

# Recovery of ectomycorrhiza after 'nitrogen saturation' of a conifer forest

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## Summary

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Received: 16 July 2010

Accepted: 19 August 2010

*New Phytologist* (2011) **189**: 515–525

doi: 10.1111/j.1469-8137.2010.03485.x

**Key words:** <sup>15</sup>N natural abundance, boreal forest, ecosystem nitrogen retention, ectomycorrhizal fungi, nitrogen deposition, soil microbial community.

- Trees reduce their carbon (C) allocation to roots and mycorrhizal fungi in response to high nitrogen (N) additions, which should reduce the N retention capacity of forests. The time needed for recovery of mycorrhizas after termination of N loading remains unknown.

- Here, we report the long-term impact of N loading and the recovery of ectomycorrhiza after high N loading on a *Pinus sylvestris* forest. We analysed the N% and abundance of the stable isotope <sup>15</sup>N in tree needles and soil, soil microbial fatty acid biomarkers and fungal DNA.

- Needles in N-loaded plots became enriched in <sup>15</sup>N, reflecting decreased N retention by mycorrhizal fungi and isotopic discrimination against <sup>15</sup>N during loss of N. Meanwhile, needles in N-limited (control) plots became depleted in <sup>15</sup>N, reflecting high retention of <sup>15</sup>N by mycorrhizal fungi. N loading was terminated after 20 yr. The δ<sup>15</sup>N and N% of the needles decreased 6 yr after N loading had been terminated, and approached values in control plots after 15 yr. This decrease, and the larger contributions compared with N-loaded plots of a fungal fatty acid biomarker and ectomycorrhizal sequences, suggest recovery of ectomycorrhiza.

- High N loading rapidly decreased the functional role of ectomycorrhiza in the forest N cycle, but significant recovery occurred within 6–15 yr after termination of N loading.

## Introduction

Industrial fixation of atmospheric N<sub>2</sub> in fertilizer production, N<sub>2</sub>-fixing crops and NO<sub>x</sub> from combustion processes in industry and vehicles now contribute more N to the world's ecosystems than natural biological N<sub>2</sub> fixation (Vitousek *et al.*, 1997; Galloway *et al.*, 2008). Hence, naturally nitrogen (N)-limited forests become 'N-saturated' when inputs of N exceed the demand by plants and soil microbes, causing high concentrations of NO<sub>3</sub><sup>-</sup> in groundwater and runoff water, and soil and water acidification (e.g. Aber *et al.*, 1998). Anthropogenic emissions of N have recently been marginally reduced in Europe, remain high in North America, and are increasing with industrial expansion in parts of Asia and South America (Galloway *et al.*, 2008).

Fortunately, experimental removal of the high N loads on N-saturated forests has shown that the concentration of

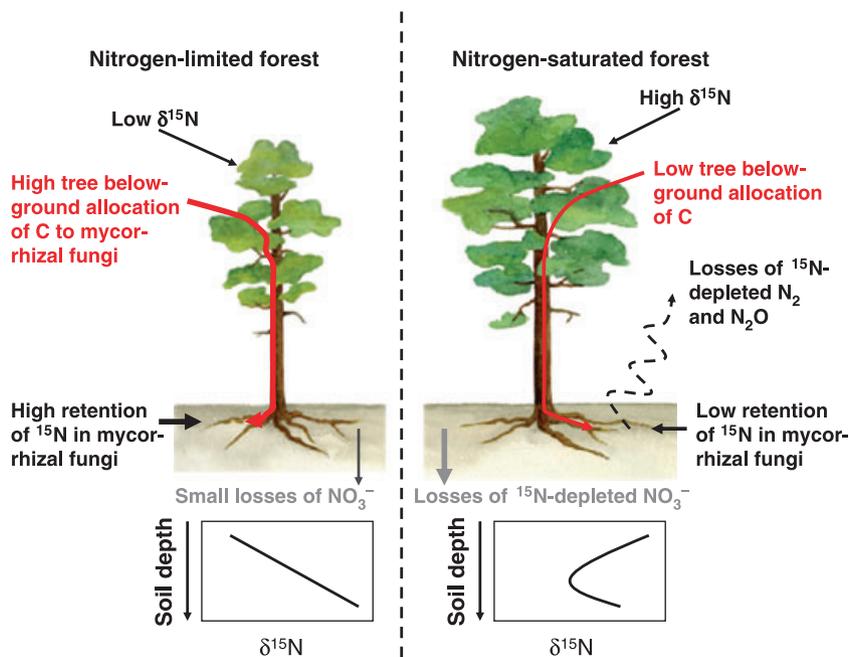
NO<sub>3</sub><sup>-</sup> in soil water declines within a few years (Boxman *et al.*, 1995; Bredemeier *et al.*, 1995; Johannisson *et al.*, 1999; Quist *et al.*, 1999). However, the contributions of different mechanisms to N retention and how it is restored in previously N-saturated forests have not been clarified. Additions of <sup>15</sup>N-labelled NH<sub>4</sub><sup>+</sup> or NO<sub>3</sub><sup>-</sup> to the forest floor have shown that much of the added N becomes immobilized in the soil (Melin *et al.*, 1983; Nadelhoffer *et al.*, 1999, 2004). The prevailing idea is that the N is bound to carbon (C) either abiotically or through the action of soil microorganisms, producing stable compounds (Stevenson, 1982; Knicker, 2004), which has motivated a focus on the C : N ratio of the soil as an indicator of N retention capacity (e.g. Dise *et al.*, 1998; Gundersen *et al.*, 1998). Clearly, there is a significant negative correlation between leaching of N and the C : N ratio of the soil (e.g. Dise *et al.*, 1998; Gundersen *et al.*, 1998), but this contains much unexplained variance, which suggests that other factors are important.

Recovery of N-saturated forests requires that N inputs are reduced and that mineral N is bound biologically to C to form stable compounds. Aber *et al.* (1998) put forward the complex hypothesis that mycorrhizal fungi take up soil inorganic N and convert it into enzymes, which are exuded back into the soil, where these proteins form stable complexes with humic compounds. This hypothesis combines their observations that in N-saturated forests additions of N do not stimulate tree growth and that, nevertheless, the soil microbial N retention capacity appears to be significant.

Like Aber *et al.* (1998), we explore the role of ectomycorrhizal (ECM) symbiosis in forest ecosystem N retention, and how this role is affected by high additions of N. We propose a simpler hypothesis, which does not involve how N is ultimately transferred to stable compounds in the soil. We hypothesize (Fig. 1) that dense ECM fungal mycelia extending from tree roots effectively trap available N in the soil in N-limited forests (Smith & Read, 2008), but do so less effectively when trees decrease their below-ground C allocation in response to a high supply of N (Wallenda & Kottke, 1998; Waring & Running, 1998; Högberg *et al.*, 2003; Nilsson & Wallander, 2003; Treseder, 2004). Thus, central to our hypothesis is the biomass and N retention capacity of the ECM mycelia.

ECM fungal mycelia may account for *c.* 40% of the microbial biomass in N-limited conifer forest (Högberg *et al.*, 2010), and are highly competitive for nutrients as a result of their large supply of C from the trees (Lindahl *et al.*, 2001; Högberg *et al.*, 2008). Moreover, they transfer N to large perennial autotrophic sinks, trees, which allocate N to tissues with high C : N ratios (Johnson, 1992). In view of the observations that ECM fungi are highly enriched in  $^{15}\text{N}$  (Gebauer & Dietrich, 1993; Handley *et al.*, 1996; Taylor *et al.*, 1997; Henn & Chapela, 2001; Trudell *et al.*, 2004) and that the  $\delta^{15}\text{N}$  of soil increases with depth, it also seems possible that ECM fungal N is an important precursor in the formation of stable organic N in the soil (Högberg *et al.*, 1996; Hobbie & Ouimette, 2009). The soil bacteria competing with fungi for N are short-lived and C-limited and maintain C : N ratios of *c.* 5 (Sterner & Elser, 2002), and they cannot immobilize comparable amounts of N in the longer term.

Additions of N reduced the allocation of tree photosynthate to soil biota, including ECM fungi, by as much as 60% in a boreal pine forest (Högberg *et al.*, 2010). A weakening of this important sink for N in the soil of this order is very likely to have profound effects on ecosystem N cycling and retention (Högberg *et al.*, 2006a). For example, the rate



**Fig. 1** Variations in the abundance of the stable isotope  $^{15}\text{N}$ ,  $\delta^{15}\text{N}$ , in N-limited (left) and N-saturated (right) conifer forests. N-limited forests: hydrological and gaseous losses of N are very small, and do not affect the isotopic signature of the N available for uptake, but there is high tree below-ground C allocation to abundant mycorrhizal mycelia, which become highly enriched in  $^{15}\text{N}$  while passing  $^{15}\text{N}$ -depleted N to their host trees. Litter depleted in  $^{15}\text{N}$  is then deposited on the forest floor. This leads to a soil profile with a substantial increase in  $^{15}\text{N}$  abundance with increasing soil depth (Högberg *et al.*, 1996; Hobbie & Ouimette, 2009). N-saturated forests: large losses of  $^{15}\text{N}$ -depleted N by leaching of nitrate and denitrification (producing the gases  $\text{N}_2$  and  $\text{N}_2\text{O}$ ) leave the remaining available N enriched in  $^{15}\text{N}$  (e.g. Handley & Scrimgeour, 1997; Högberg, 1997). Trees reduce below-ground allocation of C to mycorrhizal fungi (Högberg *et al.*, 2010), which therefore do not alter the  $^{15}\text{N}$  abundance of the N transferred from the soil to the trees. These processes lead to deposition of litter with a high abundance of  $^{15}\text{N}$  and hence a high  $^{15}\text{N}$  abundance of the surface soil (Högberg *et al.*, 1996). Note, however, that this profile, with the lowest  $\delta^{15}\text{N}$  in the intermediate horizon, reflects an early phase of N saturation; later the whole profile should become enriched in  $^{15}\text{N}$ .

of gross N mineralization is negatively correlated to the ratio of fungi to bacteria (Högberg *et al.*, 2007a), to which ECM mycelia make a large contribution (Högberg *et al.*, 2010). Conversely, recovery of ecosystem N retention after N saturation requires that free N in the soil once again is effectively coupled to the large tree below-ground flow of C to mycorrhizal fungi by being incorporated into ECM mycelium and trees.

It is difficult to study the role of ECM fungi in ecosystem N retention in the field, especially in the longer term. However, one possibility for exploring the function of ECM fungi in plant N uptake is to analyse the  $^{15}\text{N}$  natural abundance of the components soil, fungi and plants (e.g. Hobbie & Colpaert, 2003; Hobbie & Hobbie, 2006). Such analysis is based on the fact that ECM fungi become enriched in  $^{15}\text{N}$ , whereas N transferred to their plant hosts is depleted in  $^{15}\text{N}$  (Högberg *et al.*, 1999; Hobbie & Colpaert, 2003; Hobbie & Hobbie, 2006, 2008). Such small variations in the ratio  $^{15}\text{N} : ^{14}\text{N}$  are conventionally described in per mil (‰) deviations,  $\delta$ , from the standard atmospheric  $\text{N}_2$ :

$$\delta^{15}\text{N}_{\text{sample}} = ((R_{\text{sample}}/R_{\text{standard}}) - 1) \times 1000,$$

( $R$ , the ratio  $^{15}\text{N} : ^{14}\text{N}$ .)

Here, we use the natural abundance of  $^{15}\text{N}$  of tree foliage and soil to study variations in N uptake by ECM symbiosis during a 20-yr-long phase of N saturation followed by a 15-yr-long phase of recovery in an experiment in a boreal pine forest (Tamm *et al.*, 1999; Högberg *et al.*, 2006b). Our interpretation of causes of variations in  $\delta^{15}\text{N}$  in the forest is based on two established key assumptions (Fig. 1). The first assumption, as mentioned above, is that ECM fungi become enriched in  $^{15}\text{N}$  when they pass N from a source in the soil over to their tree host, because the N transferred to the host is depleted in  $^{15}\text{N}$ . The above-ground plant N then becomes deposited on the forest floor as litter, which leads to a lower  $\delta^{15}\text{N}$  of the surface soil horizon than deeper down, where ECM roots take up N. For example, during the initial 3–4 yr of needle litter decomposition in boreal pine forest, decomposer fungi were found to dominate and the  $\delta^{15}\text{N}$  of the litter did not change, but then the litter was colonized by ECM fungi and became substantially enriched in  $^{15}\text{N}$  (Lindahl *et al.*, 2007); this mechanism explains the high  $\delta^{15}\text{N}$  of the soil further down the profile. Note that, because the N is not taken up directly from the litter by the plants in the system we study, the N isotope fractionation upon uptake through ECM fungi, which occurs deeper down in the soil (Lindahl *et al.*, 2007), leads to a progressively larger difference in  $\delta^{15}\text{N}$  between the surface of the soil and deeper horizons (Högberg *et al.*, 1996; Hobbie & Ouimette, 2009). For example, the difference in  $\delta^{15}\text{N}$  between soil horizons in a ploughed agricultural field was < 2‰, but after 30 yr of ECM forest (*Pinus taeda*) develop-

ment the difference between the uppermost organic layer and the 35–60 cm soil depth was as large as 15‰ (Billings & Richter, 2006). The second assumption is that processes leading to N loss, or these processes themselves, lead to greater losses of  $^{14}\text{N}$  than of  $^{15}\text{N}$  (Handley & Scrimgeour, 1997; Högberg, 1997; Robinson, 2001). Examples of such processes are nitrification and denitrification; leaching of nitrate does not fractionate the N isotopes, but the preceding nitrification does.

In N-limited forests with small losses of N, the foliage of the trees becomes depleted in  $^{15}\text{N}$ , whereas the fungal material in the soil, and therefore the bulk soil, becomes enriched in  $^{15}\text{N}$ . Typically, the increase in  $\delta^{15}\text{N}$  between foliage and deeper soil layers is up to 5–10‰ (Riga *et al.*, 1971; Nadelhoffer & Fry 1988; Gebauer & Schulze, 1991; Högberg *et al.*, 1996; Hobbie & Ouimette, 2009), but may be larger (Billings & Richter, 2006). However, in N-saturated systems with large losses of N, and probably a reduced biomass and retention of N by the ECM mycelium, the tree foliage should become more enriched in  $^{15}\text{N}$ . Indeed, the latter has been demonstrated in field experiments and in studies along N deposition gradients (Högberg *et al.*, 1996; Emmett *et al.*, 1998; Pardo *et al.*, 2007). In Fig. 1, we describe the major differences in N cycling in N-limited and N-saturated forests, and their potential consequences for the distribution of N isotopes. We use this theoretical framework, along with analyses of fungal fatty acid biomarkers and DNA, to interpret the changes in the role of ECM fungi in N uptake in N-saturating forest and in forest recovering after N saturation. We assume that, in an ECM forest that is recovering from N saturation, a larger gradient in  $\delta^{15}\text{N}$  with low values in foliage and the uppermost soil horizon and higher  $\delta^{15}\text{N}$  deeper down in the soil will once again develop.

## Materials and Methods

The study site is located in a *Pinus sylvestris* L. forest at 64°N near Norrliden in northern Sweden (Tamm *et al.*, 1999). This forest was planted in 1953 after prescribed burning in 1952, and was thinned first in 1985 and again in 2000. The soil is a glacial till with sand as the dominant fraction. The soil type is a Haplic Podzol (FAO system) or Typic Haplocryod (US Soil Taxonomy). At this site, we studied the long-term N-loading experiment (Tamm *et al.*, 1999; Högberg *et al.*, 2006b), with control plots (N0) receiving *c.* 3 kg N ha<sup>-1</sup> yr<sup>-1</sup> by deposition, and N1, N2 and N3 plots also receiving annual additions of  $\text{NH}_4\text{NO}_3$  of on average *c.* 35, 70 and 110 kg N ha<sup>-1</sup> yr<sup>-1</sup>, respectively (Table 1). These treatments encompass the variations in N deposition across Europe (Dise & Wright, 1995). The heaviest N load, N3, was terminated after 20 yr, allowing recovery from N saturation (Tamm *et al.*, 1999; Högberg *et al.*, 2006b) to be compared with continuing N loading in N1 and N2, and the naturally N-limited conditions in N0.

**Table 1** Nitrogen addition rates ( $\text{kg N ha}^{-1} \text{ yr}^{-1}$ ) 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)

Year(s)	Nitrogen addition treatment		
	N1	N2	N3
1971–1973	60	120	180
1974–1976	40	80	120
1977–1990	30	60	90
1991	0	0	0
1992	60	120	0
1993–2006	30	60	0

Control plots, N0, received only the background deposition of N ( $c. 3 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ ).

The treatments N1 and N2 were suspended in 1990, but continued from 1992, starting with double doses of N that year (Table 1). The plots are  $30 \text{ m} \times 30 \text{ m}$  squares, and there are three replicate plots of each treatment.

Samples of current-year needles were taken from the top whorls of 10 trees per plot every year, and combined into one sample per plot and year. We have previously published data on the N% and  $\delta^{15}\text{N}$  of needles from 1970, 1971, 1975, 1980, 1984 and 1989 (Högberg, 1991) and for every year in the period 1985–1997 except for 1991 (Quist *et al.*, 1999). Here, we show data for every year in the periods 1970–1990 and 1992–2005 (no samples were taken in 1991). Samples of the three horizons S, F and H of the organic mor-layer of the soil were taken from five locations in each plot in 1992 and 2005. S is the surface horizon consisting of litter mixed with mosses and lichens. This horizon contains no plant roots. In the F horizon, decomposing fragments of above-ground plant litter are still visible, and the horizon is densely colonized by ECM roots and fungal hyphae. H is the lower organic horizon, in which the organic matter is highly decomposed; there is also an abundance of ECM roots and fungal hyphae. The mor-layer (i.e. the S, F and H horizons combined) varied in thickness between 5 and 10 cm. According to estimates based on ‘bomb- $^{14}\text{C}$ ’ analysis and modelling, the mean ecosystem ages (time since C fixation through photosynthesis) of the organic matter were 4–6, 11–15 and 27–47 yr in the S, F and H layers, respectively (Franklin *et al.*, 2003). Analyses of the  $\delta^{15}\text{N}$ , N% and C% of needles and root-free soil were carried out on elemental analysers coupled online to isotope ratio mass spectrometers (an ANCA system coupled to a 20-20 IRMS (Europa Scientific Ltd, Crewe, UK) or a Carlo-Erba EA 1110 CHN analyser coupled to a Delta Plus IRMS (ThermoQuest Finnigan, Bremen, Germany)). Analyses carried out on different instruments used the same standard materials.

For the study of the composition of the soil microbial community, the combined F + H horizon was sampled in 2004 and phospholipid fatty acids (PLFAs) in root-free soil

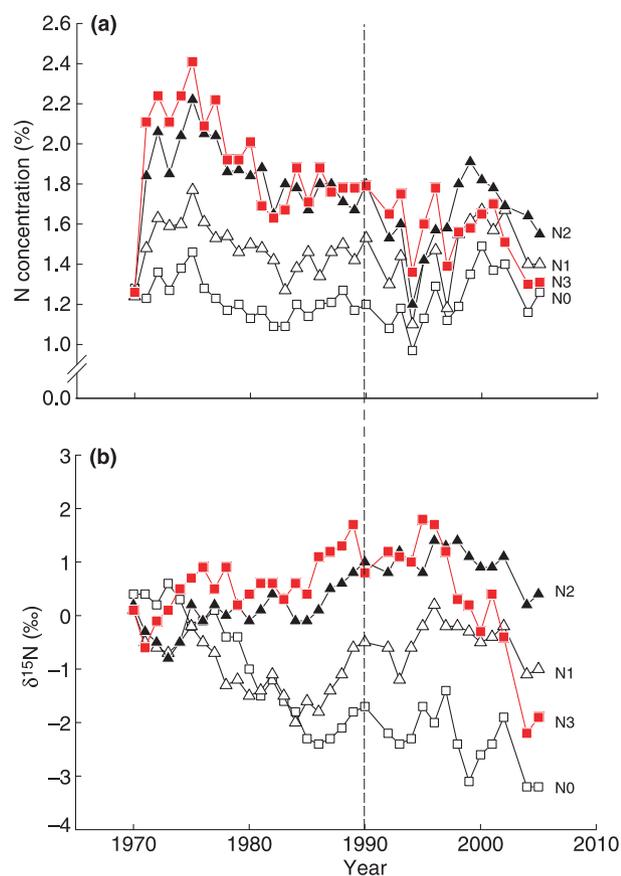
were extracted and analysed as biomarkers of bacteria and fungi; these data have been published (Högberg *et al.*, 2007b). The lipids were extracted from frozen soil using a modified Bligh & Dyer (1959) method (Frostegård *et al.*, 1991, 1993), separated on columns packed with silica gel, and eluted in sequence with chloroform, acetone and methanol. The methanol fraction was dried under  $\text{N}_2$ , dissolved, and subjected to mild methanolysis. Fatty acid methyl esters were analysed on a gas chromatograph (Agilent 6890; Agilent Technologies, Palo Alto, CA, USA). A total of 27 PLFAs were identified and quantified. The PLFA 18:2 $\omega$ 6,9 is considered the best biomarker for ECM fungi (Yarwood *et al.*, 2009), and this was supported by the DNA data obtained in this study. Moreover, tree-girdling, which terminates the tree below-ground C flow, eliminated sporocarp production by ECM fungi (Högberg *et al.*, 2001), reduced the mol% 18:2 $\omega$ 6,9 but not the mol% 18:1 $\omega$ 9 (Högberg *et al.*, 2007b).

DNA was extracted from the soil; fungal genes were amplified, cloned, sequenced and identified as described in detail by Yarwood *et al.* (2009). Briefly, DNA was extracted using a PowerSoil™ DNA isolation kit (MO BIO Laboratories, Carlsbad, CA, USA). The fungal 18S internal transcribed spacer (ITS) gene was amplified with primers ITS 1F and ITS 4. Length heterogeneity (LH)-PCR profiles were generated by Oregon State University’s Center for Genome Research and Biocomputing after fragment length analysis (ABI 3100 capillary DNA sequencer; Applied Biosystems, Foster City, CA, USA). Clones were generated using a Topo TA cloning™ kit for sequencing (Invitrogen, Carlsbad, CA, USA). Clones containing inserts were plasmid purified using the QIAprep™ Spin Miniprep kit (Qiagen Inc., Carlsbad, CA, USA) and sequenced by the High Throughput Genomics Unit at the Department of Genome Science, University of Washington, Seattle, WA, USA. ITS genes belonging to ECM fungi were confirmed using the ECM fungal sequence database UNITE (Kõljalg *et al.*, 2005). Here, we only report the contribution of ECM fungi to total fungal sequences; the effects of the N treatments on the different ECM species will be reported in detail elsewhere.

## Results

### Needle N concentration and $\delta^{15}\text{N}$

The N concentration of needles rose rapidly in N-loading treatments above the average level 1.2–1.3% found in the control (N0) treatment (Fig. 2a). The highest percentage of N, *c.* 2.4% in N3, occurred at an early stage of the experiment, and corresponded to the higher N addition rates at this stage (Table 1). Through time the differences in %N in needles between treatments decreased, and the difference was only *c.* 0.25% (in absolute terms) between the N0 and N2 plots in 2005. At this stage, the treatment N3 had been terminated for



**Fig. 2** Concentration of nitrogen (N) (a) in needles of *Pinus sylvestris* and natural abundance of <sup>15</sup>N, δ<sup>15</sup>N (b), of tree needles in plots in Experiment 55, Norrleden, Sweden, 1970–2005. Treatments are described in Table 1. The broken line indicates termination of the high N-load N3 treatment in 1990. Data are mean values based on three plots.

15 yr, during which time the needle N concentration had changed from a value comparable to that of N2 to a similar value to that found in N0; this decrease occurred between 1996 and 2005.

When the %N in needles increased rapidly in response to the first N additions, the δ<sup>15</sup>N of the needles fell, indicating that the added N had a lower δ<sup>15</sup>N abundance than the endogenous available N (Fig. 2b). After 4 yr of N loading in N3, the <sup>15</sup>N in needles started to exceed that in N0. In N2, a similar increase in δ<sup>15</sup>N above that of N0 occurred after c. 8 yr, and in the case of N1 this happened after c. 15 yr. In the control, the δ<sup>15</sup>N in needles fell more or less continuously from just above 0‰ to c. -3‰ during the 36 yr of observation, whereas the δ<sup>15</sup>N in the ongoing high-N treatments stayed above 0‰. In N3, which was terminated in 1990, the δ<sup>15</sup>N in needles stayed high for another 6 yr, until 1996, after which it declined to a value between those in the N0 and N1 plots in 2005. This decrease in N3 plots corresponded to c. 4‰; there was a

correlation between the decreases in δ<sup>15</sup>N and N% ( $r_{\text{Pearson}} = 0.71$ ,  $P < 0.05$ ,  $n = 9$ ). Increases in δ<sup>15</sup>N of 0.8, 1.4, 1.2 and 1.3‰ were seen in the needles from N0, N1, N2 and N3, respectively, during up to 3–5 yr after a thinning of the forest in 1985 (Fig. 2). A much smaller and less significant increase in the δ<sup>15</sup>N of needles occurred after a second thinning in 2000; in N0 plots this increase was 0.7‰.

### Organic soil horizon N contents, C : N ratios and δ<sup>15</sup>N

Analysis of soil samples from the NH<sub>4</sub>NO<sub>3</sub> addition treatments in 1992 showed that the N content of all three upper organic layers composing the mor-layer increased by 70–180 kg N ha<sup>-1</sup> per horizon after N loading. Thus, the total N content of the mor-layer increased by c. 400 kg N ha<sup>-1</sup> from N0 to N3 (Table 2). The increases in N content of the mor-layers corresponded to a retention of 36, 24 and 18% of the N added in N1, N2 and N3, respectively. In 1992, the δ<sup>15</sup>N values of the S and H layers were slightly lower in N1 than in N0, whereas the δ<sup>15</sup>N of the F layer was significantly lower (Fig. 3). The weighted average δ<sup>15</sup>N of the mor-layer was clearly lower in N1 than in N0 (Table 2), corroborating the previous suggestion based on needle data that the added N was isotopically lighter than the endogenous N. There was a significant increase in the weighted average δ<sup>15</sup>N of the mor-layer with increasing N additions in N-loaded plots (Table 2). At the higher N addition rates, in N2 and N3, N loading resulted in a higher δ<sup>15</sup>N of the S layer (Fig. 3), in particular. It is noteworthy that the difference between the S and H layers, 1.7‰, was comparatively small in the N0 plots at this stage.

In 2005, the differences in <sup>15</sup>N abundance among treatments had evolved further (Fig. 3), as had the difference between the S and H layers in the control, N0, which was 4.9‰. Hence, at this stage this soil profile was approaching that typical of N-limited ECM forests (Fig. 1), that is, one with an increase in δ<sup>15</sup>N of 5–10‰ with increasing soil depth. In the treatment N1, the δ<sup>15</sup>N of all three soil horizons decreased relative to 1992, suggesting that the added N had lower <sup>15</sup>N abundance than the endogenous N. In the N2 treatment, there was an increase in δ<sup>15</sup>N in the S layer, in particular, indicating progression towards N saturation (Fig. 1). In N3, in which the N loading was terminated in 1990, that is, 15 yr before this sampling, the δ<sup>15</sup>N of the S layer had greatly decreased, and there was an increase of 2.1‰ with increasing depth. There were correlations between the δ<sup>15</sup>N of the S layer and that of current needles sampled 4–5 yr earlier in both 1992 ( $R^2_{\text{adj}} = 0.5$ ,  $P < 0.01$ ) and 2005 ( $R^2_{\text{adj}} = 0.8$ ,  $P < 0.001$ ) (Fig. 4). A time-lag of 4–5 yr is consistent with the ‘bomb-<sup>14</sup>C’ age of the S layer, which was estimated to be 4–6 yr (Franklin *et al.*, 2003).

Variable	N0	N1	N2	N3
Amount of N (kg ha <sup>-1</sup> ) in the mor-layer	308 (12)a	565 (31)b	656 (47)b	700 (83)b
Weighted average $\delta^{15}\text{N}$ (‰) of the mor-layer	1.01 (0.31)a	-0.28 (0.10)b	0.24 (0.08)a,b	0.78 (0.11)a

The weighted average  $\delta^{15}\text{N}$  was calculated using data on the  $\delta^{15}\text{N}$  and the amount of N in the three horizons sampled. Row by row, significantly different mean values ( $P < 0.05$ , ANOVA followed by Holm–Sidak's test) are not followed by the same letter. Figures in parentheses are SEM.

### Relationships between $^{15}\text{N}$ of trees and abundances of soil fungi, especially ECM fungi

In 2004, when both needles and soil were sampled, there was a negative relationship between the  $\delta^{15}\text{N}$  of needles and the mol% of the fungal biomarker 18:2 $\omega$ 6,9 (Fig. 5a). This means that the N1 and N2 plots had the highest  $\delta^{15}\text{N}$  in needles, but also the lowest mol% of fungi in the soil. Moreover, the N3 treatment, which was terminated in 1990, had a position along this relationship between the N0 and N1 plots (Fig. 5a). It should be noted that total PLFA was significantly lower in N1 and N2 (but not in N3) than in N0, and that the mol% of bacterial biomarkers did not differ between treatments, whereas the mol% of 18:1 $\omega$ 9 and even more so that of 18:2 $\omega$ 6,9 were lower in N1 and N2 than in N0 (Högberg *et al.*, 2007b). Thus, we conclude that the fungal biomass was lower in N1 and N2 than in N0. The ratio fungi:bacteria were significantly lower in N1–N3 than in N0 (Högberg *et al.*, 2007b). The idea that 18:2 $\omega$ 6,9 is a good marker for ECM fungi, in particular, was supported by the strong correlation between the ratio of ECM to total fungal sequences and this PLFA (Fig. 5b). Overall, this means that there may be a negative correlation between the ratio of ECM to total fungal sequences and the  $\delta^{15}\text{N}$  in needles, but the degrees of freedom are too few to allow a conclusive analysis in this case ( $df = 3$ ,  $R^2_{\text{adj}} = 0.70$ ,  $P = 0.11$ ).

### Discussion

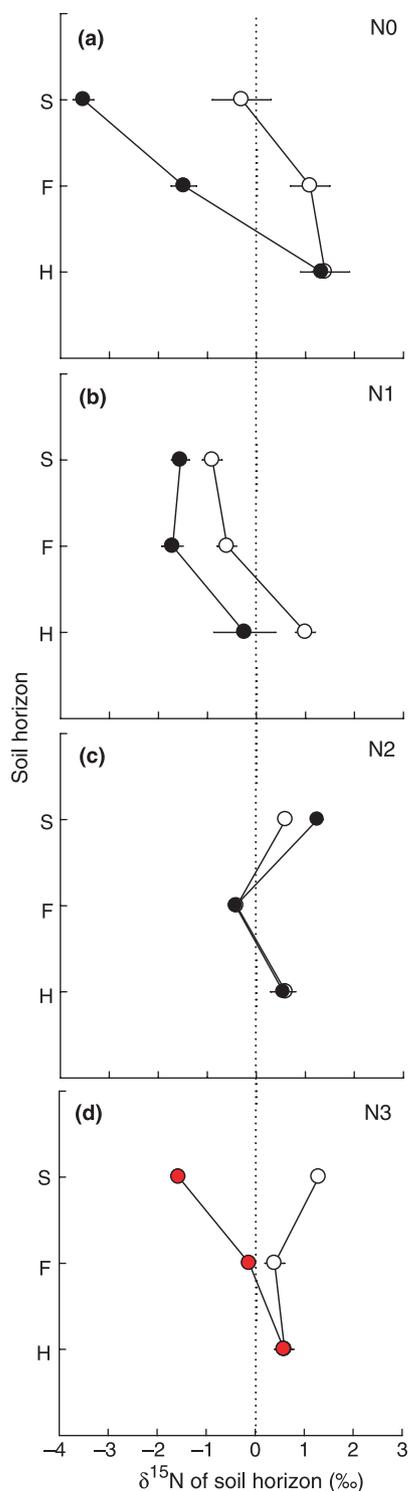
In this study, the analysis of changes in  $\delta^{15}\text{N}$  is of central importance. It is, therefore, crucial to explain the temporal changes observed in the tree needles and in the soil profile in the control (N0) treatment (Figs 2, 3), because data from this treatment serve as a baseline for our interpretation of the effects of additions of N to N1–N3 plots. The  $\delta^{15}\text{N}$  of tree foliage was between 0 and 0.4‰ the year before treatments started, but declined to *c.* -3‰ in N0 over the 35 yr of observations (Fig. 2b). We suggest that the relatively high  $\delta^{15}\text{N}$  of foliage in 1970 relates to loss of S-layer material (i.e. the horizon most depleted in  $^{15}\text{N}$ ) during the prescribed burning in 1952, which preceded planting in 1953. Moreover, we suggest that the decline in foliage  $\delta^{15}\text{N}$

**Table 2** The amounts of nitrogen (N) and the weighted average  $\delta^{15}\text{N}$  (‰) of the organic mor-layer in the different N treatments in E55 at Norrleden in 1992

reflects continuous redistribution of N isotopes within the ecosystem driven by tree N uptake through ECM fungi, that is, progressive depletion in the  $^{15}\text{N}$  of foliage and enrichment in the  $^{15}\text{N}$  of ECM fungal hyphae in deeper soil layers, as described in Fig. 1. Evidence that redistribution of N isotopes occurred over the period 1992–2005 can be found in Fig. 3, where we can observe that the difference in  $\delta^{15}\text{N}$  between the S and H layers was smaller in 1992 than in 2005 ( $1.7 \pm 0.3\text{‰}$  and  $4.9 \pm 0.0\text{‰}$ , respectively). The theory that the change in  $\delta^{15}\text{N}$  in the S layer was proximally driven by the change in the  $\delta^{15}\text{N}$  of needle litter is supported by the strong positive relationships between the  $\delta^{15}\text{N}$  values in needles and in the S layer (Fig. 4).

The high-N treatment in N3 rapidly caused N saturation in the sense that tree growth in N3 was not higher than in N0 plots (Tamm *et al.*, 1999; Högberg *et al.*, 2006b), whereas tree growth in N1 was significantly higher than in N0 (Högberg *et al.*, 2006b). Note that the definition of N saturation adopted here is widely used by ecosystem ecologists, but is not synonymous with a chemical concept of N saturation, which would require that the system does not retain added N (i.e. that N outputs = N inputs). Data on amounts of N in the vegetation and in the upper 20 cm of the soil (Tamm *et al.*, 1999) suggest that most of the N added has been lost from these ecosystem compartments in N3. However, 3–4 yr after termination of treatment N3 the leaching was only 3 kg N ha<sup>-1</sup> yr<sup>-1</sup> (Table 3) in contrast to the almost 10-times higher leaching in the ongoing treatment N2 (Table 2). Thus, there was clear evidence that N3 plots had become N-saturated before 1990, but also that they started to recover from this condition upon termination of N loading, whereas the ongoing N loading continued to move N2 towards N saturation.

Under a high N load, the  $\delta^{15}\text{N}$  of tree foliage increased after 4, 8 and 15 yr in N3, N2 and N1, respectively (as opposed to a decrease in N0 plots) (Fig. 2b). We attribute this increase in the  $\delta^{15}\text{N}$  of tree foliage to reduced retention of N by ECM fungi and to losses of N from the ecosystem (Fig. 1). A change in foliar  $\delta^{15}\text{N}$  in the N addition treatments could, in theory, also occur if the isotopic signature of the  $\text{NH}_4\text{NO}_3$  added deviates from that of available endogenous N. However, our data did not support the idea that the increase in the  $\delta^{15}\text{N}$  of tree foliage was a result of



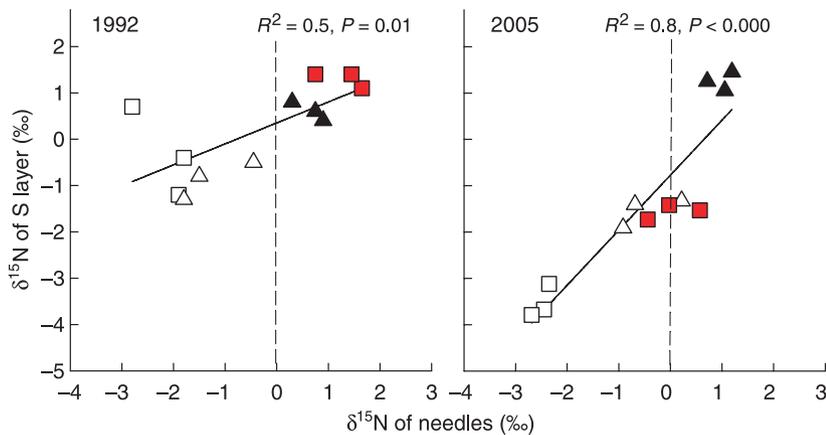
**Fig. 3** Natural abundance of  $^{15}\text{N}$ ,  $\delta^{15}\text{N}$ , of soil horizons in plots in Experiment 55, Norrleden, Sweden in 1992 (open circles) and in 2005 (closed circles). The nitrogen (N) treatments are described in Table 1. Data are mean values based on three plots ( $\pm$  SEM).

the added N being isotopically heavy. First, in all N addition treatments, the initial response was a decline in  $\delta^{15}\text{N}$  relative to  $\delta^{15}\text{N}$  in N0 plots, suggesting that the fertilizer

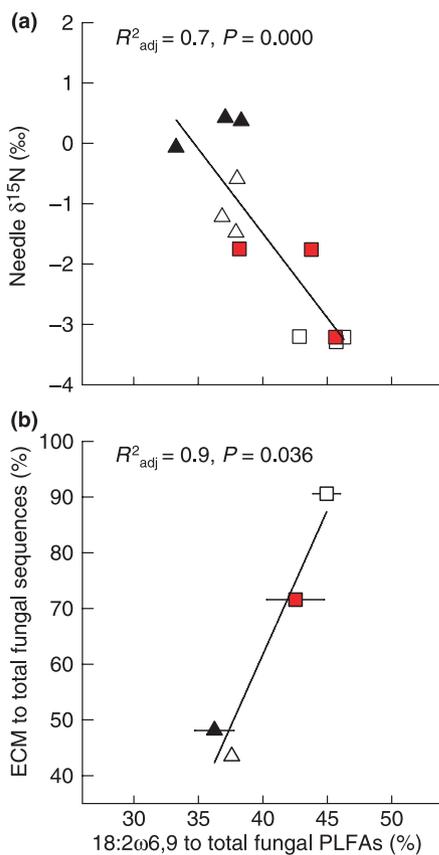
added was depleted in  $^{15}\text{N}$ . Secondly, mass balance calculations of the N in the three horizons of the upper organic mor-layer of the soil after 22 yr (in 1992) showed that, although the mor-layers of the N1–N3 plots all contained 250–400 kg N  $\text{ha}^{-1}$  more N than N0 plots, the weighted average  $\delta^{15}\text{N}$  of the mor-layer was not different in N3 from N0, whereas the fact that the  $\delta^{15}\text{N}$  was lower in N1 than in N0 (Table 2) also shows that the added N was isotopically lighter than available endogenous N. Furthermore, the  $\delta^{15}\text{N}$  of all three soil horizons decreased in N1 in 1992–2005, which shows that the  $\delta^{15}\text{N}$  of the added N continued to be low (Fig. 3).

The question remains whether the changes in  $\delta^{15}\text{N}$  in needles and in the soil profile in the different treatments were driven by changes in the N input–output balance (Högberg, 1990, 1991; Högberg & Johannisson, 1993) or whether they are primarily related to changes in the functional role of ECM fungi, which become expressed as changes in the distribution of the N isotopes within the ecosystem. Previous studies of this experiment have revealed a strong correlation between changes in the  $\delta^{15}\text{N}$  of foliage and estimated ecosystem N losses (Högberg & Johannisson, 1993). As discussed here, this also involves a change in the abundance and function of ECM fungi. In fact, our data on soils (Table 2) suggest only minor changes in the isotopic mass balance, but larger changes in the distribution of N isotopes in the soil–plant system (Figs 2, 3). Thus, in this particular case, with N added as  $\text{NH}_4\text{NO}_3$ , the effect of N additions on the function of ECM fungi appears to be more important than the effect on the N input–output balance. As shown here, at the higher N addition rates in N2 and N3, N loading resulted in comparatively high  $\delta^{15}\text{N}$  of the S layer, in particular (Fig. 3), whereas in both N0 and N1  $\delta^{15}\text{N}$  increased with depth. We have previously shown that the effect on  $\delta^{15}\text{N}$  of foliage is larger when N is added in the form of urea than when it is added as  $\text{NH}_4\text{NO}_3$  (Högberg, 1991; Högberg & Johannisson, 1993). This is attributable to isotope fractionation during  $\text{NH}_3$  volatilization from urea and a larger fraction of the N being nitrified, thus producing a greater N isotope effect for the same fraction of N lost from the ecosystem (Högberg & Johannisson, 1993).

Previously, the decline in the  $\delta^{15}\text{N}$  of foliage in N0 plots was attributed to contamination of N lost from the high N addition plots (Högberg, 1991). If this was correct, the weighted average  $\delta^{15}\text{N}$  of the mor-layer in 1992 should have been lower in N0 plots than in N1–N3 plots, but this was not the case (Table 2). Thus, changes in  $\delta^{15}\text{N}$  in the foliage of the trees were not so much driven by isotope fractionations linked to N losses altering the  $\delta^{15}\text{N}$  of available N pools in the soil as by internal redistribution of N isotopes in the ecosystem, which suggests a key role of ECM function in changing the distribution of N isotopes (Fig. 1). This suggestion is further supported by the fact that, upon termination of the highest N load treatment,



**Fig. 4** The  $\delta^{15}\text{N}$  of the uppermost soil horizon (S) vs that of tree needles in Experiment 55, Norrleden, Sweden, in 1992 and in 2005, assuming a lag time of 4–5 yr between current needles and their deposition by litter-fall on the S layer. Data are from individual plots ( $n = 12$ ). Open squares, N0 plots; open triangles, N1 plots; closed triangles, N2 plots; closed squares, N3 plots. The slopes of the lines are  $0.45 \pm 0.14$  ( $P = 0.011$ ) in 1992 and  $1.19 \pm 0.17$  ( $P < 0.001$ ) in 2005.



**Fig. 5** The relationship between the natural abundance of  $^{15}\text{N}$ ,  $\delta^{15}\text{N}$ , of needles of *Pinus sylvestris* and the mol% of the PLFA fungal biomarker 18:2 $\omega$ 6,9, and between the latter and the percentage ectomycorrhizal (ECM) to total fungal sequences in N0–N3 plots in 2004 in Experiment 55, Norrleden, Sweden. Note that the treatment N3 was terminated in 1990 (Table 1), and that trees in the N3 plots had as high  $\delta^{15}\text{N}$  as trees in N2 plots at that time (cf. Fig. 2). Data are mean values based on three plots ( $\pm$  SEM). Open triangles, N1; closed triangles, N2; open squares, N0; closed squares, N3. PLFA, phospholipid fatty acids.

N3, in 1990, the  $\delta^{15}\text{N}$  and the N concentration of foliage both remained high for  $\approx 6$  yr (Fig. 2), whereas the leaching of N diminished more rapidly (Table 3). This means that

the  $\delta^{15}\text{N}$  of needles did not change as a direct and immediate consequence of reduced N losses from the ecosystem, which were evident already after 3 yr (Table 3), but changed first when the N%, an indicator of plant N status, decreased.

In 2004, the fungal biomarker 18:2 $\omega$ 6,9 and the ratio of ECM to total fungal DNA sequences were both low in soils in the ongoing N-loading treatments N1 and N2 in comparison to the terminated high load N3 treatment and N0 (Fig. 5). Moreover, the fungal PLFA biomarker was negatively correlated with the  $\delta^{15}\text{N}$  of tree foliage. Taken together, these findings suggest a relatively low importance of ECM fungi in N uptake in N-loaded forests, but also that this function of ECM fungi recovers if the N load is terminated. Given that the concentrations of inorganic N and leaching losses of N decreased several years before the decline in the  $\delta^{15}\text{N}$  of tree foliage, we cannot explain the latter by any other mechanism than recovery of functional ECM fungi.

Why should this occur several years after the observed decrease in N availability in the soil (Table 3)? First, we know that C allocation from tree photosynthesis to roots and mycorrhizal fungi is greatly reduced after high N additions (Olsson *et al.*, 2005; Högberg *et al.*, 2010), which suggests that recovery of the below-ground C flow to roots and ECM fungi must occur if the role of ECM fungi as a sink for N is to be restored. Our data suggest that this may take some years after termination of high N loading, possibly because the N in the trees is recycled between needles of different age classes; at this site the pine trees retain up to five age classes of needles. Secondly, the importance of ECM fungi in this context is further supported by the distribution of the N isotopes in the soil in 2005, that is, the fact that in N3 there was a clear increase in  $\delta^{15}\text{N}$  with depth (Figs 3, 4). The change of this pattern is evidently driven by the decrease in the  $\delta^{15}\text{N}$  of the needles (Fig. 4), which did not occur in N3 until 6 yr after termination of N loading. Meanwhile, in the ongoing N2 treatment, in particular, the  $\delta^{15}\text{N}$  was relatively high in the S layer (Fig. 3), indicating continuing impaired N retention by ECM mycelium and losses of N.

**Table 3** Carbon (C) : nitrogen (N) ratio and concentrations of exchangeable  $\text{NH}_4^+$  and  $\text{NO}_3^-$  in the mor-layer, and leaching of these ions (zero-tension lysimeters at 20 cm depth in the mineral soil)

Year	N treatment			
	N0	N1	N2	N3
	C : N ratio			
1988 <sup>1</sup> c	36.1 (0.9)	31.3 (0.9)	31.1 (1.4)	30.0 (0.2)
2004 <sup>2</sup>	37.5 (1.2)a	31.1 (1.8)a,b	27.7 (0.6)b,c	27.2 (0.7)c
	Leaching of inorganic N ( $\text{kg N ha}^{-1} \text{ yr}^{-1}$ )			
1993–1994 <sup>3</sup>	0.3	3.3	25.0	3.2
	Exchangeable inorganic N ( $\mu\text{g g}^{-1} \text{ o.m.}$ )			
1997 <sup>4</sup>	0.4	36.2	160.0	19.0
2004 <sup>2</sup>	1 (0.3)a	41 (32)a,b	96 (11)b	4 (2)a,b

Values are means (with SEM in parentheses). Row by row, mean values not followed by the same letter are significantly different at the level  $P < 0.05$ .

<sup>1</sup>Data from Tamm *et al.* (1999).

<sup>2</sup>Data from Högberg *et al.* (2007b).

<sup>3</sup>Data from Johansson *et al.* (1999).

<sup>4</sup>Data from Quist *et al.* (1999).

Why may the role of mycorrhiza proposed here be more interesting than the soil C : N ratio as an indicator of N retention capacity? First, it seems unlikely that soil organic matter with a low C : N ratio in an N-saturated forest becomes the important and dynamic N sink implied by the rapid declines in N% and  $\delta^{15}\text{N}$  of foliage years after termination of the N3 treatment (Fig. 2, Table 3). Reductions of N inputs would only slowly alter the C : N ratio of the soil. Indeed, in 2004, the soil C : N ratios were almost identical in the N3 and N2 plots (Table 3), which offers no explanation of the precipitous declines in foliage N%,  $\delta^{15}\text{N}$  (Fig. 2) and extractable inorganic soil N (Table 3) in the terminated N3 treatment. Secondly, there is no evidence of rapidly enhanced growth (fixation of C) by the trees in N3. This leaves the mechanism proposed here, increased below-ground C allocation by the trees to their ECM fungi, as the more plausible cause of the decrease in foliage  $\delta^{15}\text{N}$ . However, this proposition needs to be supported by data confirming that the increase in ECM fungi (Fig. 5b) has occurred as a result of an increase in tree below-ground C allocation in response to reduced N availability.

In their review of the processes involved in N saturation of forests, Aber *et al.* (1998) highlighted the remarkably high N retention capacity of forests. They stated that this capacity resides in the soil, but requires that the continuum from plant canopies to roots and mycorrhizal fungi is intact. In the view of Aber *et al.* (1998), the key process is the conversion of inorganic N by mycorrhizas to extracellular enzymes, which form stable N through condensation reac-

tions between these proteins and humic compounds. We agree that the ECM symbiosis plays the key role in linking the C supply from the trees to the N supply in the soil. Our previous work in boreal coniferous forests has demonstrated that the immediate retention of labelled  $^{15}\text{NH}_4^+$  is strongly correlated to the ratio fungi:bacteria in the soil (Högberg *et al.*, 2006a), which suggests that fungi are stronger sinks for available N than are bacteria.

In contrast to the focus of Aber *et al.* (1998) on extracellular enzymes, we see tree below-ground allocation of C to ECM fungal mycelium and its responses to changes in N supply (Högberg *et al.*, 2010) as the key mechanism in forest ecosystem N retention. The high  $\delta^{15}\text{N}$  of deeper soil layers suggests that stable N is enriched in  $^{15}\text{N}$ , which coincides with the observation that ECM fungi are highly enriched in  $^{15}\text{N}$  (Högberg *et al.*, 1996; Lindahl *et al.*, 2007; Hobbie & Quimette, 2009). It may well be that the extracellular enzymes are also enriched in  $^{15}\text{N}$ , but the ECM fungal mycelium may in itself be a major precursor for stable N. Studies in agricultural settings have identified compounds in microbial cell walls as important precursors for stable soil N (e.g. Guggenberger *et al.*, 1999), but the limited evidence available from boreal forests suggests that ECM fungal cell walls have a lower  $^{15}\text{N}$  abundance than the fungal cytoplasm (Taylor *et al.*, 1997), which calls for more detailed studies of the fate of fungal N compounds and how their N isotope compositions ultimately influence that of the soil. The partitioning of N isotopes by functional ECM symbioses under N-limited conditions with  $^{15}\text{N}$ -depleted N transferred to the tree canopies and  $^{15}\text{N}$ -enriched N fungal mycelium in the soil is, however, well established and shows, in our view, the dependence of the N retention capacity of conifer forests on the supply of photosynthate C to the ECM mycelial system. We have previously shown that the ECM root tips which are the strongest sinks for C from photosynthesis are also the strongest sinks for N in the soil (Högberg *et al.*, 2008). Hence, the recovery of the capacity of ECM forests for N retention after a period of N saturation requires that the tree below-ground C flow to ECM fungi is restored. The close coupling between the tree canopy and ECM fungi was recently highlighted by Wallander *et al.* (2010), who found that production of ECM mycelium peaks at maximum tree canopy biomass. This means that relatively young fast-growing forests should be the strongest sinks for N, and leak least N, as was shown in the classical study by Vitousek & Reiners (1975).

## Acknowledgements

The experiment E55 was established by the late Carl Olof Tamm and his co-workers. Håkan Wallmark and Elin Norlin carried out the IRMS analyses. We acknowledge financial support from the former Royal College of Forestry, the Faculty of Forest Science at SLU, the

Foundation for Plant Nutrition Research, the Swedish Environmental Protection Agency, The Swedish Science Council, the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning and the foundation MISTRA (grant to the project Future Forests).

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