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	stopped. iv) Both our results (obtained using canopy N applications) and a collection of published data (obtained using soil applications) showed that generally WUEi increased in response to an increase of N applications, with the magnitude of the changes related to soil conditions and the availability of other nutrients. The opposite directions of shifts in δ15N in tree rings suggest that both the quantity of the applied N and its quality, mediated by processes occurring during canopy N retention, are important determinants of the interactions between N and C cycles, which occur in forests. Stable isotopes are useful probes to understand these processes and to put in the results of short-term experiments into context.

THE LEGACY OF ENHANCED N AND S DEPOSITION AS REVEALED BY COMBINING $\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$ IN TREE RINGS

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Abstract

This study aimed to evaluate the effects of repeated aerial nitrogen (N) and sulphur (S) misting over tree canopies of a Sitka spruce plantation in Scotland. We combined δ13C and δ18O in tree rings to evaluate the changes in CO2 assimilation (A) and stomatal conductance (gs) and to assess their contribution to variations in the intrinsic water-use efficiency (WUEi). Measurements of δ15N enabled shifts in the ecosystem N cycling following misting to be assessed. We found that: i) N applications, with or without S, increased the ratio between A and gs in favour of A, thus supporting a fertiliser effect of added N. ii) After the treatments have ceased, the trees quickly adjusted to the reductions of N deposition, but not to the reduction in S deposition, which had a negative effect on WUEi by reducing A. This indicates that the beneficial role of N deposition may be negated in forests that previously received a high load of acid rain. iii) δ15N in tree rings seems to reflect the N dynamics caused by canopy retention, with the fingerprint also present in the litter, after the experiment stopped. iv) Both our results (obtained using canopy N applications) and a collection of published data (obtained using soil applications) showed that generally WUEi increased in response to an increase of N applications, with the magnitude of the changes related to soil conditions and the availability of other nutrients. The opposite directions of shift in δ15N in tree rings suggest that both the quantity of the applied N and its quality, mediated by processes occurring during canopy N retention, are important determinants of the interactions between N and C cycles, which occur in forests. Stable isotopes are useful probes to understand these processes and to put in the results of short-term experiments into context.

Key words: annual tree rings, acid mist, CO_2 assimilation, nitrogen, Sitka spruce, stable C-O-N isotopes, stomatal conductance, sulphur, $WUE_{i,}$

1. Introduction

The extent to which forests mitigate climate change is affected by the increase in nitrogen deposition (N_{dep}) from the atmosphere, due to anthropogenic activities. Together with elevated atmospheric CO_2, N_{dep} has been recognized as one of the main drivers enhancing the terrestrial carbon 'sink' (Norby 1998; Gruber & Galloway 2008). However, the carbon (C) and nitrogen (N) cycles interact in very complex ways, with mechanisms that can be grouped under two distinct scenarios. A beneficial role of N_{dep} has been hypothesized for N-limited systems (e.g., temperate forests), since N_{dep} can act as a low-level but sustained input of fertiliser, potentially increasing tree growth and C uptake (Magnani *et al.* 2007; Högberg 2007; Sutton *et al.* 2008; Thomas et al., 2009). However, high levels of N_{dep} may result in soil acidification (Vitousek *et al.* 1997; Galloway *et al.* 2002), increased tree mortality (Magill *et al.* 2004) and declines in biodiversity (Phoenix *et al.* 2006).

The extent of the enhancement in carbon uptake by N-limited forests that can be attributed to N_{dep} has been hotly debated, with variable estimates provided (Magnani *et al.* 2008), which suggests that a deeper investigation of the crucial underlying mechanisms may be useful. One of the main issues relates to understanding whether the interactions between N_{dep} and tree canopies play an important role in linking the N and the C cycle. Most of our knowledge regarding the effects of N_{dep} on forests relies upon experiments carried out in managed fertilized forests, where the additional N was supplied as fertilizer to the soil for 1-2 years (Elhani *et al.* 2005; Brooks & Coulombe 2009) or for more than 10-years (Magill *et al.* 2004; Nadelhoffer *et al.* 2004; Högberg *et al.* 2006; Betson *et al.* 2007; Feng *et al.* 2008). The relevance of such investigations, which exclude the opportunity for canopy exchange and processing of N on the leaf surface, is debateable as it is well-known that canopy uptake occurs. The significance of the canopy uptake pathway has been shown in several studies:

Rennenberg & Gessler, 1999; Cape *et al.*2001; Gaige *et al.* 2007; Sievering *et al.* 2007. This provides an important source of N readily available for plant metabolism (Sparks 2009), whereas N applied to the soil may be rapidly incorporated into microbial biomass instead. Recently, Dezi *et al.* (2009) showed that inclusion of canopy uptake in an ecosystem level model significantly changed ecosystem C storage capacity.

Another relevant point is that deposition of N onto forest canopies occurs in combination with other pollutants, e.g., sulphur (S), which may negate the potential beneficial effects of N_{dep} and accelerate tree decline. Even though S emissions have been significantly reduced in Europe over recent years (ICP, 2009), Fowler *et al.* (2007) observed a non-linearity in the relationship between emission and deposition changes, showing that S_{dep} in remote areas has not decreased at the same rate, as closer to S sources.

Only a few experiments have examined the contribution of atmospheric N, by spraying or misting above tree canopies: the experiment at Deepsyke forest, in the UK (cf., Sheppard *et al.* 2004, 2007) and at Howland Forest, in the USA (cf., Gaige *et al.* 2007; Dail *et al.* 2009). Between the two experimental sites, the Deepsyke experiment is unique in providing the opportunity to evaluate the effects of frequent (> 50 applications during the growing season) aerial N and S misting onto the canopy of a Sitka spruce forest for a relatively long period (*i.e.*, 5 to 8-years). At this experimental site, N treatments significantly increased tree stem area increment, irrespective of whether N was added with or without S (Sheppard *et al.* 2004).

This study aimed to investigate how experimental applications of N_{dep} and S_{dep} affected the ratio between canopy CO_2 assimilation (A) and stomatal conductance (g_s), known as intrinsic water-use efficiency (WUE_i), for the Sitka spruce stand at Deepsyke. Sitka spruce is one of

the most widely planted tree species in the UK, succeeding well on in nutrient-poor soils. This species is very tolerant of low-N availability, and at the same time very responsive to small N additions (Chandler & Dale, 1995), so that a beneficial effect of N_{dep} on WUE_i, achieved through an increase in *A*, can be hypothesised.

Our approach combined the analysis of the stable isotopes of carbon ($\delta^{13}C$), oxygen ($\delta^{18}O$) and nitrogen ($\delta^{15}N$) in annual tree rings. Tree rings are very useful archives of information concerning changes in tree growth in response to environmental conditions. The combination of all three isotopes $\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$ in tree rings can provide more specific information on the underlying ecophysiological processes. The effect of different treatments on WUE_i, as assessed through $\delta^{13}C$ in tree rings, was investigated. Further, the combination of $\delta^{13}C$ and $\delta^{18}O$ allowed us to suggest which physiological traits, *i.e., A* and g_s respectively, contributed most to the variations in WUE_i. Finally, $\delta^{15}N$ provided indications of changes in ecosystem N cycling in response to the different treatments.

We addressed the following main questions: *i)* Do canopy N additions increase the balance between *A* and g_s (*i.e.*, WUE_i) in favour of *A,* and does the addition of S counteract the effect of N? *ii)* Does $\delta^{15}N$ in tree rings carry a signal of changes in the N cycle within the ecosystem in response to canopy N addition? *iii)* When the experimental applications are terminated, how fast do trees adjust to a reduction in N and S deposition? *iv)* Are the effects on tree physiological traits and N dynamics caused by canopy addition different from those predicted by studies that have employed direct application of N to the soil?

2. Materials and methods

2.1 Stable isotope theory

The relative abundance (R) of the stable isotopes, *i.e.*, $^{13}C/^{12}C$, $^{18}O/^{16}O$, $^{15}N/^{14}N$, in plant organic matter is expressed as the relative deviation from the international standards (V-PDB for $\delta^{13}C$, V-SMOW for $\delta^{18}O$ and atmospheric N_2 for $\delta^{15}N$), using delta (δ) notation, *i.e.*,

$$\delta_{sample} = \left(\frac{R_{sample}}{R_{standard}} - 1 \right) \bullet 1000 .$$

$\delta^{13}C$ and its link to WUEi

Plant material is generally depleted in the heavier isotope (^{13}C) compared to atmospheric CO_2, because of isotope fractionations taking place during the biochemical and diffusional processes leading to photosynthesis. Indeed, carbon isotope composition in tree rings ($\delta^{13}C$) reflects variation of the ratio between CO_2 concentration in leaf intercellular spaces and the atmosphere (*i.e.*, c_i/c_a ratio) related either to changes of CO_2 assimilation (A) or stomatal conductance (g_s). According to the simplified version of the Farquhar equation, $\delta^{13}C$ in plant material ($\delta^{13}C_p$) is described as follows (Farquhar *et al.* 1989):

$$\delta^{13}C_p = \delta^{13}C_a - a - (b - a)\frac{c_i}{c_a} \qquad [1]$$

where $\delta^{13}C_a$ is the isotopic signature of atmospheric CO_2, a is the fractionation for $^{13}CO_2$ as a result of diffusion through air (4.4‰), b is the fractionation during carboxylation (27‰) by the CO_2-fixing enzyme Rubisco. Due to their reciprocal link to the c_i/c_a ratio, $\delta^{13}C$ can be considered to infer changes in the intrinsic water-use efficiency (WUE_i), which is defined as the ratio between A and stomatal conductance to water vapour (g_{H_2O}), according to Ehleringer *et al.* (1993):

$$WUE_i = \frac{A}{g_{H_2O}} = \frac{(c_a - c_i)}{1.6} \qquad [2]$$

From eqn. [1] c_i can be derived as follows:

$$c_i = c_a \frac{\delta^{13}C_a - \delta^{13}C_{sample} - a}{b - a} \quad [3]$$

$\delta^{13}C_a$ and c_a for each year can be estimated according to the equations proposed by Feng (1999):

$$\delta^{13}C_a = -6.429 - 0.0060 \exp[0.0217(t - 1740)] \quad [4]$$

$$c_a = 277.78 + 1.350 \exp[0.01572(t - 1740)] \quad [5]$$

where t is the calendar year.

Finally, eqn. [2] can be solved as:

$$WUE_i = \frac{c_a}{1.6} \frac{b - \delta^{13}C_a + \delta^{13}C_{sample}}{b - a} \quad [6]$$

Hence, $\delta^{13}C$ in tree rings allows changes in the A/g_s ratio to be assessed taking into account the long-term variation of atmospheric CO_2 concentration and its isotopic signature. Although post-photosynthetic fractionations (Cernusak *et al.* 2009) may partially reduce the strength by which the primary isotope signal imprinted in leaf organic matter is recorded in heterotrophic tissues, $\delta^{13}C$ in tree rings still contains long-term information on changes of leaf physiological processes (Saurer *et al.* 2004; Gessler *et al.* 2009).

Link between $\delta^{18}O$ in tree rings and stomatal conductance

Variation in $\delta^{13}C$ can be related to changes in either A or g_s whereas $\delta^{18}O$ in organic material is specifically related to changes of $\delta^{18}O$ in leaf water in response to transpiration. Hence, when $\delta^{18}O$ of source water does not vary among studied sites, $\delta^{18}O$ in plant organic matter records changes in g_s. In fact, during transpiration molecules of water containing the lighter isotopes, $H_2{}^{16}O$, tend to diffuse faster from the sites of evaporation to the atmosphere than $H_2{}^{18}O$, so that water at the evaporative site becomes more enriched in the heavier isotopes ^{18}O

compared to water in the transpiration stream *i.e.*, coming from the soil (source of water). Variations in the oxygen isotope signature in leaf water (*i.e.*, $\delta^{18}O_{lw}$) are linked to changes in the ratio between water vapour in the atmosphere and in the intercellular spaces ($e_a/e_i \approx$ Relative humidity, Rh), according to Craig and Gordon (1965):

$$\delta^{18}O_{lw} = \delta^{18}O_s + \varepsilon^* + \varepsilon_k + (\delta^{18}O_v - \delta^{18}O_s - \varepsilon_k)\frac{e_a}{e_i} \quad [7]$$

where $\delta^{18}O_s$ and $\delta^{18}O_v$ are the oxygen isotopic compositions of soil water and atmospheric water vapour, respectively, ε_k is the kinetic fractionation during evaporative water diffusion through stomata and boundary layers, ε^* is the fractionation associated with the lower vapour pressure of $H_2{}^{18}O$ compared to that of $H_2{}^{16}O$. However, at increasing levels of transpiration, the primary ^{18}O enrichment at the evaporative site is modified by the Péclet effect (Farquhar & Lloyd 1993; Barbour *et al.* 2000a), which describes the mixing of enriched water at the evaporative sites with unenriched water pools within leaves. The signal of $\delta^{18}O_{lw}$ is then imprinted in the leaf organic molecules, due to the isotopic exchange between carbonyl oxygen and water in which they are formed (Sternberg *et al*. 1986).

Several authors have reported a negative relationship between $\delta^{18}O$ in leaf organic matter and g_s, due to their reciprocal link to vapour pressure deficit, which, in turn depends on Rh (Barbour *et al.* 2000b; Grams *et al.* 2007; Sullivan & Welker 2007; Ripullone *et al.* 2009). The signal of changes in the $\delta^{18}O$ of leaves may also be dampened in tree rings, due to isotope fractionation during translocation of sucrose and synthesis of cellulose in the tree stem (Sternberg *et al.* 1986; Saurer *et al.* 1997; Gessler *et al.* 2009). However, $\delta^{18}O$ in tree rings still carries information related to changes in g_s (McCaroll & Loader 2004; Barbour 2007).

Combining $\delta^{13}C$ and $\delta^{18}O$ in a conceptual model

Scheidegger et al. (2000) proposed a conceptual model, which helps to clarify whether changes in A, g_s or both accounted for the variations of WUE_i. The model inferred changes in c_i and therefore A from $\delta^{13}C$, assuming a negative relationship between the two parameters, according to Eqn. [1]. Further, changes in Rh were derived from $\delta^{18}O$ in tree organic matter, which, in turn, allows variations in g_s to be predicted. As a further extension of the model, Grams et al. (2007) showed that g_s can be inferred directly from $\delta^{18}O$ in leaf cellulose, which was confirmed also by comparing stable isotopes with gas exchange measurements. In both conceptual approaches, an important prerequisite is similar $\delta^{18}O$ of source water and vapour water for the investigated trees.

$\delta^{15}N$ and ecosystem N cycle

The theoretical understanding of $\delta^{15}N$ in tree pools is not as comprehensive as for $\delta^{13}C$ and $\delta^{18}O$. This is due to the complexity of the N cycle and the possibility for plants to use different forms of N, *i.e.*, ammonium (NH_4^+), nitrate (NO_3^-) and dissolved organic N. Overall, the $\delta^{15}N$ in tree pools reflects the isotopic signature of the main N sources (soil and atmosphere) and isotope fractionations occurring during N uptake by roots and foliage, and assimilation within plant compartments (Robinson *et al.* 1998; Comstock 2001). On the other hand, $\delta^{15}N$ in the soil reflects the relative contribution of different N compounds and is strongly affected by fractionations during biogeochemical processes. Indeed, nitrification produces ^{15}N-depleted NO_3^-, leaving in the soil ^{15}N-enriched NH_4^+. Ammonia volatilization can also enhance the ^{15}N enrichment of the NH_4^+ remaining in the soil and therefore, available for plants, while atmospheric deposition of NH_3 may correspondingly supply ^{15}N depleted N (Tozer *et al.* 2005). NO_3^- can also be lost via denitrification and leaching, leaving ^{15}N-enriched NO_3^- in the soil (Nadelhoffer & Frey, 1994; Högberg 1997). Fractionations during

mineralization of the substantial pool of soil organic matter have been reported to reduce (Létolle, 1980), albeit not significantly (Högberg 1997), the $\delta^{15}N$ of the product NH_4^+.

Atmospheric N can be a significant source of N for trees. Before reaching the soil, atmospheric N (e.g., NO_x and NH_x forms) interacts with tree canopies in such a way that a consistent part can be retained and taken up by canopies, leading to a $\delta^{15}N$ signal which depends on the relative contribution of the exchanged N compounds (NH_4^+ *vs.* NO_3^-) (Heaton *et al.* 1997). As a consequence, the amount of atmospheric N reaching the soil as wet and dry deposition and the isotope signature are modified by the interaction with forest canopies.

Overall, it can be hypothesised that, at very low levels of N_{dep}, when mineralization is the chief source of available N, and provided canopies have also reached full closure, $\delta^{15}N$ in tree pools should be relatively stable and most closely related to soil $\delta^{15}N$ with little long-term variation. By contrast, when an increase in available N occurs, e.g., an increase of N_{dep} from the atmosphere or from fertilisation, variations of $\delta^{15}N$ in tree pools should reflect: *i)* the contribution of the added N, according to its $\delta^{15}N$ and *ii)* the variations in soil $\delta^{15}N$ because of changes in the degree of closure of the N cycle (*i.e.*, losses caused by NO_3^- leaching, denitrification and NH_3 volatilization), the prevailing effect of which is to enrich the $\delta^{15}N$ signal of tree pools.

2.2 Experimental site

The study was carried out in a *Picea sitchensis* (Bong.) Carr. plantation at Deepsyke (290 m asl, latitude 55°46'N, longitude 3°18'W) in the Scottish Borders (Scotland, UK), with a low background of N_{dep} (8-10 kg N ha^{-1} yr^{-1}). A mixture of provenances (identities unknown) was

planted in 1986 at 2 m spacing on drained deep peat soil, on mounds formed from the inversion of organic horizon removed to form the drainage ditches (Sheppard *et al.* 1999).

Within the forest stand, an area of 1.5 ha was divided into four replicated blocks, each containing six different spraying treatments: nitrogen (N), sulphur (S), NS (as a single dose), 2NS (as a double dose of both N and S), wet control (e.g., only water), no treatment (e.g., no spraying). Treatments increased the amount of precipitation by 20% (for N, NS and wet control) or 40% (for 2NS). In this study, we considered only N, NS, 2NS and wet control (Table 1, Fig. 1a-b). Acid mist was applied approximately twice weekly during the growing season (> 50 applications from April until November) from 1996 to 2000, with four replicates for each treatment. In a second phase (2001-2003), two plots (selected at random, irrespective of the block) were maintained on the original treatment, while in the other two S or N addition of the original treatment was removed in order to simulate the recovery from reduced S and N (Table 1) (cf., Sheppard *et al.* 2004, 2008).

Within each plot, five of the 10 trees were selected for sampling wood cores (two per tree) in June 2009, using a 0.5 cm diameter increment borer. The five trees were selected according to the diameter variation relative to the diameter distribution of the wet control. Therefore, trees showing extreme growth rates (exceptionally low or high) were excluded. The undecomposed litter and the organic soil (0-10 cm) were sampled from five locations in the furrows along the planting lines.

2.3 Sample preparation and isotope analyses

Before isotopic measurements, wood cores were subjected to a chemical pre-treatment in order to remove N mobile compounds, which may affect the detection of the $\delta^{15}N$ signal. For

this purpose, we employed the rapid procedure proposed by Sheppard and Thomson (2000): 4 hours in a mixture of toluene: ethanol; 4 hours in ethanol; 4 hours in distilled water. After drying, each wood core was dated and thereafter annual rings were identified and separated from bark to pith under a stereoscope. Along a chronology of 15-years (1995-2009), annual rings were split in to the following sections: **I)** the year 1995 (before the treatments); **II)** the single annual rings from 1996 to 2003 (during the treatments); **III)** the group of annual rings from 2004 to 2009 (after the treatments, *i.e.*, all six rings together with no annual separation). Within a plot, all the samples from the five trees were ground with a centrifugal mill and pooled by year, in order to get an average isotopic signal.

Soil and litter samples were dried in the oven for 12 hours at 105°C. After removing traces of fine root and litter, soil samples were ground with a ceramic mortar. For litter samples, a centrifugal mill was used to produce a fine powder for the analysis.

For $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$ measurements, 0.6-0.8 mg, 15-20 mg and 0.5-0.7 mg of wood powder, respectively were weighed in tin capsules (silver capsules for oxygen). An amount of 4-5 mg of soil and litter samples was weighed into tin capsules. Further, 4-5 mg of the ammonium nitrate (NH_4NO_3) used for the spraying and stored from 1996 was weighed into tin capsules (n=6) for the $\delta^{15}N$ analyses. We measured a $\delta^{15}N$ value of 1.6±0.42 ‰.

For $\delta^{13}C$ and $\delta^{15}N$ analyses, each determined in a separate measurement, samples were combusted under an excess of oxygen with an elemental analyzer (EA-1100, Carlo Erba, Milano, Italy), connected to an isotope ratio mass spectrometer (Delta - S Finnigan MAT, Bremen, Germany) through a variable open split interface (ConFlo II, Finnigan MAT, Bremen, Germany). For $\delta^{18}O$, the weighed bulk organic material was decomposed to CO by

thermal pyrolysis at 1080 °C in a different elemental analyzer (EA-1108, Carlo Erba, Milano, Italy), which was connected to the continuous flow mass spectrometer (Delta - S Finnigan MAT, Bremen, Germany).

Ground bulk samples obtained from 1-year old needles collected in December 1997 and used in previous investigation for measuring nutrient content in needles (e.g., Sheppard *et al.* 2004) were used for determinations of $\delta^{13}C$ and $\delta^{15}N$. An amount of 3-4 mg of needle samples was weighed into tin capsules for combustion in the CE Instruments NA2500 Elemental Analyzer and for determination of both $\delta^{13}C$ and $\delta^{15}N$ by VG Prism III Isotope ratio mass spectrometer.

The isotope values were expressed in the δ-notation (in per mil; ‰) as relative deviation from the international standard (atmospheric N_2 for $\delta^{15}N$, V-PDB for $\delta^{13}C$ and V-SMOW for $\delta^{18}O$).

2.4 Meta-analysis

Among all the studies in the literature reporting on tree growth responses to increased N_{dep}, mainly simulated as ground fertilization, we selected those experiments that satisfied the following main criteria: *i)* were carried out on tree species; *ii)* did not involve a ^{15}N-labelling experiment; *iii)* were conducted in the field; *iv)* measured $\delta^{15}N$ in tree rings and $\delta^{13}C$ in either needles or tree rings. These studies are summarised in Table 2. Most of the sites considered in our meta-analysis were located in areas with low-level of N_{dep}. Elhani *et al.* (2005) reported values ranging between 9 and 13 kg ha^{-1} yr^{-1}, for the years after the fertilization experiment. The $\delta^{15}N$ signal of the mineral N-fertiliser supplied during the experiments had values close to that of the atmospheric N_2 (*i.e.*, 0‰) (Nadelhoffer *et al.* 2004; Elhani *et al.* 2005). We measured a $\delta^{15}N$ value of the ammonium nitrate added during the spraying and found it to be

slightly enriched in ^{15}N, *i.e.*, 1.6‰. Overall, we assume no differences in the $\delta^{15}N$ of added N either in the soil or to tree canopies.

From each study, data points and corresponding error bars of $\delta^{13}C$ and $\delta^{15}N$ values were extracted using an image analysis programme (UTHSCSA Image Tool). We considered only the years when the experimental treatments were undertaken. For given $\delta^{13}C$ values, WUE_i's were calculated, according to equation [6]. Values are presented as differences between treated and control treatments, for both WUE_i and $\delta^{15}N$ values, in order to highlight the effect of the treatment and to account for differences in tree age and/or climate effects across studies.

2.5 Statistical analyses

Pre-treatment (1995) and post-treatment (2004-2009) differences for $\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$ across plots were tested by ANOVA, considering the assigned treatments as fixed factors. For the pre-treatment year (1995) we found no significant effect of 'block' as the random factor, so block was excluded from the subsequent analyses. The effect of different treatments was evaluated by repeated-measures ANOVA for the years 1996-2000, when replication of the experiment was balanced (four replicates for each treatment). Soil moisture was included as a covariate (Sheppard *et al.* 2004) and with an interaction term with treatments. The year-by-year differences between the treatments and the wet control were investigated by ANOVA, using the Least Significant Difference (LSD) test. Due to the experimental design not being balanced during the recovery phase (2001-2003), differences between recovery (n=2) and treatment (n=2) for each treatment, were assessed using independent sample t-test.

The *t*-tests were employed for investigating the difference between needles collected in 1997 and the annual ring formed in the same year for $\delta^{13}C$ and $\delta^{15}N$ values, while differences among treatments for either $\delta^{13}C$ or $\delta^{15}N$ in needles and annual rings were tested by ANOVA. All statistical analyses were carried out with SPSS 16.0 statistical package (SPSS, Chicago, IL). Where not differently specified, the level of significance for statistical tests was $\alpha = 0.05$.

3. Results

3.1 Effect of treatments on $\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$ in tree rings

Before the spray treatments began, $\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$ in 1995 showed similar values for all plots with different assigned treatments (Tables 3, 4, 5). However, over the five years of the experiment, $\delta^{13}C$ and $\delta^{15}N$ were most affected by the addition of nitrogen (N) alone or in combination with sulphur (S). While $\delta^{13}C$ showed a decreasing trend over time in all the treatments (Fig. 2, panels a, b, c), repeated-measures ANOVA from 1996 to 2000 also revealed a significant effect of treatment, with 2NS and N having higher $\delta^{13}C$ values than NS and wet control (Table 3). The tests of between-subject effects showed a non-significant effect of the covariate (*i.e.*, the variability in soil moisture among plots) on changes in $\delta^{13}C$ (F= 1.2; P=0.3).

The effect of simulated N and S deposition to the tree canopy for $\delta^{18}O$ was not as clear as for $\delta^{13}C$ (Fig. 2 panels d, e, f). Overall, there was a tendency for $\delta^{18}O$ to decrease for the first few years after the spraying started, with a trough in 1998, which was also the wettest year of the experimental period. Repeated-measures ANOVA did not show any significant treatment effect, even when variations in soil moisture were taken into account as a covariate (Table 4).

Overall, $\delta^{15}N$ values showed a slight tendency to increase over time (Fig. 2 g, h, i), with a higher variability among years for the wet control compared to plots receiving the different treatments. Changes in $\delta^{15}N$ were not affected by treatment, when N was in combination with S, with trees in NS and 2NS showing similar $\delta^{15}N$ values to the wet control. By contrast, N alone led to a more negative $\delta^{15}N$ values compared to the wet control and the NS treatment (Table 5). The covariate soil moisture was not significant for $\delta^{15}N$ (F=0.06, P=0.8).

For the 1997 annual ring (*i.e.,* after one year's treatment), $\delta^{13}C$ and $\delta^{15}N$ measured in wood could be compared with the corresponding values measured in needles formed in the same year (sampled in December 1997). When both values were plotted as differences from the wet control (Fig. 3) a significant increase in $\delta^{15}N$ in needles, but not in annual rings was observed which seemed to be dose related (Fig. 3a). By contrast, the effect of treatment was not obvious for $\delta^{13}C$ measured in needles, while it was significant (P<0.1) in the case of the corresponding annual ring, with the 2NS treatments showing an increase in $\delta^{13}C$ within one year of treatment (Fig. 3b). It is noteworthy that, the difference between the needle and annual ring for C and N isotopes has a different direction, significant only for 2NS: for $\delta^{15}N$, more positive values were observed in needles compared to the annual ring, while the opposite was true for $\delta^{13}C$.

The removal of S or N addition from the original treatment (Table 1) did not significantly affect stable isotope trends in the second phase of the experiment, with similar values measured for the recovery and the treatment plots (Tables 3, 4, 5). There was a tendency for $\delta^{13}C$ in the wet control to increase, while $\delta^{13}C$ tended to decrease in 2NS, NS and N (Fig. 2 a, b, c). Variation of $\delta^{18}O$ in treated plots followed the same time trend as in the wet control,

with the exception of 2003, when an increase of $\delta^{18}O$, with particular reference to N and 2NS, was observed.

After the experiment had ceased, in all the treatments $\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$ values approached those measured in the wet control (Tables 3, 4, 5), bearing in mind that stable isotopes were measured in the pooled annual ring from 2004 to 2009. With particular reference to $\delta^{15}N$, wood was ^{15}N-depleted compared to the soil samples, but ^{15}N-enriched compared to the litter sampled in June 2009 (Fig. 4).

3.2 Changes in WUE_i and its determinants

For each treatment, variations of WUE_i were evaluated as differences from the wet control (Fig. 5 a, b, c), without distinguishing between recovery and original treatments in the last 3-years of the experiment. The three treatments (*i.e.*, 2NS, NS and N) showed different temporal trends, with particular reference to the first phase of the experiment (*i.e.*, 1996-2000). The 2NS treatment led to a rapid but short-lived increase in WUE_i, followed by a decline from 1998. Furthermore, for the post-treatment phase (2004-2009) this decline strengthened producing a larger reduction in WUE_i compared to the control (Fig. 5 a). In the NS treatment, WUE_i showed a pattern similar to that observed for 2NS, but with a lower magnitude of variation and only a small non-significant decrease post treatment (Fig. 5 b). By contrast, for the N treatment, WUE_i kept increasing over the first phase of experiment and also for the beginning of the second phase. However, during the post-treatment years, WUE_i in the N treatment also decreased but only by a small amount, falling back to that measured in the wet control (Fig. 5 c).

The combination of $\delta^{13}C$ and $\delta^{18}O$ values, expressed in terms of their differences from the wet control, is shown in the main panel of Fig. 6. Values relative to the 8-years of the experiment (*i.e.*, 1996-2003) were averaged, without distinction between recovery and treatment periods. This allows the different effects of N alone and N in combination with S to be assessed for the combined variations of $\delta^{13}C$ and $\delta^{18}O$. N addition led to an increase in $\delta^{13}C$, with a marginally significant effect on $\delta^{18}O$. By contrast, N combined with S (irrespective of the double or single dose) led to an increase in $\delta^{13}C$ combined with a decrease in $\delta^{18}O$. For the post-treatment phase (*i.e.*, treatment *vs.* post-treatment phase), we observed a tendency to decrease for both isotopes, with a higher magnitude of change for $\delta^{13}C$ than $\delta^{18}O$. Different trends are discussed later in the paper in terms of variations in CO_2 assimilation and stomatal conductance, according to the conceptual model of Scheidegger *et al.* (2000) (Fig. 6, side panels).

3.3 Canopy vs. soil applications for WUEi and $\delta^{15}N$: a meta-analysis of data in the literature

We compared our results with those reported in previous studies, where N was added to the soil as either ammonium nitrate or urea, sometimes also in combination with S or other nutrients (e.g., K, P, Ca).

All values were considered as differences from the corresponding control, to standardise the differences in climate and soil conditions across sites. Overall, both above-canopy and below-canopy fertilisation showed that addition of N leads to an increase in WUE_i, with a magnitude that was equal to or greater for canopy compared to soil applications (Fig. 7). In two cases, no change (Balster *et al.* 2009) or even a reduction (Heaton & Crossley 1995) of WUE_i was observed with applied N. This latter study interestingly used the same NS combination as employed here.

A different dynamic was observed for $\delta^{15}N$, when the two different experimental approaches (canopy *vs.* soil N applications) were compared (Fig. 8). With increasing N dose applied to the soil, the $\delta^{15}N$ in tree rings became more ^{15}N-enriched compared to the control, whereas canopy applications seemed to lower the ^{15}N enrichment, although the low number of points prevents statistical analysis.

Interestingly, irrespective of the approach considered, the combination of N and S appears to restrict the difference between treatment and control for $\delta^{15}N$. In fact, when N alone was added either to the soil (Nadelhoffer *et al.* 2004) or to the canopy (*i.e.*, Deepsyke forest), the difference in $\delta^{15}N$ from the control was always larger than in the case of combined N+S applications, with a higher difference in the case of soil fertilization. Changes in the $\delta^{15}N$ in tree rings in response to N application seemed to be species-specific (conifer *vs.* deciduous species) and compound-specific (ammonium nitrate *vs.* urea fertilizer). Conifers were more sensitive than deciduous trees to N application. For the former group, the ^{15}N enrichment in tree rings compared to control was lower when N was added in combination with other nutrients (Elhani *et al.* 2005).

4. Discussion

4.1 Changes in WUEi and its determinants following N and S acid mist above tree canopies.

One year after the experiment started (*i.e.*, 1997) a clear effect of the treatment on the C isotope signal in the annual ring was already present, although no signal was detected in 1-year old needles produced in the same year (Fig. 3 b). 5 years exposure to acid mist affected the isotopic signature in tree rings significantly, leading to higher $\delta^{13}C$ values (Table 3), thus a higher WUE_i, for N and 2NS treatments. This result is consistent with the increase in stem area increment reported by Sheppard *et al.* (2004) at the same site. Despite the similarity in all

treatments with the positive response for tree growth rates, $\delta^{13}C$ enabled a treatment-specific change in terms of WUE_i to be identified. In fact, although both N and 2NS showed an increase of WUE_i, the different temporal trends (Fig. 5) demonstrate that the N application led to a long lasting positive effect of N availability on the ratio between assimilation (A) and stomatal conductance (g_s), whereas the 2NS application resulted in a short lived pulse only. The short lived increase in WUE_i with the 2NS treatment was followed post-treatment by a significant decrease during 2004-2009 compared with the control, a tendency which was also apparent (but not significant) for the NS treatment, but not for the N treatment. Combined with the lower WUE_i values for the NS compared with the N treatment during the experiment (Fig. 5), this indicates that S deposition had a longer lasting detrimental effect on WUE_i. We are aware that by grouping the annual rings from 2004 to 2009 we might have lost a year-to-year trend of WUE_i after N and S were removed, hiding some nutrient "payback" effect due to the treatments.

The different patterns for WUE_i suggest different physiological mechanisms behind the responses observed for the three treatments. Combining $\delta^{13}C$ and $\delta^{18}O$ in a conceptual model (Fig. 6), according to Scheidegger *et al.* (2000), enabled us to better understand how N treatments with and without S affects N use in the tree. An important prerequisite of the conceptual model is similar $\delta^{18}O$ of source water and vapour water for the investigated trees. In our study, trees were growing in the same area with closed canopies giving rise to similar Rh and treated with rainwater collected on site, so we assume that possible differences in g_s, as inferred by $\delta^{18}O$ in tree rings, can be attributed to the treatments alone.

The increase in $\delta^{13}C$ observed in all treatments during the experiment (*i.e.*, 1996-2003) indicates a reduction in the c_i/c_a ratio, explained by either an increase in A, at constant g_s or by

a reduction of g_s at constant *A*. $\delta^{18}O$, combined to $\delta^{13}C$, helps to clarify whether changes in *A*, g_s or both accounted for the variations of WUE_i.

In the case of N addition, only small changes in $\delta^{18}O$ were observed (compared to the pre-treatment time), therefore variation of WUE_i were mostly related to an increase of *A*, in response to a fertiliser effect of N (Fig. 6, side panels). In another experiment where spruce branches were sprayed with NH_4NO_3, N was shown to contribute significantly not only to enhance CO_2 assimilation but also to reduce the thermally dissipated light, suggesting an increase of photosynthetic efficiency (Tomaszewski & Sievering, 2007).

In the case of 2NS and NS treatments, the reduction of $\delta^{18}O$ compared with the control suggests that together with *A*, g_s might have increased too. Acid mist has been shown to alter cuticular wax structure (Fowler *et al.* 1980; Shepherd & Griffiths 2006 and references therein) and to affect stomatal opening, with a mechanism related to changes in apoplastic pH and in the electrochemical potential gradient across the guard cell plasmalemma (Eamus & Fowler 1990).

Based on our measurements, the beneficial effect of N on *A* tended to disappear the experiment stopped (Fig. 6, sides panels), especially in the 2NS treatment. As suggested by the larger reduction of $\delta^{13}C$ compared to $\delta^{18}O$, g_s did not change as much as *A*, which decreased after the experiment stopped. This means that excess of S in the system affected negatively tree physiological traits even six years after the spray treatment stopped, suggesting a low resilience of the forest to recover from excess S_{dep} to either tree canopies and/or soil.

Non-treatment effects on stable C and O isotope trends

Long dendro-isotope chronologies have often reported an increase of $\delta^{13}C$ in tree rings during the juvenile stage of tree growth (Duquesnay *et al.* 1998; McCaroll & Loader 2004), although this was not observed in our study. In fact, variations in $\delta^{13}C$ during the investigated period were quite consistent, with a reduction of about 4‰ over a period of six years (Fig. 2a-c). Since we considered a short chronology (*i.e.*, 15-years) data were not corrected for the ^{13}C-depletion of atmospheric CO_2, and thus may partially explain the decreasing trend for $\delta^{13}C$ in tree rings. Another explanation could be related to the age of the plantation. In fact, when the experiment started in 1996, trees were about 10-year old, in a stage of pre-canopy closure (Sheppard *et al.* 2004), which could have played a significant role, by increasing shading, which, in turn, reduced the $\delta^{13}C$, by increasing the c_i/c_a ratio, due to a low CO_2 assimilation rate for a giving g_s (Farquhar, 1980).

$\delta^{18}O$ in tree rings reflects both $\delta^{18}O$ of source water and the changes in $\delta^{18}O$ in leaf water during transpiration, modified by the Péclet effect (Craig & Gordon 1965, Farquhar & Lloyd 1993, Barbour *et al.* 2000a), in response to changes in environmental factors, e.g., precipitation and changes in summer relative humidity conditions (McCaroll & Loader, 2004). Because the treatment plots were small and randomly distributed, we can assume no differences among treatments for the oxygen isotopic signature of soil water. Further, although high variability in soil moisture was reported between plots (Sheppard *et al.* 2004), this did not significantly affect the $\delta^{18}O$ in the tree rings. We also assume that the water used for the spraying had a similar $\delta^{18}O$ in all treatments, since it was derived from the same source, *i.e.*, precipitation. Therefore, $\delta^{18}O$ in tree ring records changes in g_s due to variations in atmospheric evaporative conditions.

It is worth underlining that treatments (including the wet control) supplied an additional 20% (e.g., NS, N and wet control) and 40% (e.g., 2NS) of water to the precipitation. Therefore, the general decreasing trend observed (Fig. 2d-f) for $\delta^{18}O$ in all treatments during the first years of the experiment might be a spraying-related effect. In fact, frequent spraying (> 50 applications) over the growing season contributed to maintaining a low vapour pressure deficit, enabling the needles to maintain optimal hydration states, with no limitation to stomata opening.

Despite the fact that the additional water added during the spray treatments having possibly reduced the detection of the climatic signal, $\delta^{18}O$ proved to be a valuable archive for changes in evaporative demand from the atmosphere. In fact, the trough of $\delta^{18}O$ values in 1998, corresponding to the wettest year of the experiment, may reflected "over watering" conditions for trees, with particular reference to NS and 2NS. Moreover, the response recorded in 2003 for both $\delta^{13}C$ and $\delta^{18}O$ might be related to drought conditions all over Europe, with particular reference to trees in the treatment plots where recovery phase started (*i.e.*, the misting with S had ceased), suggesting that S might have affected tree responsiveness to seasonal changes in weather conditions.

4.2 Generalization of our findings: comparison with other studies

Only a few studies met the most relevant conditions for inclusion in the meta-analysis: fertilizer response was assessed using $\delta^{13}C$ and $\delta^{15}N$ in tree rings. It was beyond our aim to provide an estimation of the changes of absolute values of WUE_i and $\delta^{15}N$ for soil *vs.* canopy N application, rather we were interested in the extent of variations in relation to the two approaches, in order to account for differences in the underlying mechanisms.

Changes in WUEi: same direction and magnitude?

We are conscious that different parameters might account for variations of WUE_i at each of the sites considered in the meta-analysis, such as age, tree species, and different climatic and environmental conditions. However, by considering the difference from the control, we should be able to extract the influence of the treatment on WUE_i, through an effect on *A*, at a given g_s, given that the contribution of ambient N_{dep} is similar in all the sites considered.

The increase in N availability, through either soil or canopy application, enables trees to enhance the efficiency by which they assimilate CO_2, thus confirming the "almost axiomatic and strongly positive relationship" (Warren and Adams, 2005) between *A* and N supply. Overall, there seems to be a steep increase of WUE_i up to 150 kg N ha^{-1} yr^{-1} (Fig. 7). At higher doses, the ratio between *A* and g_s tends to stabilize, though it must be noted that this conclusion is contradicted by the results of Balster *et al.* (2009), where urea was used as a fertiliser supply.

Although results for both above-canopy and below-canopy fertilisation followed a similar trend for WUE_i, the magnitude tended to be higher in the case of canopy applications. In fact, for a similar dose of N applied (e.g., 48-50 kg N ha^{-1} yr^{-1}), the difference from the control for WUEi was higher for trees receiving N as mist application over tree canopies (Fig. 8), compared to those receiving the same amount supplied to the soil (Betson *et al.* 2007). At higher dose (e.g., 96-100 kg N ha^{-1} yr^{-1}), the increase of WUE_i compared with the control was in the same range reported by Elhani *et al.* (2005), where however, N was added in combination with other nutrients (e.g., K, Ca and P). Lower responses were observed by Elhani *et al.* (2005) when N was added alone (Fig. 8).

It must be acknowledged that differences in the N use efficiency and N-C allocation in young (*i.e.*, the present study) and mature (Betson *et al.*, Elhani *et al.*) trees might account for the differences in the WUE_i following N additions.

In the case of Balster *et al.* (2009) and Heaton and Crossley (1995), values deviated from others reported in Fig. 7. In the latter investigation, the spraying with acid mist resulted in a reduction of CO_2 assimilation (as assessed by the more negative $\delta^{13}C$ in the treated trees compared to the control), which was supported also by a decrease of radial growth increment during the experiment. However, in the investigation of Heaton and Crossley, the Sitka spruce at the Glencorse forest (Scotland), was potentially P limited, as a result of the acid treatment, which mobilized Al (Carreira *et al.* 1997). This result has important implication for forest management, indicating that in general, at low-level of N availability, an increase of N_{dep} can hardly match with a fertiliser effect on CO_2 assimilation and growth if other conditions are not met (e.g., other nutrients or water availability).

Footprint of N applications on $\delta^{15}N$ in tree rings: what do they tell us about the N cycle in forests?

The fertiliser applied to both soil and canopy had a similar $\delta^{15}N$ values, thus we can assume that the differences between the treatment and the control for $\delta^{15}N$ measured in the tree rings reflect changes in soil and canopy processes, rather than differences in isotope signature of the N applied.

Results of our meta-analysis show an enrichment in the heavier isotope for $\delta^{15}N$ in tree rings, with cumulative added N in the soil (Fig. 8 a). Although data in our meta-analysis are scarce,

we observed a tendency for conifers to respond more strongly than deciduous trees to N supply (Nadelhoffer *et al.* 2004), suggesting a difference in the NH_4^+ *vs.* NO_3^- uptake (Gebauer *et al.* 2000) and their specific N isotope signature. The differences observed for the $\delta^{15}N$ values measured in tree subjected to ammonium nitrate and urea applications could reflect a different isotopic signature of these compounds as well as different N processing.

Soil N applications speed up many biogeochemical processes related to N, *i.e.*, N microbial immobilization and/or losses, which in turn not only modify the immobilized N in a form more readily available to trees, but also its isotopic signature. In particular, losses of ^{15}N-depleted compounds, through denitrification, NO_3^- leaching or ammonia volatilization, which increase when the N dose exceeds the requirements of the trees', are reflected in plant material as an increase in $\delta^{15}N$ (Johannison & Högberg 1994) (Fig. 8b).

If N losses had occurred also at our experimental site in response to the spraying, they would have resulted in a significant enrichment of $\delta^{15}N$ in tree rings compared to the control. However, this was not the case. In fact, spraying of N over tree canopies tended to lower the $\delta^{15}N$ in tree rings compared to the wet control and the NS treatments (Fig. 2 g-i, Fig. 8 a). We can exclude a difference in the isotopic signature of the ammonium nitrate salt used during the experiment, which had a $\delta^{15}N$ value of 1.6‰. Likewise, the $\delta^{15}N$ value of the treatment was unlikely to have been diluted by the $\delta^{15}N$ of background N_{dep}, given that the rate of background N deposition (8 kg N ha^{-1} yr^{-1}) was much smaller than the added rates (48 and 96 kg N ha^{-1} yr^{-1}). Also, based on $\delta^{15}N$ values of NH_4^+ (-3.8±2.6 ‰) and NO_3^- (-3.8±2.4 ‰) in rainfall, measured for six different sites across Scotland (Heaton, pers. comm.), only a small dilution effect would be expected. Hence, assuming no changes in the $\delta^{15}N$ of the atmospheric

N source, the long-term trend measured for $\delta^{15}N$ in the different treatments reflect different dynamics of canopy N exchange rather than changes of N in the soil. This suggests that soil applications fail to capture all the dynamics and isotope fractionation that occur when deposition takes place on the canopy.

Before reaching the soil, the atmospheric N can be modified by the interaction with tree canopies, which can retain most of the N added by the spray treatments, leaving only a small amount to reach the soil. Chiwa *et al.* (2004) reported significant levels of canopy N retention at our experimental site, with 2NS and N showing higher N retention than NS (*i.e.*, around 80%). Further, the dynamics of canopy N exchange varied according to whether the N addition also contained S. In fact, the absolute amount of N retained as NH_4^+ and NO_3^- was similar in the 2NS treatment, whereas tree canopies in the N treatment retained more NH_4-Nthan NO_3-N (Chiwa *et al.* 2004). The $\delta^{15}N$ measured in needles (for the year 1997) and tree rings at each treatment would therefore reflect the proportion of NH_4^+ and NO_3^- ions taken up and their relative isotope signature, which can be very different (Heaton *et al.* 1997).

The substantial level of canopy N retention would have restricted the amount of N washed through to the soil, limiting any soil N accumulation or loss, thus explaining why the $\delta^{15}N$ shift goes in the opposite direction to that found when N fertilizer was added directly to the soil. In fact, no clear indication of excess N was apparent after 5 years, except in the 2NS treatment where needle fall was significantly increased (reducing canopy N uptake) and N_2O emissions were recorded (Sheppard *et al.* 2004).

Six years after the experiment stopped, $\delta^{15}N$ in the soil did not reveal any clear signal of the treatments (Fig. 4). This further suggests that only a fraction of the N reached the soil, which

eventually was diluted in relation to the large soil N pool. Treatment effects, with particular reference to the N addition, were not detectable in tree rings once treatment ceased (Fig. 2 g-i, Fig. 4). However, it must be recognized that pooling the annual rings from 2004 to 2009 precluded our ability to track the rate of loss of the treatment signal. Interestingly, a carry-over effect of the treatment-specific contribution to canopy N dynamics was still present in undecomposed litter sampled 5 years after the experiment stopped, *i.e.* more negative $\delta^{15}N$ values in the plot that received N treatment.

Conclusions

The combination of $\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$ in tree rings enhanced our understanding of how variations in atmospheric N and S deposition affect some key processes underpinning the C and N cycle in a Sitka spruce. Specifically we have shown that:

i. N independent of the presence of S, increased the ratio between assimilation (A) and stomatal conductance (g_s) in favour of A, thus supporting the fertiliser effect of N. However, the combination of N and S, as a double dose, counteracted the duration and the strength of the positive effect of N on WUE_i, likely related to a negative effect of S on stomatal control.

ii. Based on a mean value of WUE_i, trees largely returned to pre-treatment values within 5 years (2004-2009) after the cessation of N_{dep}. However, this was not the case for S_{dep}, where WUE_i continued to fall. A carry-over effect of S excess could affect negatively the balance between A and g_s, by reducing A for trees subjected to 2NS. This suggests that the beneficial role of N_{dep} is counteracted by the negative effect of S, which may still be operating in forests that have received high loads of acid rain. It is possible that such a 'payback' effect after reduction in NS deposition was also

present for the N treatment, but this was not clear from the bulk measurements for the 6-year period (2004-2009).

iii. Treatment-specific variations in the $\delta^{15}N$ in tree rings carried the effect of different canopy nitrogen dynamics, either in both the short (in needles) and the long-term (tree rings), with an isotope signal imprinted also in the litter, even after treatment stopped.

iv. Soil and canopy N applications showed an increase of WUE_i in response to increased N_{dep}. However, for the same N dose applied, spraying over tree canopies led to a larger change in WUEi compared to soil applications. By contrast, the two application methods produced different results with regard to the changes in the N dynamics. This suggests, on one hand, that experiments considering soil application might underestimate the contribution of N_{dep} on the ecosystem N cycle, since they exclude possible N exchanges at the canopy level. On the other hand, they might overestimate the effect of N_{dep} to the soil, since canopy N retention tends to reduce the quality and quantity of N reaching the soil, thus contributing to slowing down soil bioactivity and preventing N accumulation and losses.

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Table 1. Treatments of acid mist applied on a young Sitka spruce plantation at Deepsyke forest (Scotland, UK) from 1996 to 2003. In the first phase of the experiment (1996-2000) all treatments had 4 replicates, while in the second phase (2001-2003), two plots were maintened with the original treatments, while in the other two the recovery from acidity was started. Adapted from Sheppard *et al.* 2004.

Number of plots	Treatments (1996-2000) [I]	Treatments (2001-2003) [II]	Components	Doses (kg ha^{-1} yr^{-1}) N	S	pH
2	NSacid	NSacid	NH_4NO_3+H_2SO_4 [I-II]	48	50	2.5
2	NSacid	NS-recovery (N)	NH_4NO_3+H_2SO_4 [I]	48	50	2.5
			NH_4NO_3 [II]	48		4.5
2	2NSacid	2NSacid	NH_4NO_3+H_2SO_4 [I-II]	96	100	2.5
2	2NSacid	2NS-recovery (2N)	NH_4NO_3+H_2SO_4 [I]	96	100	2.5
			NH_4NO_3 [II]	96		4.5
2	N	N	NH_4NO_3 [I-II]	48		4.5
2	N	N-recovery	NH_4NO_3 [I]	48		4.5
		(wet control)	None [II]	-	-	4.5
4	Wet control	No changes	None	-	-	4.5

Table 2 Research papers included in the meta-analysis.

Authors	Location	N_{dep} (kg ha^{-1} yr^{-1})	Species	Age (years)	Fertiliser	Application	Dose applied (kg ha^{-1} yr^{-1})	Duration of the experiment	Isotope
Balster *et al.* (2009)	Interior NW, USA	N/A	*Pseudotsuga menziesii*	85	Urea	Soil	178 357	Dose applied twice over a 6-years interval	$\delta^{13}C$ and $\delta^{15}N$ in tree rings
Betson *et al.* (2007)	Norrliden, Sweden	3	*Pinus sylvestris*	~50	Ammonium nitrate	Soil	30 60 90	32 years: dose applied from 1971 to 2002	$\delta^{13}C$ in needles
Brooks & Coulombe (2009)	Washington, USA	N/A	*Pseudotsuga menziesii*	85	Ammonium nitrate	Soil	157 314 471	1 year: dose applied only once, in April 1964	$\delta^{13}C$, WUEi in tree rings
Choi *et al.* (2005)	Scotland County, NC, USA	N/A	*Pinus taeda*	15	Urea	Soil	101	7 years: dose applied every year from 1992 to 1998	$\delta^{13}C$ in needles
Elhani *et al.* (2005)	Brittany, France	9-13	*Fagus sylvatica*	80	Ammonium nitrate	Soil	100 100with PKCa	2 years: dose applied once (e.g., in May 1973 and 1974)	$\delta^{13}C$, $\delta^{15}N$ in tree rings
Heaton & Crossley (1995)	Scotland, UK	10	*Picea sitchenisis*	20	Ammonium nitrate	Canopy	48	3 years: dose applied twice per week during the grwoing season from 1991 to 1993.	$\delta^{13}C$ in needles
Nadelhoffer *et al.* (2004)	Massachusetts, USA	8	Deciduous species	~80	Ammonium nitrate	Soil	8 58 158	16 years: 6 montly additions during the growing season from 1988 to 2004	$\delta^{15}N$ in tree rings
Nadelhoffer *et al.* (2004)	Massachusetts, USA	8	*Pinus resinosa*	~80	Ammonium nitrate	Soil	8 58 158		

Table. 3. Results of the statistical analyses of the differences between treatments in $\delta^{13}C$ for the period 1995, 1996-2000, 2001-2003 and 2004-2009. Differences for the first and last period were tested by univariate ANOVA, with four replicates for each treatment. The effect of different treatments during the second period was explored by repeated-measures ANOVA, with four replicates for each treatment. Finally, differences in the recovery period were assessed by independent sample *t*-tests, with two replicates for each treatment.

Treatments	1995 Pre-treatment	1996-2000 Treatment I	2001-2003 Treatment II			2004-2009 Post-treatment
			Recovery		Treatment	
2NS	-25.91 a (±0.24)	-27.85 a (±0.14)	-29.43 (±0.13)	n.s.	-29.02 (±0.20)	-29.56a (±0.11)
NS	-26.05a (±0.15)	-28.15 a (±0.15)	-29.47 (±0.19)	n.s.	-29.27 (±0.29)	-29.29a (±0.32)
N	-26.14a (±0.09)	**-27.74 b** (±0.12)	-28.99 (±0.12)	n.s.	-29.29 (±0.14)	-29.12a (±0.02)
Wet control	-26.14a (±0.11)	**-28.46 b** (±0.12)				-29.05a (±0.15)
Statistical tests	ANOVA	Repeated-measures	Independent *t*-test			ANOVA
	F=0.479; *P*=0.7	*F*=5.912 *P*<0.05	*t*=-1.73, *P*=0.11 *t*=-0.60, *P*=0.56 *t*=1.63, *P*=0.13			*F*=1.6 *P*=0.239

Table 4. Results of the statistical analyses of the differences between treatments in $\delta^{18}O$ for the periods 1995, 1996-2000, 2001-2003 and 2004-2009. Differences for the first and last period were analysed using univariate ANOVA, with four replicates for each treatment. The effect of different treatments during the second period was explored by repeated-measures ANOVA, with four replicates for each treatment. Finally, differences in the recovery phase were assessed by indipendent sample *t*-test, with two replicates for each treatment. For the treatment I (1996-2000), estimated marginal mean are provided (*i.e.*, adjusted for the variability in the soil relative water content among plots).

Treatments	1995 Pre-treatment	1996-2000 Treatment I	2001-2003 Treatment II			2004-2009 Post-treatment
			Recovery		Treatment	
2NS	24.89a (±0.18)	24.31a (±0.14)	24.26 (±0.15)	n.s.	24.06 (±0.07)	24.07a (±0.09)
NS	25.0a (±0.07)	24.26a (±0.14)	24.15 (±0.14)	n.s.	24.08 (±0.17)	24.12a (±0.10)
N	25.02a (±0.18)	24.38a (±0.15)	24.46 (±0.16)	n.s.	24.37 (±0.13)	24.33a (±0.12)
Wet control	24.91a (±0.22)	24.54a (±0.14)				24.22a (±0.13)
Statistical tests	ANOVA	Repeated-measures	Independent *t*-test			ANOVA
	F=0.145 *P*=0.9	*F*=0.793 *P*= 0.5	*t*=1.156, *P*=0.8 *t*=0.319, *P*=0.3 *t*=0.409, *P*=0.7			*F*=1.587 *P*=0.2

Table 5. Results of the statistical analyses of the differences between treatments in $\delta^{15}N$ for the periods 1995, 1996-2000, 2001-2003 and 2004-2009. Differences for the first and last period were analysed using univariate ANOVA, with four replicates for each treatment. The effect of different treatments during the second period was explored by repeated-measures ANOVA, with four replicates for each treatment. Finally, differences in the recovery phase were assessed by indipendent sample *t*-test, with two replicates for each treatment.

Treatments	1995 Pre-treatment	1996-2000 Treatment I	2001-2003 Treatment II			2004-2009 Post-treatment
			Recovery		Treatment	
2NS	-3.63a (±0.31)	-2.89ab (±0.15)	-2.85 (±0.13)	n.s.	-2.78 (±0.27)	-2.32a (±0.37)
NS	-3.28a (±0.36)	-2.64a (±0.57)	**-1.64 (±0.49)**	*.	**-3.29 (±0.17)**	-2.33a (±0.62)
N	-3.49a (±0.31)	**-3.46 b (±0.12)**	-3.06 (±0.12)	n.s.	-2.94 (±0.12)	-2.25a (±0.54)
Wet control	-3.08a (±0.359)	-2.43a (±0.18)				-2.77a (±0.23)
Statistical tests	ANOVA	Repeated-measures	Independent *t*-tests			ANOVA
	F=0.504 *P*=0.69	*F*=2.92 *P*= 0.07	*t*=-2.17, *P*=0.83 *t*=3.22, *P*=0.009 *t*=-0.30, *P*=0.77			*F*=0.198 *P*=0.89

Figure 1. (a) Distribution of replicates for each treatment, modified from Chiwa et al. (2004). **(b)** Picture of the experimental site with spraying system set up. The different colours visible in panel (a) refer to different treatments: black=2NS, red=NS, green=N and blue=Wet control. Black lines delimite the 4 replicated blocks designed South, West, North and East. Picture in the panel (b) was provided by Dr. Alan Crossley (CEH).

Figure 2. Trend of $\delta^{13}C$ (panels a, b, c), $\delta^{18}O$ (panels d, e, f) and $\delta^{15}N$ (panels g h i) in tree rings from 1995 to 2009 for the different treatments (NS, 2NS, N and wet control) applied at Deepsyke forest. For the years 1995-2000 each points is the mean±SE of *n*= 4, while for years 2001-2003, means were calculated over *n*=2 plots for the recovery and *n*=2 plots for the original treatment. Please note that for $\delta^{18}O$ relative to the year 2002, in NS we show only a single value measured in one plot. Shaded areas mark the pre-treatment and post-treatment periods and the vertical line, the start of the recovery period. A year-to-year significant (*P*<0.1) effect of the treatment as assessed by LSD test is indicated by the star. The trough in 1998 for $\delta^{18}O$ in all the treatments and in the wet control corresponds to the wettest year of the experiment.

Figure 3. Differences between treatments (2NS, NS and N) and control for $\delta^{15}N$ (panel a) and $\delta^{13}C$ (panel b) measured in needles (open points) and the corresponding annual ring (closed points) in 1997. For each isotope, stars ((*) for *P*<0.1 and (**) for *P*<0.05) indicate significant differences between annual rings and needles. Capital and small letters refers to ANOVA tests of differences across treatments for needles and tree rings, respectively. Each point represents the mean± confidence interval (*n*=4).

Figure 4. $\delta^{15}N$ values measured in soil and litter sample collected in June 2009 and in the group of annual rings from 2004 to 2009. Each value is the mean ± SE for *n*=4 plots.

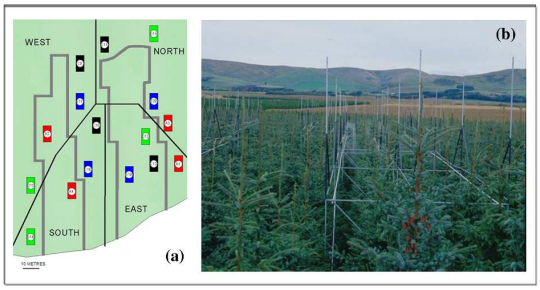
Figure 5. Differences from the wet control (*i.e.*, treatment-control) for WUEi values measured in 2NS (panel a), NS (panel b) and N (panel c) treatments. Each point represents the mean of the values measured in *n*=4 plots for each treatments; bars are confidence interval. Here, we avereged the values measured for each year from 1996 to 2003 in *n*=4 plots within each treatment, without distinguishing between recovery and original treatment in the last 3-years of the experiment. Shaded areas indicate the pre and post-treatment periods.

Figure 6. Main panel. Combination of $\delta^{13}C$ and $\delta^{18}O$ values for 2NS, NS and N treatment, evaluated as difference from the wet control (treatment-wet control). For the pre-treatment year (e.g., 1995), we considered the mean of the differences from the wet control for $\delta^{13}C$ and $\delta^{18}O$ calculated over all the values measured in each treatments (*n*=12). Bars represent the confidence intervals. Values relative to the eight years of the experiment (*i.e.*, 1996-2003) were averaged for each treatment, without separating recovery from the original treatment. Post-treatment refer to the grouped annual rings from 2004 to 2009 in each treatment (*n*=4). **Side panel.** Interpretation of the $\delta^{13}C$-$\delta^{18}O$ trends based on the conceptual model (Scheidegger *et al.* 2000). According to the model, variations of c_i and therefore A are derived from $\delta^{13}C$, while changes in g_s were predicted from $\delta^{18}O$.

Figure 7. Difference between treatment and control for WUE_i calculated from $\delta^{13}C$ values measured in either tree rings or needles. Soil and canopy N application are indicated with brown and blue symbols, respectively. Full symbols refer to deciduous species while open symbols to conifers. We used crossed squares and circles when WUE_i was calculated from $\delta^{13}C$ in needles. Each point is the mean calculated from values measured during the fertilization experiments

considered in the meta-analysis and published in the papers indicated in the legend. Bars are confidence intervals.

Figure 8. (a) Difference between treatment and wet control for $\delta^{15}N$ measured in tree rings, compared across five studies. Soil and canopy N addition are indicated with brown symbols and blue colours, respectively. Full symbols refer to deciduous species while open symbols to conifers. Each point is the mean calculated over the values measured during the fertilization experiments considered in the meta-analysis and published in the papers indicated in the legend. (**b)** Example of ^{15}N- enrichment of $\delta^{15}N$ measured in plant (full symbols) and soil (open symbols) at increasing level of N supplied to the soil (source: Johannison & Högberg 1994). The picture in the panel (b) is provided as valuable example of the ^{15}N-enrichment of $\delta^{15}N$ in plant and soil at higher doses of N supplied to the soil, mainly related to the increase of loss of ^{15}N-depleted N compounds through denitrification, ammonia volatilization or nitrate leaching (Johannison & Högberg 1994).



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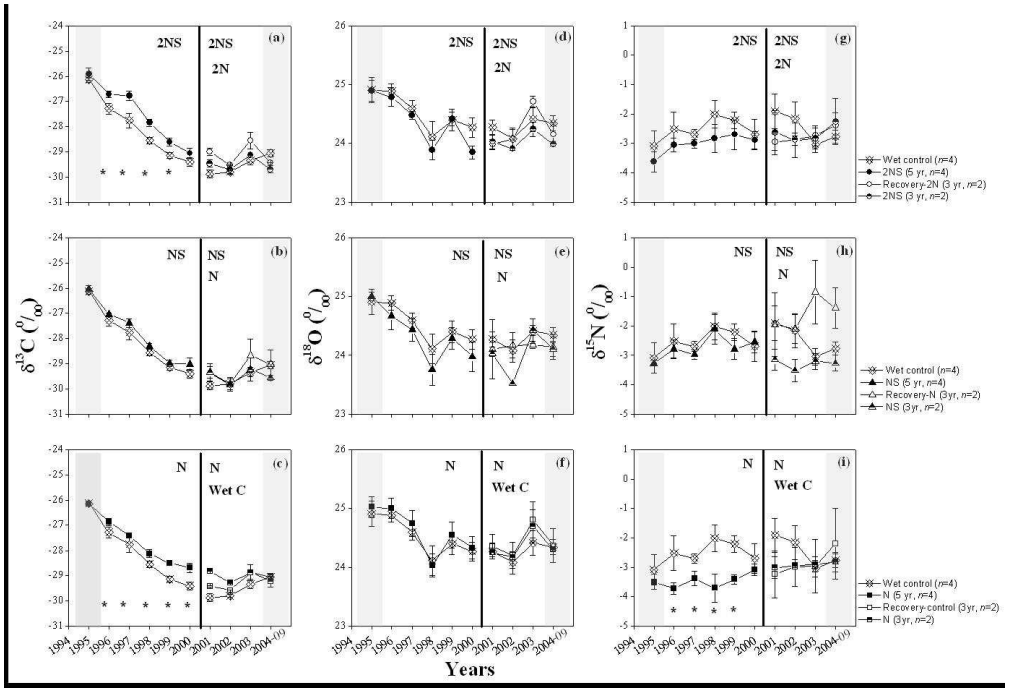

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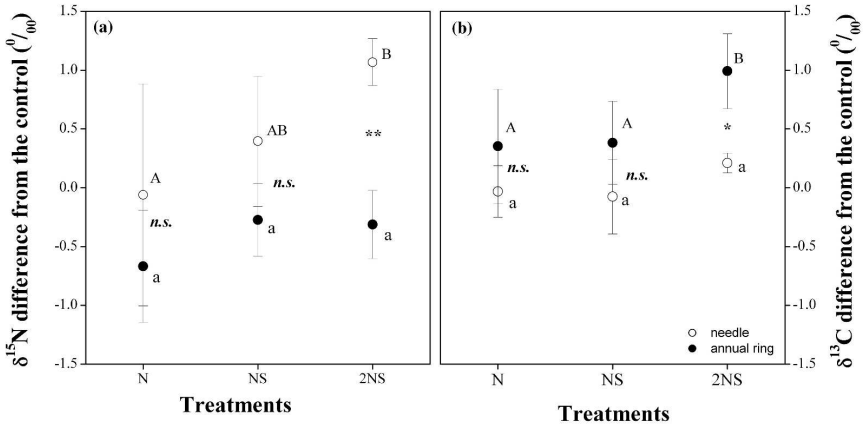

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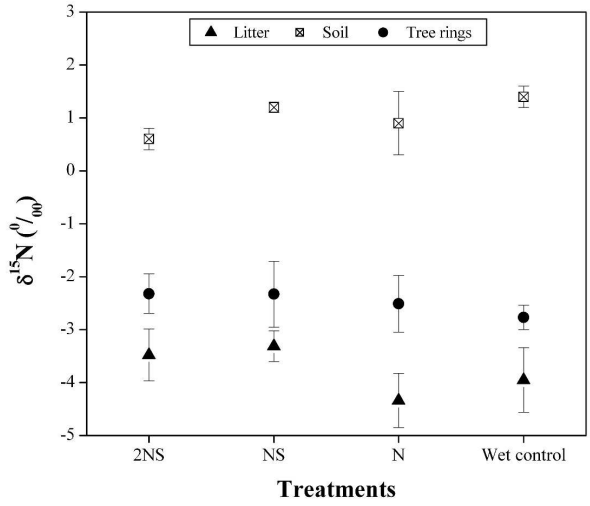

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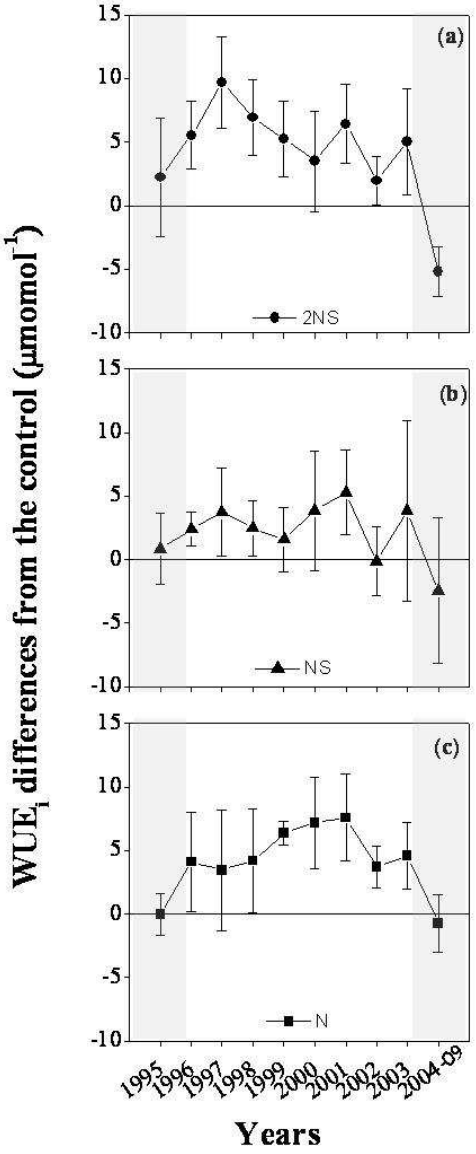

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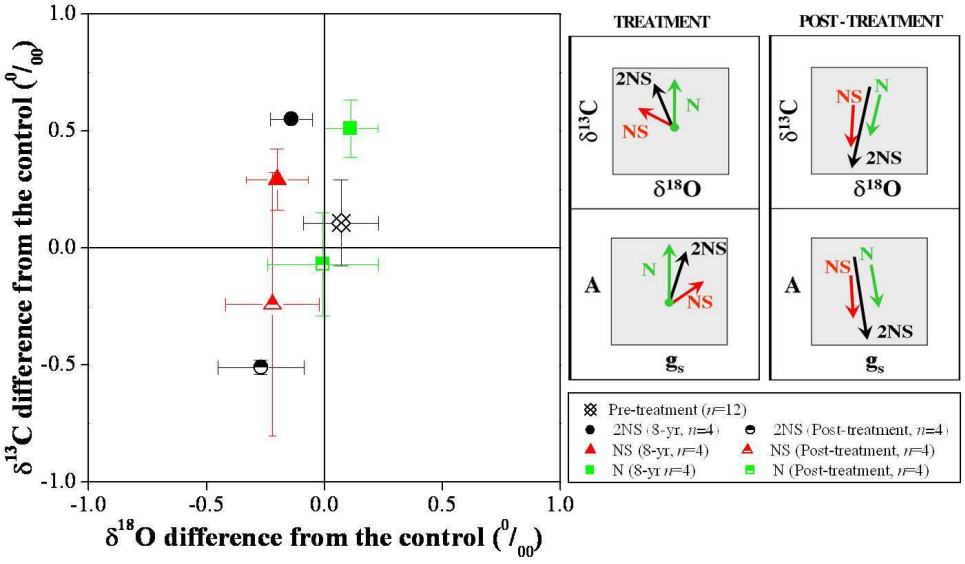

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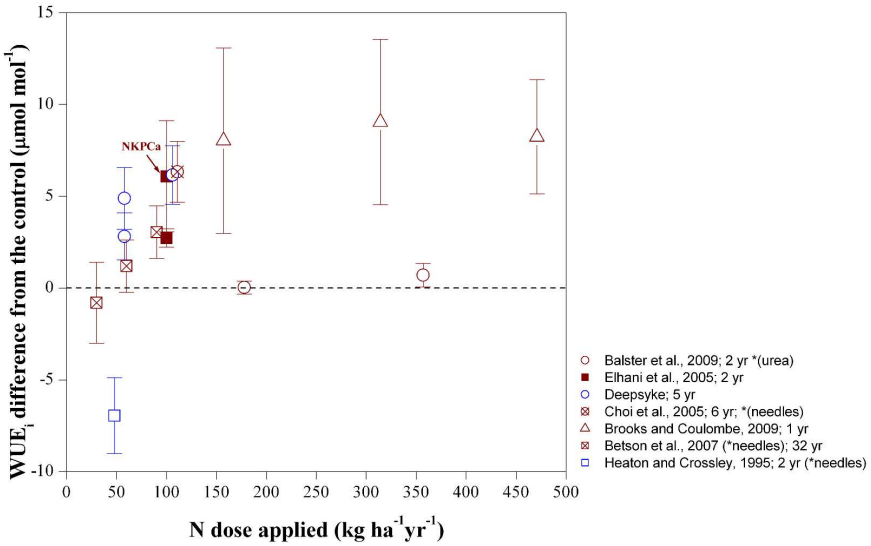

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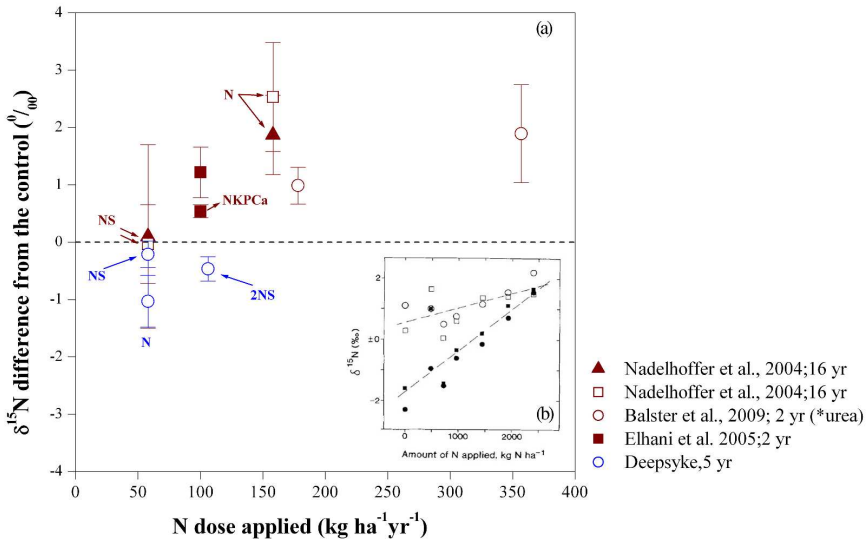

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