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Isotopic evidence for the occurrence of biological nitrification and nitrogen deposition processing in forest canopies

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Abstract

This study examines the role of tree canopies in processing atmospheric nitrogen (N_{dep}) for four forests in the UK subjected to different N_{dep}: Scots pine and beech stands under high N_{dep} (HN, 13-19 kg N ha^{-1} yr^{-1}), compared to Scots pine and beech stands under low N_{dep} (LN, 9 kg N ha^{-1} yr^{-1}). Changes of NO_3-N and NH_4-N concentrations in rainfall (RF) and throughfall (TF) together with a quadruple isotope approach, which combines $\delta^{18}O$, $\Delta^{17}O$ and $\delta^{15}N$ in NO_3^- and $\delta^{15}N$ in NH_4^+, were used to assess N transformations by the canopies. Generally, HN sites showed higher NH_4-N and NO_3-N concentrations in RF compared to the LN sites. Similar values of $\delta^{15}N$-NO_3^- and $\delta^{18}O$ in RF suggested similar source of atmospheric NO_3^- (*i.e.*, local traffic), while more positive values for $\delta^{15}N$-NH_4^+ at HN compared to LN likely reflected the contribution of dry NH_x deposition from intensive local farming. The isotopic signatures of the N-forms changed after interacting with tree canopies. Indeed, ^{15}N-enriched NH_4^+ in TF compared to RF at all sites suggested that canopies played an important role in buffering dry N_{dep} also at the low N_{dep} site. By using two independent methods, based on $\delta^{18}O$ and $\Delta^{17}O$, we quantified for the first time the proportion of NO_3^- in TF, which derived from nitrification occurring in tree canopies at the HN site. Specifically, for Scots pine all the considered isotope approaches detected biological nitrification. By contrast for the beech, only by using the mixing model with $\Delta^{17}O$ we were able to depict the occurrence of nitrification within canopies. Our study suggests that tree canopies play an active role in the N cycling within forest ecosystems. Processing of N_{dep} within canopies should not be

neglected and needs further exploration, with the combination of multiple isotope tracers, with particular reference to $\Delta^{17}O$.

Key words: *Nitrogen deposition, $\delta^{15}N$, $\delta^{18}O$, $\Delta^{17}O$, NH_4-N, NO_3-N, DON, forest canopy interception, canopy nitrification, Scots pine, beech*

Introduction

Forest canopies play a significant role in regulating carbon and water exchanges with the atmosphere, with profound effects on climate (Bonan 2008; Candell et al., 2007; Schulze, 2006). However, the contribution of tree canopies in altering the chemical composition of precipitation and, consequently, the nutrient cycling within a forest has been less investigated. In particular, it is unclear whether the deposition of reactive nitrogen species (N_{dep}) to canopies is retained, re-emitted and/or altered by chemical or biological reactions, and what portion and chemical form of deposited N eventually reaches the soil as washed out N-compounds. Interception of N_{dep} by forest canopies contributes to the cycling of N in the terrestrial biosphere, thereby affecting plant health, community structure and biodiversity, nutrient cycling, greenhouse gas balance, soil pH and water quality (Cape and Pearcy, 1998; Galloway et al., 2004; Lindberg et al., 1986; Pitcairn et al. 1998; Pitman et al., 2010; Prescott, 2002; Rennenberg and Gessler, 1999; Vanguelova et al., 2011; Vitousek et al,. 1997).

Understanding the interactions taking place between atmospheric N and forest canopies, under different environmental conditions and N_{dep} levels, for various forest types (*e.g.*, conifer *vs.* broadleaf forests) and tree species remains complex. Systematic monitoring of the main N chemical species (*i.e.*, NH_4^+, NO_3^-, dissolved organic N) in rainfall (RF) and throughfall (TF) has now been carried out for almost two decades in a network of experimental European forests (*i.e.*, Level II network of ICP plots http://icp-forests.net/).

While these measurements quantify the atmospheric N inputs to forests and soils, they have not been sufficient to allow assessing in-canopy processes that may be affecting changes in N compounds.

Forests are particularly efficient at scavenging pollutants via dry and occult deposition due to their aerodynamically rough canopies (Fowler et al. 1989). As a consequence, the total N speciation and N concentrations in RF differ from those in TF. Fluxes of N in TF reflect a mixture of wet, occult (fog/cloud), and dry deposition, that may also be chemically or biologically modified during canopy exchange and uptake. Commonly, TF has a higher N-compounds concentration compared with RF, particularly in areas subjected to high N input from the atmosphere, which provide indication of dry N_{dep} inputs (Fang et al. 2011; Lovett, 1994; Lovett et al., 2000; Lovett and Lindberg 1993; Tietema and Beier, 1995; Vanguelova et al., 2010). Occult deposition can also be marked in areas where seasonal fogs and N pollution sources coincide. This has resulted in very large N inputs (25–45 kg ha^{-1} yr^{-1}) in some areas such as the most highly exposed forests of the Los Angeles air basin (Bytnerowicz and Fenn, 1996). Using a labelled N approach, foliar uptake of aqueous N was recently proved to occur in beech and birch, with NH_4^+ more readily taken up than NO_3^- (Wuyts et al., 2015). Ammonia is readily absorbed directly onto foliage (see the review by Pearson and Stewart, 1993) and TF N fluxes are enhanced in forests that are near NH_3 sources such as agricultural and farming areas (Vanguelova and Pitman, 2009). Moreover, in very low N_{dep} areas (*e.g.*, total N_{dep} of 2–3 kg ha^{-1} yr^{-1}), such as in Finland, tree canopies tend to retain much of the N they capture by dry deposition due to uptake by epiphytic lichens, microbial immobilization within the canopy, N absorption into foliage and assimilation by leaves and stems (Mustajärvi et al., 2008). A recent study conducted in Italian forests reported an apparent canopy consumption of N for sites at low N_{dep}, *i.e.,* < 4-6 kg N ha^{-1} yr^{-1} (Ferretti et al., 2014). Similarly, in a study conducted in three National Parks in Washington State (USA) subjected to low N_{dep}, up to 90% of the atmospheric N, mostly in the form of NO_3-N, was found to have been consumed by the forest canopies (Fenn et al., 2013).

The stable nitrogen isotope composition ($\delta^{15}N$) of wet N_{dep} has helped to characterize the sources of atmospheric N (Freyer, 1991; Heaton, 1987, Kendall et al. 2007 and references therein) and its transformations when interacting with the biosphere, as assessed through measurements of $\delta^{15}N$ in plants and soil (Ammann et al., 1999; Guerrieri et al., 2009, 2011; Nadelhoffer et al., 1999; Saurer et al., 2004; Savard et al., 2009). In addition, observations have been made of changes in the $\delta^{15}N$ of NO_3^- in TF that suggested the occurrence of nitrification processes (*i.e.*, from NH_4^+ to NO_3^-) in the canopy of Norway spruce of central Europe (Sah and Brumme, 2003) and of a montane rain forest in Ecuador (Schwarz et al., 2011). Teuber et al. (2007) found evidence that autotrophic nitrifiers were present in the needles of a spruce forest exposed to high levels of N_{dep} (but not in needles of tree canopies exposed to low levels of N_{dep}), and proposed that canopy N transformations may partly be bacterial. However, a broad range of processes can lead to similar alterations of TF isotopic composition, so distinguishing between various processes using a single-isotope approach is challenging.

The application of the dual isotope approach, *i.e.*, the combined measurement of $\delta^{15}N$ and $\delta^{18}O$ in NO_3^- in bulk precipitation and stream water has provided another important step towards a better understanding of the importance of N_{dep} and of its cycling in forests. For example, $\delta^{18}O$ can help assess whether the NO_3^- in the soil solution derives from atmospheric N or from nitrification processes. This is possible because of the large difference between the isotopic signature of the atmospherically-derived NO_3^- (between 20 and 80 ‰) and the signature for the NO_3^- derived from nitrification (between -10 and +10 ‰, Kendall, 1998; Burns and Kendall, 2002).

An even more powerful approach has been proposed by Michalski et al. (2002, 2003) and Costa et al. (2011) based on the measurements of $\delta^{17}O$, together with $\delta^{18}O$, to characterize the sources of NO_3^-. Mass-dependent isotope fractionation leads to a consistent relationship between $\delta^{17}O$ and $\delta^{18}O$, *i.e.*: $\delta^{17}O \approx 0.52 \times \delta^{18}O$ (Matsuhisa et al., 1978; Miller, 2002; Young et al., 2002). However, in the case of ozone-mediated nitrate formation in the atmosphere, mass-independent oxygen isotope compositions are observed (Michalski et al., 2002). This 'excess' of ^{17}O is quantified by $\Delta^{17}O = \delta^{17}O - 0.52 \times \delta^{18}O$. This means that ozone-derived NO_3^- has a $\Delta^{17}O > 0$, while mass-dependent nitrification produces NO_3^- with $\Delta^{17}O = 0$. These new tools offer the possibility to test some of the hypotheses previously proposed in the literature, in particular to determine the relative contribution of occult dry deposition and of bacterial-mediated nitrification in tree canopies to the chemical composition of canopy TF and the N input to the soil.

This study investigated whether N transformations occurred within the tree canopies of four different forests in the UK subjected to different levels of N_{dep}. The NO_3-N and NH_4-N and Dissolved Organic Nitrogen (DON) concentrations in RF and TF were used to assess the role of canopy in filtering, retaining and processing atmospheric N. Furthermore, we used $\delta^{15}N$-$\delta^{18}O$ and $\Delta^{17}O$ in NO_3^- and $\delta^{15}N$ in NH_4^+, to assess if and how atmospheric N is processed within the canopy. In particular we tested the following hypotheses: 1) In forests with low to intermediate levels of N_{dep} (*i.e.*, about 10 kg ha^{-1} yr^{-1}) no differences exist between RF and TF for either ions concentrations or their isotopic signature, regardless the tree species. In cases when most of the atmospheric N is retained in the canopies, the isotopic signatures of NO_3^- and NH_4^+ in TF should still reflect that of atmospheric N in RF, as a result of low canopy processing and canopy uptake. 2) At high N_{dep} sites, exceeding critical N loads (*i.e.*, 20-30 kg ha^{-1} yr^{-1}), significant differences exist between RF and TF for both NH_4-N and NO_3-N concentrations and their isotopic signature, as a result of isotope fractionations during N processing within the canopy and enhanced by the high input of wet and dry N_{dep}. For the first time we used two independent approaches, based on $\Delta^{17}O$ and $\delta^{18}O$ in NO_3^- to determine the occurrence of bacterial nitrification from NH_4^+ to NO_3^- in forest canopies at high N_{dep} levels.

Materials and Methods

Site description and sampling

Two Scots pine (*Pinus sylvestris* L) and two beech (*Fagus sylvatica* L.) stands were studied. The pine stands were within the UK Forest Monitoring network (http://www.forestry.gov.uk/fr/INFD-67MEVC; Vanguelova et al., 2010), which is part of the ICP European Forest Network. The two beech stands are part of long term experiments on monitoring of the effects of N_{dep} on forest and soil biochemical cycling in the UK (Vanguelova and Pitman, 2009, 2011). Two forests, one for each tree species, were situated at Alice Holt and Rogate (6 km apart) in South East England and the remaining two at Thetford (< 8 km apart), East England. They were chosen on the basis of similarity in stand (age, density, and management history), climate, and soil conditions, but at contrasting levels of ambient N_{dep} (Table 1). In particular, the pine and beech stands at Thetford are subjected to higher background levels of N_{dep} (13 kg N ha^{-1} yr^{-1} and 19 kg N ha^{-1} yr^{-1}, respectively) compared to forest stands at Alice Holt and Rogate (9-10 kg N ha^{-1} yr^{-1}) (Table 1). Thetford in East Anglia, is known to be among the areas with highest atmospheric N inputs in the UK (RoTAP report, 2012; Vanguelova et al., 2010), mostly in the reduced form, coming mainly from the intensive livestock farms (in particular pigs and chickens). Therefore, the two forest stands in Thetford will be referred to as HN (high nitrogen) and the forests in Rogate and Alice Holt as LN (low nitrogen) sites. Rainfall (RF) and throughfall (TF) sampling and analysis have been carried at the sites over a number of years by means of two bulk RF collectors and ten TF collectors per site. Sampling and analytical procedures followed the level II protocols described in detail in the ICP Forests manual (2010). In this study only samples collected bi-weekly during the 2011 growing season, from June until November, were considered.

Chemical and isotope analyses of water samples

After collection, RF and TF water samples were filtered through a 0.45 μm membrane filter and then analysed for NH_4-N, colorimetrically, and total N by Carbon analyser (Shimadzu 5000, Osaka, Japan) and for NO_3-N by Ion Chromatography (Dionex DX-500). Dissolved organic nitrogen (DON) was calculated from the difference between measured total and inorganic nitrogen forms. Chemical analyses were carried out on water samples collected from each of the RF and TF collectors. The RF and TF elemental fluxes were calculated using measured water volumes at the sites and measured elemental concentrations. Dry N_{dep} values were estimated as the difference between TF and RF for each of the N-forms according to European ICP forest monitoring manual, which assumed zero canopy exchange (ICP, 2010) (Table 1-2). To check this assumption, we compared values measured at our sites with the 5x5 km grid modelled N_{dep} dataset for the UK, as used in the RoTAP review (2012). The estimate included wet and dry NH_x-N (NH_4, NH_3) and NO_y-N (NO_2, NO_3, HNO_3) deposition, modelled with FRAME upon 2005 emissions data (RoTAP review, 2012 - chapter 4).

A sub-sample of the water analysed for ion concentrations was used for stable isotope measurements. Based on measured concentrations, we worked out the volume of water needed to obtain NH_4-N and NO_3-N concentrations > 0.5 mg. For this reason, we combined water collected from June until August and then from September until November and we considered (on average between the two time windows considered) 1.5 l for RF and 1 l for TF in the case of forests at HN, while 2 l for RF and 3-4 l for TF in the case of forests at the LN. Pooling was also necessary for RF water samples collected at the two LN and the two HN sites because not enough volume of water was available for each of the two forests at the LN. We assumed that pooling RF water samples within each level of N_{dep} was not likely to have an impact on the characterization of the isotopic signature of the atmospheric N, due to similar atmospheric N input and source. Indeed, no significant differences was found in the amount of NO_3-N and NH_4-N in RF at either of the two sites, except at Thetford where the NH_4-N was significantly ($p<0.05$) higher in the beech relative to the pine stand at the HN site. This was likely the result of the beech site being located only a few hundred meters away from a chicken farm that generates NH_3 concentrations as high as ~73 µg/m^3 (Vanguelova and Pitman, 2009, 2011).

Each RF and TF sample was composited as described above and then passed through cation and anion exchange resins. Ammonium from the cation resin was eluted with hydrochloric acid and converted to ammonium sulfate on a quartz filter paper using an alkaline diffusion method (Heaton, 2001). Nitrate from the anion resin was eluted with hydrobromic acid, and processed to silver nitrate (Chang et al., 1999; Heaton et al., 2004). The $^{15}N/^{14}N$ ratios of the ammonium sulfate and the silver nitrate were analysed by combustion in a Flash EA on-line to a Delta Plus XL mass spectrometer (ThermoFinnigan, Bremen, Germany), with $\delta^{15}N$ values versus air (atmospheric N_2) calculated by comparison with standards calibrated against IAEA N 1 and N 2 assuming these had values of +0.4‰ and +20.3‰, respectively. $^{18}O/^{16}O$ ratios of the silver nitrate were analysed by thermal conversion to CO gas at 1400°C in a TC-EA on-line to a Delta Plus XL mass spectrometer (ThermoFinnigan, Bremen, Germany), with $\delta^{18}O$ values calculated versus SMOW by comparison with IAEA-NO_3 assuming it had a value of +25.6‰. Analytical precisions (1 SD) were typically <0.3‰ for $\delta^{15}N$ and <0.6‰ for $\delta^{18}O$. Finally, a sub-sample of the composite RF and TF water as described above was used for $\delta^{17}O$ measurements by Delta V Plus ratio mass spectrometer. The NO_3^- was converted to O_2 and N_2 using the denitrifier method (Casciotti et al., 2002; Kaiser et al., 2007). Analytical precisions (1 SD) for $\Delta^{17}O$ were <1.0‰ based on replicate analysis of the reference material USGS35.

Statistical analyses

Concentrations of NH_4-N, NO_3-N were log-transformed to account for non-normality and variance heterogeneity, as assessed through Shapiro and Levene test, respectively. Independent sample *t*-tests were employed to test for differences between deposition levels (*e.g.*, HN and LN) and water samples (i.e., RF and TF) for NH_4-N and NO_3-N, while, within each water sample, differences between concentrations of different compounds were tested through paired-samples *t*-tests (*t*). The non-parametric Wilcoxon test (*W*) was employed when log-transformed data did not conform to a normal distribution. Given the small sample size available for the isotopic data (*i.e.*, n= 2 for RF and n= 4 for TF per level of N_{dep}, as a result of pooling the water samples collected from June until August and then September until November), we calculated the difference in isotopic fractionation between TF and RF without separating beech and pine stands and used a *t*-test to verify the significance of the difference between LN and HN stands. The level of significance of all statistical tests was set as $p \leq 0.05$. R project statistical computing (vers. 3.0.2; R Core Development Team, 2014) was used for all the analyses.

Mass balance calculations based on $\Delta^{17}O$ *and* $\delta^{18}O$

To assess the proportions of atmospheric *vs.* biologically derived NO_3^- collected in the TF underneath tree canopies at the HN site (*i.e.*, Scots pine and beech forests), we considered two independent methods as described in Riha et al., (2014). The methods were only employed at the HN sites as our data suggested little to none canopy processing at the LN sites (cf., Results section, Fig.1).The first one is a mass balance approach based on the use of $\Delta^{17}O$ in the following equation:

$$\Delta^{17}O_{Tf} = f_{Bio}(\Delta^{17}O_{Bio}) + f_{Atm}(\Delta^{17}O_{Atm}) \qquad (1)$$

where $\Delta^{17}O_{Tf}$ is the measured isotopic composition of NO_3^- in TF, while $\Delta^{17}O_{Bio}$ and $\Delta^{17}O_{Atm}$ indicate the isotopic signatures of the biologically and atmospherically-derived NO_3^-, respectively. The f_{Bio} and f_{Atm} are the two unknown NO_3^- flux fractions from the two different sources, the sum of which is 1. The f_{Atm} included the fractions of both the wet (f_{wet}) and the dry (f_{dry}) NO_3^- deposition washed out from the canopy, net of the fraction retained and/or taken up by the canopies (f_U), *i.e.*, $f_{Atm}=f_{Wet}+f_{Dry}-f_U$. Assuming that $\Delta^{17}O_{Bio}=0$ (Michalski et al. 2003) and that $\Delta^{17}O$ in RF reflected both wet and dry N_{dep}, equation 1 can be reduced to:

$$f_{Atm} = (\Delta^{17}O_{Tf} / \Delta^{17}O_{Atm}) \qquad (2)$$

and

$$f_{Bio} = 1 - f_{Atm} \qquad (3)$$

The assumption of similar $\Delta^{17}O$ values for wet and dry N_{dep} stems from the fact that $\Delta^{17}O$ in atmospherically-derived nitrate is mostly determined by photochemical oxidation of NO_x by tropospheric ozone (Michalski et al., 2011), not the phase (gaseous, solid, or liquid) into which it is partitioned. Measurements of aerosol nitrate and rain NO_3^- collected during the same season do not have significant differences in $\Delta^{17}O$ values (Riha 2013, Michalski et al., 2011). Hence it is not related to specific point emission sources and it is not affected by mass-dependent isotope fractionations, which, in fact play a significant role in the case of the other two isotope ratios, *i.e.*, $^{18}O/^{16}O$ and particularly $^{15}N/^{14}N$.

The second method considered the $\delta^{18}O$ measured in NO_3^- based on the following equation:

$$f_{atm} = (\delta^{18}O_{Tf} - \delta^{18}O_{Nitr})/(\delta^{18}O_{Atm} - \delta^{18}O_{Nitr}) \tag{4}$$

where $\delta^{18}O_{Tf}$, $\delta^{18}O_{Atm}$ and $\delta^{18}O_{Nitr}$ are the oxygen isotopic signatures of the NO_3^- in TF, atmospheric deposition (combined RF and dry N_{dep}) and produced from nitrification, respectively. $\delta^{18}O$ of NO_3^- derived from nitrification was calculated by considering that two oxygen atoms in the formed NO_3^- were derived from atmospheric water (*i.e.*, RF) and one from atmospheric O_2 as described in the following equation (Mayer et al., 2001):

$$\delta^{18}O_{nitr} = \frac{2}{3}(\delta^{18}O_{Rf} + \varepsilon_{Rf}) + \frac{1}{3}(\delta^{18}O_{O_2} + \varepsilon_{O_2}) \tag{5}$$

Assuming negligible the isotope fractionation during water (ε_{Rf}) and O_2 (ε_{O_2}) incorporation (Mayer et al., 2001), $\delta^{18}O$ of NO_3^- from nitrification was obtained from $\delta^{18}O$ of atmospheric O_2 ($\delta^{18}O_{Atm}$= 23.9 ‰, Barkan and Luz, 2005) and the oxygen isotopic signature of the RF. We have assumed this latter to have values of about -5.5‰ (for June-August) and -8.5‰ (for September-November), based on the weighted mean $\delta^{18}O$ values for June-August 2011 and September-November 2011 rainfall at a site near Oxford in the UK (W.G. Darling, personal communication).

Results

Concentrations of NH_4-N, NO_3-N and DON in RF and TF

The concentration of N compounds varied between LN and HN sites and between RF and TF. At the two LN forests the concentrations of ions in RF were not significantly different (Fig. 1A and B) and the RF and TF had similar NH_4-N and NO_3-N concentrations, (Scots pine: t=1.78, 7.97 and p=0.11, 0.56, respectively; beech: W=163, 125 and p=0.73, 0.48, respectively). In contrast, at the HN forests, the NH_4-N and NO_3-N concentrations were significantly higher in TF compared to RF, for both Scots pine (t=6.42, 6.26, respectively; all

$p<0.001$) and beech ($W=265$, 250, respectively; all $p<0.001$) (Fig. 1A and B). Ion concentrations in both RF and TF were significantly higher at the HN than at LN sites, with the exception of RF in the beech stands, which had similar NO_3-N concentrations. Concentrations of DON in both RF and TF were significantly (RF: $W=32$, $p<0.05$; TF: $W=34$, $p<0.01$) higher for the beech stand (Fig. 1D) at the HN compared to LN site. By contrast, Scots pine (Fig. 1D) subjected to different atmospheric N loads from the atmosphere showed similar values of DON concentrations in both RF and TF. However, DON concentrations in RF did not show a significant difference when comparing beech and Scots pine stand at the LN site, while concentrations were slightly higher ($W= 35$, $p=0.05$) at the beech compared to the Scots pine stand at the HN site. Concentrations of DON in TF were similar at the two LN forests, while they were higher ($W= 33$; $p<0.05$) at the beech than the Scots pine stand at the HN site (Fig. 1D).

Extrapolation of our seasonal measurements over time and model validation of estimated dry N_{dep} fluxes

The mean of total N fluxes during the 6 months we considered in this study (*i.e.,* June to November 2011) are reported in Table 2. TF-N fluxes were higher than RF fluxes at the two forests at the HN, with particular reference to the NH_4-N at the beech site. By contrast, at the LN site RF N-fluxes were higher than TF-N fluxes for both species. An independent estimate of the dry N_{dep} at our sites can be obtained using the modelling approach outlined in RoTAP (2012). Figure S1 (in Supplementary materials) shows a comparison of the measured wet N and estimated dry N fluxes (*i.e.,* as a difference between TF and RF fluxes) at the two level of N_{dep} and shown in Table 1, with the fluxes of wet and dry N_{dep} obtained from the 5x5 km grid UK map, based on modelled N_{dep} with FRAME upon 2005 N emissions data (RoTAP, 2012). Interestingly, a reasonably good agreement was found between the on-site measurements and the modelled values of wet N_{dep}. However, the fluxes of dry N_{dep} predicted using the RoTAP modelling approach were much higher than those estimated as the difference between TF and RF fluxes at our sites (Fig. S1).

Values of $\delta^{15}N$-NH_4

Values of $\delta^{15}N$-NH_4^+ in RF (Fig. 2A) ranged from positive at the HN site (+ 1.49 ±3.5 ‰) to very negative at the LN site (-9.14‰±0.2). Due to the limited number of RF measurements (*i.e.*, n=2 per level of N_{dep}), statistical analyses of isotope data were performed per level of N_{dep}, combining data for both tree species and focussing on the differences between RF and TF. However, TF values measured separately for beech and Scots pine are presented in Figure 2A, to show the species-specific changes in the isotope compositions in N compounds collected below the canopies. More positive values were measured for $\delta^{15}N$-NH_4^+ in TF compared to RF at both HN (t=-2.85, p<0.05) and LN (t=-15.16, p<0.001) sites. The TF-RF difference for $\delta^{15}N$ in NH_4^+ was much higher (t=-2.65, p<0.05) at the LN compared to the HN site (Fig. 2B).

Values of $\delta^{15}N$, $\delta^{18}O$ and $\Delta^{17}O$-NO_3

The $\delta^{15}N$ in NO_3^- of RF (Fig. 3A) showed similar negative values at the HN (-3.4 ‰ ± 1.4) and LN sites (-2.8‰± 1.7). Albeit lower, the $\delta^{15}N$-NO_3^- values in TF at the HN site (diff= -4.9 ‰ ± 3.4) were only slightly different (t= -1.72, p=0.06) compared to the LN sites (diff= +1.1 ‰ ± 0.54) (Fig. 3D). Despite differences between RF and TF for $\delta^{15}N$ in NO_3^- not being significant within each level of N_{dep}, $\delta^{15}N$ in NO_3^- showed more negative values in TF than RF at the HN site at the Scots pine stand (Figure 3A).

The $\delta^{18}O$ in NO_3^- of RF showed similar values at the two different levels of N_{dep}, *i.e.*, LN = 63.9 ‰ ± 0.88; HN =64.1 ‰ ± 3.2 (Fig. 3B). Within each level of N_{dep}, $\delta^{18}O$ values did not significantly differ between RF and TF. However, similarly to $\delta^{15}N$, we observed lower $\delta^{18}O$ in TF compared to RF in the case of the Scots pine at the HN. A significant contrast (t=-2.34, p<0.05) was found in the difference between the $\delta^{18}O$ values of NO_3^- in TF compared with RF across levels of N_{dep} (Figure 3E), with lower $\delta^{18}O$-NO_3^- values at HN than LN site.

$\Delta^{17}O$ values measured in RF at our sites ranged from 23.14 (±0.58) ‰ at the LN site to 25.53 (±0.76) ‰ at the HN site. A significant difference was found in the $\Delta^{17}O$ of NO_3^- in the TF vs. RF at the HN sites (W=16, p<0.05), but not at the LN sites. Within individual species, it is worth pointing out that beech showed lower $\Delta^{17}O$ values than Scots pine (Figure 3C). When we considered the difference between RF and TF, $\Delta^{17}O$ values in NO_3^- had lower values on average at the HN sites (t = -1.86, p=0.05) than LN sites (Fig. 3F), but the difference was not significant.

Combined plots for the three isotopic species of NO_3^- at the Scots pine and beech sites are given in Figure 4 as trajectories of change from RF to TF values, to emphasise the consequences of canopy processing for the three tracers, with particular references to forests at HN levels. For Scots pine (Fig. 4 A and B), only in the case of HN sites did $\delta^{15}N$, $\delta^{18}O$ and to a less extent $\Delta^{17}O$ values in TF diverge from those measured in RF. For beech (Fig.4 C and D), distinct changes in $\delta^{18}O$ *vs.* $\delta^{15}N$ were not observed, and only in the case of HN site, did $\Delta^{17}O$ become substantially lower from RF to TF.

Assessing the source of NO_3^- in the TF at the sites with high atmospheric N loads

Two mixing models, partitioning fluxes based on either $\Delta^{17}O$ or $\delta^{18}O$, were used to estimate the relative contributions of atmospheric *vs.* nitrification-derived NO_3^- collected underneath tree canopies. Using the two-end-member mixing model with the $\Delta^{17}O$ (Eq. 2 and 3 in the Materials and Methods) values measured in TF and RF (Table S1), the fractions of NO_3^- in TF coming from nitrification (f_{bio}) ranged from 0.17 for the Scots pine up to 0.59 for the beech (*i.e.*, 17 to 59%) at the two HN sites (Fig. 5A). Most of the NO_3^- collected in the TF at the Scots pine stand derived from the atmosphere (mean of f_{Atm}= 0.83±0.002), with only a minor contribution from nitrification (mean of f_{Bio}=0.17±0.002). By contrast, biologically-derived NO_3^- seemed to be the dominant fraction of the NO_3^- in TF of the beech stand (f_{Bio}=0.59±0.03), at least for the time period considered in this study (Fig. 5A).

When using the mixing model based on $\delta^{18}O$ partitioning (equations 4 and 5 in the Materials and Methods), a higher fraction of NO_3^- in TF was estimated to derive from the atmosphere (Scots pine: f_{Atm}= 0.62 ± 0.07; beech: f_{Atm} = 0.90 ± 0.09) than from nitrification (Scots pine: f_{Bio}= 0.38 ± 0.07; beech: f_{Bio} = 0.10 ± 0.09) (Fig. 5B). The two approaches were more consistent for the Scots pine, while they did lead to opposite results in the case of beech. Averaging across the two methods, the proportion of the biologically-derived nitrification was 27% for Scots pine (range of 17 to 38%) and 34% for beech (range of 10 to 59%).

Discussion

Four forests (two Scots pine and two beech stands) subjected to contrasting levels of N_{dep} in the UK were selected to assess whether and how tree canopies altered N_{dep} and its isotopic signature in TF. To our knowledge, this is the first study that combined measurements of NO_3-N and NH_4-N fluxes together with their relative isotope signatures, *i.e.*, $\delta^{15}N$ in NO_3^- and NH_4^+ and $\delta^{18}O$ and, specifically, $\Delta^{17}O$ in NO_3^- to determine the role of canopy processing of atmospherically-derived N_{dep}. In the following sections we discussed changes in TF fluxes at the HN and LN sites and how stable isotopes helped assessing the different processes taking place on tree canopies exposed to different atmospheric N loads.

Atmospheric N and its isotopic signatures at the contrasting N_{dep} levels

Both beech and Scots pine forests at HN sites were subjected to air masses with high NH_3-N/NH_4-N concentrations and had higher NH_4-N deposition relative to the LN sites. The HN beech site, which is right next to an intensive chicken farm, is trapping the farm's NH_4/NH_3 emissions along a very distinct 200-m-long N gradient where concentrations decrease to levels similar to those in the near-by Scots pine stand (Vanguelova and Pitman, 2009). This is showed by the higher NH_4-N concentrations in RF at the beech than the Scots pine stand at the HN, while no difference was found for NO_3-N concentrations (Fig. 1). Fluxes relative to the 2011 growing season indicated that at the beech stand NH_4-N is the dominant component of wet N_{dep}, while NH_4-N and NO_3-N contributed almost similarly to wet deposition at the Scots pine (Table 2). These results are in line with the data from long-term monitoring within the ICP forest network (Table 1), which showed that Thetford is among the sites receiving the highest N_{dep} in the UK (RoTAP report, 2012; Vanguelova et al., 2010), mostly in the reduced form, coming mainly from the intensive livestock farms (in particular pigs and chickens). Records over more than 10 years also suggest that the overall total N_{dep} at the Thetford pine site has decreased over time, because of reductions in wet (in both forms NH_4-N and NO_3-N) rather than dry deposition (Vanguelova et al., 2010), confirming the national trend (RoTAP, 2012).

The relative contributions of dry *vs.* wet N_{dep} at the site-level were broadly in agreement with modelled deposition rates obtained at the 5×5 km scale (RoTAP report, 2012, cf., Figure S1). For example, the modelled data suggested similar values for the total (wet plus dry) oxidized N forms (NO_3-N, NO_2-N and HNO_3-N) at the HN vs. LN sites, which is consistent with a similar impart of traffic-derived emissions at these sites. The large difference in dry NH_x deposition between LN and HN is also consistent with the effects of the numerous pig and chicken farms at HN. In addition, the rates of modelled NO_x and NH_x wet deposition were similar to the long-term measurements (Table 1) in RF at both the HN and LN sites. However, the modelled values of dry NO_x and NH_x deposition at both the HN and LN sites were substantially higher compared to the estimated dry deposition as difference between RF and TF. While this suggests significant canopy uptake at the HN sites, it must be remembered that the model estimates also include NH_3-N together with NH_4-N deposition, and NO_2-N-HNO_3-N together with NO_3-N, which were not directly account for in either the data previously published and reported in Table 1 or the current study. In addition, it is possible that the 5x5 km model of RoTAP (2012) fails to capture the small scale variability in N_{dep}, and especially in dry deposited NH_3.

Isotopic signatures measured in NO_3^- and NH_4^+ in RF (Fig. 2, 3A, B) at our sites were in the same range found in previous analyses of monthly rainfall samples from a range of sites in the UK (Heaton et al, 1997; Curtis et al., 2012; Heaton, unpublished data; Table 3). Overall, δ^{15}N values in NH_4^+ measured across the UK ranged from negative to slightly positive values (-12.6‰ to +2.8‰), with a mean of -4.3‰. The positive values observed at the Thetford sites are likely reflecting the contribution of NH_4/NH_3 emissions coming from the intensive chicken farms. Indeed, Heaton et al. (1997) reported that the δ^{15}N value of TF ammonium in part of a Scots pine plantation artificially fumigated with ammonia gas was 17‰ higher than the value for TF in the non-fumigated part of the plantation. Moreover in a recent study, Yeatman et al. (2001) measured δ^{15}N values of + 13.5‰ in aerosol-NH_4^+ sampled near chicken, cow and pig livestock enterprises and positive δ^{15}N values in bulk precipitation were also reported by Emmett et al. (1998) for two conifer stands near livestock feed lots in the Netherland.

The δ^{15}N values of NO_3^- were similar to those reported in the study by Heaton et al. (1997). However, a high range of values was measured across the UK (-8.2‰ to +4.3‰) (Table 3), with a mean δ^{15}N-NO_3 values of -2‰. A similar range of δ^{15}N values in NO_3^- from -11 ‰ to

+3.5 ‰ was reported in studies across the USA (Kendall et al., 1998; Kendall et al., 2007; Elliott et al., 2007), while Tobari et al. (2010) measured $\delta^{15}N$ values in bulk precipitation ranging from -7 to +15.4 ‰ across different watersheds in Japan. Moreover, a number of studies in the literature used $\delta^{15}N$ to assess the anthropogenic NO_x source. For instance, very negative (-13 to -2‰) $\delta^{15}N$-NO_x values were reported in the case of emissions coming from traffic, while positive values (between 4 and 16 ‰) were measured for emissions from coal-fired power plants (Heaton, 1990). Similar values of $\delta^{15}N$-NO_3 in RF at HN and LN sites in our study suggest a similar anthropogenic NO_x source, most likely emissions coming from local road traffic, consistent also with the absolute concentrations measured in RF at both HN and LN. This is confirmed also by the similar values we measured for $\delta^{18}O$-NO_3 in RF, irrespective of site. Moreover, $\Delta^{17}O$ in RF at the HN was 2‰ higher than at the LN sites, possibly suggesting that NO_x went through slightly different oxidation processes (Michlaski et al., 2003). $\Delta^{17}O$ values measured at our sites (ranging from 22 ‰ to 26 ‰) were similar to those reported by Costa et al. (2011) for NO_3^- in rain samples (23.1 ‰ ± 1.8) collected in Michigan and by Michalski et al. (2004) in aerosol (26 ‰ ± 3) sampled in Southern California.

Processes affecting throughfall N at contrasting N_{dep} levels: canopy retention, dry N_{dep} and biological transformation

Our data showed that at the LN TF-N fluxes were lower than RF N-fluxes (Table 2), suggesting that most of the atmospheric N was retained by tree canopies, as observed also in other studies (Fenn et al., 2013, Ferretti et al., 2014; Houle et al., 2015, Lindberg et al., 1986; De Schrijver et al., 2004: Staelens et al. 2007). Epiphytic lichens, fungi and microorganisms on the canopy may contribute to the higher N retention and subsequent processing at the LN sites, a possibility supported by the significant increase of DON concentrations in TF (Fig. 1C), as also reported in other studies (Woods et al., 2012).

By contrast, at the HN sites NH_4-N and NO_3-N concentrations and fluxes were higher in TF than RF, irrespective of tree species (Fig. 1 and Table 2). We also found higher NH_4-N in TF underneath beech than Scots pine, with the former receiving higher NH_x-N atmospheric inputs than the latter, while both NO_3-N and NH_4-N TF fluxes increased underneath the Scots pine. These last results are in line with previous studies in the literature (De Vries et al., 2014, Fenn et al. 2000; Lovett and Lindberg, 1993; Vanguelova et al., 2010) and they suggest that in areas with high dry N_{dep}, canopy filtering and rain washing will contribute to increasing the N inputs to TF and hence to the soils, compared to areas subjected to low atmospheric N loads, in particular dry N_{dep}. Nevertheless, the different proportion of the N-compounds in TF underneath the two forests could also be related to species-specific canopy N retention, which, however, is difficult to quantify by looking only at the difference between TF and RF.

The more positive values for $\delta^{15}N$ in NH_4^+ collected in TF are consistent with the dry NH_{dep} washed off the canopies and contributing to increasing NH_4-N in TF at the Thetford site (Fig. 1-2). Indeed, the $\delta^{15}N$ values of NH_4^+ in dry deposition tend to be higher than those measured in bulk precipitation (Heaton, 1997), suggesting that a fraction of the measured TF originated from dry N_{dep}. Interestingly, while the NH_4-N concentration did not vary significantly from RF to TF and the N fluxes were lower in TF *vs.* RF at the LN forests, a fingerprint of dry N_{dep} was still detected by the ^{15}N enrichment in NH_4^+ underneath the canopies.

The higher NO_3-N in TF at the HN sites for both Scots pine and beech could in principle result from a combination of dry deposition and canopy nitrification processes. As in the case of NH_4^+, higher values of $\delta^{15}N$ of NO_3^- in TF compared to RF could be expected (Heaton, 1997), but were not found at these sites (Fig. 3A). Nitrification of NH_4^+ leads to the production of ^{15}N depleted NO_3^- leaving behind more ^{15}N enriched NH_4^+ (Högberg, 1997). Indeed, we measured more negative (but not significantly so) $\delta^{15}N\text{-}NO_3^-$ in the TF-RF differentials at the HN compared to the LN site. The ^{15}N depletion of NO_3^- in TF was particularly detected for Scots pine, but not for beech, at the HN site (Fig. 3A, 4A). A decrease in $\delta^{15}N$ in NO_3^- from RF to TF was reported in studies in a spruce forest in Germany by Sah and Brumme (2003) and in a montane rain forest in Ecuador by Schwarz et al. (2011), explained in both cases by isotope fractionation during nitrification of NH_4^+ to NO_3^- in the canopy leaves. However, none of these previous studies could unequivocally attribute the shifts in $^{15}N\text{-}NO_3$ to biological NH_4^+ nitrification. In this study, evidence of nitrification occurring within the canopy was clearly provided by using two independent methods, based on $\Delta^{17}O$ and $\delta^{18}O$. Our results showed that although atmospheric NO_3^- was the dominant source of NO_3^- in TF at the Scots pine stand, a considerable proportion (varying between 17 and 38%, depending on which isotope was employed for the mass balance) derived from biological nitrification. The two approaches broadly agreed, but $\Delta^{17}O$ lead to higher f_{Atm} estimates than those obtained by $\delta^{18}O$ (Fig. 5). Significantly, both methods detected the contribution of biologically-derived NO_3^-.

Interestingly, similar values of $\delta^{15}N\text{-}NO_3$ in TF and RF did not provide a clear signal of canopy transformation for the beech at the HN (Fig. 4C). In contrast, the mass balance approach using $\Delta^{17}O$ and $\delta^{18}O$ proved that biological activity contributed to higher NO_3^- underneath beech canopies, with quite different estimate of f_{Bio} though. Indeed, based on $\Delta^{17}O$, isotopes biologically-derived NO_3^- was as a much as from atmospherically-derived NO_3^- (Fig. 5A). Whereas the mixing model based on $\delta^{18}O$ estimated that 90% of the NO_3^- in TF derived from the atmosphere and only a small fraction from nitrification (Fig. 5B).

Moreover, the significant increase of DON concentrations in TF at both Scots pine and beech sites provide evidence of transformation of dissolved inorganic N to DON within tree canopies (Gaige et al., 2007). Higher DON concentrations in TF can be related to leaching from leaves and needles and/or release by bacterial epiphytes in the phyllosphere (Müller et al., 2004).

The fact that only in the case of the Scots pine we found consistency between $\delta^{15}N\text{-}NO_3^-$ and the mixing model approaches based on $\delta^{18}O$ and $\Delta^{17}O$ could be partially related to differences between species in the canopy structure and phenology. Conifers are more efficient in scavenging aerosol and atmospheric deposition than broadleaf species (Augusto et al., 2002; De Schrijver et al., 2007) due to the greater canopy surface area and roughness. Furthermore, conifer evergreen phenology implies a higher canopy retention capacity than in deciduous species (De Schrijver et al. 2000), as in the case of Scots pine, whose needles can remain in the canopy for 2-3 years. This means that atmospheric N deposited onto tree canopies and cumulated over multiple growing years and not taken up by needles could undergo several biological transformations, which imply isotope fractionations leading to a distinct isotopic signature between the atmospheric N source and the final produced N specimen. However, assessing the differences between two forests at HN for canopy N transformation goes beyond the aim of this study, due to low replicates per species. Nevertheless, our results certainly shade light on species-specific dynamic of biological activity in tree canopies, which deserves further investigation.

Effectiveness of the two mass balance approaches based on $\delta^{18}O$ and $\Delta^{17}O$

The use of $\Delta^{17}O$ in nitrate was successfully applied to assess the contribution of atmospheric *vs.* microbiologically derived NO_3^- in a forest catchment (Costa et al., 2011) and lately, in combination with $\delta^{18}O$, in an urban environment (Riha et al., 2014). Both studies looked at the isotopic composition in N specimens in the runoff water *vs.* RF. Processes occurring in the soil, with particular reference to nitrification, and isotope fractionations associated with them, are very well described (see among the others, Högberg et al., 1997). While canopy N retention and transformation are widely acknowledged as important pathways for trees to acquire N (Sparks, 2009, Pennisi, 2015), the underlying mechanisms are still not understood. This is particularly true for the isotope fractionations, which may occur during nitrification in the canopies or N uptake. One potential limitation of the mixing model based on $\delta^{18}O$ is in the estimation of the $\delta^{18}O_{Nitr}$ (see Materials and Methods). First, precipitation intercepted by tree canopies might be subjected to evaporation, which, in turn affects the $\delta^{18}O$ of the precipitation-derived water available for nitrification (*e.g.*, water remaining on the canopy might be more ^{18}O-enriched than precipitation itself). Second, the assumptions underlying the use of Equation 5 may not be always valid. In some environments oxygen isotope exchange between a nitrification intermediary, nitrite and water may invalidate the two thirds and one thirds proportions of Equation 5. In addition, the possible influence of an equilibrium isotope fractionation during NO_2^- and H_2O exchange at the enzyme (+14‰) and an inverse kinetic isotope effect during NO_2^-oxidation into NO_3^- have also been proposed and would lead to higher $\delta^{18}O$ values than those predicted by the simple isotope mass balance model (Buchwald et al., 2013; Casciotti et al.; 2010, 2011; Snider et al., 2010). Third, N deposited onto canopies could be more reactive and subject to further transformations before being processed within the canopies or washed-off (*e.g.*, NH_3 volatilization, NO reaction with ozone or denitrification). Thus, improper calculation of $\delta^{18}O_{Nitr}$ might affect the estimation of f_{Atm} and f_{Bio}. While mass-dependent isotope fractionations related to NO_3^- transformation can significantly affect the $\delta^{18}O$, they have no effect on $\Delta^{17}O$. For this reason, using $\Delta^{17}O$ seems a more robust approach, leading to a better estimate of f_{Atm} *vs.* f_{Bio} (Michalski et al., 2003). However, more studies are needed in order to assess the $\Delta^{17}O$ of wet *vs.* dry N_{dep} and how they change over time.

Synthesis

Our results partially confirmed the initial hypotheses 1) that at the LN sites, ion concentrations in TF and their respective isotopic signatures reflected the input of atmospheric N as derived from RF. However, isotope data revealed that even with a low atmospheric N load, canopies played an important role in intercepting and retaining dry N_{dep} (with particular reference to the reduced N-form), which represents an additional (but often overlooked) N source relative to wet N_{dep} as assessed through RF. Differences in the RF and TF fluxes together with an increase in TF DON concentrations provided evidence of canopy N retention and possible uptake. At the HN sites, the passing of atmospheric N through canopies affected both ion concentrations and their isotopic signature (which confirmed our hypothesis 2). The occurrence of dry deposition explained the higher NH_4-N concentrations and ^{15}N enrichment in NH_4^+ measured below the canopy in TF water *vs.* RF. As for the higher NO_3-N in TF vs. RF, the isotopes $\delta^{15}N$ and $\delta^{18}O$ could not provide clear indications of its origin, even though for Scots pine $\delta^{15}N$-NO_3^- provided some indications of biologically-derived NO_3^-. The unambiguous response came however from $\Delta^{17}O$, which allowed to detect that a consistent fraction of the NO_3^- recovered underneath the canopies derived from biological nitrification, with an especially large magnitude at the beech stand (where the other isotopes, particularly $\delta^{18}O$, failed to provide conclusive evidence).

We acknowledge that the conclusions of this study rely on a limited number of isotope measurements at each site and a limited selection of forest stands, which did not allow detailed investigations of the tree species-specific pattern of canopy N transformations. However, by combining multiple isotopes the study identified canopy processing of atmospheric deposition (and especially canopy biological nitrification) as a major process that should not be neglected and needs further exploration. This has important implications for policy-related emission abatement strategies, which aim to manage forests and landscape not only for enhancing C-sequestration, but also for atmospheric N capture.

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Table 1. Site, climatic and atmospheric N_{dep} characteristics of the four forests included in the study. Climate data are mean values calculated over the years 1960-2010 and deposition data are mean values over a number of years (*e.g.*, Alice Holt / Beech stand: 2006-2008; Rogate / Scots pine stand: 2010-2012; Thetford / Scots pine stand: 1995-2010; Thetford / Beech stand: 2006-2008, Vanguelova et al., 2010, Vanguelova and Pitman, 2011). Soil type is provided according the Word Reference Base for Soil Resources (IUSS, 2007).

Site	**Location**	**Forest stand**	**Stand age (yrs)**	**Soil type (WRB, 2006)**	**Precipitation (mm yr $^{-1}$)**	**T (°C)**	**NH_4^+/NO_3^- Dry dep. (kg ha $^{-1}$ yr $^{-1}$)**	**NH_4^+/NO_3^- Wet dep. (kg ha $^{-1}$ yr $^{-1}$)**	**Tot N_{dep} Dry/ Wet (kg ha $^{-1}$ yr $^{-1}$)**	**Tot N_{dep} (kg ha $^{-1}$ yr $^{-1}$)**
LN	Alice Holt	Beech	70	Cambisol	800	11.6	2.7/0.2	3.7/3.2	2.9/6.9	9.8
	Rogate	Scots pine	60	Cambisol	800	11.6	4.1/0.6	3.1/2.9	4.8/5.9	10.7
HN	Thetford	Beech	70	Arenosol	600	11.3	4.9/4.6	7.5/2.7	9.5/10.2	19.7
		Scots pine	45				3.2/1.8	5/3.3	5.0/8.4	13.4

Table 2. NH4-N, NO_3-N and DON fluxes measured over the 6 months considered in this study (*i.e.,* June to November 2011) at the two forests at LN (*i.e.,* Alice Holt - Beech stand; Rogate-Scots pine stand) and tow forests at the HN site (Thetford Scots pine stand; Thetford-Beech stand).

Site	Location	Forest stand	NH_4-N/NO_3-N RF (kg ha^{-1})	NH_4-N/NO_3-N TF (kg ha^{-1})	DON RF (kg ha^{-1})	DON TF (kg ha^{-1})
LN	Alice Holt	Beech	1.54/1.52	1.05/1.26	1.00	1.68
	Rogate	Scots pine	1.31/1.41	1.08/0.94	0.91	1.69
HN	Thetford	Beech	3.14/0.97	9.96/2.12	1.15	2.48
		Scots pine	1.92/1.42	3.67/3.83	0.65	1.19

Table 3. Range of δ^{15}N and δ^{18}O values for NO_3^- and NH_4^+ in monthly rainfall samples from mainly remote upland areas in north and west mainland Britain (Heaton et al, 1997; Curtis et al., 2012; Heaton, unpublished data)

Isotope	Total range	Mean	Interquartile range	N
δ^{15}N-NO_3	-8.2‰ to +4.3‰	-2.0‰	-3.8‰ to -0.5‰	117
δ^{18}O-NO_3	+50‰ to +82‰	+69‰	+65‰ to +73‰	117
δ^{15}N-NH_4	-12.6‰ to +2.8‰	-4.3‰	-6.2‰ to -2.8‰	86

Figure 1 NH_4–N (panel **a**), NO_3–N (panel **b**) and DON (panel **c**) concentrations in Rainfall (RF) and Throughfall (TF) for beech (BE) and Scots pine (SP) at the LN (*i.e.*, Alice and Holt and Rogate, respectively) and HN (*i.e.*, Thetford) sites. Each symbol represents the mean (± SE) for ions concentrations measured in water samples collected bi-weekly from June until November 2011 in n=2 RF and n=10 TF collectors.

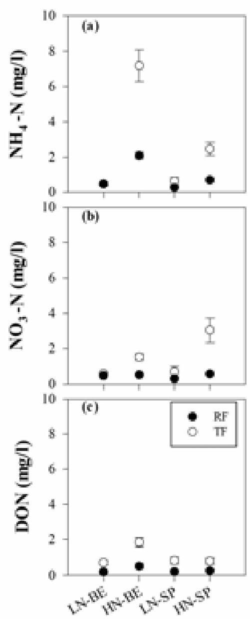
Figure 2 a) $\delta^{15}N$-NH_4^+ in Rainfall (RF) and Throughfall (TF) for Scots pine (SP) and beech (BE) forests at the LN (*i.e.*, Alice and Holt and Rogate, respectively) and HN (*i.e.*, Thetford) sites. Each symbol represents the mean (± SE) for isotope measurements carried out in water samples collected from June-August and September- November 2011. **b)** Differences (mean ± CI, calculated on *n*=4 observations) between TF and RF for $\delta^{15}N$-NH_4^+ values measured at the LN and HN sites, without distinguishing between tree species.

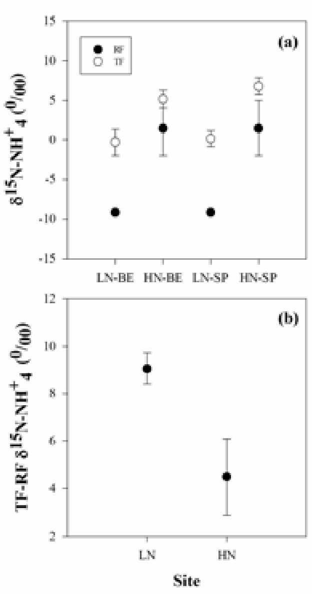
Figure 3 a) $\delta^{15}N$, **b)** $\delta^{18}O$ and **c)** $\Delta^{17}O$ values of NO_3^- in rainfall (RF) and throughfall (TF) for Scots pine (SP) and beech (BE) at the LN *(i.e.*, Alice and Holt and Rogate, respectively) and HN (*i.e.*, Thetford). Each symbol represents the mean (± SE) for isotope measurements carried out in water samples collected from June-August and September-November 2011. Differences (diff., mean ± CI, calculated on *n*=4 observations) between TF and RF for **d)** $\delta^{15}N$-NO_3^-, **e)** $\delta^{18}O$-NO_3^- and **f)** $\Delta^{17}O$-NO_3^- values measured at the LN and HN sites, without distinguishing between tree species.

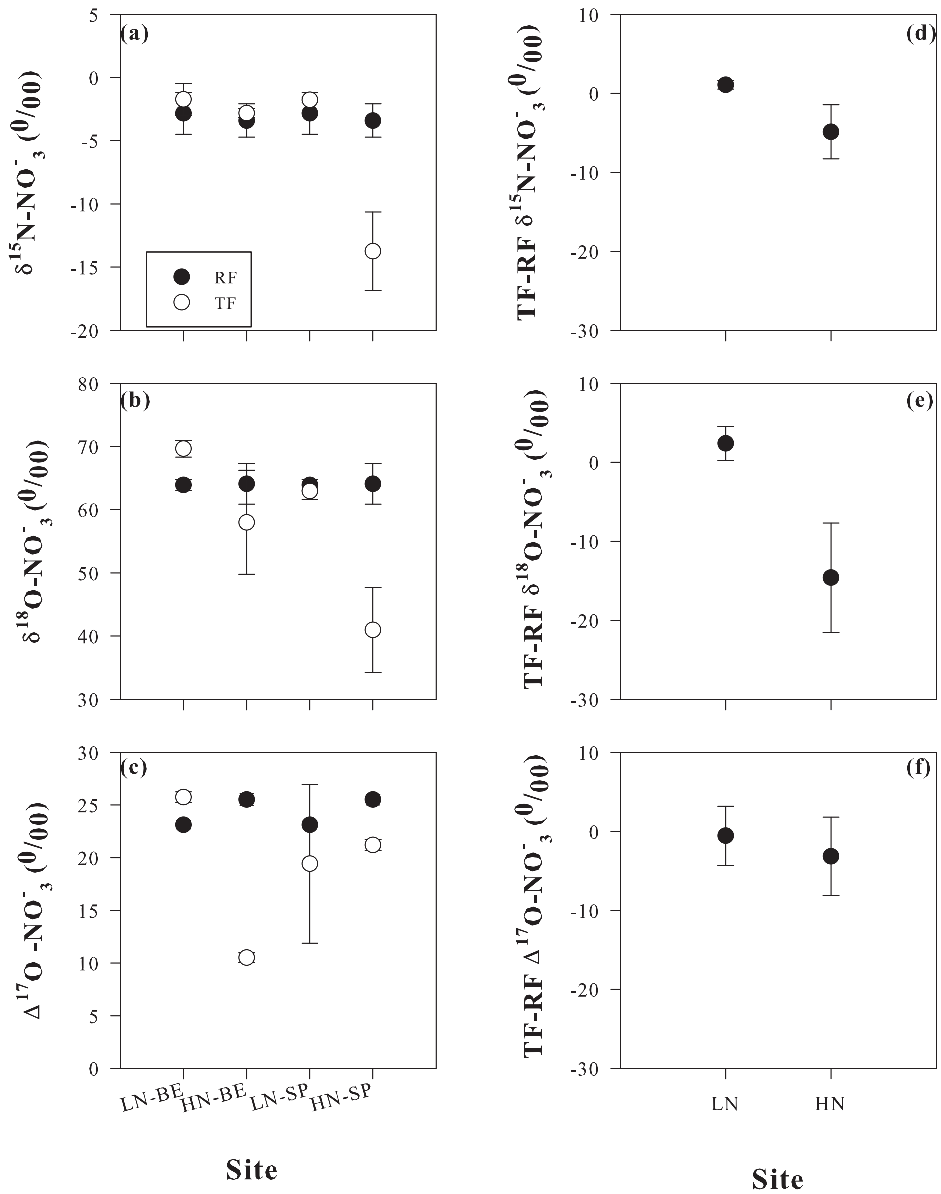
Figure 4 $\delta^{15}N$ *vs.* $\delta^{18}O$ and $\delta^{15}N$ *vs.* $\Delta^{17}O$ for Scots pine (**a** and **b**, respectively) and beech (**c** and **d**, respectively) measured in RF and TF. Each symbol represents the mean ± SE for isotope measurements carried out in water samples collected from June-August and September- November 2011 at the LN (Rogate and Alice Holt for the Scots pine and Beech, respectively) and HN (Thetford, for both tree species). Arrows depict dramatic changes from RF to TF for the isotope values.

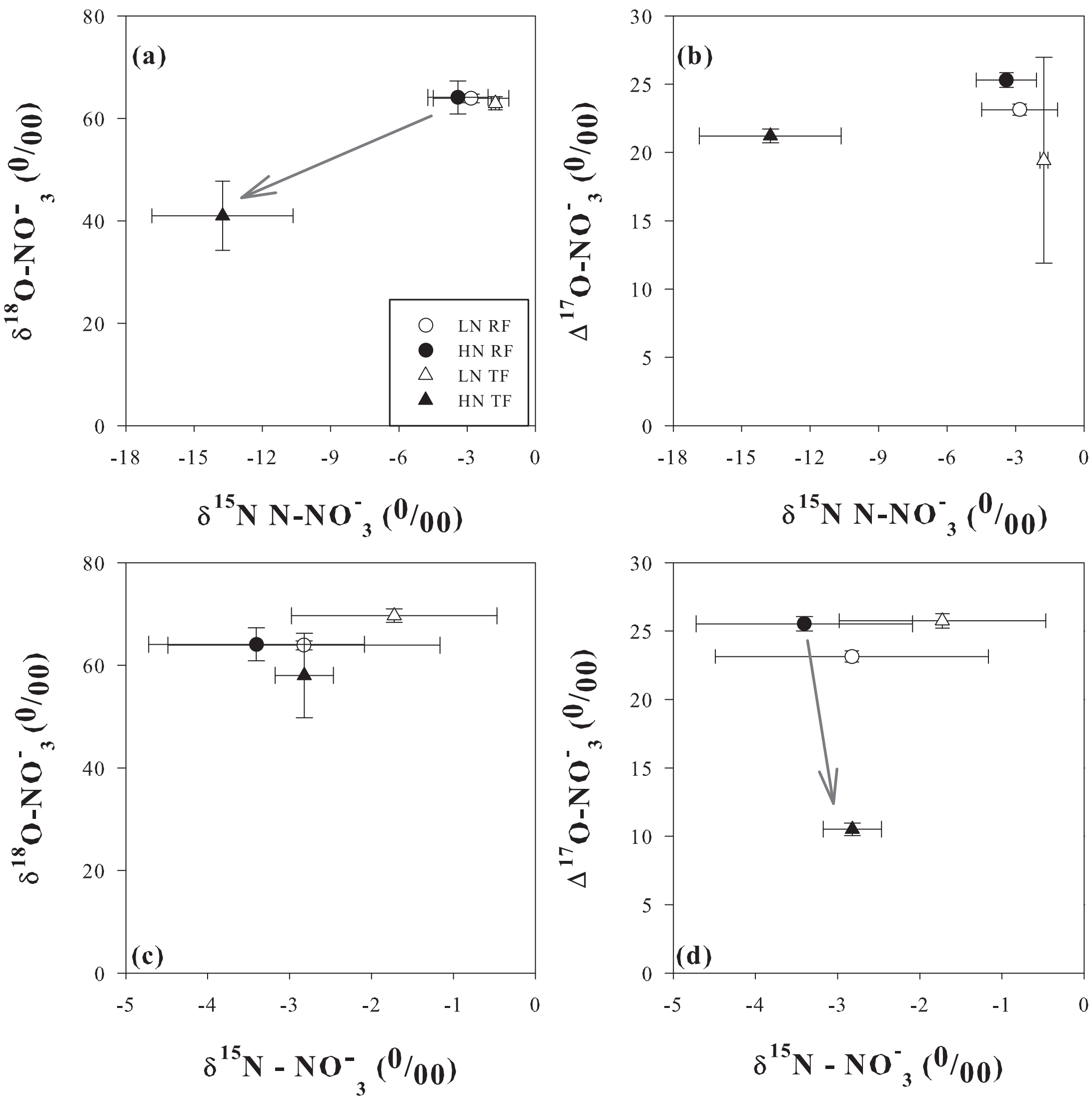
Figure 5 Mean (± SE) of the NO_3^- fraction derived from the atmosphere (f_{Atm}) and nitrification (f_{Bio}) based on mixing model using $\Delta^{17}O$ (panel **a**) and $\delta^{18}O$ (panel **b**) measured in NO_3^- for the June-August and September-November months at the two HN forest stands. SP and BE indicate Scots pine and Beech, respectively.

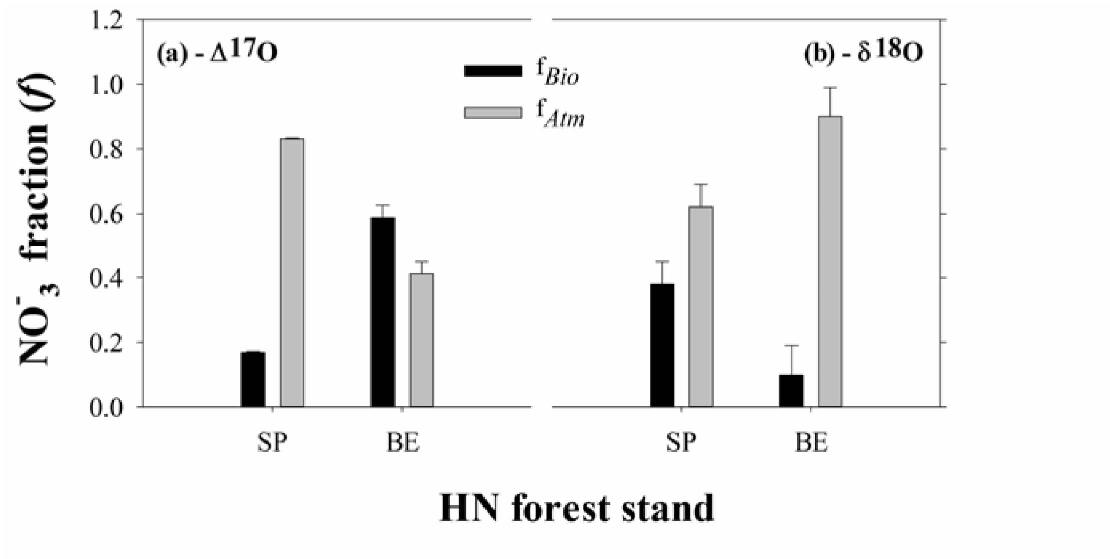
Figure S1. Comparison between measured and modelled N_{dep} at the four forests subjected to different level of N_{dep}. Measured values are obtained from long-term monitoring at the four sites as reported in the Table 1: NH_4-N and NO_3-N concentrations in RF were used to determine the Wet N_{dep}, while dry Ndep were estimate as difference between TF and RF fluxes (ICP, 2010). Modelled values were obtained by 5x5 km grid modelled N_{dep} with FRAME upon 2005 emissions data (RoTAP review, 2012 - chapter 4). Given that for each level of N_{dep} (*e.g.*, what we defined HN and LN sites) there were two forests, which were 6-8 km apart to each other, we extractacted the grid with the closer coordinate to our sites and then we consider 2 grids before and 2 after and we calculated the average of N_{dep} data, to compare with our values reported in the Table 1.


(a)
NH4-N (mg/l)
(b)
NO3-N (mg/l)
(c)
DON (mg/l)
RF
TF
LN-BE
HN-BE
LN-SP
HN-SP


(a)
RF
TF
LN-BE
HN-BE
LN-SP
HN-SP
(b)
LN
HN
Site


(a)
(b)
(c)
(d)
(e)
(f)
RF
TF
δ^{15}N-NO$_3^-$ ($^0/_{00}$)
δ^{18}O-NO$_3^-$ ($^0/_{00}$)
Δ^{17}O -NO$_3^-$ ($^0/_{00}$)
TF-RF δ^{15}N-NO$_3^-$ ($^0/_{00}$)
TF-RF δ^{18}O-NO$_3^-$ ($^0/_{00}$)
TF-RF Δ^{17}O-NO$_3^-$ ($^0/_{00}$)
LN-BE
HN-BE
LN-SP
HN-SP
LN
HN
Site
Site


(a)
(b)
(c)
(d)
LN RF
HN RF
LN TF
HN TF
δ18O-NO-3 (0/00)
Δ17O-NO-3 (0/00)
δ15N N-NO-3 (0/00)
δ15N - NO-3 (0/00)


(a) - Δ17O
(b) - δ18O
fBio
fAtm
NO-3 fraction (f)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
SP
BE
SP
BE
HN forest stand