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Is the high ^{15}N natural abundance of trees in N-loaded forests caused by an internal ecosystem N isotope redistribution or a change in the ecosystem N isotope mass balance?

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Abstract High $\delta^{15}N$ of tree foliage in forests subject to high N supply has been attributed to ^{15}N enrichment of plant available soil N pools after losses of N through processes involving N isotope fractionation (ammonia volatilization, nitrification followed by leaching and denitrification, and denitrification in itself). However, in a long-term experiment with high annual additions of NH_4NO_3, we found no change in the weighted average $\delta^{15}N$ of the soil, but attributed the high $\delta^{15}N$ of trees to loss of ectomycorrhizal fungi and their function in tree N uptake, which involves redistribution of N isotopes in the ecosystem (Högberg et al. New Phytol 189:515–525, 2011), rather than a loss of isotopically light N. Here, we compare the effects of additions of urea and NH_4NO_3 on the $\delta^{15}N$ of trees and the soil profile, because we have previously found higher $\delta^{15}N$ in tree foliage in trees in the urea plots. Doing this, we found no differences between the NH_4NO_3 and urea treatments in the concentration of N in the foliage, or the amounts of N in the organic mor-layer of the soil. However, the foliage of trees receiving the highest N loads in the urea treatment were more enriched in ^{15}N than the corresponding NH_4NO_3 plots, and, importantly, the weighted average $\delta^{15}N$ of the soil showed that N losses had been associated with fractionation against ^{15}N in the urea plots. Thus, our results in combination with those of Högberg et al. (New Phytol 189:515–525, 2011) show that high $\delta^{15}N$ of the vegetation after high N load may be caused by both an internal redistribution of the N isotopes (as a result of change of the function of ectomycorrhiza) and by losses of isotopically light N through processes fractionating against ^{15}N (in case of urea ammonia volatilization, nitrification followed by leaching and denitrification).

Keywords Ectomycorrhiza · Forest soils · Nitrogen deposition · N-15 natural abundance

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Introduction

Under many natural conditions, the supply of nitrogen is limiting plant production in large areas of temperate and boreal forests (e.g., Tamm 1991). In parts of Europe and N. America, nitrogen deposition is now slowly removing this limitation (Galloway et al. 2008). Consequently, forests become N saturated as the supply of N exceeds the combined plant and microbial demand for N (e.g., Aber et al. 1998). When this occurs, leaching losses of nitrate become high, as do losses of nitrous oxide, a potent greenhouse gas.

Processes associated with N loss, e.g., nitrification and denitrification, discriminate against the heavier N

isotope, ^{15}N (Shearer and Kohl 1986; Högberg 1997). Thus, the N lost is isotopically light, leaving the remaining ecosystem enriched in ^{15}N. As a consequence the ^{15}N abundance may be a useful indicator of N losses from forests (Vitousek et al. 1989; Högberg 1990). Indeed, Högberg and Johannisson (1993) reported a correlation between the fractional loss of added N and an increase in $\delta^{15}N$ over time in tree foliage in a long-term N-loading experiment. Likewise, enrichment in ^{15}N of tree foliage has been found in field surveys of forests subject to high N loads in both Europe and N. America (e.g., Högberg et al. 1996; Emmett et al. 1998; Pardo et al. 2007). Högberg (1991) and Högberg and Johannisson (1993) also found that trees in plots to which urea, $CO(NH_2)_2$, had been applied became more enriched in ^{15}N than did trees in plots to which NH_4NO_3 had been added. They ascribed this greater isotope effect to ammonia volatilization and higher rates of nitrification in the urea plots. The enrichment in ^{15}N was thought to first occur in labile pools of N in the soil, and, therefore, soon also in the foliage of trees, while the larger pool of less labile N in the soil should change its isotopic composition more slowly (Johannisson and Högberg 1994).

In addition to these differences in overall ecosystem $\delta^{15}N$ there are also distinct patterns of the N isotope ratio within ecosystems. In forests dominated by ectomycorrhizal (ECM) tree species, which are typical of strongly N-limited temperate and boreal forests (Smith and Read 2008), there is commonly an increase in $\delta^{15}N$ with increasing soil depth (Riga et al. 1971; Nadelhoffer and Fry 1988; Melillo et al. 1989; Gebauer and Schulze 1991; Högberg et al. 1996; Billings and Richter 2006; Sah et al. 2006; Lindahl et al. 2007; Hobbie and Ouimette 2009; Wallander et al. 2009). The classical explanation of how this increase develops is that there is a fractionation against the heavier N isotope during plant N uptake, which leaves the remaining soil N enriched in ^{15}N, while subsequently litter depleted in ^{15}N becomes deposited on the forest floor (Nadelhoffer and Fry 1988). After the discovery that ECM fungi are highly enriched in ^{15}N relative to their host plants (e.g., Gebauer and Dietrich 1993; Handley et al. 1996; Högberg et al. 1996), we proposed the additional explanation that ECM fungal material may be a precursor of the biologically more stable N in the lower horizons (Högberg et al. 1996). The high $\delta^{15}N$ of ECM fungi has been attributed to isotopic fractionation during transaminations taking place in the fungi when N is transported from the soil, through the hyphae and into the plants (Hobbie and Ouimette 2009; Hobbie and Högberg 2012). This leaves the fungal material enriched in ^{15}N, while the N passed on to the host plant is depleted in ^{15}N relative to the N source (Högberg et al. 1999). Importantly, the $\delta^{15}N$ of ECM plants is variable dependent on variations in the efficiency of transfer of N through the fungal symbiont along this pathway (Hobbie and Hobbie 2006).

Moreover, we found that in forests subject to high rates of N deposition or experimental N loading the uppermost soil horizon could be isotopically heavier than the lower horizons (Högberg et al. 1996), i.e. a pattern contrary to that found under natural N-limited conditions. We assumed that this reflected N losses from the plant available N pools through pathways (nitrification followed by denitrification or leaching of nitrate) leading to isotopic enrichment of the remaining plant available N.

Since the early exploratory phase, many studies have confirmed the observation of a higher $\delta^{15}N$ in ECM fungal N as compared to the N in their host plants (e.g., Taylor et al. 1997, 2003; Högberg et al. 1999; Hobbie et al. 2000; Henn and Chapella 2001; Hobbie and Hobbie 2006; Kohzu et al. 1999; Trudell et al. 2004). The difference of around 10 ‰ in N-limited forests (Taylor et al. 2003) coincides with the difference between the uppermost and lower soil horizons (e.g., Högberg et al. 1996; Lindahl et al. 2007; Hobbie and Ouimette 2009). Recently, Lindahl et al. (2007) studied the relation between the species composition of the fungal community and the variation in $\delta^{15}N$ down the soil profile in a boreal forest. They found that there was no change in $\delta^{15}N$ during the initial stage of litter decomposition representing a decrease in C:N ratio from c. 130 to 45, during which saprotrophic fungi dominated the fungal flora. Further down, ECM fungi became dominant and simultaneously the $\delta^{15}N$ of the organic material increased from −4.5 to +6.0 ‰, indicating uptake of N by ECM trees. This supports the hypothesis that uptake of N through ECM fungi drives the development of the ^{15}N enrichment with increasing soil depth (Högberg et al. 1996). Indeed, in N-loaded forests, in which the biomass of ECM fungi was reduced (Högberg et al. 2007), there was a high $\delta^{15}N$ in the foliage and the uppermost soil horizon, but lower $\delta^{15}N$ in both 6–15

years after the N load was terminated, which was also associated with recovery of the ECM fungi (Högberg et al. 2011).

Thus, according to the above there may be two major reasons why plant N in N-loaded forests becomes enriched in ^{15}N. These are (1) losses of N associated with processes fractionating the stable N isotopes, and (2) changes in the function of ECM fungi, especially their role in redistributing N isotopes in the ecosystem. The two are likely interrelated. However, while most papers in the past have focussed on (1), a recent study of effects of high additions of NH_4NO_3 provided little evidence of N losses as cause of high foliar $\delta^{15}N$, which was primarily attributed to change in the function of ECM symbiosis in redistributing N isotopes in the ecosystem, i.e. (2)(Högberg et al. 2011). Here, we use the urea treatments in the same long-term field experiment, in which case previous studies have shown a higher $\delta^{15}N$ in foliage than in plots to which was NH_4NO_3 added, to test the hypothesis that the high $\delta^{15}N$ in foliage of trees in the high N urea plots is the result of a change in the soil N isotope mass balance rather than a result of a change in the role of ECM symbiosis in the redistribution of N isotopes in the ecosystem.

Materials and methods

Experiment E55 at Norrliden is located in a *Pinus sylvestris* L. forest in northern Sweden (64°N). The forest was planted in 1953 after a prescribed burning in 1952. It is located on gently sloping glacial till, and the soil type is an Orthic Podzol; detailed descriptions of the site, stand, soil, and the experiment, which was established in 1970, are given by Tamm et al. (1999) and Högberg et al. (2006). We use data from the part of E55 involving additions of NH_4NO_3 or as urea. The urea plots are situated immediately to the east and upslope relative to the NH_4NO_3 plots. The plots are quadratic, 30 m by 30 m each, and there are three replicate plots of each treatment. These are N0, N1, N2, and N3. N0 is the control, to which no N has been added experimentally; the N deposition in this area is around 3 kg N ha^{-1} yr^{-1}. The N additions to N1–N3 started in 1971 at high levels, but were reduced in two steps (Table 1), leading to average N addition rates per year of 36 kg N ha^{-1} in N1, 72 kg N ha^{-1} in N2 and 108 kg N ha^{-1} in N3 through the period 1971–1990.

Table 1 Nitrogen addition rates (kg N ha^{-1} yr^{-1}) in the experiment E55, Norrliden, Sweden, over the time period 1971–1990 (there were no additions in 1991 or before the soil sampling in 1992)

Years	N1	N2	N3
1971–1973	60	120	180
1974–1976	40	80	120
1977–1990	30	60	90
Total 1971–1990	720	1,440	2,160

Control plots, N0, received only the background deposition of N (~3 kg N ha^{-1} yr^{-1})

The $\delta^{15}N$ of fertilizer N derived from N2 is usually close to 0 ‰ (Högberg 1997); we have asked the one supplier for samples to analyze, but such material has not been archived. Data on KCl-extractable soil NO_3-N from 1988 reported by Aronsson et al. (1999) showed no differences between the two forms of N added; comparing urea with NH_4NO_3 plots, the amounts of NO_3-N kg^{-1} ha^{-1} in the humus layer were 6.0 versus 1.0, 19.5 versus 14.9 and 33.6 versus 27.5, in the N1, N2 and N3 plots, respectively.

Every year samples of current needles have been taken from 10 trees per plot (located within the inner 400 m^2 of the plot) and pooled into one sample per plot. The leaf samples were dried (70 °C, 24 h) and archived. Previously, we have reported analytical data on %N and $\delta^{15}N$ of current needles in the urea plots from every fifth year from 1970 through to 1990 (Högberg 1991). Here, we report data on these variables for every year for the same period and the treatment effects on N content and $\delta^{15}N$ of the mor-layer soil as sampled in 1992. Data from the NH_4NO_3 plots were reported by Högberg et al. (2011), and are shown here to provide a comparison to the data from the urea plots. Samples of the mor-layer were taken, using a 10 cm diameter auger from five locations within the central 400 m^2 of each plot in 1992. Note that the N treatments were all suspended in 1991, and that the sampling in 1992 was conducted before the N additions to the treatments N1 and N2 that year (N3 was terminated in 1990). The mor-layer was divided into its three characteristic horizons, the S-layer (surface horizon comprising litter mixed with mosses and lichens; this horizon contains no plant roots or active ECM mycelium, see Lindahl et al. 2007), the F-layer (so called fermentation-layer containing identifiable fragments of decomposing litter and an abundance of plant roots and fungal mycelium, especially of ECM fungi), and the H-layer (which also contains abundant roots and

ECM mycelium, but in which the organic matter is of an amorphous character). The mean ecosystem age, the time since the C was incorporated through photosynthesis, was estimated at 4–6, 11–15 and 27–47 years, for the S-, F- and H-layer, respectively (Franklin et al. 2003). In this forest, the pine trees retain around five age-classes of needles. Thus, the organic matter in the S- and F-layers was composed of organic matter produced during the period of the experiment studied here, while the H-layer contains older material. The N added has contributed to all horizons. Each sample from each soil horizon was weighed separately after drying (70 °C, 24 h).

Dried and finely ground samples of tree needles and soil were analysed for %N and $\delta^{15}N$ on an elemental analyser coupled on-line to an isotope ratio mass spectrometer (an ANCA system coupled to a 20–20 IRMS, Europa Scientific Ltd., Crewe, England). We calculated the weighted average $\delta^{15}N$ for each soil horizon, and for the entire mor-layer, using the data on soil mass, %N and $\delta^{15}N$. We used ANOVA to test for differences between treatments.

Results

Needle N concentration and amounts of N in the mor-layer

The N concentration of needles rose rapidly in N-loading treatments above the average level 1.2–1.3 % in the control, N0, treatment (Fig. 1a). The highest percentage of N, around 2.4 % in N3, occurred at an early stage of the experiment, and corresponded with the higher N addition rates through this stage (Table 1). There were no differences in %N of needles as a result of applying the two different forms of N; only the dose mattered (Fig. 2). The total N content of the mor-layer roughly doubled after the N additions irrespective of dose or form of N applied (Table 2). The increases in N content of the mor-layers in the urea plots corresponded to a retention of 38, 28 and 16 % of the N added in N1, N2 and N3, respectively.

$\delta^{15}N$ of needles

An initial decline in $\delta^{15}N$ in N1–N3 indicated that the added N was isotopically lighter than endogenous

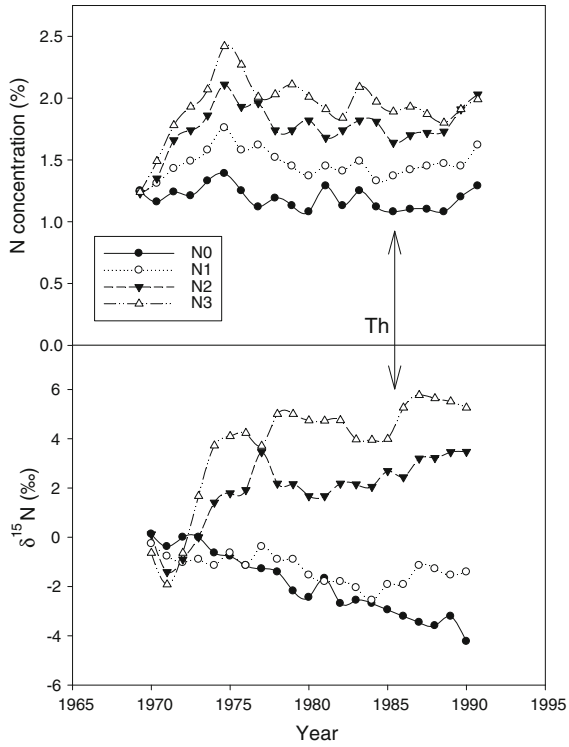

Fig. 1 Concentration of N (*upper panel*) and $\delta^{15}N$ (*lower panel*) of needles of *P. sylvestris* trees in the urea plots, 1970–1990, experiment 55, Norrliden, Sweden. Data are plot means. Treatments are described in Table 1. Th, forest thinning

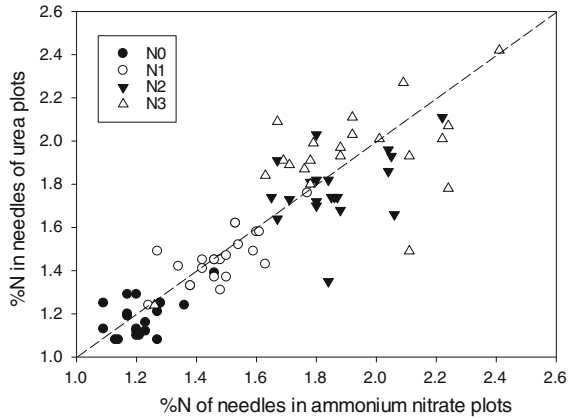

Fig. 2 Concentration of N in needles in *P. sylvestris* trees in plots treated with NH_4NO_3 and urea, 1970–1990, experiment 55, Norrliden, Sweden. Data are plot means for the same year and treatment. Data for the NH_4NO_3 plots are from Högberg et al. (2011). The *broken line* shows $y = x$. Treatments are described in Table 1

available N (Fig. 1b). The $\delta^{15}N$ of needles in the N-loading treatments started to exceed that in the N0 after 3, 4 and 14 years of N-loading in N3, N2 and N1,

Table 2 Amount of N (in kg ha^{-1}) in the organic mor-layer in the NH_4NO_3 and urea treatments in the experiment E55, Norrliden, Sweden in 1992

N form added	N0	N1	N2	N3
NH_4NO_3	308 ± 12a	565 ± 31b	656 ± 47b	700 ± 83b
Urea	332 ± 43a	606 ± 48b	727 ± 35b	682 ± 10b

There are 3 replicate plots per treatment. The treatments are described in Table 1. Significantly different mean values ($P < 0.05$, two-way ANOVA followed by Holm–Sidak's test) are not followed by the same letter. Data are mean ± SE Data for the NH_4NO_3 treatment from Högberg et al. (2011)

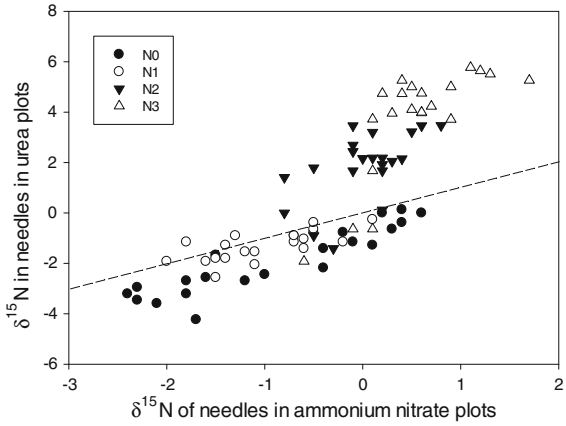

Fig. 3 Natural abundance of ^{15}N in tree foliage in plots treated with NH_4NO_3 and urea, 1970–1990, experiment 55, Norrliden, Sweden. Data are plot means for the same year and treatment. Data for the NH_4NO_3 plots are from Högberg et al. (2011). The *broken line* shows $y = x$. Treatments are described in Table 1

respectively. The changes in $\delta^{15}N$ were larger in N2 and N3 urea plots than in the corresponding NH_4NO_3 treatments (Högberg et al. 2011, Fig. 3), with increases of up to +6 ‰ in N3, and a total difference between N3 and N0 of 10 ‰ in 1990. In the N0 plots, the $\delta^{15}N$ of needles fell about 4 ‰ in 20 years, i.e. faster than the 2 ‰ in the control plots among the NH_4NO_3 treatments (Högberg et al. 2011).

$\delta^{15}N$ of organic soil horizons

The data on $\delta^{15}N$ of the mor-layers from 1992 showed larger differences among urea treatments than among the NH_4NO_3 treatments, corresponding to the greater above-mentioned differences in the needles (Table 3). The weighted average $\delta^{15}N$ of the entire mor-layers in the urea plots were −0.8 ± 0.4, −0.7 ± 0.3, 1.4 ± 0.1 and 2.7 ± 0.1 for N0, N1, N2 and N3, respectively. Note that while the amounts of N retained in the mor-layers were similar for the two forms of N added (Table 2), the $\delta^{15}N$ differed substantially, also with regard to the control plots in the two N treatment blocks; we are unable to explain the difference between control plots, and have chosen to focus on potential differences within the two N form treatments in our statistical analysis. Note also that the lack of effect in the N1 treatment indicates that the $\delta^{15}N$ of the urea fertilizer must have been close to that of endogenous soil N (Table 3; Fig. 4). In both N0 and N1 $\delta^{15}N$ increased with increasing soil depth, while the reverse occurred in N2 and N3 (Fig. 4).

Discussion

There was no difference in %N of needles between the urea and the NH_4NO_3 plots (Fig. 2), nor was there any difference in accumulation of N in the mor-layer that was related to the form of N applied (Table 2). Hence, we assume in the following that the plots in the urea treatments were as N-limited or N-saturated as the corresponding plots treated with NH_4NO_3. Furthermore, data on KCl-extractable NO_3^- from the soil in N treated plots in 1988 (Tamm et al. 1999; Aronsson et al. 1999; the data are given in the "Materials and

Table 3 Weighted (by mass N) average $\delta^{15}N$ for the organic mor-layer in the NH_4NO_3 and urea treatments in the experiment E55, Norrliden, Sweden in 1992

N form added	N0	N1	N2	N3
NH_4NO_3	1.0 ± 0.3a	−0.3 ± 0.1b	0.2 ± 0.1a,b	0.8 ± 0.1a
Urea	−0.8 ± 0.4a	−0.7 ± 0.3a	1.4 ± 0.1b	2.7 ± 0.1c

There are 3 replicate plots per treatment. The treatments are described in Table 1. Row by row, significantly different mean values ($P < 0.05$, one-way ANOVA followed by Holm–Sidak's test) are not followed by the same letter. Data are mean ± SE Data for the NH_4NO_3 treatment from Högberg et al. (2011)

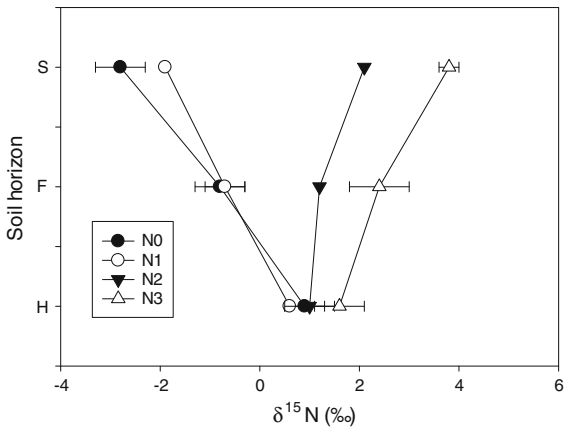

Fig. 4 The $\delta^{15}N$ in soil horizons in the urea plots in 1992, experiment 55, Norrliden, Sweden. Data are plot mean ± SE Treatments are described in Table 1

methods" of this paper) showed no differences between the urea and the NH_4NO_3 plots (two-way ANOVA with form of N and N dose as factors). This is especially interesting, since in the case of NH_4NO_3 half of the N was added as NO_3^-, while NO_3^- in the urea plots must have been produced by nitrifying organisms (Popovic 1985) through a process known to leave the remaining NH_4^+ enriched in ^{15}N (Högberg 1997).

In the NH_4NO_3 treated plots we have previously demonstrated that the major cause of the ^{15}N enrichment of tree needles in the high N treatments, in particular, was a loss of the role of ECM fungi in tree N uptake, and thus in redistributing the N isotopes in the ecosystem (Högberg et al. 2011). Nitrogen losses occurred in the high N treatments, but were presumably mainly through leaching (Johannisson et al. 1999), i.e. mass flow, which does not cause isotope fractionation. We previously also observed that the $\delta^{15}N$ of the new needles decreased after the termination of the N3 treatment, in which case also DNA sequencing and studies of fatty acid biomarkers also revealed a recovery of ECM fungi. Thus, we (Högberg et al. 2011) observed that the $\delta^{15}N$ in the soil profile became similar to that in the control plot and also found no evidence that the N isotope mass balance had changed; we only saw that the role of ECM symbiosis in redistributing the N isotopes in the ecosystem was affected by the N treatments.

In case of the urea plots studied here, the N mass balance appeared to be roughly the same as for the corresponding NH_4NO_3 N dose treatments (Table 2), but the isotope effect of N treatments was clearly larger than in the NH_4NO_3 N2 and N3 treatments (Table 3; Fig. 3). A small effect could be attributed to volatilization of NH_3 directly from the surfaces of the urea fertilizer pellets (Nömmik 1973); we assume that the elevated partial pressures of NH_3 in air around individual urea fertilizer pellets do not overlap, and hence that this process is not dependent on amount of N applied. Nor does the acidity of the soil matter, since the volatilization occurs before the pellets are dissolved by rain or dew, and thus before the added N enters the soil. The larger effect on $\delta^{15}N$ of foliage seen in the N2 and N3 treatments must, therefore, be explained by the other processes, e.g., enrichment of the remaining pool of NH_4^+ through isotope fractionation during nitrification followed by leaching or denitrification.

Our data from the NH_4NO_3 treatment (Högberg et al. 2011) provided clear evidence that the high $\delta^{15}N$ of foliage in N-loaded forests may not necessarily involve increased N losses from the system, but can be a result of internal redistribution of N isotopes. It may, thus, be important to repeat that the losses of N from the urea and NH_4NO_3 treatments were similar, which suggests that the losses from the latter were not associated with N isotope fractionation, as expected if leaching of N added as NO_3^- is the major agent of loss. However, as shown in the urea treatment, in which case isotopic fractionation during nitrification precedes leaching of NO_3^-, the N isotope mass balance cannot be ignored. A good example of this is the very high $\delta^{15}N$ of soils at seabird rookeries, where ammonia volatilization and nitrification are important (e.g., Mizutani et al. 1986). With regard to N deposition on forests, which mostly is composed of both NH_4^+ and NO_3^-, i.e. should be compared with our NH_4NO_3 treatment, we propose that internal N isotope redistribution is the more important driver of early changes in $\delta^{15}N$ of tree foliage, after the initial change towards the $\delta^{15}N$ of the added N (Högberg et al. 2011, Fig. 1 here).

Global surveys show a smaller difference in $\delta^{15}N$ between trees and soil in tropical as compared to temperate and boreal forests, with plant foliage being particularly low in $\delta^{15}N$ relative to soil at the higher latitudes (e.g., Martinelli et al. 1999; Craine et al. 2009). In boreal ECM forests the N cycle is tightly closed, while in tropical rainforests, where trees with arbuscular mycorrhizal symbiosis are more common,

the higher $\delta^{15}N$ of foliage (Martinelli et al. 1999) is an indicator of a more open N cycle, especially with regard to the important role of denitrification in N losses from these ecosystems (e.g., Houlton et al. 2006). We propose that the pivotal role of ECM symbiosis, as shown in the previous study of the NH_4NO_3 plots (Högberg et al. 2011) and elsewhere in affecting the distribution of isotopes of N in ecosystems (e.g., Craine et al. 2009; Hobbie and Ouimette 2009; Hobbie and Högberg 2012), needs to be taken into account if $\delta^{15}N$ is used to study N losses from forest ecosystems.

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