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DOI
10.1016/j.scitotenv.2020.144356

Publication date
2021

Document Version
Final published version

Published in
Science of the Total Environment

Science of the Total Environment 768 (2021) 144356

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Long-term stabilization of ^{15}N-labeled experimental NH_4^+ deposition in a temperate forest under high N deposition

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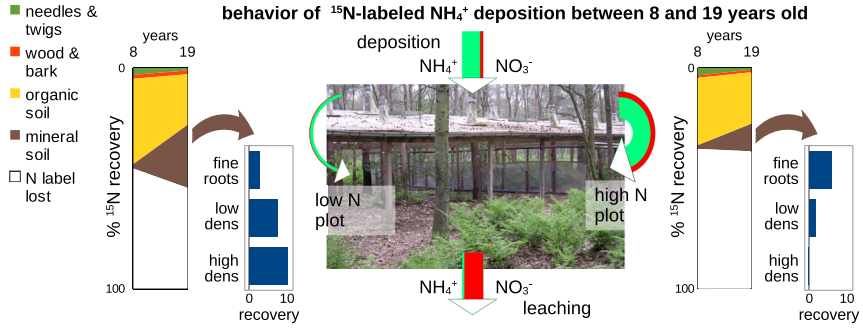
HIGHLIGHTS

- We studied N dynamics in a forest under high N deposition using labeled N deposition.
- N retained after 8 years was still there after 19 years despite strong N leaching.
- After 19 years labeled N was mostly present in stable but also in more labile pools.
- After 19 years N deposition remained mainly in the organic and mineral soil.
- Mineral soil density fractions retained less N deposition if N deposition was high.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 23 September 2020
Received in revised form 30 November 2020
Accepted 1 December 2020
Available online 28 December 2020

Editor: Elena Paoletti

Keywords:
^{15}N tracer
N deposition
Scots pine
Stabilization
Organo-mineral associations
N leaching

ABSTRACT

High nitrogen (N) deposition levels, currently present in many industrial and agricultural regions of the world, can strongly affect the functioning of forest ecosystems. In a pine forest with strong N leaching, located in the Netherlands, we studied the long-term fate of a year-long NH_4^+ deposition cohort labeled with ^{15}N. A high ambient and a low N deposition treatment had been established at the site by means of a roof and sprinklers. Resampling the N pools 19 years after labeling and 11 years after the last sampling, we found similar ^{15}N deltas in needles, twigs and the LF1 organic soil layer of each treatment, indicating intensive N cycling among these pools. In the last 11 years, label recovery decreased in these labile pools, while recovery remained constant in wood and increased in bark. Together these aboveground vegetation pools retained less than 3% of the labeled N. In the organic layers, label recovery after 19 years decreased to 23% in both treatments, while in the mineral soil it increased from 4% to 13% (high N) and from 3% to 29% (low N treatment). Within the mineral soil of the high N treatment the labeled N was mainly found in fine roots, while in the low N treatment most N was incorporated in the two soil density fractions, shifting to the high density fraction with depth. This suggests a low capacity of the mineral soil at high N deposition to incorporate N. After the labeled N had been lost substantially in previous years, especially in the first, its presence remained constant in the last 11 years at 38% (high N) and 54% (low N treatment). Apparently, even in this strongly N leaching ecosystem, N once incorporated, was retained well and did not affect the input-output fluxes of the system.

1. Introduction

Nitrogen (N) is considered to be a limiting nutrient in most temperate forests (LeBauer and Treseder, 2008; Vitousek and Howarth, 1991). However, global emissions of reactive N and its deposition on terrestrial

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https://doi.org/10.1016/j.scitotenv.2020.144356

ecosystems have greatly increased during the last decades as a result of human activities such as fossil fuel combustion, synthetic fertilizer application, and intensive livestock farming (Erisman et al., 2011a, 2011b; Galloway et al., 2008). This has had a profound effect on the availability of N to trees and other organisms and the N cycling in forests, especially in industrialized regions (Aber et al., 1998; Lovett and Goodale, 2011). Leaching of N as NO_3^- leads to soil acidification and loss of other essential nutrients and pollution of groundwater and surface waters, while the N retained can lead to eutrophication, and affect species composition and biodiversity, depending on the extent the N retained is available to the vegetation (Erisman et al., 2011b).

Changes in the N dynamics of forests can occur on very long time scales (Gilliam et al., 2019; Groffman et al., 2018; McLauchlan et al., 2007). Bernal et al. (2012) for example identified a forest cutting in the early 1900s as an important factor contributing to a recent decrease in N leaching in a temperate forest (McLauchlan et al., 2007). Spohn and Sierra (2018) presented cases in which average residence times of N in forest ecosystems reach 100 years. Changes in N dynamics can also take a long time before they have effects on other processes. In a model study of a forest N fertilization experiment the effect of N fertilization on C sequestration was predicted to start with a delay of ten years and to subsequently diminish again after thirty years (Currie et al., 2004). In a meta-analysis of the responses of north temperate forests to N inputs Nave et al. (2009) concluded that changes in N inputs can lead to shifts in ecosystem properties lasting several decades, e.g. in the forest floor C:N ratio.

Nitrogen retention and availability depends on the pools the N resides in and their characteristics. The stability of forest ecosystem N pools can vary widely: the canopy and fine roots of the vegetation are turned over in few years or less, while wood and bark have much longer turnover times. Mineral soil also contains very stable pools together with more labile ones. To distinguish between less and more stable soil organic matter (SOM) pools, soil can be split in different density fractions (Cerli et al., 2012). As a result of such a fractionation, SOM can be divided into largely pure and free organic matter on the one hand and organic matter occluded into aggregates or associated with minerals (Moni et al., 2012) on the other hand. The two latter fractions are relatively stable (Baldock and Skjemstad, 2000).

To monitor the distribution of N in forest N pools, tracer experiments have been carried out with the stable isotope ^{15}N. In field experiments N deposition was enriched in ^{15}N, followed by monitoring the ^{15}N presence in the various N pools (Nadelhoffer et al., 2004; Tietema et al., 1998). Most of these experiments have lasted only months or a few years. From a meta-analysis of such short-term experiments (Templer et al., 2012), it was concluded that in forests about 75% of the labeled N deposition was retained by the ecosystem and the majority of this N went into the organic and mineral soil layers. To better assess the behavior of N longer term experiments are needed (Fuss et al., 2019; Gurmesa et al., 2016), of which there are only a few (Cheng et al., 2019; Goodale, 2017). These show that in later years retention in these sites remained mostly stable, while still most of the labeled N deposition was found in the soil (Cheng et al., 2019; Schleppi et al., 2017; Veerman et al., 2020). The sites in which these experiments were carried out had N depositions between 8 and 20 kg N ha^{-1} yr^{-1}, while N leaching was absent or at most 50% of the N deposition. To study the effects of higher N depositions in many of these sites chronic fertilizations have been carried out, together with ^{15}N labeling. Templer et al. (2012) found that fertilizer increased N retention below 45 kg N ha^{-1} yr^{-1}, while it decreased retention above this fertilization rate. Such experiments lasting longer than 3 years showed a lower label retention in the soil of fertilized treatments than in that of unfertilized treatments, while vegetation had similar values between treatments (Cheng et al., 2019). However, as in these experiments ^{15}N labeling started at the same time or shortly after fertilization started, these experiments are not directly comparable to sites with a legacy of chronically high N deposition.

Forests in the Netherlands have been subjected to high N deposition levels, which could reach 60 kg N ha^{-1} yr^{-1}. Trends of reactive N emissions in the Netherlands have shown a doubling between the early 1960s and 1980, while remaining at that level in the next decade (Erisman et al., 2011b; Thomas et al., 1988; Thomas and Erisman, 1990). High N depositions are found in particular in regions with intensive agricultural activities. These regions mostly have poor sandy, well drained soils and losses of N in such forests through leaching of nitrate to the subsoil are usually considerable, while at the same time these ecosystems have accumulated substantial amounts of N (Meesenburg et al., 2016). As such they can be considered to be at the extreme end of forests subjected to adverse effects of increased N deposition (Gundersen et al., 1998).

In 1992–1993 a ^{15}N deposition labeling (Koopmans et al., 1996) was carried out in a long term field experiment in a strongly N leaching forest in the Netherlands (Boxman et al., 1995, 1998). This experiment consisted of two treatments: one with ambient N deposition and one with a strongly reduced N deposition. The objective was to study whether the effects of high N deposition upon the forest were reversible. In this study we report how much of the labeled N deposition remained in this ecosystem 19 years after labeling compared to results 8 years after labeling (Wessel et al., 2013), and in which compartments the labeled N resided, including different soil density fractions. Our aim was to establish to what extent the labeled N deposition of 19 years before was still retained within this forest ecosystem, subjected to substantial N leaching losses, and how the labeled N retained was distributed between more labile N pools, such as the needles, and more stable N pools, such as bark and wood and the high density fraction of the mineral soil. Our hypotheses were that between 8 and 19 years after labeling the N retained would have decreased, and relatively more of the labeled N would be present in the stable pools.

2. Material and methods

2.1. Study area

The study was carried out in a forested stand near Ysselsteyn (52°13′ N, 5°39′E) in the southeastern part of the Netherlands. The stand was surrounded by agricultural land and intensive livestock farms. The stand consisted mainly of *Pinus sylvestris* L. trees planted between 1945 and 1950 on plowed heathland, which probably had been under cultivation in the past (Boxman and Roelofs, 2006). It had a scattered undergrowth of ferns (*Dryopteris dilatata* (Hoffm.) A. Gray) and brambles (*Rubus sp.*). The soil was classified as a Humic Haplorthod. The humic top layer (5–9 cm) and the organic rich mineral soil down to 50 cm had a C concentration of 4.7% in the top 10 cm to 2.4% in the layer of 25 to 50 cm. At least part of this organic material originated from before the time of the present forest, possibly from the former heathland or from fertilization with sods, when it was in use as arable land. Ambient atmospheric N deposition (NH_4^+ and NO_3^-) was more than 60 kg N ha^{-1} yr^{-1} in throughfall in the 1980s (84% as NH_4^+-N), decreasing to approximately 45 kg N ha^{-1} yr^{-1} by 2004 (65% as NH_4^+-N) following the introduction of emission regulations (Boxman and Roelofs, 2006). At the time of sampling in 2012, N deposition in throughfall was 42 kg N ha^{-1} yr^{-1} (59% as NH_4^+-N). Tree height was 12–15 m during the experiment (Boxman et al., 1998; Boxman and Roelofs, 2006). Trees increased in stem size (DBH), but probably not in canopy mass, as litter fall did not change during the experiment (Boxman et al., 1998). The site was considered N saturated, as it leached NO_3^- from the well-drained soil. Yet, as a result of the high N deposition, the ecosystem still showed a net gain of N (Boxman et al., 1995, 1998; Koopmans et al., 1996). The region has a temperate climate with a mean annual precipitation of approximately 750 mm. Average daily minimum and maximum temperatures are −0.2 and 5.2 °C in January and 12.2 and 22.6 °C in July. Boxman et al. (1998) give a more detailed description of the site.

2.2. Experimental treatment

As part of a study to investigate the effect of a reduction of N deposition on the functioning of forests, a roof was built in the Ysselsteyn forest to intercept the throughfall. The transparent roof measured 14×28 m and was built at 2–3 m height underneath the canopy in May 1989 (Van Dijk et al., 1992). Under this roof two plots were established of 10×10 m with buffer zones of 2 m around each plot. The 100 m^2 plots each contained 8 or 9 pine trees. In one plot artificial throughfall similar to normal throughfall but with a low N (NH_4^+ and NO_3^-) and S (SO_4^{2-}) content was sprinkled underneath the roof, leading to deposition levels of approximately 5 kg N ha^{-1} yr^{-1} (low N treatment). The other plot served as a control (high N treatment). Here the throughfall on the roof was collected and sprinkled underneath the roof. Sprinkling was done with fully automated real time sprinklers, installed about 60 cm above the forest floor (Boxman et al., 1995, 1998).

In 2001 the treatment in the high N deposition plot was changed. The level of N deposition was lowered with the same method as described above from an ambient level of on average 45 to 20 kg N ha^{-1} yr^{-1}. N deposition remained at this level until sampling in 2012. This 20 kg N ha^{-1} yr^{-1} is considered the critical N load for this type of forest (Boxman and Roelofs, 2006). Furthermore, this plot was split into four 5 by 5 m subplots: one control subplot and three others receiving the treatments sod-cutting, fertilization and liming (Boxman and Roelofs, 2006).

Between May 1992 and May 1993, ^{15}N was applied to both the low and the high N plot under the roof by automatically pumping a 99.4% enriched $(^{15}NH_4)_2SO_4$ solution into the water supply of the sprinkling installation. The excess ^{15}N for the low and the high N plots was 0.46 and 0.44 kg ^{15}N ha^{-1}, respectively.

In this study we focused on the behavior of the NH_4^+ deposition, which was labeled with ^{15}N. Although the natural deposition contains NO_3^- as well (Boxman et al., 1995; Koopmans et al., 1996), we assumed that its influence on the results was limited, as NH_4^+ was the dominant N species in the deposition, and NO_3^- is the more mobile of the two. In addition, this system contained a lot of N, and part of the NO_3^- leached from the system probably originated directly from the deposition (Kjønaas and Wright, 2007).

2.3. Sampling and laboratory analyses

In February 2012, 19 years after labeling, we took five soil samples in the low N deposition plot. The organic soil was sampled with a metal frame of 25 × 25 cm pressed into the surface. For the mineral soil layers of 0–10 and 10–25 cm a PVC tube (6.7 cm diameter) was used. For the mineral soil layer of 25–50 cm each replicate consisted of three bulked subsamples taken with a gouge auger (1.2 cm diameter) from the same hole as was used for the 0–10 and 10–25 cm samples. The organic soil was visually separated into a LF1 and a F2 layer, where the LF1 contained needles that were still recognizable and the F2 contained needles that had started to fragment and decompose or were not recognizable anymore. Cones, large twigs and roots (>1 mm) were removed from the samples. To evaluate ^{15}N recovery in the soil of the high N deposition plot in the period of 2001 to 2012, we sampled the control subplot only, because all three other treatments affected directly soil conditions and consequently ^{15}N recovery. Because of the limited dimensions of 5 by 5 m, we took fewer replicates than in the low N deposition plot (three instead of five).

Despite the change in N deposition level from 45 to 20 kg N ha^{-1} yr^{-1} in 2001 and the sod-cutting, fertilization and liming treatments in the high N deposition plot, yearly DBH measurements of this plot indicated a linear increase in the period of 1991 to 2012 in all trees (Boxman, unpublished results). Therefore, we concluded that both the decrease in N deposition of 45 to 20 kg N ha^{-1} yr^{-1} and the treatments in the subplots had not affected tree growth, and we used all trees in both N deposition plots to evaluate the recovery of ^{15}N in the vegetation in 2012.

We sampled needles, twigs, bark, and stem wood of all trees in both plots. Needles were split into current year and 1-year-old needles. Older needles were not present. Twigs sampled were the current year twigs and 2-year-old twigs. The latter were considered to be one fifth of the total of branches in line with Koopmans et al. (1996). The remainder of the branches was ignored (Koopmans et al., 1996). A 16 cm^2 patch of bark was sampled from each tree at breast height. Wood was sampled with a 0.5 cm internal diameter corer at the same location as the bark sample. Initially, fine roots were not sampled separately from the soil samples, so they must be considered to be part of the soil samples. However, as part of the density fractionation of the mineral soil samples (see below), fine roots were separated afterwards.

All samples were dried at 70 °C for at least 48 h, mixed and ground to a very fine powder in a planetary mill before analysis. Samples were then analyzed for C and N concentration using a CNS analyzer (Vario EL analyzer, Elementar) and for ^{15}N using an elemental analyzer (PDZ Europa ANCA-GSL) interfaced to a continuous flow isotope ratio mass spectrometer (IRMS) (PDZ Europa 20–20). All samples were analyzed in duplicate and averages were used.

A density fractionation of the organic matter in the mineral soil was carried out according to Cerli et al. (2012). Originally this method was aimed at separating three organic fractions; (1) organic debris neither attached to minerals nor occluded within aggregates, (2) organic debris occluded within aggregates and released upon disruption of the aggregates and (3) organic matter strongly bound to minerals (Cerli et al., 2012). A pre-test with soil from the Ysselsteyn site showed that the occluded fraction was too low to allow for any determination. Therefore, the fractionation method was adapted to separate only two fractions, a low density (<1.6 g cm^{-3}) and a high density fraction (>1.6 g cm^{-3}). The light fraction consisted of free, almost pure organic matter, while the latter consisted of both occluded (organic debris released upon disruption of aggregates) and organic matter associated to minerals. All mineral soil samples were sequentially sieved (d=1, 0.5 and 0.425 mm) to separate fine roots. To 5 g of the remaining mineral soil, 25 ml of a Na polytungstate (NaPT) solution with a density of 1.6 g cm^{-3} was added, and the samples were centrifuged for 30 min at 20,000 rpm. The floating material (low density fraction) was separated and collected on a filter by using a rubber spatula and by carefully decanting and vacuum filtering the solution. This low density fraction was rinsed with deionized water and flushed from the filter and subsequently freeze-dried. The remaining soil was re-suspended in deionized water and the resulting slurry was centrifuged again. This procedure was repeated several times until the conductivity of the solution was lower than 500 μS cm^{-1}. The sediment, representing the high density fraction, was then separated, washed and freeze-dried, as described for the low density fraction (Cerli et al., 2012). The fine roots and both density fractions were analyzed for C and N concentration; for ^{15}N they were analyzed using an IRMS (ISOPRIME 100). Both Isotope Ratio Mass Spectrometers used in this study (PDZ Europa 20–20 and ISOPRIME 100) were extensively calibrated and results of the analyses were found comparable. Adding the results of the three fractions together (Crow et al., 2007; Kramer et al., 2009) yielded on average 101 ± 1% of the dry mass, 91 ± 14% of the N, and 72 ± 16% of the delta ^{15}N of the original samples (±standard deviation, $n = 24$).

2.4. Data analysis

2.4.1. N pools and C:N ratios

Pool sizes of N were determined the same way as done by Koopmans et al. (1996). Dry mass per area of the soil layers was calculated by dividing the mass of the soil samples by the area of the used sampling tool. Then, the dry matter per surface area of the compartment was multiplied with its N concentration. The dry mass per area of the various woody tree compartments in the plots was calculated from the average diameter at breast height (DBH) values and allometric relationships (Dik, 1984). At the earlier samplings a constant tree density of

705 trees ha^{-1} was used, however, some of the trees in the low N deposition plot had died in between the sampling at 8 years and at 19 years, but were still standing upright. We carried out our calculations with the same tree density (705 trees ha^{-1}) assuming that the retained ^{15}N in the dead trees was not released in between measurements. The mass of needles and twigs at 19 years was assumed to be similar to the mass 8 years after ^{15}N labeling. A constant canopy mass seems reasonable, because this is a mature forest with a closed canopy and no thinning was carried out since the start of the experiment in 1989. Furthermore, although DBH increased, yearly litter input remained constant during the years until 1995 (Boxman et al., 1998), and subsequent years (unpublished results).

C:N ratios of the various compartments were calculated using their C and N concentrations. To calculate the C:N ratios of the aboveground vegetation compartments in 1994 average C concentrations from 2001 and 2012 were used, as the C concentrations of these compartments did not vary between treatments and years (data not shown).

2.4.2. ^{15}N recoveries

The recovery of the applied ^{15}N in each pool i ($\%^{15}N_{rec,i}$) was calculated using the following equation derived from Nadelhoffer et al. (2004):

$$\%^{15}N_{rec,i} = \frac{m_i(\text{at}\%^{15}N_i - \text{at}\%^{15}N_{i,\text{init}})}{m_{\text{label}}} \quad (1)$$

where $\text{at}\%^{15}N_i$ is the atom percentage of ^{15}N in N pool i; $\text{at}\%^{15}N_{i,\text{init}}$ is the atom percentage of ^{15}N in pool i before ^{15}N was added (natural abundance); m_i the N mass of the pool (kg N ha^{-1}); and m_{label} is the mass of the ^{15}N added to N deposition (kg ^{15}N ha^{-1}) (Wessel et al., 2013). The delta ^{15}N at natural abundance of the various pools were obtained from Koopmans et al. (1996). Because no data were available on delta ^{15}N at natural abundance of the fine roots and the low and high density soil fractions in the different mineral soil layers, we calculated the percentage ^{15}N recovery in these fractions using the natural ^{15}N abundance of the total soil layer considered (Koopmans et al., 1996). About 60% of the ^{15}N label recovered in the total soil layers was found in the three fractions together. This lower yield was probably due to the fact that the delta values of the fractions were an underestimate (see above, the weighted yield in delta was 72%), while the natural abundances were still the original values from the total soil layers. Such differences have been encountered in other studies, and were possibly due to discrimination effects of the dissolution of the soil material for the density fractionation, while the ^{15}N in the total soil was determined directly on the solid material (Crow et al., 2007; Kramer et al., 2009). We decided not to correct for this, as our analysis was focused on the relative contribution of the fine roots and the density fractions to the retention of labeled N.

Averages per plot and standard errors of the mean were calculated for deltas and ^{15}N recoveries. As the trees varied substantially in size and because distances between the trees varied as well, it was impossible to estimate the variation in the dry mass per area of the vegetation from the individual tree samples. Therefore, this variation was not included in the calculation of the errors of the label recoveries. The variation in ^{15}N natural abundances before ^{15}N labeling were not available, partly because single composite samples had been used (Koopmans et al., 1996).

2.4.3. Statistical analysis

To evaluate the effects of the N deposition treatment and the time after application of the ^{15}N tracer on the delta ^{15}N and ^{15}N recovery of each of the N pools, a 2-way ANOVA was applied. Additive as well as interaction effects were assessed. Time after application of the tracer was considered as a numerical predictor. If time was considered as a categorical predictor results of the analysis were mostly similar (data not shown), the only exception being the ^{15}N recovery in the LF1 layer. Samples within the research plots were treated as replicates.

Differences in N pool sizes, C:N ratios, summed ^{15}N recoveries and mineral soil carbon content were tested for significance using Welch's unequal variances t-test for independent samples. Differences in C:N ratios between high and low density fractions were tested with a paired two-sample t-test. The relationships of mineral soil C content and C:N ratio with depth were tested using multiple linear regression with depth and location as independent variables. A significance level of 0.05 was used. For all statistical analyses, the R statistical computing program was used (R Core Team, 2018).

3. Results

3.1. N pools

The N pool sizes of the different compartments are presented in Table 1. In the high N plot the N in the needles and twigs decreased significantly between 2001 and 2012. This was due to the N concentration in the material, as the N pool was calculated under the assumption that the needles and twigs mass did not change between 2001 and 2012. In both plots the N present in the stems on an area basis, increased significantly between 2001 and 2012 (Table 1). Furthermore, the N in the stems was significantly larger in the low N plot than in the high N plot, as a result of the larger growth of the tree stems in the low N plot (Boxman et al., 1998).

In the low N plot, the total soil N pool differed significantly between 2012 and 2001. Comparing the total soil N pools of all years of both plots, shows that results of 2001 in the low N plot were lower than the others, and this was due to a small N pool size in the mineral soil. The N pool of the organic F2 layer was significantly larger in the high N plot in 2012 than in 2001 and also larger than in the low N plot in 2012. In contrast with this, the N pool in the 0–10 cm mineral soil layer below it was largest in 2012 in the low N plot, although only the difference with the N pool of the low N plot in 2001 was significant. The three mineral soil layers we distinguished were not of equal thickness: the N concentrations of the layers on a 10 cm depth basis decreased from about 1300 to 750 kg N ha^{-1} 10 cm^{-1}.

C:N ratios of the different compartments are presented in Table 2. C:N ratios in the aboveground vegetation and organic soil compartments were generally higher in the low N plot than in the high N plot, but this was only significant for the bark compartment. In the high N plot the C:N ratio showed a significant increase between 2001 and 2012 in three of the four needles and twigs compartments, the bark compartment and the LF1 organic soil layer. In the low N plot the increases were only significant in the 2-year old twigs and the bark compartment and the LF1 soil layer. In the mineral soil the C:N ratio mostly also increased between 2001 and 2012, but this was only significant in the two deeper layers of the low N plot. In the organic soil, C:N ratios decreased with depth, and this was significant in 2012 in both plots ($p < 0.05$). In the mineral soil, the ratio generally increased with depth. In the low N plot in 2012, this increase was significant ($p < 0.05$).

3.2. Delta ^{15}N values

The presence of the labeled N varied across the compartments of each plot (Fig. 1). Of the aboveground vegetation, the youngest compartments, the needles and twigs, had significantly decreasing delta ^{15}N values with time. In each treatment, these delta values converged to similar values during the experiment. Bark delta values did not show a significant effect with time, while those of wood decreased significantly with time in a similar pattern as the needles and twigs.

In the upper and lower organic layers, LF1 and F2 respectively, the delta values also decreased considerably with time, just like the needles and twigs. In 2012 delta values in the LF1 and those in the needles and twigs all had converged to similar values. In the F2 layer, the decline was

Table 1
N pool sizes of plant and soil compartments of 2 plots under high and lowered N deposition in a Scots pine stand in Ysselsteyn, the Netherlands.

	High N plot									Low N plot								
	1994			2001			2012			1994			2001			2012		
	kg N ha^{-1}	SEM	n	kg N ha^{-1}	SEM	n	kg N ha^{-1}	SEM	n	kg N ha^{-1}	SEM	n	kg N ha^{-1}	SEM	n	kg N ha^{-1}	SEM	n
Needles																		
0–1 year	92.0	0.9	3	89.7	3.5	9	70.1*	3.0	7	78.3	1.0	3	80.0	4.1	8	73.0	5.3	6
1–2 year	26.0	0.5	3	23.8	0.7	9	21.0*	0.7	7	22.6	0.4	3	20.9	1.1	8	21.2	1.8	5
Twigs																		
0–1 year	2.7	0.1	3	2.8	0.1	9	2.6	0.1	7	2.3	0.0	3	2.5	0.1	8	2.4	0.1	6
2 year	22.4	0.7	3	15.1	1.5	9	7.2*	0.7	7	17.9	0.6	3	11.7	1.3	8	7.0*	0.6	5
Needles & twigs	*143.1*			*131.4*	*5.4*	*9*	*100.9**	*3.6*	*7*	*121.1*			*115.1*	*6.1*	*8*	*102.6*	*10.1*	*5*
Stem																		
Bark	30.5	0.4	3	41.9	1.9	9	46.7	1.7	7	32.4	0.7	3	47.7	1.5	8	67.0*†	2.2	7
Wood	32.3	0.5	3	41.1	1.7	9	74.1*	12.3	7	34.3	2.9	3	63.2	2.9	8	94.9*	4.7	7
Total stem	*62.8*			*83.1*	*2.1*	*9*	*120.8**	*12.4*	*7*	*66.7*			*110.9*	*3.5*	*8*	*161.9*†*	*4.9*	*7*
Organic soil																		
LF1	49.1	6.9	3	523.5	39.8	5	273.9	72.4	3	38.5	5.8	3	297.0	38.3	5	244.8	39.8	5
F2	828.2	120.6	3	849.0	128.0	5	1665.2*	140.1	3	987.4	155.9	3	992.0	120.1	5	841.0†	239.6	5
Total organic soil	*877.3*			*1372.5*	*109.0*	*5*	*1939.1**	*137.0*	*3*	*1025.9*			*1288.8*	*98.1*	*5*	*1085.9†*	*274.3*	*5*
Mineral soil																		
0–10 cm	1519.0	139.6	3	1027.0	186.7	5	1242.9	67.1	3	1494.5	81.4	3	950.0	166.7	5	1665.7*	226.3	5
10–25 cm	1435.1	268.1	3	1323.0	186.5	5	1393.8	273.4	3	1289.1	207.0	3	920.0	147.8	5	970.5	72.8	5
25–50 cm	2031.8	237.7	3	1942.0	181.4	5	1525.3	40.8	3	2042.5	28.4	3	1491.0	220.5	5	2180.2	292.3	5
Total mineral soil	*4985.9*			*4292.6*	*497.6*	*5*	*4162.0*	*269.1*	*3*	*4826.1*			*3361.0*	*288.3*	*5*	*4816.5**	*481.5*	*5*
Total soil	*5863.1*			*5665.2*	*561.4*	*5*	*6101.1*	*351.2*	*3*	*5851.9*			*4649.9*	*321.0*	*5*	*5902.4**	*377.8*	*5*
Total system	*6069.0*			*5878.9*			*6322.8*			*6039.8*			*4876.0*			*6167.8*		

1994 data from Koopmans et al. (1996), 2001 data from Wessel et al. (2013); SEM = Standard error of the mean; * indicates a significant difference between 2001 and 2012 ($p < 0.05$); † indicates a significant difference between high N and low N plot in 2012 ($p < 0.05$).

slower than those in the vegetation and LF1 compartments. As a result, the delta in the F2 of the low N deposition plot was higher than any other compartment measured at that time.

In the mineral soil, the delta values of all layers showed similar changes over time. The changes in two upper layers were not statistically significant, while those in the 25–50 cm layer increased significantly with time. The lowest delta values were found in 2001, but one has to be careful when comparing these to the values in 1994 because in the two deeper layers only one, combined, sample was measured at that time, hence the accuracy of these measurements cannot be assessed.

Delta ^{15}N values of the two treatments cannot be directly compared, as they depend on the applied delta in the labeled throughfall, which was strongly different in the two treatments (35,500 in the low N plot and 2865 in the high N plot). However, the results showed that, while the delta values of the organic soil layers and the deepest mineral layer were all significantly higher in the low N plot, the delta values of the aboveground vegetation pools were not significantly different between plots, and even tended to be lower in the low N plot (Fig. 1).

3.3. ^{15}N label recoveries

There were clear differences in ^{15}N label recovery between compartments, just as for the delta results (Fig. 2). The needles and twigs showed very similar behavior, all four showing a significant decrease with time and an interaction effect. Only the older twigs showed a

Table 2
C:N ratios (g C g^{-1} N) of plant and soil compartments of 2 plots under high and lowered N deposition in a Scots pine stand in Ysselsteyn, the Netherlands.

	Site values	High N plot						Low N plot					
		1994	2001			2012			1994	2001			2012		
				SEM	n		SEM	n			SEM	n		SEM	n
Needles															
0–1 year		18	20.0	0.8	9	25.7*	1.1	7	22	22.9	1.2	8	25.1	1.7	6
1–2 year		18	21.1	0.7	9	23.9*	0.8	7	23	24.5	1.2	8	24.3	2.3	5
Twigs															
0–1 year		27	30.0	1.3	9	32.0	1.4	7	35	33.7	1.4	8	34.2	1.5	6
2 year		55	68.2	5.6	9	141.6*	14.4	7	73	86.7	7.5	8	135.7*	12.3	5
Stem															
Bark	86	-	78.1	4.2	9	94.9*	3.4	7	-	92.8	3.1	8	114.1*†	4.0	7
Wood	586	-	615.3	25.8	9	513.9	58.8	7	-	554.8	29.4	8	632.7	30.0	7
Organic soil															
LF1	28	-	21.4	1.1	5	27.7*	1.3	3	-	25.0	1.1	5	30.3*	1.0	5
F2	20	-	19.5	0.4	5	20.0	1.1	3	-	20.5	0.2	5	22.0	0.8	5
Mineral soil															
0–10 cm	32	-	29.7	0.5	5	31.7	2.4	3	-	26.7	0.5	5	26.3	0.6	5
10–25 cm	42	-	34.9	2.3	5	42.4	2.6	3	-	29.7	1.2	5	34.2*	0.5	5
25–50 cm	47	-	31.6	1.9	5	37.0	4.2	3	-	31.5	0.5	5	35.1*	1.0	5

Site values from Koopmans et al. (1996); 1994 data calculated from N concentrations from Koopmans et al. (1996) and average C concentrations from later years; - indicates no data available; 2001 data from Wessel et al. (2013). SEM = standard error of the mean; * indicates significant difference between 2001 and 2012 ($p < 0.05$); † indicates significant difference between high N and low N plot in 2012 ($p < 0.05$).

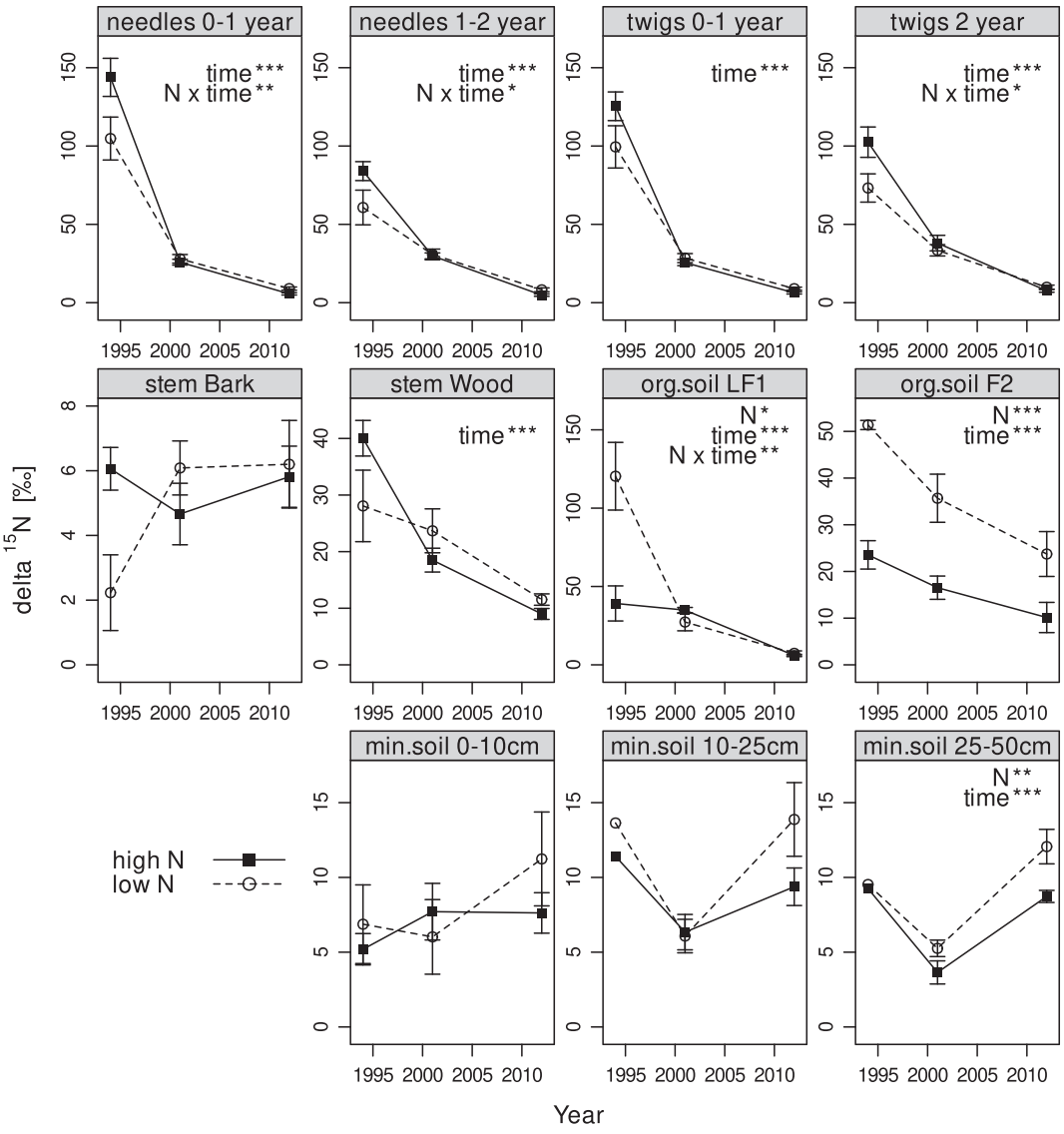

Fig. 1. Delta ^{15}N in N pools of a high and low N deposition plot in a Scots pine forest, 1, 8 and 19 years after labeling. Error bars are standard errors of the mean; no standard errors for the two lower mineral soil layers in 1994. See the upper-right corner of the sub-panels for the significant treatment effects: N is the effect of the N deposition treatment, time of the change over time and N x time the interaction of these two factors: * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$. Data 1994 from Koopmans et al. (1996), data 2001 from Wessel et al. (2013).

significant effect of the N deposition treatment: recovery was larger in the high N deposition plot. This was mainly apparent in 1994 and 2001, as in 2012 the results had converged to similar values. The results of the needle and the other twig compartments, though not significant, showed a similar pattern with N deposition treatment. In contrast with these compartments, bark and wood compartments did not decrease with time. For both compartments recovery was significantly larger in the low N deposition plot, while the recovery in bark also increased significantly with time. The increase with time was stronger in the low N deposition plot and this resulted in a significant interaction effect. As a result of the growth of the trees, the N pools of both compartments increased substantially during the experimental period, with the growth being larger in the low N deposition plot (Table 1). An increase in N pool size increases the recovery if the presence of label in the pool remains the same.

The organic layer represented the main sink for the ^{15}N label in most cases (Table 3). Especially in the F2, the lower organic layer, the recovery was large. In the LF1, recovery in the high N deposition plot was significantly larger than in the low N plot. In this respect it differed from the wood and bark compartments, but was similar to the trend in the needle and twigs compartments. Recoveries in the LF1 compartment increased between 1994 and 2001, and decreased between 2001 and 2012. However, a time effect was insignificant because only a linear effect of time was considered in our model. In contrast with the LF1 layer, recovery in the lower F2 organic layer was significantly larger in the low N deposition plot. There was also a significant decrease with time, although this was only apparent in the low N deposition plot, as the recoveries in the high N deposition plot were more or less constant. As a consequence, a significant interaction effect between time and N deposition treatment was found.

In the mineral part of the soil, only the recovery in the 25–50 cm layer showed a significant increase with time and N deposition treatment. In the two lower layers recovery was zero or even negative in 2001, but in 2012 the recovery had increased again.

The ^{15}N label recovery of an N pool is the result of its size (in kg N ha^{-1}) times its ^{15}N label content (also called the "specific ^{15}N labeling of an N pool" (Schleppi et al., 1999)). This is illustrated in Fig. A1 (See Appendix A), where the % recovery in an N pool is represented by an area of which the vertical side is the size of the N pool and the horizontal side the ^{15}N label content of the N pool (as a percentage of the total N in the pool). It shows that the aboveground vegetation and the LF1 layer only made a small contribution to the ^{15}N recovery despite their

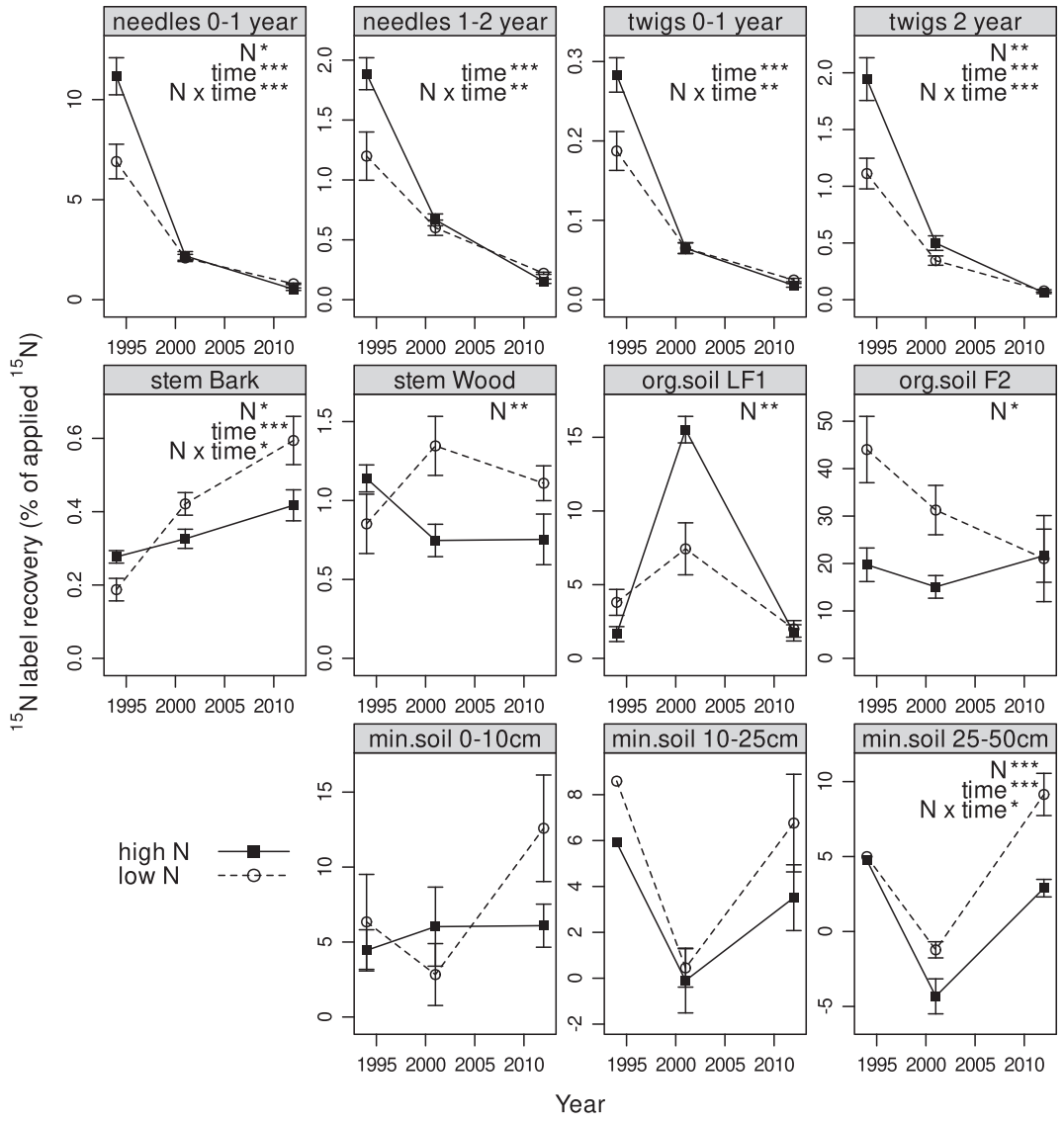

Fig. 2. Recovery of ^{15}N (as percentage of applied) in N pools of a high and low N deposition plot in a Scots pine forest, 1, 8 and 19 years after labeling. Error bars are standard errors of the mean; no standard errors for the two lower mineral soil layers in 1994. See the upper-right corner of the sub-panels for the significant treatment effects: N is the effect of the N deposition treatment, time of the change over time and N x time the interaction of these two factors: * = p<0.05, ** = p<0.01, *** = p<0.001. Data 1994 from Koopmans et al. (1996), data 2001 from Wessel et al. (2013).

relatively large label content, because their N pool sizes were small. The mineral soil pools on the other hand contributed substantially to the ^{15}N label recovery as a result of their large pool size, while their label content remained relatively modest. The importance of the F2 layer for the label recovery was a result of both a substantial label content and a large N pool size. The increase in ^{15}N recovery in the bark and

Table 3
^{15}N label recoveries (%) in aggregate N pools of 2 plots under high and lowered N deposition in a Scots pine stand in Ysselsteyn, the Netherlands.

	High N plot							Low N plot						
	1994	2001			2012			1994	2001			2012		
			SEM	n		SEM	n			SEM	n		SEM	n
Aboveground vegetation	16.7	4.5	0.4	9	1.9*	0.2	7	10.4	4.9	0.4	8	2.8*†	0.2	6
Organic soil	21.4	30.6	2.9	5	23.4	5.2	3	47.8	38.7	6.1	5	23.0	9.6	5
Mineral soil	15.1	1.6	3.9	5	12.5	3.3	3	19.9	2.1	3.1	5	28.5*†	5.7	5
Total soil	*36.5*	*32.2*	*5.9*	*5*	*35.9*	*8.1*	*3*	*67.8*	*40.8*	*7.7*	*5*	*51.5*	*14.4*	*5*
Total system	*53.2*	*36.7*	*5.9*		*37.8*	*8.1*		*78.2*	*45.6*	*7.7*		*54.4*	*14.4*	
Leaching	17.3							10.1						

1994 data from Koopmans et al. (1996); 2001 data from Wessel et al. (2013). SEM = standard error of the mean; * indicates a significant difference between 2001 and 2012 ($p < 0.05$); † indicates a significant difference between high N and low N plot in 2012 ($p < 0.05$).

wood and the LF1 pools with time was the result of an increase in pool size, as their label content decreased with time. Of course the label content of the high N deposition was in general much larger than that of the low N deposition plot, as a result of the much larger labeled NH_4^+ deposition applied.

Total recoveries in 2012 were equal to or larger than those in 2001. For the low N plot total recovery was 45% in 2001 and 54% in 2012, while for the high N plot it was 37% in 2001 and 38% in 2012 (Table 3). These differences in the total recoveries between 2001 and 2012 were not significant ($p < 0.05$) for neither plot. There was a significant decrease between 2001 and 2012 in the recoveries of the aboveground vegetation in both plots. As can be seen in Table 3, the soil always retained the most label in both plots, while in both 1994 and especially 2001 the recovery in the organic soil was always higher than in the mineral soil. However, in 2012 the recovery in the mineral soil had increased, which increase was significant in the low N plot and almost significant in the high N plot ($p = 0.08$). As a consequence, the recovery in the mineral soil exceeded the recovery in the organic soil in the low N plot in 2012, while in the high N plot the mineral soil had gained also strongly in importance in 2012 compared to 2001. Differences in recoveries between the two plots were mostly not significant in 2012 except for the aboveground vegetation and the mineral soil, of which the recoveries were significantly higher in the low N plot than in the high N plot.

3.4. Density fractionations of the mineral soil layers

Carbon content and C:N ratios of the two density fractions and the fine roots are presented in Table 4. We tested for differences in these parameters between plots, and for trends with depth of these parameters in each plot. We also tested for differences in C:N ratios between high and low density fractions in the individual soil layers of each plot. There were no significant differences between plots in C content of the density fractions or fine roots. The C:N ratio was usually lower in the low N than in the high N plot, but this difference was only significant for the high density fraction of the 10–25 cm layer. A generally lower C:N ratio in the low N plot was also found in the total soil layers (Table 2), but for these the differences were never significant. The carbon content of the low density fraction in the different mineral soil layers decreased significantly with depth in the low N plot and tended to decrease in the high N plot ($p = 0.08$). The latter was also true for the fine roots in the low N plot ($p=0.08$). The carbon content of the high density fraction never showed a trend with depth. The C:N ratio of both the low and high density fractions in the low N plot decreased significantly with depth, as did the low density fraction in the high N plot. The C:N ratio of the high density fraction of each layer of the two plots was always higher than the ratio of the low density fraction, indicating that these fractions consisted of different types of organic matter. In five of the six cases this difference was significant, the only exception being the 25–50 cm layer in the high N plot.

The delta values of the fine roots and the density fractions of the mineral soil layers measured in 2012 are presented in Fig. 3. In both plots the fine roots had much higher delta values than the two density fractions. The delta values in the fine roots even exceeded the delta values of all aboveground vegetation and organic soil compartments measured in 2012 (Fig. 1). The difference in delta values between the fine roots and the two density fractions was generally smaller in the layers of the low N deposition plot than those in the high N deposition plot. There were no clear differences in delta values between the two density fractions, except for the 25–50 cm layer of the low N deposition plot where the delta values of the high density fraction were higher than that of the low density fraction.

The ^{15}N label recoveries of the two density fractions differed strongly between the two N deposition treatments (Fig. 4). In the high N deposition plot, only in the upper mineral soil layer there was a substantial recovery in the two soil density fractions. In the two lower layers, recoveries in the two density fractions were absent or very small and the fine roots dominated the recovery. In the low N deposition plot on the other hand, most label in the upper mineral soil layer was recovered in the low density fraction while in the deeper layers the high density fraction dominated the recovery. Because for all fractions the average natural abundance of each layer was used to calculate the recoveries, the recovery in the fine roots was probably underestimated and the recovery in the two density fractions overestimated, as the ^{15}N natural abundance of fine roots is usually lower than that of the organic material in the mineral part of the soil (Feng et al., 2008; Högberg et al., 1996).

Fig. A2 (Appendix A) shows the relationships between label recovery, label content and N pool size of the fine root and density fractions similar to Fig. A1. The figure shows that in the high N deposition plot a high label content coincided with a relatively large recovery in the fine root pools, whereas in the low N deposition plot label content was also highest in the fine roots but the other pools had larger recoveries as a result of their larger pool sizes. The figure makes clear that the shift with depth of the label recovery from the low density to the high

Table 4
C concentrations and C:N ratios of fine roots, low density and high density fractions in the mineral soil layers of a Scots pine forest at high and low N deposition, 19 years after ^{15}N labeling. Numbers are means with standard error of the mean (SEM); n=3 (high N plot) and n=5 (low N plot).

Mineral soil	High N plot				Low N plot			
	C concentration mg C g^{-1} soil		C:N ratio g C g^{-1} N		C concentration mg C g^{-1} soil		C:N ratio g C g^{-1} N	
		SEM		SEM		SEM		SEM
0–10 cm								
Roots	2.0	1.1	34.8	3.6	2.7	0.7	30.6	0.9
Low density fraction	14.9	4.6	29.1	2.4	32.8*	8.3	24.1*	0.4
High density fraction	16.6	1.6	33.9¶	3.1	13.6	1.2	27.2¶*	0.3
Total	*33.5*	*4.7*	*32.0*	*2.6*	*49.1*	*9.6*	*25.3*	*0.4*
10–25 cm								
Roots	1.3	0.4	32.3	0.5	0.7	0.1	35.4	2.8
Low density fraction	4.1	0.6	35.3	1.8	3.2	0.4	29.8	0.6
High density fraction	22.8	3.7	43.8¶	2.4	9.2	1.2	32.7†¶	0.8
Total	*28.1*	*4.7*	*41.7*	*2.2*	*13.2*	*1.2*	*32.1*	*0.6*
25–50 cm								
Roots	1.9	0.6	35.9	0.8	1.0	0.2	35.2	1.9
Low density fraction	3.9	1.3	33.0	2.5	3.7	0.6	30.7	1.0
High density fraction	12.7	3.4	37.9	3.3	14.3	3.0	34.8¶	2.1
Total	*18.5*	*3.8*	*36.9*	*3.1*	*19.0*	*2.9*	*33.8*	*1.8*

Tests were carried out for differences in these parameters between plots († indicates a significant difference, $p < 0.05$), for trends with depth of these parameters in each plot (* indicates a significant relationship, $p < 0.05$), and for differences in C:N ratios between high and low density fractions in the individual soil layers of each plot (¶ indicates a significant difference, $p < 0.05$).

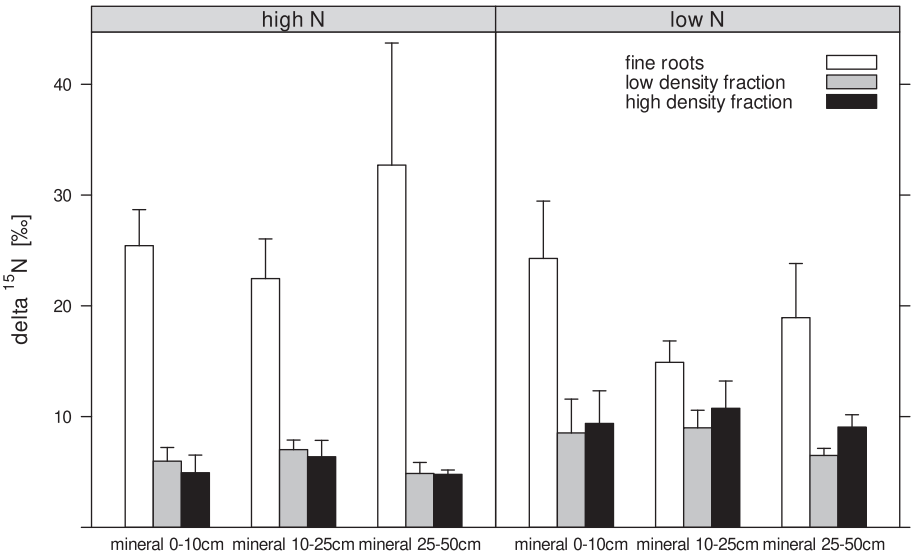

Fig. 3. Delta ^{15}N in the fine roots (d < 0.5 mm), in the low density fraction (<1.6 g cm^{-3}) and in the high density fraction (>1.6 g cm^{-3}) in three layers of the mineral soil of a high and low N deposition plot in a Scots pine forest 19 years after ^{15}N labeling. Error bars represent standard errors of the mean, $n = 3$ (high N deposition plot) and $n = 5$ (low N deposition plot).

density fraction observed in the low N plot (Fig. 4) is the result of an increase in N pool size of the high density fraction with depth and a stronger reduction in label content of the low density fraction with depth.

4. Discussion

4.1. Vegetation and LF1 organic soil layer

The needles, twigs and LF1 organic soil layer compartments were of limited importance for the retention of the labeled N in 2012. The continuing (Wessel et al., 2013) loss of label from the N pools of these compartments was shown by the decreasing ^{15}N recovery in these compartments (Fig. 2), while recovery in the whole ecosystem did not decrease (Table 3). The identical dynamics of delta ^{15}N values between 8 and 19 years after ^{15}N labeling, and its convergence to a narrow range of 5 to 10‰ delta ^{15}N in all these compartments (Fig. 1) corroborated previous conclusions that their N pools with a relatively short turnover time have an intensive and fast exchange of N among each other (Feng et al., 2008; Wessel et al., 2013).

The recovery of the labeled N in the bark increased between 2001 and 2012, while it remained constant in the wood compartment. The N pools of these two compartments increased in size as a result of the growth of the trees (Table 1) (Boxman et al., 1998). Although the delta ^{15}N values either remained constant or decreased for these compartments (Fig. 1), this increase in N pool size was sufficient for the label recovery to remain constant or even increase. This development in time can also be viewed in Fig. A1 (Appendix A), where the shape of the rectangles representing the combined N pool of wood and bark changed with time. The width of the rectangle, representing the content of labeled N decreased with time, and the height of the rectangle, representing the size of the pool, increased with time. As a result, the area of the rectangle, representing the label recovery, retained more

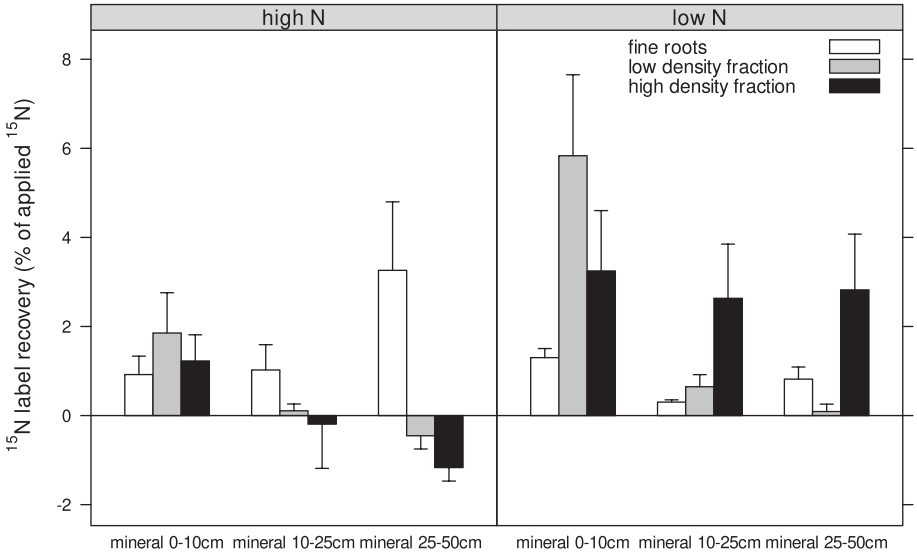

Fig. 4. Recovery of ^{15}N (as percentage of applied) in the fine roots (d<0.5 mm), in the low density fraction (<1.6 g cm^{-3}) and in the high density fraction (>1.6 g cm^{-3}) in three layers of the mineral soil of a high and low N deposition plot in a Scots pine forest 19 years after ^{15}N labeling. Error bars represent standard errors of the mean, n = 3 (high N deposition plot) and n = 5 (low N deposition plot).

or less the same size. As the trees in the low N deposition plot grew faster than those in the high N plot (Boxman et al., 1998) the recovery in the low N plot increased faster than in the high N plot.

4.2. Soil

Of the ^{15}N label retained in the ecosystem, the organic layer constituted the major sink during the first few years of the experiment (Feng et al., 2008; Tietema et al., 1998). Although 19 years after labeling the retention in the organic soil layer was still relatively large, the mineral soil had also become a major sink with a recovery of 12.5% and 28.5% of the labeled N at high and low N deposition, respectively (Table 3). These results were in line with those found in other studies. The dominance of the soil over the vegetation in retaining labeled N deposition has been found in most field experiments with ^{15}N label during the first 1.5 years after labeling (Templer et al., 2012), and several tracer experiments have shown that this pattern is stable over much longer periods (Krause et al., 2012; Nadelhoffer et al., 2004; Veerman et al., 2020). Nadelhoffer et al. (2004) carried out a tracer experiment in a mixed hardwood and a red pine stand at Harvard Forest, in the North-Eastern US. After 7 years, they found recoveries in the top 20 cm of the mineral soil ranging from 15% to 17% in the hardwood and from 22 to 34% in the pine stand. Krause et al. (2012) reported results of an experiment in a Norway spruce forest at Alptal, Switzerland. In this experiment, ^{15}N recovery in the control plot was 43% in the organic soil layer and 13% in the mineral soil 9 years after labeling, and in the N addition plot 40% in the organic soil layer and 19% in the mineral soil 14 years after labeling. On an even longer timescale of 2 decades after ^{15}N labeling in 4 different European forests of the NITREX project, spanning a range of N deposition levels and N addition experiments (Dise and Wright, 1995), recovery in organic and mineral soil layers was still substantial, varying between 19 and 109% of the labeled N (Veerman et al., 2020).

While the label content of the LF1 layer between 2001 and 2012 was similar to that of the needle and twig compartments, the F2 layer was quite different from these: delta values were higher and in the high N plot there was no decrease in recovery. This stronger retention of labeled N between 1994 and 2012 in the F2 layer of the high N deposition plot could have been due to a larger supply of labeled N from the aboveground vegetation and the LF1 layer of this plot, as in this plot a larger part of the labeled N deposition was lost from these compartments between 1994 and 2012. Alternatively, as after one year in the high N plot more label had been incorporated into the vegetation while in the low N plot the soil was a more important sink (Fig. 2, Koopmans et al. (1996)), the labeled N in the high N plot may have become to a larger extent part of the structural part of the organic material and thus more resistant to loss once it had moved to the F2 layer (Nair et al., 2017).

After all label had been lost completely from the two deeper mineral layers in 2001, some labeled N reappeared there in 2012. Apparently, the N label had not been strongly bound in these layers in 1994. N mining from the mineral soil layer has been suggested as a loss process of N from mineral soil (Cosby et al., 1997; Currie et al., 2004; Lovett et al., 2018), but this seems less likely in this forest with its high overall availability of N. However, transient N immobilization on various time scales has been found in soils. Fuss et al. (2019) found that $^{15}NO_3^-$ label could be bound to the mineral soil and lost again within weeks, and ascribed this to microbial activity. Residence times of soil low density fractions have been quantified to be less than 10 years, so it is possible that this N was bound in such organic material (Baisden et al., 2002).

In contrast with the F2 layer, a stronger retention of labeled N in the mineral layers of the low N deposition plot was observed between 2001 and 2012, though this higher recovery in the low N deposition plot was only statistically significant in the deepest mineral layer. In turn this labeled N in the low N deposition plot may have originated from the F2 layer, of which the labeled N content decreased much between 2001 and 2012. The decrease of the F2 alone was not enough to equal the concurrent increases in the mineral layers, but decreases in labeled N in the LF1 layer and the vegetation may also have contributed.

Although the increase in the recovery of labeled N in the mineral soil layers between 2001 and 2012 occurred in both plots, the density fractionation results showed clear differences between them. Therefore, it seems likely that different processes were responsible for the changes in each plot. In the high N plot the recovered label in the two deepest layers was only present in the fine roots fraction (Fig. 4). In the low N plot the fine roots contributed only a minor part of the recovery of the label, although this may have been an underestimate, as the fine root natural abundance was probably lower than the average natural abundance used for the calculation of the recovery in the fine roots. The higher recovery in the roots in the high N plot and the higher recovery in the soil density fractions in the low N plot in 2012 was preceded in 1994 by a larger recovery of label in the aboveground vegetation in the high N plot versus a larger recovery of label in the soil layers in the low N plot. Apparently, during the experiment more labeled N was present in the vegetation or material originating from it in the high N plot, while in the low N plot the labeled N moved more directly to the soil material, where it was immobilized. The increase in label observed in the mineral layer of the high N plot may be the result of the ingrowth of roots containing label between 2001 and 2012.

The smaller retention of the labeled N in the density fractions of the mineral soil layer in the high N plot could be because the capacity of the mineral soil in the high N plot to immobilize N was smaller. The cause for this may be that since 1989 the low N plot had received less N deposition, resulting in a larger capacity to immobilize N. Furthermore, as the labeled amount of N deposition in the high N plot was about 10 times that in the low N plot, a 10 times larger amount of N would have to be immobilized in the high N plot for a similar fraction of the labeled N deposition to be immobilized in the high N plot as in the low N plot (cf. the different scales of the x-axes in Figs. A1 and A2).

In the low N plot, the recovered label was mainly present in the two soil density fractions (Fig. 4). There was a shift from low to high density material with profile depth (Table 4) and a shift in delta values from the low density fraction to the high density fraction with depth (Fig. 3). Assuming that the SOM becomes older with depth, these results suggest that the labile, low density material gradually transformed into the more stable, high density material and that the label also moved from the labile to the more stable material. However, it is not certain that the soil organic material at all depths was of the same origin and went through the same transformations: the present forest is only 65 years old, and the land was previously in use as heathland or arable land (Boxman and Roelofs, 2006). As soil organic material ages and gets increasingly transformed, it is expected to decrease in C:N ratio (Hatton et al., 2012; Kramer et al., 2017), but here the C:N ratio in the mineral soil increased with depth (Table 2). The cause for this increase in C:N ratio could be that the deeper soil organic matter originated not from the *Pinus* forest but from heather vegetation, either directly or through the fertilization with sods from heathland. The dominant plant species on such heathland, *Calluna vulgaris* (L.) Hull has a litter C:N ratio much higher than that of *Pinus* forest litter (Beier et al., 2009). Evidence exists that N reaching the soil can be bound to the high density soil fraction directly, and not only through transformation of N containing low density material (Fuss et al., 2019; Kramer et al., 2017; Zhu and Wang, 2011).

4.3. Retention of N deposition

Leaching of N from this site is substantial in both plots, as has been measured in 1994 by Koopmans et al. (1996) (Table 3). The presence of large amounts of NO_3^- in the subsoil of both plots in this well drained forest in later years (Boxman et al., 2008) indicates that N loss through leaching remained considerable. Losses of labeled N deposition occurred mostly in the first year after labeling (Koopmans et al., 1996). Large initial losses have been observed in other terrestrial ecosystems

as well (Calvo-Fernández et al., 2015; Kjønaas and Wright, 2007; Schleppi et al., 1999; Wang et al., 2018), especially in case of high N deposition (Curtis et al., 2011). The results from 2001 (Wessel et al., 2013) indicate that these losses subsequently decreased. Our results from 2012 showed that the retention of the labeled N did not decrease further between 2001 and 2012 (Table 3), and thus our first hypothesis that this would occur has to be rejected. The absence of any detectable loss of labeled N deposition between 12 and 19 years was unexpected, considering this forest has an extremely high N status compared to other forests (Boxman et al., 1998; Tietema et al., 1997; Veerman et al., 2020). Apparently, over a time period of two decades this nutrient can be retained extremely well, once it has been incorporated in some way into the vegetation and the soil. In a forest in the state of New York, similarly a complete retention of labeled N deposition was found 5–6 years after labeling, but the N status of this forest must have been much lower, considering total N deposition was never higher than 10 kg N ha^{-1} yr^{-1} (Goodale, 2017). In the previously discussed ^{15}N labeling experiment in a Norway spruce forest at Alptal, Switzerland (Krause et al., 2012), tree felling 20 years after labeling strongly increased NO_3^- leaching, but this NO_3^- contained none (N addition experiment) or very little of the labeled N (control experiment) (Schleppi et al., 2017). In order to explain the absence of N export from a mature non-growing forest, Lovett et al. (2018) put forward a conceptual model in which is described that after a maturing forest does no longer accumulate any biomass the soil may still accumulate N for some time, because the trees have mined the soil for N during their growth phase and this N pool is then replenished in the next phase. Although mining the soil for N seems less likely to occur in our forest with its high N status, the deeper SOM originating from high C:N ratio -heathland- material may play a similar N immobilizing role as the mined SOM in their conceptual model, at least in case of the low N plot. However, taking the whole experiment into consideration, it rather seems that the fluxes of N in the system were not all directly coupled to each other, and the forest ecosystem was able to retain N once incorporated, while at the same time other, not yet incorporated N was less well retained in the system. This would be in agreement with the way Lovett and Goodale (2011) describe how in forest ecosystems added N can flow simultaneously to all sinks and losses in the system, while the fate of the added N and the temporal patterns of flow of N depend on the strength of the sinks. A hysteresis effect by which N is generally incorporated faster into the forest vegetation and soil than it is mobilized again from these pools (Gilliam et al., 2019) probably further contributed to the retention of incorporated N and to the loss of recently added N. The strong retention of the labeled N deposition in the various pools is consistent with the fast improvement of the forest ecosystem Boxman et al. (1995, 1998) found after reduction of the N deposition, such as a change in soil solution chemistry, less leaching of base cations, decreased N concentration of the needles and an increased growth of the trees.

While the total labeled N retained did not decrease between 2001 and 2012, there were clear shifts from labile to more recalcitrant parts of the N in the ecosystem. In the aboveground vegetation labeled N in the needles and twigs decreased with time, while that in the bark and wood increased or remained the same. In the mineral soil in the low N plot, the increase in label in the two deepest layers between 2001 and 2012 was mainly present in the high density fraction, which is considered the more recalcitrant fraction of the soil. The observed shift towards the high density fraction with depth might be interpreted as a transformation of N from the low to the high density fraction with time. The decreasing presence of labeled N in the needles and twigs between 2001 and 2012 also indicated that the increase in labeled N in the soil did not occur in fractions available to the vegetation. Thus, our second hypothesis that stable N pools would become more important for the retention of the labeled N was partly confirmed. Nevertheless, also in less recalcitrant pools label could be retained for 19 years in this strongly N leaching forest. Cycling between needles, twigs and LF1 kept some label in these compartments, while in the high N plot the fine roots were the most important compartment in the two deeper layers of the mineral soil. Templer et al. (2012) concluded from their meta-analysis of forests of which the N deposition was labeled with ^{15}N that the contribution of the N deposition to C sequestration is probably small, as most labeled N deposition ended up in the soil and not in the vegetation. This study corroborates this conclusion, as after 19 years the soil was the main sink for the labeled N deposition as well, especially if the soil is compared to the bark and wood compartments, the compartments with the longest residence times of the vegetation.

4.4. Consequences for N cycling

Despite N leaching was more or less equal to the N deposition in this forest under ambient circumstances, the majority of the labeled N was retained within the ecosystem after one year. A large retention of labeled N was found by Gurmesa et al. (2016) after one year in a forest in China with similar N deposition and leaching. One would expect that in subsequent years the rest of the labeled N would have to be leached from such an ecosystem, otherwise leaching could not equal deposition in the longer term. However, leaching of the remaining labeled N did not occur in the 19 years of our experiment. This would mean that either transformation of N in the ecosystem is extremely slow, or the labeled N of our experiment, and probably other N deposited in adjacent time periods is more strongly bound than N that is much older. Either way, it is clear that steady state conditions for the N dynamics in a forest ecosystem are not easily reached (Gilliam et al., 2019).

The current N deposition in the region of the Ysselsteyn forest exceeds its critical load strongly and this exceedance is among the highest in Europe (Forsius et al., 2021). Critical N loads have originally been developed with respect to soil acidification, but are used now as well for effects on eutrophication and biodiversity (Dise et al., 2011). Critical loads for ecosystems are estimated under the assumption that there are steady-state conditions (Posch et al., 2008). The strong retention we found of the labeled N deposition can have consequences for the time it takes the ecosystem to reach steady state conditions, after the N deposition has been lowered to critical load levels. During this time, the so-called recovery delay time (Slootweg et al., 2008) the previously accumulated N may still be released and affect the functioning of the ecosystem. Boxman et al. (1995) found that some conditions improved within a few years after lowering of the N deposition in this forest, such as changes in soil solution chemistry, and the N concentration of the canopy (Schmitz et al., 2019). However, N processes in forest ecosystems occur at different time scales (Moldan et al., 2006), and the N loads that have accumulated in the previous years may determine whether recovery occurs (Dise et al., 2011). The accumulated, strongly-retained N may have a lasting effect upon the N conditions in the ecosystem. This may happen even if the N deposition has been strongly reduced, e.g. through increased gross N mineralization immobilization fluxes, with consequences for the N availability, competitive interactions between species, and species composition (Bobbink et al., 2010).

5. Conclusions

Many studies concerning the dynamics of N in forests and concepts explaining the behavior of N in these ecosystems are based on budget studies following input output fluxes and net changes in sizes of N pools of the system (e.g. Gosz et al., 1976; Johnson and Lindberg, 1992; Tietema and Verstraten, 1991). Using the ^{15}N labeling technique, this study followed a one-year cohort of N deposition to examine the fate of this cohort in the forest ecosystem on a decadal scale. Short term cohort studies of N deposition already showed that substantial parts can be leached quickly in the first stage (in a leaching system), while losses in a subsequent phase can be much smaller. This long term study makes clear that also on a decadal scale N deposition, once

incorporated in vegetation and soil, can be retained inside the system despite strong N leaching fluxes. Apparently, internal processes and input-output fluxes are to some extent separated. As a consequence changes in the external N fluxes may have less impact upon the internal N dynamics than assumed, and cohort studies such as this one can constitute a useful addition to budget studies of forest ecosystems in order to understand their N dynamics (Rosi-Marshall et al., 2016).

CRediT authorship contribution statement

Wim W. Wessel: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Andries W. Boxman:** Investigation, Supervision. **Chiara Cerli:** Methodology, Validation, Investigation, Resources. **E. Emiel van Loon:** Formal analysis, Writing – review & editing. **Albert Tietema:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank Laura Fernández Fraile, Rachael Chambers, Lennart Stam, Michiel Nilwik and Roy Peters for their excellent help in the field and laboratory. In addition, we thank John Parsons for critically reading an earlier version of the manuscript. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.144356.

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