a small model system experiment where we attempted to simulate in a controlled fashion two different scenarios of nitrogen deposition - the common ^{15}N-labelled N_{DEP} trace applied to soil (c.f. Nadelhoffer *et al.*, 1999c), and an alternative where the entire N_{DEP} load was applied to the canopy. We used ^{15}N-labelled litter in this experiment to try and separate the fate of N from this natural recycling from uptakes of mineral forms but found no significant differences in ^{15}N return. **Chapter 5** continued the second thread of the potted, mesocosm experiment and focused on the soil system, contrasting nitrogen deposition applied to the soil, with nitrogen derived from the litter in a mature commercial conifer stand. In contrast to Chapter 4, a strong and persistent ^{15}N release was observed from the litter via a high ^{15}N return in root and soil compartments which was not observed in the nursery experiment.

In this chapter I discuss a synthesis of the main research output of this thesis, and the implications of different methods of ^{15}N traces and ^{15}N sources to estimates of forest ΔC/ΔN in relation to the overall literature.

This concerns our inferences that i) a 2 to 3 times greater N assignment to wood was made under canopy N_{DEP} treatments compared to soil N_{DEP}, and ii) a greater return of ^{15}N estimated to be released from litter was returned in soil horizons than N_{DEP} tracers. We observed both of these results using established methods, but included a direct control in our experiment which allowed this comparison previously not available in the literature. Both of these may feed back into the overall forest ΔC/ΔN effect of nitrogen deposition, if found on a larger scale, but there are many potential conditions when inferring the relevance of this result. Here, I will address these limitations and uncertainties, which I follow with a consideration of the overall inferences of ΔC/ΔN from the experiments described in this thesis in Section 6.6, taking into account these concerns and synthesising the overall output of the research chapters to explain their contribution to understanding of the effects of anthropogenic nitrogen deposition on forest growth.

6.1 Why was a Litter-derived ^{15}N Signal so Different Between Experiments?

Kawaletz *et al.* (2014) suggest linking pot and field studies as a method to improve estimates gained from field studies. In the experiments in Chapters 4 and 5 we attempted this by only performing the technically limiting canopy fertilization in the nursery but applying similar soil N_{DEP} treatments in both studies. In the latter chapter, while only the belowground system was available for study, it remained in stratified soil horizons and under field conditions, offering greater comparability to the real world than the nursery study. A comparison of matched treatments is provided in Table 6.1.

Table 6.1: Comparison of treatments between Chapters 4 and 5.

Nursery Experiment (Chapter 4)	Field Experiment (Chapter 5)
NA_{litter}-Water	Water Control
NA_{litter}-$^{15}N_{soil}$	$^{15}N_{Dep}$s
NA_{litter}-$^{15}N_{canopy}$	-
$^{15}N_{soil}$-Water	-
$^{15}N_{soil}$-NA_{soil}	$^{15}N_{Litter}$s
$^{15}N_{soil}$-NA_{canopy}	-
-	$^{15}N_{Dep}$u

It is noticeable that while there was a strong recovery of the ^{15}N-label in the soil of the field experiment in Chapter 5 (exceeding return of ^{15}N added in deposition treatments), there was no corresponding evidence of a clear distinction between ^{15}N-litter treatments and the water control in the nursery experiment of Chapter 4. In this first section I will discuss possible explanations for this discrepancy, focusing on differences in decomposition rate, litter layer mass, soil types and methodological differences in litter preparation.

6.1.1 Estimates of Decomposition Rates

Both the nursery and field experiment experienced problems relating to litter mass, although these problems were not the same between the two experiments.

Our estimated rates of decomposition in the nursery (based on the litter bag mass loss in Section 5.3.5) were clearly overestimates when compared to the mass remaining in this layer when separated and dried, while in the field experiment basing the litter mass in the unswapped plots (which was not recovered) at the end of the experiment on the mass of litter removed from the swapped plots at the start of the experiment appeared unrealistic when compared to the literature as this generated an implausibly strong litter sink (< 100 % of ^{15}N when the δ^{15}N observed was applied across the entire expected mass.

In both cases we decided to make a conservative assumption in terms of a overall ^{15}N release/retention and based litter N release in Chapter 4 on values which fit the variable mass return from litter at the end of the experiment (a 5 % N release Titus and Malcolm, 1999; van Huysen *et al.*, 2013) and in Chapter 5 on a reasonable UK average mass for similar plots (Morison *et al.*, 2011). These had opposite effects on the total ^{15}N return calculated; adjusting the plot litter mass reduced the total N pool which a measured δ^{15}N expression was applied to in the field, while the estimated total ^{15}N available from the litter was decreased in the nursery experiment by reducing the estimated rate of release, so deviations from the control therefore produced a greater estimated ^{15}N retention in the labelled litter treatments (using this reduced rate N release did not affect the amount of ^{15}N applied in the deposition treatments). However, as litter ^{15}N return was so low and inconsistent this had no overall effect on the statistical significance of the return observed. Using literature masses may have generated a source of bias in the field experiment rather than relying on experimental measurements but as this brought estimates of total ^{15}N return from litter to within possible values given that there were no additional ^{15}N sources available it was considered preferable than the impossible litter sink which would otherwise have been calculated. Despite these amendments, a strong ^{15}N return from litter was observed in the field study, while no significant trend in ^{15}N was found in the pots.

Some technical constraints from conclusions drawn from pot studies are discussed further in section 6.2.1, and the effects of sample sizes on possibilities of type I and type II errors are further discussed in section 6.2.2. For now, it is assumed that the different ^{15}N responses were not due to inherent weaknesses of one method of study over another, nor insufficient replication, and that explanations for this difference can arise from inherent methodological differences despite the design which aimed for comparability between both studies.

In the field experiment, net N release from litter was assumed to be equivalent to litterbag N loss and have a similar ^{15}N content to the decomposing litter, while in the nursery experiment, this N loss was calculated using values derived from the literature. Here, the litter had a significantly lower average ^{15}N content, but due to differences in masses of litter applied to each experiment, the total available ^{15}N was similar (Table 6.2). If litterbag values from Chapter 5 had been used in the nursery experiment, total N release would have been calculated to be larger, but as the litter ^{15}N return was otherwise non-significant no changes would be made to the conclusions. If literature values for decomposition rate had been applied to the field experiment this would have reduced total N release predicted and raised observed percentage ^{15}N recovery based on observed ^{15}N abundance.

The implications of the assumptions which governed these estimated N losses are covered in the relevant chapters and are not discussed here beyond noting that using measured values would increase the dissimilarity between observed ^{15}N return. While there is a difference of approximately 2:1 in total ^{15}N availability in favour of the field experiment this is both insufficient to explain the low and irregular ^{15}N return in the nursery soil compartments (in the spring and summer 2013 a degree of highly variable enrichment was seen among the ^{15}N-litter treatments in this experiment, suggesting some degree of uptake, albeit an unsustained and heterogenous expression), and subject to a high degree of uncertainty. Therefore we also consider three further important distinctions between the experiments:

Table 6.2: Comparison of N availability between the two ^{15}N-labelled litter experiments. Litter was relatively more enriched in Chapter 5 but overall ^{15}N availability was similar between experiments when we applied the decomposition rates fitting changes in litter mass observed at each site.

Experiment	Nursery Experiment Chapter 4	Field Experiment Chapter 5
Mass (kg m^{-2})	4.42	1.39
N % Litter	1.15	1.05
Atom % ^{15}N	0.94	1.82
Relative Total ^{15}N in Litter	~ 2	~ 1
N release (% Total N)	5	~ 20
Relative Available ^{15}N	1	2

differences in litter density and associated thermal and hydrological properties, differences in litter type, and differences in litter provenance.

6.1.2 Litter Mass and Isotope Retention and Losses

The nursery experiment had over three times as much litter as the field experiment per area (Table 6.2). In the former, all the available litter at the time was used, as we recognised that we may have had differences in detecting the labelled ^{15}N release in some pools due to the lower than ideal mean ^{15}N atom % of the litter. In the latter experiment we spread the litter as broadly as possible between four plots which were at the minimum size we were comfortable with in relation to the structure of the plantation (a three by three tree grid with a single central tree). As ^{15}N abundance in the litter for this experiment was greater, the lower mass applied per area was less of a concern, but this difference may have resulted in differences in the thermal and hydrological properties of the litter layer as it was considerably thicker in the nursery than at the field site.

Differences in litter depth and their impact on estimates of litter layer ^{15}N release were mentioned in section 5.4 in relation to possible variation in N incorporation and decay rates within the litter layer. These same factors (Lindahl *et al.*, 2007; Wang *et al.*, 2013) may have caused a difference in rates of decomposition and N release between treatments, affecting the total amount of N decomposed in the thicker litter layer in the nursery, or been compounded by the pot-bound status of these individuals. Litter intercepts throughfall (Putuhena and Cordery, 1996), but retains heat and reduces evapotranspiration from the soil (Kelliher *et al.*, 1986; Schaap *et al.*, 1997). Changes in litter thickness may also affect soil temperatures (Ogée and Brunet, 2002), but despite the thermal properties of a thicker litter layer, the soil system in the nursery experiment was much more exposed than in the field. Pots in nursery experiments are sometimes buried, which may reduce heat fluctuations in the soil system, while exposure and thermal capacity of dark pots may caused differences in soil temperatures (Passioura, 2006). Soil microbes are known to respond to temperature (e.g. Zogg *et al.*, 1997) and numerous temperature effects are also observed in roots (Pregitzer *et al.*, 2000). These may affect overall ^{15}N retention in roots and soil and by implication also affect N losses such as losses from soil water and trace gas emissions if sink strength into biotic pools differs. Emissions of N-containing trace gasses such as N_2O (Smith *et al.*, 1998) are affected by soil water content and would have changed if retention of water increased (Sato *et al.*, 2004) in this layer, but overall little information is available regarding the drainage and water storage capacity in litters in general (Guevara-Escobar, 2007) and emissions as N_2O were probably low (from ^{15}N-labelled beech litter, Eickenscheidt and Brumme (2012) calculated less than 1 % total ^{15}N loss as nitrous oxide over a decade). The potted experiment may have generally been more open to ^{15}N losses as it was homogenised, lacking distinct soil layers at the start of the experiment and may also have experienced differences in soil N cycling due to the disruption to networks of mycorrhiza fungi within the soil system; in the field experiment these were largely undisturbed but the separation of small volumes of highly disturbed forest soil in the pots may have meant that these species were not present or at low abundance. Thus, overall it is possible that the lack of a ^{15}N-return from the labelled litter in the nursery was simply due to the substrate structure of this soil system, although Weatherall *et al.* (2006a) observed a ^{15}N return on similar *Picea sitchensis* seedlings grown on sand.

In addition to the possible artefacts of soil and litter layer preparation in the pots, it is also possible the thicker litter layer may have been more able to retain more ^{15}N released from decomposition as much of this denser litter was more spatially separate from pools within the soil and lower litter. We aimed to quantify this in the litterbag experiment in chapter 5 but did not measure any notable ^{15}N return in the relevant treatment. The reasons for this are discussed in section 5.4 and may be an artefact of the experimental design. We were not able to directly measure this in the nursery.

A final difference which may have affected ^{15}N retention and losses in the soil system was differences in C inputs from above ground. Soil respiration is driven by C inputs from recent photosynthesis (Högberg *et al.*, 2001) which may have differed if the ratio of above-ground C fixation to soil volume differed between trees. Differences in C inputs to the soil may have affected differences in rhizosphere activity and thus overall rates of decomposition, which may have been higher at the field site due the closed canopy of the forest being able to assimilate most of the incoming solar radiation, compared to the sparser branches and lower Leaf Area Index expected at the nursery site (Turner *et al.*, 2000; Tobin *et al.*, 2006).

As soil water and gaseous N losses, litter ^{15}N retention and C inputs to the soil were not measured in either experiment, and literature is limited on these subjects, it is difficult to fully assess how important these factors may have been in reducing

^{15}N return in the potted treatment. While speculative, these potential impacts on N release and return are summarised in table 6.3. As demonstrated, effects are multidirectional and thus we can make no clear inference about how these effects of litter decomposition may have affected ^{15}N return.

Table 6.3: Summary of potential unmeasured effects on decomposition rate and ^{15}N losses to unmeasured pools which may have affected availability for litter-derived ^{15}N to root and soil pools in the nursery experiment. Directional arrows indicate probable increase, or decrease of ^{15}N availability. Arrows in the column titles indicate expected increase or decrease in the Nursery Experiment (Chapter 4), relative to the field (Chapter 5)

	↑ Depth	↑ Exposure	↓ Rhizosphere Priming
Decomposition Rate	↑	↓	↓
Litter N incorporation	↑	?	?
N losses (litter)	↑	↑	?
N losses (soil)	↓ ?	↑ ?	?

6.1.3 Soil Types

A difference between the experiments may also have occurred in soils used in the pots when compared to the field site. As well as being homogenised and lacking distinct soil layers at the start of the experiment, the potted soil was derived from a different site (Griffin Forest) than the field experiment (Cloich Forest), due to logistical problems within the project. This may have played a part in ^{15}N retention (or indeed, other differences in treatment responses between the experiments) as the soil from Cardrona is a mineral stagnohumic gley (Clement, 2004), while at the field site used for Chapter 5 the soil is of much more organic shallow peat (Sheppard *et al.*, 1995). While the Griffin Forest soil has an organic topsoil, which was likely homogenised with some mineral-derived soil from lower horizons and was also diluted with a low N/P/K compost (10 %) when preparing the soil for the pots. Various studies applying ^{15}N labels have found differences in retention in the soil system on different soil types (e.g. Müller, 1988; Clough *et al.*, 1998) and although soil type differences are most reported in direct comparisons in agricultural systems, it seems reasonable to assume that this may have been a factor affecting ^{15}N return between the two experiments.

6.1.4 Labelled-Litter Preparation Effects

We replaced the litter in both of our soil system experiments with prepared artificial litter, created by drying live leaves rather than harvested from litterfall, which is made up largely of leaves which have undergone senescence. Amino acids and other compounds are recycled from leaves during senescence, conserving N and other sparse nutrients within the tree (Hörtensteiner and Feller, 2002), and may have remained in our artificially created litter substitute. At the start of the field experiment (Figure 5.1, top panel), N content of the ^{15}N litter was 1-2 times higher than N content of the litter layer, and, while this difference rapidly decreased, this treatment remained most N enriched for the entire study. This higher N content likely reflected N remaining in structures which would otherwise have been digested and remobilised during senescence. If these differed in recalcitrance compared to natural litter (more easily broken down compounds may be more likely to be mobilised), this artificial litter may have released more ^{15}N early in decomposition compared to natural litters, and possibly contributed to short term peaks in ^{15}N contents (e.g. Figure 5.2, O_h horizon) early in the experiment. Evidence for such an effect is, however, limited, and litter N content behaved similarly in all treatments aside from this early difference (Figure 5.1). Other differences between real and prepared artificial litter layers may have been related to water content or colonisation by soil communities before the experiment started, which we controlled for in Chapter 5 by subjecting one of the unswapped litter treatments ($^{15}N_{Dep}$s) to oven drying treatments similar to the labelled litter to reduce its water content and attempt to sterilise any pre-existing decomposer communities.

It is also worth considering potential differences between the experiments due to provenance of the litters used to supply the ^{15}N label. Both of these were derived from Sitka Spruce stem injection experiments in forests other than the sites at which the litter was used (see Churchland *et al.* (2012) and Chapter 3), and differed slightly in nutrient content (Table 6.2), but were processed in similar manners and stored dry for a period before application. As well as this, neither of the applicatons were of litter produced by senescence, rather harvests of live leaves prepared to allow the ^{15}N label trace.

We cannot make any reasonable inferences about differences in site of origin but did not expect there to be any major differences in decomposability of needles between the two sites as these were from similar stands, had fairly similar N content, and were both from adult trees considerably older than the 6-8 year lifetime of *Picea sitchensis* needles (Norman and Jarvis, 1974), thus with a reasonably comparable range of needle ages available in a whole canopy harvest. However, as due to differences in plot size, we applied mixes of litter to the field plots and split the litter from a single tree between multiple pots in the nursery, it could be expected that differences in decomposition rates or ^{15}N retention in the litter could have arisen due to differences in litter mixes; from species mixtures in the literature it appears that a non-additive enhancement of total decomposition rates often occurs (Gartner and Cardon, 2004). However this trend appears to be reversed in single-species mixtures of different N content (Smith and Bradford, 2003) which decompose slower than single-quality litters. As this is the only study addressing variation in litter mixes within, rather than between species, and studies only grasses, there is no information if variation between individuals, as is possible when litter is derived from whole trees, may affect rates of decomposition. Literature rates between same-species litter are both only 10-30 % (Smith and Bradford, 2003) and in the opposite direction to that which we would expect if mixed source litter produced higher ^{15}N release in the field. The potential difference between mixed and unmixed litter is therefore unlikely to fully explain the lack of a discernible difference in ^{15}N-litter in the nursery.

Finally, it is also worth considering if injection experiments which produced the litter itself could have influenced release of ^{15}N between studies. Both Churchland *et al.* (2012) and our Chapter 3 method injected trees with a ^{15}N-enriched compound, left them to stand for a similar period of time (5 months in our study, 3 months in Churchland *et al.* (2012), A. Weatherall, pers. comm), over autumn. After felling, trees in both studies were left outside overwinter, and processed in spring. Due the the volume of biomass, much of our material was air, rather than oven-dried (as in the case of the litter for the nursery experiment), but we did not expect any differences in major losses of ^{15}N to occur in the 80° oven treatment to dry the litter. As we recovered $\sim$ 100 % of the injected N the Chapter 3 field experiment we expected there to have been virtually no losses of ^{15}N over the storage and processing period. Unless the injection substrate (aspartic acid in Churchland *et al.* (2012) affected this, we expected this to also be the case in the litter used in Chapter 4, and subsequent N losses during the our experiments were based on a measured N pool at the start of our experiments rather than the total injected N in previous experiments so it was unlikely that ^{15}N losses over the storage period could have affected results.

A greater total amount of ^{15}N was delivered in our injection experiment, but total injected ^{15}N was a relatively small amount of the total tree N pool in both studies (we estimated this at 0.3 to 0.8 % depending on tree size compared to 0.25 to 0.5 % total ^{15}N in Churchland *et al.* (2012), and the the average litter enrichment from our trees was only two times that of the litter derived from Churchland *et al.* (2012) (Table 6.2). In both of these injection experiments the majority of N available to the canopy was therefore N from the background, unlabelled soil N pool or existing N stores within the tree and we did not expect any differences in assignment of the different isotopes of N, beyond minor differences due to fractionation, which we did not expect to be large enough to cause such a major difference in ^{15}N return in the soil pools. Differences in the quantity of delivered ^{15}N for enrichment and individual needle ^{15}N abundance were therefore unlikely to be a determinant on release of this ^{15}N in decomposition in our studies.

A more major difference may be that aspartic acid was used as a ^{15}N injection carrier in Churchland *et al.* (2012), while we used ammonium nitrate. Organic molecules such as amino acids are the main N transport molecules in the xylem (Pate, 1973), and the aspartic acid is a common component of xylem sap (one of the 5 most common amino acids in scots pine), and unlike other molecules, is not expressed in a seasonal cycle (Näsholm and Ericsson, 1990). On the other hand, our NH_4NO_3 is the main mineral forms of N uptake. Literature on stem injection experiments is very sparse (we consider this further in Chapter 3.1) and unfortunately it is difficult to assess the scope to which foliar ^{15}N expression may differ when these two different compounds are used for injection as comparison experiments with different ^{15}N-vectors have not been published. Certainly, the $NO_3{}^-$ group must be reduced before it can be assimilated into biological compounds, which occurs in leaves in some species, and in roots in others (Smirnoff *et al.*, 1984; Black *et al.*, 2002). Once it is assimilated, this may be transported as a different amino molecule than aspartic acid. The ^{15}N label may therefore arrive in the canopy in different forms between these two treatments, which may affect how this ^{15}N is assigned within the needles. If N in this form is incorporated in greater amounts into more recalcitrant ^{15}N components of foliage than mineral NH_4NO_3, this may influence the rate of ^{15}N release from these litters when considered over only one stage of overall decomposition. Weatherall *et al.* (2006b), traced ^{15}N successfully from Sitka Spruce litter to Sitka Spruce trees, and used NH_4NO_3

as the injection compound to prepare enriched litter, like our successful trace. However without more evidence on differences in assignment of these compounds it is difficult to determine whether this may have had an effect on ^{15}N fate in our experiments.

Overall, there is no single factor which alone explains the lack of a ^{15}N return from the litter in the nursery experiment in Chapter 4, when a substantial ^{15}N return was observed in Chapter 5. A combination of several of these may have combined to reduce ^{15}N availability in the Nursery study. Such limitations when compared to field experiments are common in potted studies, and this approach, along with other potentials, is discussed in the next section.

6.2 Upscaling Limitations

Based on the potted sapling experiment we were able to estimate that with the allocation of canopy N_{DEP} to wood we observed, $\Delta C/\Delta N$ estimates may more than double. In Chapter 5, we also found a greater ^{15}N return from a litter-derived ^{15}N tracer than conventional ^{15}N deposition. However, an important issue which immediately arises from such results is how well such studies can scale to regional and global ecosystem responses. Like our work, field $^{15}N_{dep}$ studies are often un-replicated among sites due to high financial and logistical costs, and meta-analyses are particularly important to draw together the work of different authors (e.g. Nadelhoffer *et al.*, 1999c; Templer *et al.*, 2012), whereas unlabelled N_{DEP} experiments are much more likely to be replicated between sites (e.g. Gentilesca *et al.*, 2013).

Approaches to experimental study of ecology can vary to anywhere between ecosystem level manipulations of natural systems to microcosms in greenhouses and growth chambers. Field experiments are most akin to the real world by taking a fraction of a natural ecosystem for manipulations, but generate financial, logistical and analytical constraints due to the scale and complexity of systems studied. On the other hand, while smaller scale nursery or glasshouse experiments are poorer abstractions of the real world, they allow control of biotic and abiotic variation in the field, an isolation of the variables of interest without confounding effects of other heterogeneity, and for problems to be addressed on a manageable scale.

In our two N_{DEP} experiments we attempted to maximise generality, inter-comparability between our studies and applicability of our experiments to wider understanding, by maintaining the same study species and close to ambient levels of N availability between treatments. The nursery experiment had a simpler soil system and was able to receive canopy treatments, while the field experiment was an established plantation with little disturbance. In some cases soil systems in nursery experiments may be created to better mimic the natural environment (e.g. pre-stratifying soil horizons in the pots), but as they are removed from the site of interest these microcosms cannot perfectly model local environmental conditions. Both of these studies were outside and open to local climate heterogeneity, while experiments in Chapter 4 were constrained to pots and contained 3-4 year old *Picea sichensis*, while Chapter 5 was established in a 42 year old *Picea sichensis* plantation forest. While this was only on a single species, and our two experiments were on soils from different sites, this allows our results to be compared and combined with greater certainty, and was also appropriate as *Picea sichensis* is the most important forestry species by area in the UK ($\sim$ 29 % of all forestry, and 49 % of conifers (Smith and Gilbert, 2003)).

Differences in scale were constrained between a desire to work on adult trees which make up the majority of a $\Delta C/\Delta N$ response, but also to be able to apply treatments and representatively sample as required. This necessitated splitting work between a nursery and field experiment, although the number of replicates in both experiments was limited by the availability of labelled litter. It is both expensive and labour-intensive to produce labelled litter on a large scale which both explains the relative paucity of studies using labelled litter in the literature (most ^{15}N-enriched field studies in the literature involve litter created in a single fertilization (Zeller and Colin-Belgrand, 1998)) and the complete litter-swap of a forest catchment or large scale plot is logistically implausible. Even with ~ 100 dry kg of litter available from our ^{15}N-injection experiments we were only able to replace four 4 x 4 m^{-2} plots in Chapter 5. Similarly, we were constrained to a mesocosm experiment to study foliar N_{DEP} treatments as, as well as the high cost of ^{15}N-enriched material for isotope traces, canopy-fertilization treatments require a method to apply ^{15}N to the canopy. This is hugely logistically challenging, and in the literature only a single canopy-level fertilization study has been performed using a ^{15}N-isotope tracer (Dail *et al.* (2009)) and others performed on trees are from pots (e.g Wilson and Tiley, 1998; Ammann *et al.*, 1999) or single branches (Bowden *et al.*, 1989).

In this section I will examine two of the major factors which limit comparability both between our experiments and with the rest of the literature: i) that our $\Delta C/\Delta N$ estimates are obtained from potted studies, and ii) that our sample sizes were fairly small within each experiment.

6.2.1 Limitations of Potted Nursery Studies

Our conclusions about a reasonable strong $\Delta C/\Delta N$ effect due to high wood return of N_{DEP} in our nursery experiment was limited as this was performed on young, pot-bound trees. Pot studies can be useful for woody species, but experimental design must be considered carefully as trees have longer lived tissue and can grow to require larger volumes of soil than contained in pots, while they also often require longer periods of study than herbaceous species due to their longevity and lower growth performance than non-woody species (Kawaletz *et al.*, 2014). Poorter *et al.* (2012) recommend a root size to pot size ratio of < 1 g L^{-1} to avoid deleterious effects on both below- (available space) and above- (limitations imposed by soil nutrients, water, and temperatures) -ground growth in longer experiments. Pot-bound trees also face increased limitations as biomass accumulates, which needs to be balanced with an adjustment period to pot conditions to ensure equal starting conditions for all individuals, and accommodate the potential for mortality during the experiment (Kawaletz *et al.*, 2014). In our nursery experiment, we left the trees for a year before treatments started, and at the end of our experiment, the average root to volume ratio in our pots was ~ 0.6 g L^{-1}, well within the recommended size to avoid effects of a pot-bound root system. Over this time we also suffered a 6-7 % mortality (2 trees) from the entire cohort of 30 treatment trees, which was probably due to aphid-induced defoliation before the experiment began. While these trees appeared reasonably healthy at the start of the experiment and remained in the treatments, studies in nursery environments may be particularly vulnerable to such problems and compound the vulnerability of small trees to such stochastic events.

Transferability in pot studies is also limited by ontagenic shifts between young and old trees (e.g. Weih and Nordh, 2005), and conclusions drawn from young individuals often poorly match with older ones. However, in the case of our nursery experiment, a strong $\Delta\text{C}/\Delta\text{N}$ effect depends on the allocation of N to woody biomass, and as trees age the proportion of high C/N (stem, old branches, coarse roots) to low C/N biomass (foliage, twigs, fine roots) increases (Helmisaari and Makkonen, 2002; Peichl and Arain, 2007) and annual growth tends to increase, so the allocation to wood found in Chapter 4 cannot be due to relatively higher growth rates of wood in seedlings than comparable older trees.

It is also worth noting that in older trees, despite annual greater mass accumulation, the proportion of wood laid down in the annual increment is increasingly smaller due to accumulating size of older pools of biomass (Stephenson *et al.*, 2014). If, as expected, ^{15}N assignment from canopy-level fertilization is primarily to the currently growing pool in short-term ^{15}N fertilization studies and the relative proportion of this pool to the rest of the woody biomass in the tree declines over time, the strong ΔC/ΔN effect we saw would need to be maintained over the entire lifetime of the tree for similarly strong effect on overall C uptake. While this may seem a reasonable assumption, as these older trees make up the majority of C uptake due to their size, confirmation of a high canopy uptake input to N_{DEP} both in the field and in large trees is critical to assessing if this uptake occurs on the scale necessary for a major effect on CO_2 uptake when applied to a whole-ecosystem scale.

6.2.2 Small Sample Sizes

Another major limitation to our studies is the small sample size of our experiments ($n = 5$ per treatment in the potted experiment, $n = 3$ in the field study). These sample sizes reduced the degrees of freedom available for statistical analysis and while this did not invalidate the statistical tests used, did increase the potential for both Type I and Type II errors. Low sample sizes are often used due to logistical limitations (for example, in Chapter 3, our assessment of ^{15}N assignment within 12 stem injected trees was the first time this has been attempted with statistical replication in the literature) or required due to the cost (large-scale ^{15}N-fertilization studies (e.g. Gaige *et al.*, 2007) are often unreplicated on the plot level). In our two ^{15}N fertilization experiments, it was critical to have enough treatments to compare the different sources of ^{15}N we were interested, as well as providing sufficient controls. In both of these we were limited in sample size due to available ^{15}N-enriched material and logistical capacity.

Sample size was less of a problem in Chapter 3 where we were able to inject 12 trees, the limit on this being largely the effort required to maintain multiple stem injections simultaneously and the cost of large amounts of 98 % ^{15}N-enriched ammonium nitrate to achieve our desired high enrichment, which we justified as we needed a high enrichment for further experiments. We took the litter produced in this experiment and applied $\sim$ 1/3 of the 300 kg to our field site (the remaining litter was deployed at another site and is not covered in this thesis). This only allowed us to replace four 4 x 4 m^{-2} plots for the ^{15}N-labelled litter swap treatment, and, as one of these plots was required for burying litter bags (which would entail frequent disturbance), the number of plots for this treatment was limited to three. Similarly, in the potted experiment we felt the logistical challenges and analytical costs for ^{15}N analysis of 30 trees was the limit to what could be practically achieved, limiting sample size across six treatments to n = 5.

This problem of small sample sizes in the two N$_{DEP}$ chapters is apparent in the high heterogeneity at a single time, but was relieved somewhat by the ability to measure many of the pools of interest over a time series. This required incorporating a serial correlation structure into statistical models to account for sequential measurements pseudo-replicated over time. Despite this, we also observed high variation within the sample trees in both experiments, which was often likely due to heterogeneity within the pools of interest (soil (Högberg, 1997) and litter (Weber and Bol, 2008)) δ^{15}N are known to be spatially heterogeneous. Within saplings in Chapter 4, ^{15}N partitioning may also have occurred on levels beyond the age classes measured, such as spatially or laterally within the canopy as apparent in the large trees in Chapter 3. This variation is evident in time series graphs for both of these chapters, but we assumed overall trends to be linear (see section 6.3), and in both experiments had reasonable R^2_m when trends over time were apparent. Nonetheless, a larger sample size would likely have reduced this variation.

6.3 Timescale of N_{DEP} Experiments

Ecological studies where processes can occur over long periods or with pronounced seasonal and interannual variability are often difficult to study within the realities of funding and field logistics (Callahan, 1984). These issues may have been especially true in this project where the limits of a PhD timeframe imposed constraints which meant that the pot system and field experiment were studied for periods of 14 months and 16 months respectively while the overall objectives of this thesis concern issues that occur the entire management cycle of a forest stand. These two traces provide different challenges to projecting the effect of an experiment which has been measured in the short term to make long term predictions and the effect of time can be split into individual, but interacting demands: long-term responses and inter seasonal variability.

6.3.1 Seasonal Changes in N Pools

Seasonal cycles in tree growth (Weinstein *et al.*, 1991) and nutrient translocation between pools (Millard and Grelet, 2010) are well established and discussed in Chapter 3. N content of tissues is constrained by biologically possible C/N ratios but as N uptake and C fixation potential vary through the year, the C/N ratio can change to optimise immediate photosynthesis (Hirose and Werger, 1987) and maximise reserves of N for future growth (Millard and Grelet, 2010) and long-term reproductive success. Plants respond to changing available N and C and by remobilising N-containing compounds from storage pools (Millard and Grelet, 2010), while a similar process occurs to conserve limiting nutrients by withdrawing nutrients from leaves prior to senescence (Chapin *et al.*, 1990), although not all N is removed before shedding and even mobile amino acids such as arginine may remain in senescent foliage of a high N content, which may function as a defence, or removal mechanism under N saturation (Näsholm, 1994).

We discussed the implications of these seasonal patterns on N_{DEP} return in canopy pools earlier in the thesis (Chapter 3.4) and measured trends in N content over more than a year in foliage (Chapter 4.3) and roots (Chapter 5.3). The resolution at which we measured ^{15}N return and needle N content is much higher than as is previously reported, with many field studies looking at, at best, one or two time points 6-21 months (e.g. Nadelhoffer *et al.*, 1995, 1999a; Tietema and Emmett, 1998; Schleppi and Bucher-Wallin, 1999; Dail *et al.*, 2009) after sampling. As resolution in such studies is extremely limited, our high resolution ^{15}N traces to needles, roots, and soil across the two deposition experiments represent a useful addition to scientific detail in this area, showing that ^{15}N responses are rapid, sustained, but also fairly variable at low ^{15}N enrichment, which may necessitate a high resolution sampling for accurate measurements of ^{15}N return, especially at relatively small variation in $\delta^{15}N$. In this study, a strong cyclical trend of N appeared to be occurring in the foliage, consistent with filling and emptying of needle N overwinter, while no overall trend (and high variability) was found in roots. This was consistent with the N storage pool in the needles in Sitka Spruce (Millard and Grelet, 2010). No seasonal trend was apparent in root N content in chapter 5, consistent with the literature that roots are not a N store in this species.

In *Picea sitchensis*, woody N pools are a sequestration, rather than storage pool for nitrogen, while other species, particularly deciduous broadleaves, store N in the bark (Millard and Grelet, 2010). Due to this particular facet of the species' ecology we felt that this single time-step, necessary due to the destructive harvest, was sufficiently representative of long-term N assignment in the stem and branches. In other species with different N storage habit a single measurement at an arbitrary point in the growing season would not have been sufficient.

Aside from total nitrogen content, seasonal changes in ^{15}N content of the needles may also have occurred in our resolution needle time-series (there seems to be a peak of foliar ^{15}N early in the year in the NA_{canopy} treatment (Figure 4.2), which may have also occurred in other treatments but was not visible due to the high variability compared to the magnitude of excess ^{15}N expression.

For statistical analyses we treated the ^{15}N time-series in both the respective chapters as linear as the durations of these experiments were too short to tell if these cycles were indeed varying throughout the year and using a mean trajectory in a linear model would be enough to determine differences between treatments. If this apparent seasonal trend in ^{15}N-isotope content was genuine, this may also have been due to seasonal variation in ^{15}N storage and uptake as it occurred close to peak [N] in this time-series. ^{15}N obtained earlier in the year may have been assigned to storage pools in the needles, where N is stored in metabolically active proteins such as RuBiSCO (Millard *et al.*, 2007), rather than contributing the structural and long-term N, as N uptake may peak early in the growing season (Jaeger *et al.*, 1999). This N stored before periods of peak demand could be mobilised when this N is needed, resulting in the peak of ^{15}N at the same time as N content was at its maximum. However, to confirm that this is the case a longer time series would be needed at a similar resolution, where repeat instances of this peak could be observed.

6.3.2 Long Term Responses to Nitrogen Deposition

Over the longer term, our study species, Sitka Spruce, is typically grown in rotations of around 35-40 years in the United Kingdom (Moore, 2011), although many stands are considerably older. Nitrogen deposition studies are typically much shorter, while changes in background N_{DEP} concentration may occur over decades (Waldner *et al.*, 2014).

While estimates of the N_{DEP} effect based on short-term studies (such as our work) take part on only a fraction of the whole lifespan of the a tree, the duration of our two studies were not particularly short when compared to other ^{15}N-tracer studies in the literature and we were particularly interested in not inducing a long-term N fertilization effect, which may be dependant on first saturating storage compartments in the tree (Sheppard *et al.*, 2004). Plots summarised in Nadelhoffer *et al.* (1999c) have an average duration of 14.5 months and several of these studies only applied ^{15}N fertilizers over a single growing season. With no ^{15}N-labelled tracer, longer term studies have been performed at natural abunadance, both with traditional soil fertilizations (e.g. Magill *et al.*, 2004; Hyvönen *et al.*, 2008; Gentilesca *et al.*, 2013; Savage *et al.*, 2013), as well as responses to slightly shorter term canopy fertilization (e.g. Guerrieri *et al.*, 2011).

As mentioned in Chapter 1, a shortcoming in understanding N_{DEP} responses still exists in reference to long-term feedbacks in litter quality and N recycling from the soil. High ^{15}N returns in needles may reflect short-term storage rather than sequestration. Sitka Spruce stores N in needles overwinter (discussed in depth in Chapter 3) so initially high foliar ^{15}N returns do not necessarily mean that this N remains in needles and i) changes litter quality or ii) is not eventually relocated to woody sequestration. However, C/N ratios in foliage usually decrease under chronic N_{DEP} (McNulty *et al.*, 1991; Magill *et al.*, 2004) and following senescence, low C/N foliage is still shed with a relatively high absolute nitrogen content (Näsholm, 1994; Magill *et al.*, 2004), which affects the C/N content of the litter layer (McNulty *et al.*, 1991) in addition to the effects of direct N_{DEP} on forest floor chemistry. Litter quality is well known to affect decomposition rates (McNulty *et al.*, 1991; Cotrufo *et al.*, 1994; Coûteaux *et al.*, 1995), and interacts with N availability (Knorr *et al.*, 2005), and little layer thickness, so changes in foliar N under long-term N_{DEP} may have impacts on N recycling from the soil and long-term N assignment beyond that which was considered in this study. This may interact with the differences observed between litter-derived and mineral N in our field study to change $\Delta C/\Delta N$ beyond the additive effect of ^{15}N partitioning following foliar and soil ^{15}N uptake and changes in litter N release from a N_{DEP} effect on litter decomposition.

Decomposition is a complicated and sequential process (Berg and McClaugherty, 2008) and in both experiments with labelled litter we could only trace recently decomposed fractions of litter. In a particularly extreme example, Wardle *et al.* (1997) found almost 3000 year old mor humus layers in boreal regions which had not burned during this time. It is impossible to introduce a ^{15}N label to litter and humus without labelling other pools, and the potential to test the effects of N_{DEP} on the fate of older fractions of litter is therefore limited as this must be introduced as fresh litter and sinks for released ^{15}N may already have obtained ^{15}N from quickly decomposing fractions before measurement. Similarly, a second long-term effect of litter decomposition is that, in our litter swap experiment, the entire N layer was the same age, and applied at the same time. This meant that all of the N losses from decomposition were from fast-decomposing fractions of litter. More recalcitrant, older fractions may decompose to different compounds, with different fates, and N concentrations may have also different effects on litter decomposition at different stages of decomposition (Berg and McClaugherty, 2008). As we aimed to not manipulate N_{DEP} significantly in all experiments these interactive effects between magnitude and ^{15}N source were not a major concern, and in a much longer-term experiment, the former issue could be addressed by storing additional labelled litter and sequentially adding it to the plots over time.

6.4 Comparision to Other Studies

6.4.1 Diversity of Deposition Methods

This is not the only study where N_{DEP} retention in the canopy nor litter ^{15}N return has been assessed in different ecosystem pools, but we believe that Chapters 4 and 5 display novel controlled comparisons against standard soil fertilization experiments.

The diversity of methods in the limited number of foliar ^{15}N-N_{DEP} studies (c.f Wilson and Tiley, 1998; Ammann *et al.*, 1999; Boyce *et al.*, 1996; Dail *et al.*, 2009) and elsewhere in the literature (c.f. Schulze, 2000; Sievering *et al.*, 2007) makes it very difficult to assess the total contribution to nutrition. This process has been reported to vary from 5 % (Wilson and Tiley, 1998) to 50 % (Sievering *et al.*, 2007) of total N required by trees. We have added to this with our method of ^{15}N-application in Chapter 4 and found a very strong (64 %) ^{15}N return from ^{15}N applied direct to the canopy canopy, although it was also possible for this ^{15}N to have been taken up by the roots if washed out onto the soil. We chose a direct application to the canopy in water and since making a comparison against estimated litter N_{DEP} was important in the experimental design, we wanted to be as certain as possible about the amount of N_{DEP} delivered to the canopy.

Other ^{15}N-N_{DEP} studies have measured immediate ^{15}N return (Wilson and Tiley, 1998), estimated N uptake ^{15}N-applications to a single canopy branch (Boyce *et al.*, 1996), or in relatively unrealistic conditions (Lumme, 1994), and it is difficult to draw major conclusions about the importance of this process to $\Delta C/\Delta N$ from such results if changes occur over a long term or at the whole canopy level. Only Dail *et al.* (2009) has addressed this problem on the scale of an ecosystem and long timespan but from a single study it is difficult to draw overall conclusions. Our relatively strong ^{15}N return in saplings does not match the low wood allocation in this study and foliar uptake of N remains an important subject to explore with reference to $\Delta C/\Delta N$. Greater numbers of studies at ecologically relevant scales, closer attention to timing of measurements and longer term experiments (discussed in section 6.3) may contribute greatly to our understanding of foliar effects on forest $\Delta C/\Delta N$ and confirm if the strong effect seen in our foliar experiment translates to a strong effect on forest C uptake.

6.4.2 Diversity of Labelled Litter Methodology

There is more comparability among studies using ^{15}N labelled litter, but only a few experiments where this has been performed. ^{15}N-labelled litter used in field experiments has primarily been drawn from a single source (Zeller and Colin-Belgrand, 1998) for almost every investigation on an field scale (e.g. Zeller and Colin-Belgrand, 2001; Caner *et al.*, 2004; Hatton *et al.*, 2012; Eickenscheidt and Brumme, 2012; Bimüller *et al.*, 2013), notable exceptions being Blumfield and Xu (2004); Fahey *et al.* (2011); Weatherall (2005). In these studies a rapid transfer of ^{15}N to organic matter is observed as seen in our (Chapter 5) but we are one of the few using more recalciatrant conifer litter (except Fahey *et al.*, 2011; Weatherall, 2005), and first to attempt to compare this to N_{DEP}. Litter quality varies between species and understanding of ^{15}N release from litter is vital on material aside from the common *Fagus sylvatica* litter of Zeller and Colin-Belgrand (1998).

As our objectives were for a direct comparison we applied only a low magnitude of highly enriched ^{15}N, but litter decomposition is altered by nitrogen deposition (Knorr *et al.*, 2005) and ultimately the implications of a higher retention of organic-derived ^{15}N than mineral N is only of interest in the context of $\Delta C/\Delta N$ as a difference in partitioning implies that the effects of N_{DEP} on litter decomposition are not simply an additional percentage of mineralised N available under changing N_{DEP} intensities. To test this similar experiments could be performed comparing

N_{DEP} against ^{15}N-litter but also manipulating total N additions which affect the rate of litter N release. Maintaining ^{15}N content at similar magnitudes could be obtained by using a relatively less enriched addition to match predicted litter release in such experiments, ensuring comparable ^{15}N amounts while N_{DEP} is raised.

6.5 Representativeness of Nitrogen Deposition in Experiments

Aside from consideration of how our work relates to other studies, it is also worth mentioning that uptake of wet, mineral N_{DEP} is only a portion of nitrogen deposition to forests as there are also sizeable dry deposited and organic fractions in deposition which receive a great deal less research attention.

Inputs of inorganic N from deposition are predominately in the form of $NH_4{}^+$ and $NO_3{}^-$ (Galloway *et al.*, 2004) and are deposited in both wet and dry forms. Many field experiments deploy ^{15}N traces in wet deposition as this is considerably easier to retain expensive inputs on the ecosystem in question (e.g Nadelhoffer *et al.*, 1999c; Dail *et al.*, 2009; Perakis *et al.*, 2005). We followed a similar methodology in both of our N_{DEP} experiments , applying deposition inputs as dissolved NH_4NO_3.

Dry deposition, on the other hand, is more difficult to simulate and is most often studied using budgets rather than manipulations (e.g Lovett and Lindberg, 1984). Studies using automatic field fumigation systems as have been used in other ecosystems (e.g. Leith *et al.*, 1995; Sheppard *et al.*, 2013), even before the cost of a ^{15}N label is calculated are expensive to operate in forests with dense above-ground biomass and the major surface areas for interception in the canopy, high above the ground. However as forest structure makes them particularly susceptible to N_{DEP}, their interaction with dry N_{DEP}, particularly canopy uptake, are very interesting.

Consequently much less information is known about dry nitrogen deposition and its $\Delta C/\Delta N$ effect is usually assumed to be the same, despite constituting 40-50 % of total N_{DEP} (Dentener *et al.*, 2006). The lack of consideration of dry N_{DEP} was one of the criticisms levelled at Magnani *et al.* (2007) by Sutton *et al.* (2008) and it is assumed that wet N_{DEP} scales in the same way as dry N_{DEP} in many studies tracing ^{15}N additions and inferring a carbon effect of nitrogen additions. As N processes in the canopy may change N_{DEP} uptake, some validation of this assumption would be useful if the costs of full-scale ecosystem fumigation are impractical. Studies such as Ammann *et al.* (1999) can demonstrate dry uptake of N_{DEP} from strong background sources which could provide a method with which assessment of the relative importance of wet and dry N_{DEP} could be compared.

There is also a portion of the literature which suggests that organic nitrogen compounds compose $\sim$ 30 % of water-soluble N and also a possible fraction of organic N in the atmosphere (Cape *et al.*, 2011; Cornell, 2011), but there is still considerable uncertainty in what exactly the organic fraction of N_{DEP} consists of. Typically, a ‘number of organic compounds’ consisting of a mix of urea, amino acids, amines, peptides (Cape *et al.*, 2011) are assumed to make up this proportion of deposition, and there is very little information about the magnitude, composition, and importance of this fraction of nitrogen deposition which can relate to forest growth or biological effects of deposition of these compounds at all (Jickells and Baker, 2013). Various forms of organic nitrogen may be transported different distances in the atmosphere and may have differing effects on the forest system, and at manipulation levels, organic applications are understudied apart from various urea fertilization experiments (e.g. Zeller and Colin-Belgrand, 1998; Mugasha and Pluth, 1994). As plants can acquire some organic N, if these organic inputs are in an appropriate form for uptake then logically this proportion of N_{DEP}

may contribute to plant growth by bypassing the microbial bottleneck in a similar manner as we have shown for organic N from decomposition and is observed in numerous studies (e.g. Näsholm *et al.*, 2009). This clearly poses many questions that are yet to be answered.

6.6 Overall Estimates of ΔC/ΔN

The objectives of this thesis were to investigate some of the assumptions in ^{15}N-N$_{DEP}$ experiments to understand how these may have an effect on differences in ΔC/ΔN estimates between these and other, correlative studies. In order to compare the differences in ^{15}N return in our two N$_{DEP}$ experiments to literature estimates of ΔC/ΔN (see Table 1.1), we have applied the calculations used to assess the total N$_{DEP}$ effect to the simple ΔC/ΔN calculation from Nadelhoffer *et al.* (1999c), which synthesised ^{15}N-tracer addition partitioning between several studies and applied it to a mathematical model calculating ΔC/ΔN effect from the relative allocation of N to four pools (woody biomass, non-woody biomass, soil, and N losses) with different C/N ratios. In Nadelhoffer *et al.* (1999c), an overall ΔC/ΔN effect of 49.8 kg C kg N $^{-1}$ (49.8:1) can be calculated by dividing the total CO$_2$-C uptake calculated by total applied N$_{DEP}$. Similarly, we can calculate individual ΔC/ΔN of trees and wood of 28.8 kg C kg N $^{-1}$ and 21 kg C kg N $^{-1}$ by dividing the CO$_2$-C assimilated by total N$_{DEP}$ to calculate individual ΔC/ΔN for trees and soil (Table 6.4). Nadelhoffer *et al.* (1999c) report that their values represent < 20 % of a total 1.5 to 1.9 Pg terrestrial C uptake at the time of this study but calculations of a total ΔC/ΔN effect are independent of both total N$_{DEP}$ and the global C sink strength which have been refined in subsequent studies (e.g. Pan *et al.*, 2011). For ease of comparison, we retained the C/N ratios used in Nadelhoffer *et al.* (1999c) in the following estimates, and report only ΔC/ΔN changes from different N sources to assess relative importance in relation to this study. The ΔC/ΔN presented in separate pools in the following tables (6.4 and

6.5) is also relative to total ecosystem inputs of N, rather than the fraction of this acquired by the pool itself.

6.6.1 Nitrogen Uptake from Canopy Deposition

To calculate ΔC/ΔN from our canopy N_{DEP} treatments (Chapter 4), we modified the calculations from Nadelhoffer *et al.* (1999c) by altering the tree pools to the values we calculated in our nursery experiment mass balance (Table 4.7) for Canopy (NA_{litter}-$^{15}N_{canopy}$) and soil treatments (NA_{litter}-$^{15}N_{soil}$). We assumed that soil ^{15}N partitioning of N applied to the canopy which reached the soil did not change, and removed the extra N assigned to above-ground pools in equal parts from N which would otherwise be assigned to soil, or lost via leaching.

Table 6.4: Comparison of ΔC/ΔN effect between values calculated from (Nadelhoffer *et al.*, 1999c) and our experiment. Adjustments to overall budgets presented in (Nadelhoffer *et al.*, 1999c, Table 2) were made by altering the Woody and Non-Woody pools to recovery calculated in our experiment (Table 4.7), with additional N drawn proportionally from soil (forest floor + mineral) and leeching + gaseous losses. Our woody pools were both stem pools, and 2011-2012 branches, while our non-woody pools were the needle pools and 2013 branches. Errors are standard deviations from our study propagated with CN ratios of Nadelhoffer *et al.* (1999c).

	Nadelhoffer *et al.* (1999c)	Soil Deposition	Canopy Deposition
Tree	28.8	23.8 ± 18	104.6 ± 16
Soil	21	20.4 ± 2	9.4 ± 2
Total	49.8	44.2 ± 18	113.9 ± 16

Our soil N_{DEP} treatment was similar to Nadelhoffer *et al.* (1999c), estimates drawn from C and N budgets (de Vries *et al.*, 2006) with a total C sink of 44.2 kg C kg N $^{-1}$ split fairly evenly between trees and soil. The error on this (which we present as standard deviation from our mass balance propagated to the overall Tree and Soil pools we calculated, but without errors on data from Nadelhoffer *et al.* (1999c), as these are not given in this theoretical calculation) were fairly large (18 kg C kg N $^{-1}$), which was mainly due to the very high uncertainty on stem assignment (Table 5.5). Our estimates of $\Delta C/\Delta N$ from the canopy N_{DEP} treatment was 113.9 ± 16 kg C kg N $^{-1}$, which was more than double the estimates from Nadelhoffer *et al.* (1999c), and more than 2.5 times that drawn from our soil treatment which was comparable to the methods used in these other isotope studies. The majority of this $\Delta C/\Delta N$ effect was due to a high response from the tree pool which, in total, returned 46 % of N in non-woody pools and 18 % in woody pools. This reduced ^{15}N assignment to the soil and $\Delta C/\Delta N$ resulting in a slightly lower $\Delta C/\Delta N$ in this pool. The substantial return in wood, while smaller than non-woody pools (Table 4.7), had a much greater $\Delta C/\Delta N$ effect due to high C/N ratios, but the overall differences were both due to this and the effect of a higher total ^{15}N recovery in trees under the canopy treatment; if $\Delta C/\Delta N$ is recalculated using the same total ^{15}N return above-ground, but the 3:1 ratio of assignment between woody and non-woody biomass used by Nadelhoffer *et al.* (1999c) is maintained, a 20 % lower $\Delta C/\Delta N$ is calculated (101 kg C kg N $^{-1}$, while using the 9:2 ratio from our soil N_{DEP} treatment the effect is 55 % lower (70 kg C kg N $^{-1}$). Similarly, our $\Delta C/\Delta N$ calculation assumes that the N_{DEP} application to the canopy was representative of both interception and uptake together. Dezi *et al.* (2010) in their incorporation of canopy uptake into the G'DAY model used literature values of 80 % uptake (Sievering *et al.*, 2007) and 60 % N retention by the canopy (Chopping *et al.*, 2008). It was difficult to assess how well our application made directly to the canopy related to these two factors as our N return from canopy applications was from a ^{15}N application which may have been subject to some losses of N subsequently washed out of the canopy. To upscale based on crown closure, Dezi *et al.* (2010) used a crude single value from a single study. As these are difficult to reliably estimate we have assumed our 64 % return is inclusive of both of these losses to the soil while these would need to be assessed more thoroughly in a canopy-level ^{15}N-fertilization study to make a more thorough upscaling of N return to ecosystem level. As crown cover is likely to be below 100 % in most forests, we may have overestimated the ΔC/ΔN effect but view the literature on this as too uncertain at the current time to reliably improve estimates.

Overall, the difference in our treatments raised ΔC/ΔN from values similar to Nadelhoffer *et al.* (1999c) or de Vries *et al.* (2006) to, when using our observed N partitioning as well as total N return, a similar magnitude as values from Dezi *et al.* (2010), who used a model which incorporated foliar uptake., or Thomas *et al.* (2009), which used a similar correlative approach to Magnani *et al.* (2007) on US forest inventory data. While our ^{15}N estimate is drawn from pot-based saplings (which have already been discussed in section 6.2.1), and the ΔC/ΔN effect calculated using Nadelhoffer *et al.* (1999c) with conservative (high) C/N ratios and N partitioning, our results suggest that overall ΔC/ΔN may be substantially underestimated when based on ^{15}N traces direct to the forest floor and canopy uptake of N may result in a much higher effect on woody C sequestration than is seen from soil N_{DEP} treatments in the soil.

6.6.2 Nitrogen Uptake from Litter

Our field experiment returned 36 % more ^{15}N in roots (in combined O_h and A_h horizons) when the ^{15}N source was derived from litter, than when the ^{15}N was applied in deposition (Table 5.5). We calculated a potential ΔC/ΔN effect of litter-derived N by assuming that increase in plant N occurred similarly throughout the plant (there is some evidence that organic N sources affect plant biomass partitioning in favour of the roots in seedlings (Cambui *et al.*, 2011; Gruffman *et al.*, 2012), which may affect later performance and morphology, but no current evidence of longer term effects or N form effects on adult trees. We also felt that removing this additional N acquired by trees from soil pools was unrealistic as a greater soil ^{15}N return was also found from our labelled litter treatment (Table 5.5), and assumed that this ^{15}N was drawn mostly from the fraction otherwise leached or lost as trace gases, leaving forest floor N return unchanged. The fraction of N_{DEP} lost is primarily made up of NO_3^- in N_{DEP} studies due to the much higher cation retention capacity of soils), and precursors to these mineral N products are better retained in soils than mineral N (see section 5.4.3). This method may underestimate soil return if this follows the increases in soil ^{15}N recovery under $^{15}N_{Litter}$s in chapter 5, but, as we were unable to assess litter to litter ^{15}N transfer, the the higher soil ^{15}N recovery under the labelled litter treatments could be balanced by a lower litter N retention in litter when N is derived from this source). Using these assumptions, the overall N retained in the soil and trees is 97 % of litter-derived N, producing an overall $\Delta C/\Delta N$ if 60.12 kg C kg N $^{-1}$, $\sim$ 20 % greater than N_{DEP}-N based on Nadelhoffer *et al.* (1999c) (Table 6.5).

Table 6.5: Comparison of $\Delta C/\Delta N$ effect between values calculated from Nadelhoffer *et al.* (1999c) and litter-derived decomposition. Adjustments to overall budgets presented in (Nadelhoffer *et al.*, 1999c, Table2) were made by altering the Woody and Non-Woody pools by a proportion based on ^{15}N recovery in our field experiment (Table 5.5). Additional N assigned from this pool is drawn from estimated N losses and does not influence forest floor ^{15}N retention, which appeared higher in our study although we were not able to quantify litter-litter transfer. Errors are not given due to the high uncertainty in soil N return due to the missing litter-litter transfer from our experiment.

	$^{15}N_{Dep}$s (Nadelhoffer *et al.* (1999c))	$^{15}N_{Litter}$s (Litter-derived N)	% increase
Tree	28.75	39.12	36 %
Soil	21	21	0 %
Total	49.75	60.12	20 %

Overall ecosystem estimates are fairly uncertain both due to our limited sample size and lack of a complete quantification of soil ^{15}N return. It is also worth noting that this overall increase in ΔC/ΔN of 20 % is also not an additional effect of N_{DEP} on litter N retention under a litter addition to the soil, in the same manner as our canopy and soil N_{DEP} treatments, but rather a relative importance of N to C uptake; as all biomass growth requires N the entire temperate 0.6-0.7 Pg C y^{-1} net uptake (Goodale *et al.*, 2002) is ultimately dependent on N nutrition but the calculations from Nadelhoffer *et al.* (1999c) examine the extent to which an excess C sink can be attributed to a direct N_{DEP} effect. Consequently,the relatively greater accountancy of litter ^{15}N than N_{DEP}-^{15}N in this study and 20 % higher 'ΔC/ΔN' represents a relative difference in the importance of nutrition of litter-derived N with implications for the overall ΔC/ΔN effect of decomposition-N dependent on the changes in decomposition rate. As these are very complex (Knorr *et al.*, 2005), we have not made an direct inference of overall effects of nitrogen deposition on litter pool N, and the contribution of the change in mobilised, internal N on ecosystem ΔC/ΔN in this thesis, but note that the fate of forest floor N under N_{DEP} may be an area where much more work is necessary to understand overall ecosystem ΔC/ΔN effects.

Chapter 7

Conclusions

The work in this thesis was designed to test some of the assumptions about N assignment made by commonly cited ^{15}N nitrogen fertilization experiments which may explain some of the difference in estimates of C uptake based on isotope tracer studies (Nadelhoffer *et al.*, 1999c) and multi-site correlations of C uptake from NEP against nitrogen deposition. Using the ^{15}N return in wood we found with a method applying N_{DEP} to the canopy, rather than the soil, and pool C/N ratios used in Nadelhoffer *et al.* (1999c), we can calculate an effect size of 114 $\Delta C/\Delta N$, more than twice that when applying the ^{15}N partitioning found in our soil trace.

We also estimated $\Delta C/\Delta N$ from the higher root retention of ^{15}N from organic sources in our field study as approximately 20 % higher than that of our N_{DEP} treatment. While this is not directly comparable against the effect of mineral N_{DEP} in terms of $\Delta C/\Delta N$ effect, as this N is already in the ecosystem and not an additional surplus with a cumulative effect on tree $\Delta C/\Delta N$, this difference suggests that more N from litter decomposition is available to trees than mineral ^{15}N applications and the effect of these sources of N on $\Delta C/\Delta N$ may not be additive if decomposition effects of N_{DEP} on litter and associated changes in N availability are taken into account.

Many questions remain about the effect of nitrogen deposition on forest ecosystems and this studies was necessarily limited in scope, but we note that these results suggest that a N_{DEP} response may tend towards the higher estimates recently published from larger scale correlative and modelling studies (e.g. Dezi *et al.*, 2010; Thomas *et al.*, 2009), although not as high as revised estimates from Magnani *et al.* (2008).

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