Different responses of ash and beech on nitrate *versus* ammonium leaf labeling

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Abstract

The effects of tree species on the N cycle in forest systems are still under debate. However, contradicting results of different ^{15}N labeling techniques of trees and N tracers in the individual studies hamper a generalized mechanistic view. Therefore, we compared $Ca(^{15}NO_3)_2$ and $^{15}NH_4Cl$ leaf-labeling method to investigate: (1) N allocation patterns from aboveground to belowground, (2) the cycles of N in soil-plant systems, and (3) to allow the production of highly ^{15}N enriched litter for subsequent decomposition studies.

20 beeches (*Fagus sylvatica*) and 20 ashes (*Fraxinus excelsior*) were ^{15}N pulse labeled from aboveground with $Ca(^{15}NO_3)_2$ and 40 beeches and 40 ashes were ^{15}N pulse labeled from aboveground with $^{15}NH_4Cl$. ^{15}N was quantified in tree compartments (leaves, stem, roots) and in soil after 8 d.

Beech and ash incorporated generally more ^{15}N from the applied $^{15}NH_4Cl$ compared to $Ca(^{15}NO_3)_2$ in all measured compartments, except for ash leaves. Ash had highest ^{15}N incorporation [45% of the applied with $Ca(^{15}NO_3)_2$] in its leaves. Both tree species kept over 90% of all fixed ^{15}N from $Ca(^{15}NO_3)$ in their leaves, whereas only 50% of the ^{15}N from the $^{15}NH_4Cl$ tracer remained in the leaves and 50% were allocated to stem, roots, and soil. There was no damage of the leaves by both salts, and thus both ^{15}N tracers enable long-term labeling *in situ* field studies on N rhizodeposition and allocation in soils. Nonetheless, the ^{15}N incorporation by both salts was species specific: the leaf labeling with $^{15}NH_4Cl$ results in a more homogenous distribution between the tree compartments in both tree species and, therefore, $^{15}NH_4Cl$ is more appropriate for allocation studies. The leaf labeling with $Ca(^{15}NO_3)_2$ is a suitable tool to produce highly enriched ^{15}N leaf litter for further long term *in situ* decomposition and turnover studies.

Key words: $Ca(^{15}NO_3)_2$ labeling / deciduous forest / $^{15}NH_4Cl$ labeling / nitrogen cycles / species effects / tree rhizodeposition

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1 Introduction

Plant-available N in soil originates from various sources: microbially fixed atmospheric N_2, atmospheric N deposition, mineralization of plant litter and root exudates and mineralization of soil organic matter (*Millard* and *Grelet*, 2010). N cycling in forest ecosystems has been intensively studied during the past decades, especially regarding the expected climate change scenarios (*Heinrich* et al., 2015) and ^{15}N labeling techniques are frequently used to study N transformations and allocation in agroecosystems, but seldom in forest ecosystems. Studies on N rhizodeposition in deciduous forest ecosystems are still scarce and deliver contrasting results (*Brumme* et al., 1992; *Hertenberger* and *Wanek*, 2004).

Investigating N rhizodeposition requires ^{15}N labeling of the tree from aboveground. One reason for the high variability in results on N allocation is the use of different ^{15}N labeling methods of plants (*Jones* et al., 2009). Three labeling approaches are frequently used to investigate N rhizodeposition: (1) shoot labeling, (2) leaf labeling, and (3) split root system. Ammonium nitrate or urea have been used for the shoot labeling (also called wick method) (*Russell* and *Fillery*, 1996; *Mayer* et al., 2003; *Yasmin* et al., 2006; *Wichern* et al., 2011; *Glaser* et al., 2012). For example, *Glaser* et al. (2012) used ^{15}N shoot labeling by drilling a hole through the stem and inserting a fiber glass wick, covered with a PVC tube at both sides of the hole. The cut surface between hole and wick was sealed with PVC glue and the glass fiber wicks were saturated with sterile water and connected with a reservoir containing the $^{15}NH_4{}^{15}NO_3$ tracer solution. The produced labeled plant material was then used to trace and quantify N from litter decomposition, such as in other studies (*Schmidt* and *Scrimgeour*, 2001; *Bimüller* et al., 2013; *Benesch* et al., 2015). Leguminous trees stem-labeled with $K^{15}NO_3$ solution showed limited ^{15}N transfer to associated grass in an agroforestry system and indicated that transfer of the added ^{15}N was limited in space (*i.e.*, up to 1m from the trees) and delayed in time (*i.e.*, ^{15}N reached the tree roots more than 3 months after labeling), which prevented estimation based on the stem-^{15}N

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labeling method (*Sierra* and *Daudin*, 2010). However, injecting ^{15}N directly into the vessel elements also requires special equipment and foliar fertilization delivers the nutrients directly to the leaves and increases N use efficiency. Therefore, a ^{15}N foliar application could be a useful tool for labeling tree leaf material (*Ta* et al., 1989). Urea and NH_4NO_3 have frequently been used for leaf labeling to investigate the distribution and recycling of canopy N storage reserves (*Ayala* et al., 2014). *Ayala* et al. (2014) bagged cherry trees to isolate them from the rest of the soil and then sprayed a solution of ^{15}N-urea onto the leaves. Their approach is similar to the method used by *Zeller* et al. (1998) and *d'Annunzio* et al. (2008), who sprayed beech trees also with ^{15}N urea to produce ^{15}N-labeled litter. ^{15}N litter labeling experiments conducted in two European forests revealed after a decade that 60% of the ^{15}N tracer from the litter was retained in soil aggregates, while plant debris still contained 40% of the tracer (*Hatton* et al., 2012). Varying concentrations of $^{15}NH_4^+$ and $^{15}NO_3^-$ were also used for leaf labeling to study the preferential uptake of NH_4^+ and NO_3^- by aboveground parts of beech trees and as simulated rain on red maple and white oak and revealed that the foliar uptake of $^{15}NH_4^+$ from the tracer by deciduous tree leaves exceeded the uptake of NO_3^- (*Garten* and *Hanson*, 1990; *Brumme* et al., 1992).

The N fluxes in the xylem of trees are regulated by three processes: remobilization from internal reserves, root uptake of N from the soil, and phloem–xylem recycling (*Dambrine* et al., 1995; *Grassi* et al., 2003). Species-specific patterns of C and N allocation in the tree compartments might be due to differences in remobilization and recycling processes and, therefore, differences in the amount of rhizodeposition into the soil (*Sommer* et al., 2016). However, the labeling of grey alder leaves following root fertilization and leaf fertilization with either $^{15}NH_4^+$ or $^{15}NO_3^-$ revealed that root fertilization gave better labeling efficiency, uniformity and repeatability than leaf labeling in both ^{15}N labeling forms (*González-Prieto* et al., 1995). $(NH_4)_2SO_4$ and KNO_3 have been used in the split root system, where the root systems of one seedling are split between two soil chambers. Labeling one soil chamber with a ^{15}N-enriched N source enables observing the N loss from the portion of the root system growing in the unlabeled soil chamber (*Sawatsky* and *Soper*, 1991; *Jensen*, 1996; *Schmidtke*, 2005). It can be concluded that a tracer including NH_4^+ will take up higher amount of N and might therefore have advantages in further leaf labeling experiments.

Picea abies was labeled with $^{15}NO_3^-$ and $^{15}NH_4^+$ through soil application addressing the question of preferential N source uptake and investigated 94% recovery for the applied $^{15}NH_4^+$ and 100% for the applied $^{15}NO_3^-$ for the entire stand in the first year (*Buchmann* et al., 1995). The main sink for both N forms was the soil, where 87% of the $^{15}NH_4^+$ and 79% of the $^{15}NO_3^-$ were found, and surprisingly eight months after labeling, 9% of the ammonium and 15% of the nitrate label were found in the understory in shrubs and the perennial grass of *Picea abies*, whereas *Picea* retained only 3% of the $^{15}NH_4^+$ and 7% of the $^{15}NO_3^-$ (*Buchmann* et al., 1996). Besides mineral N labeling of soils, dual isotope labeling (^{15}N and ^{13}C) of amino acids is a widely used approach quantifying the intact uptake of amino acids by plants (*Näsholm* et al., 1998; *Hodge*, 2004; *Moran-Zuloaga* et al., 2015).

$^{15}NH_4Cl$ has also only been chosen for belowground labeling up to day and alongside NO_3^-. *Trogisch* (2012) and *Zeugin* (2010) used three chemical N tracers dual-labelled glycine, $K^{15}NO_3$, and $^{15}NH_4Cl$ for ^{15}N soil labeling under tropical broadleaf tree species to study N uptake patterns. Those ^{15}N labeling experiments allow to study the preferences of species for different chemical N sources (*Zeugin*, 2010; *Liu* et al., 2016) and clearly demonstrated that multidimensional N-use complementarity can facilitate species coexistence (*Xu* et al., 2011). However, ^{15}N labeling experiments from belowground, if not constructed as split-root experiments, are not considering N sources of rhizodeposition, whereas leaf labeling might be an adequate tool for investigating differences in N allocation and exudation strategies (*Wichern* et al., 2008). However, leaf-labeling with ^{15}N-urea should not be considered a pure pulse-labeling method because *Gasser* et al. (2015) showed quantitative evidence of overestimated rhizodeposition on red clover.

The aim of this study was to investigate whether $Ca(^{15}NO_3)_2$ or $^{15}NH_4Cl$ can be used as tracers for the leaf labeling, providing an alternative to urea or ammonium nitrate to trace N allocation by trees. Beech and ash were chosen as model trees as their relevance in temperate broad leaf forests is high and a deviation N cycling induced by these trees was already observed (*Guckland* et al., 2009; *Langenbruch* et al., 2014). We hypothesized that leaf labeling with $Ca(^{15}NO_3)_2$ and $^{15}NH_4Cl$ results in (1) chemical species specific differences and (2) tree species specific differences. Furthermore, certain potential advantages over the classical labeling approaches, including (1) no damage to the leaves by the tracer, (2) fast ^{15}N distribution of the N tracer due to high mobility of nitrate, and (3) possibility to produce highly ^{15}N enriched litter for further decomposition studies will be investigated and discussed.

2 Material and methods

The study is based on two experiments: (1) ^{15}N labeling with $Ca(^{15}NO_3)_2$ and (2) ^{15}N labeling with $^{15}NH_4Cl$.

2.1 ^{15}N labeling with $Ca(^{15}NO_3)_2$

The experimental site (10°05′ N, 10°30′ E, 300 asl) was located in the southwest of Weberstedt, which belongs to the province of Thuringia (Germany) in the northeastern part of the Hainich National Park. The mean annual temperature is 7.5°C and the mean annual precipitation is 670 mm. The Hainich is the largest continuous broad-leaved forest of Germany, dominated by beech, and grows on a Stagnic Luvisol (WRB 2006) developed from loess that is underlaid by Triassic limestone. The *in situ* ^{15}N pulse labeling of 20 beeches (*Fagus sylvatica*) and 20 ashes (*Fraxinus excelsior*) was conducted in August 2011 on trees with 3–4 m height and were ;compared with 10 reference trees of each species. All trees including the reference trees were chosen within an area of uniform light intensity under a closed beech canopy and scattered with a maximum distance of 300 m from the center of the site.

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2.2 ^{15}N labeling with $^{15}NH_4Cl$

The experimental site Göttinger Wald (51°35′ N, 9°58′ E, 362 asl) was located in the southwest of Göttingen, within the province of Lower Saxony, Germany. The Göttinger Wald is a 130–145 year old beech forest scattered with ashes and maple also on a Triassic limestone plateau. The mean annual temperature is 7.7°C and the mean annual precipitation 610 mm (*Maraun* et al., 2001). 40 ashes and 40 beeches were chosen by height of approximately 1 m and compared with 20 reference trees of each species. Ashes and beeches were taken from the forest with undisturbed soil and placed into pots with sufficient space for the entire rooting system (size: 23 × 23 cm; depth: 26 cm). The trees had a reestablishing time of two months and were kept under the canopy of mature beech trees in the Göttinger Wald and transferred to an outdoor greenhouse instantly before labeling. The seedlings were irrigated regularly and herbs were removed by cutting the shoots at soil surface. The ^{15}N pulse labeling pot experiment of 20 beeches and 20 ashes was conducted mainly in August 2012, *i.e.*, at the same season.

2.3 Labeling method

Glass vials were used as reservoir containing the ^{15}N-labeled calcium nitrate solution (99.23 at% ^{15}N, Campro Scientific GmbH, Berlin, Germany) or the ^{15}N labeled ammonium chloride solution (98 at% ^{15}N, Campro Scientific GmbH, Berlin, Germany). The ^{15}N solutions were applied per gram aboveground biomass with 3×10^{-5} mol in both experiments and stayed on the trees for 72 h. The aboveground biomass is listed in Tab. 1. Three leaves of each beech and three leaflets of each ash with a similar area were mechanically roughened to allow the uptake of the solution and then placed directly in the vials with the tracer solution. Three vials were fixed on the branches at different heights in each tree. The vials were closed with Parafilm and additionally covered with a transparent bag to avoid spilling on the ground. At the end of the labeling, on day three, the vials were removed with a cut behind the leaves to avoid contamination of the ground or other leaves. Leaf, stem, root, and soil samples were analyzed to quantify the allocation of ^{15}N. This experiment compares data of samples taken 8 d after the start of the labeling. Therefore, four beeches and four ashes for the labeling with $Ca(^{15}NO_3)_2$ and ten ashes and ten beeches for the labeling with $^{15}NH_4Cl$ were compared, respectively. The other trees samples were not compared in this study because they different in sampling time.

Table 1: Mean aboveground biomass.

Tree species / ^{15}N tracer	Leaf Biomass (g) Mean +/– SEM	Stem Biomass (g) Mean +/– SEM
Beech / $Ca(^{15}NO_3)_2$	80 ± 6	400 ± 60
Beech / $^{15}NH_4Cl$	8.5 ± 0.6	37 ± 2
Ash / $Ca(^{15}NO_3)_2$	55 ± 7	180 ± 10
Ash / $^{15}NH_4Cl$	4.3 ± 0.4	24 ± 2

All leaves were sampled and stems were cut 10 cm above the soil, in the middle of the tree, and the top part. Root samples were taken 10–15 cm from the main root after the tree was entirely uprooted to make sure it belongs to the labeled tree and matches the soil samples. Soil sampling was also performed in up to 15 cm distance to the tree with a split tube in three replicates. The intact core was divided into two to three depth segments but only the top segment of 0–10 cm was used for comparison in this study. The soil was removed from the column, weighed, homogenized, and the water content was determined in a subsample.

For the analysis of N, content and $\delta^{15}N$ signature in plant tissue and soil, leaves, stem, root, and bulk soil samples were freeze-dried, ground in a ball mill (Retsch Schwingmühle MM2, Haan, Germany), and an aliquot (approx. 2 mg for plant tissue and 12 mg for soil) was filled into tin capsules. Relative N isotope abundances in leaves, stems, roots, and soil samples were measured using an elemental analyzer NA1500 (Fison-Instruments, Rodano, Milano, Italy) coupled to a Delta plus isotope ratio mass spectrometer (Finnigan MAT, Bremen, Germany) through a ConFlo III interface (Thermo Electron Cooperation, Bremen, Germany). $\delta^{15}N$ values were calibrated based on co-measured certified IAEA Standards (IAEA-600, USGS26, USGS40, USGS41, IAEA-N-1, IAEA-N-2 and IAEA-NO-3).

2.4 Calculation of ^{15}N uptake

The ^{15}N uptake by plants from sources of different isotopic composition alters their $\delta^{15}N$ value, which follows a two-component mixing model between the ^{15}N natural abundance isotopic signature and the signature of the incorporated tracer-derived ^{15}N according to *Gearing* et al. (1991), as shown in Eq. (1):

$$[N]_{incTracer} = [N]_{compartment} \times \frac{at\%_{labelled} - at\%_{ref}}{at\%_{15N-Tracer} - at_{\%ref}}, \quad (1)$$

with $[N]_{compartment}$ = the nitrogen content of sample (mmol $g_{freeze\text{-}dried\ soil;leaf;stem;root}^{-1}$), $[N]_{incTracer}$ = total amount of ^{15}N incorporated into the plant in (mmol $g_{freeze\text{-}dried\ soil;leaf;stem;root}^{-1}$), $at\%_{labelled}$ = ^{15}N values of the labeled sample of the tree (leaf, stem, root, soil), $at\%_{ref}$ = ^{15}N values of the non-labeled reference sample of the tree (leaf, stem, root, soil), $at\%_{15N\text{-}Tracer}$ = ^{15}N enrichment of the added $Ca(^{15}NO_3)_2$ / $^{15}NH_4Cl$.

Allocation of the incorporated ^{15}N was calculated by dividing the incorporation into a certain plant or soil compartment through the sum of total ^{15}N recovered in all plant and soil pools. This value was displayed as % of ^{15}N allocation by multiplying it with 100%.

2.5 Statistics

Field replications were corrected for outliers using the Nalimov outlier test. All plant compartments and soil data were tested with a factorial analysis of variance (ANOVA). A Tukey HSD (honest significant difference) tests for post hoc compar-

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ison were used to compare ^{15}N incorporation or ^{15}N allocation as dependent variables, while tree species, ^{15}N tracer and within-individual variation were used as independent variables (significance level of $p < 0.05$). The error bars show a standard error of the mean (SEM) in all graphs.

3 Results

3.1 ^{15}N incorporation into plant tissues and soil

Significant differences in ^{15}N incorporation were shown between the chosen N compounds and the tree species in the leaves 8 d after the start of the labeling. The ^{15}N values in the field study had an average of 4.8 ± 2.3 at% in beech and 3.5 ± 0.4 at% in ash, and the ^{15}N values in the small trees reached in beech 2.0 ± 0.1 at% and in ash 1.1 ± 0.2 at%. Whereas N form and tree species itself but also the interactions between these factors affected the ^{15}N incorporation into leaves, only the ^{15}N species affected the allocation to stem and soil significantly.

Beech and ash recovered between 0.812% to 5.940% ^{15}N from the applied $^{15}NH_4Cl$ into roots, stem, and soil. The pulse labeling experiment with $Ca(^{15}NO_3)_2$ resulted only in 0.001% to 0.316% ^{15}N from the applied tracer in roots, stem, and soil (Fig. 1). Beech incorporated 1.9% ^{15}N from the applied $^{15}NH_4Cl$ in leaves and 9.6% ^{15}N from the applied $Ca(^{15}NO_3)_2$ (Fig. 1). Ash showed the highest incorporation with 45% of the ^{15}N applied as $Ca(^{15}NO_3)_2$ in its leaves, but only 6% of the ^{15}N applied in the labeling with $^{15}NH_4Cl$ (Fig. 1).

3.2 ^{15}N allocation into plant tissues and soil

Beech and ash allocated with over 90% of all fixed ^{15}N of the $Ca(^{15}NO_3)_2$ almost everything into the leaves (Fig. 2), *i.e.*, did not allocate any relevant ^{15}N portion belowground. Beech allocates ten times more ^{15}N of the assimilated $Ca(^{15}NO_3)_2$ in its stem in comparison to ash, in which the applied ^{15}N was nearly exclusively recovered in the leaves. In contrast, both tree species allocated only approx. 50% of the applied $^{15}NH_4Cl$ in the leaves 8 d after the start of the labeling, whereas the other half was incorporated in other plant compartments and soil. 25–35% of ^{15}N were allocated to stem and 5–15% to the root. However, ash allocated three times more ^{15}N in its roots than beech (Fig. 2). A similar pattern was found for soil, where also ash released by factor of two higher amounts of the incorporated ^{15}N compared to beech. For any labeling study based on ^{15}N tracing of rhizodeposits, it is important to consider that beech allocated by the factor ten and ash with 15% even by the factor 500 more ^{15}N in the soil in the $^{15}NH_4Cl$ approach in comparison to the labeling with $Ca(^{15}NO_3)_2$.

The ANOVA demonstrates that this strong effect of the N tracer form on allocation pattern is not only highly significant in soil, but also for all other plant compartments. Comparing effects of tree species on ^{15}N incorporation and allocations shows that relative allocation in the compartments is similar between both tree species.

4 Discussion

The ability of plants to take up nutrients directly by leaf tissues is well known (*Tuckey* et al., 1962; *Brumme* et al., 1992). Nevertheless, we used, for the first time, the ^{15}N leaf-labeling method based on $Ca(^{15}NO_3)_2$ on beech and ash trees in a field experiment and a ^{15}N leaf-labeling method based on $^{15}NH_4Cl$ on the same tree species in a pot experiment. Despite an identical labeling procedure and identical season for labeling, we are aware that the ecophysiological conditions for trees in field and—even if larger sized—in pots are

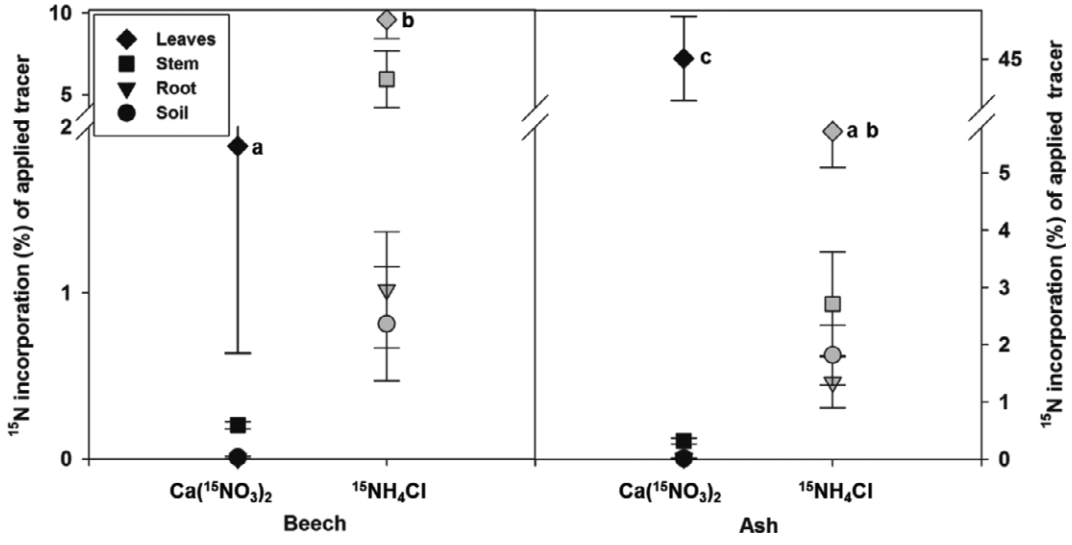

Figure 1: ^{15}N incorporation of applied $Ca(^{15}NO_3)_2$ (black symbols; $n = 4$) and $^{15}NH_4Cl$ (grey symbols; $n = 10$) 8 d after the start of the labeling for beech (left) and ash (right) in leaves (diamonds), stem (squares), roots (triangle), and soil (circles). Error bars show SEM. Small letters show significant ($p < 0.05$) differences of ^{15}N in leaves between the tree species and between the ^{15}N forms. Markers without letters display no significant differences between tree species and ^{15}N forms.

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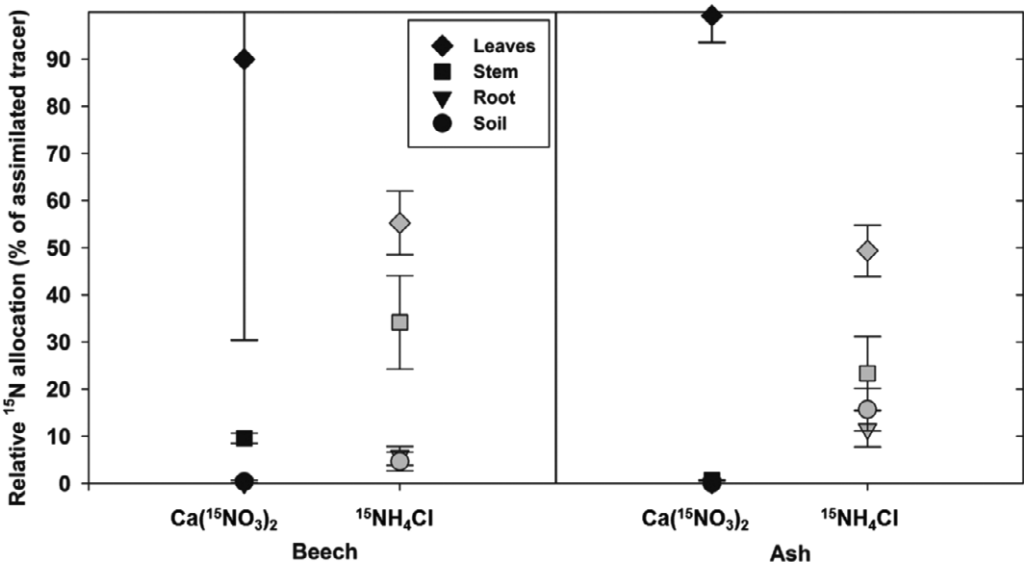

Figure 2: Relative ^{15}N allocation of applied Ca(^{15}NO$_3$)$_2$ (black symbols; n = 4) and ^{15}NH$_4$Cl (grey symbols; n = 10) 8 d after the start of the labeling for beech (left) and ash (right) in leaves (diamonds), stem (squares), roots (triangle), and soil (circles). Error bars show SEM. No statistical differences between tree species and between the N forms.

not identical and are likely to have an effect on the N allocation pattern presented in this study. Therefore, differences in ^{15}N allocation cannot be solely interpreted by N species effect. However, the relative differences of beech and ash, observed in this study for the two N species, suggests that there is a strong interaction between species specific allocation patterns and the ^{15}N species and, thus, that N speciation plays a role for the ^{15}N allocation pattern following labeling.

Previous studies preferentially used urea or NH_4^+ for leaf labeling to investigate the distribution and recycling of canopy N storage reserves (*Ayala* et al., 2014) or to produce ^{15}N labeled plant material to trace and quantify the N stabilization and N released from litter decomposition (*Schmidt* and *Scrimgeour*, 2001; *Bimüller* et al., 2013). Importantly, urea foliar fertilization can cause leaf damage and negatively influence plant growth and allocation patterns. Both leaf labeling approaches did not show any damage to the unlabeled leaves. Therefore, N leaf-labeling with Ca(^{15}NO$_3$)$_2$ and ^{15}NH$_4$Cl will enable long-term labeling studies on N rhizodeposition, N turnover and stabilization in soils. This is particularly important for *in situ* experiments in forest stands, in which long-term N balance is one of the future challenges for forest ecosystem research.

The absolute ^{15}N enrichments (at%) depend on the amount of biomass, the amount of tracer, and the enrichment of the ^{15}N tracer. Thus, to compare the efficiency of the approaches, especially for not identically sized trees, we focused on the relative uptake and incorporation of ^{15}N (in % of applied label) by the trees in this experiment. Beech assimilates 10% and therefore five times more ^{15}N from the applied ^{15}NH$_4$Cl in its leaves in comparison to the applied Ca(^{15}NO$_3$)$_2$. Ash showed the highest uptake with 45% of the ^{15}N applied with Ca(^{15}NO$_3$)$_2$ in its leaves, which was eight times more ^{15}N than in labeling experiment with ^{15}NH$_4$Cl. *Glaser* et al. (2012) labeled *via* stem injection with ammonium nitrate (99.25 at% ^{15}N) various broad-leaved trees and the uptake also varied between 14% in *Croton macrostachys* to 63% in *Cupressus lusitanica* in the leaves. This supports our results and demonstrates that absolute N uptake, also *via* leaf, is strongly species dependent. However, the final ^{15}N enrichment achieved in the litter in the experiment of *Glaser* et al. (2012) and in the two leaf labeling approaches presented here are sufficient for subsequent litter turnover studies. None of the two labeling methods (leaf *versus* stem) could be given a clear preference if the production of ^{15}N labelled litter is the objective for ^{15}N tree labeling. Similarly, both N forms chosen for leaf labeling produced highly enriched litter and, therefore, we suggest that the Ca(^{15}NO$_3$)$_2$ labeling method as well as the ^{15}NH$_4$Cl labeling approach are suitable tools to gain highly ^{15}N enriched litter.

Brumme et al. (1992) labeled young beeches with ^{15}NH$_4$^{15}NO$_3$ and ^{15}NH$_4$NO$_3$ and stated that the NH_4 uptake was 27% higher than the NO_3^- uptake. Our results confirm this observation at least for beech, because beech showed a 12% higher NH_4^+ than the NO_3^- uptake summing up all measured compartments. However, ash in contrast incorporated four times less NH_4^+ than NO_3^- into all measured compartments. Therefore, we conclude that there is likely a strong species effect on N allocation and, consequently, for each ^{15}N leaf labeling a careful selection of the N species has to be done as the absolute amount of uptake is affected by the N species and the tree species. Limited knowledge on these interactions for many not yet investigated tree species requires careful consideration of this aspect for all further investigations and potential pre-experiments with the undescribed tree species.

The N distribution in higher plant results not only from the mineral N uptake by the roots and the reduction of oxidized N

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species (*Gavrichkova* and *Kuzyakov*, 2010), but also from xylem translocation, phloem cycling, and short and long term storages as amino acids or proteins (*Clarkson* et al., 1986; *Laine* et al., 1994). *Rennenberg* et al. (1998) discovered that the total soluble non-protein N content of xylem sap within beech trees is dominated by organic N rather than by inorganic N and that the assimilation of inorganic N in beech trees takes place mainly in the roots. Furthermore *Rennenberg* et al. (1998) mentioned that Arginine appears to be the main storage compound and accumulates in beech compartments in comparison to spruce, for example. Glutamine is present in beech trees in high amounts in all tissues and transport systems during the entire growing season (*Rennenberg* et al., 1998). The N storage in the stem depends highly on the season, is closely linked to tree phenology, and operates at temporal scales of months to years, with remobilization being source driven (*Millard* and *Grelet*, 2010). Although we already found a remarkable allocation of ^{15}N from $^{15}NH_4Cl$ into stem, the production of highly enriched stem, *e.g.*, for wood decomposition studies, would presumably be even more successful in case of multiple pulse labeling at various seasons.

Glutamine was presented by *Rennenberg* et al. (1998) as N compound circulating between the shoot and the roots in beech, but there are no comparable studies on ash. However, ammonium and nitrate are not incorporated similarly into universal N transporters such as glutamine, mainly because nitrate should be reduced before it can be used for amination of an amino acid. The reduction of NO_3^- to NH_4^+ is catalyzed by nitrate and nitrite reductase enzymes and is among the most energy-intensive processes in the plants and can implicate additional respiration (*Gavrichkova* and *Kuzyakov*, 2010). Thus, it is likely that the observed differences in the allocation of ammonium and nitrate-derived ^{15}N to belowground arise from their deviating applicability to be transferred on the organic N transporting molecules, *i.e*., mainly amino acids.

Beyer et al. (2013) showed in the National Park Hainich that the longevity of fine roots of ash is significantly higher than of beech, which is one of the reason explaining differences in the rhizodeposition of theses tree species. However, it is unclear whether fine roots re-translocate significant N amounts to other plant compartments before root death and whether re-translocation from senescent fine roots varies with N availability (*Nadelhoffer*, 2000). The strongly deviating ^{15}N incorporation between soil and root in case of $Ca(^{15}NO_3)_2$ labeling suggests that nitrate remains in a highly mobile N form. This N form might not be lost with the fine root turnover to the soil but kept, presumably by re-allocation, in the tree biomass. However, higher ^{15}N incorporation from $^{15}NH_4Cl$ into soil points towards an increased N loss *via* exudation or fine root turnover.

Glaser et al. (2012) discovered in their *in situ* wick labeling approach with $^{15}NH_4^+$ that only a part of the labile N fraction in the leaves was ^{15}N enriched. This was similarly observed in the comparison of ^{15}N species of the present study: beech and ash kept over 90% of the fixed ^{15}N from $Ca(^{15}NO_3)_2$ in their leaves but only 50% of the applied $^{15}NH_4Cl$, whereas the other 50% were allocated to stem, roots, and soil. We assume that the ^{15}N from the $Ca(^{15}NO_3)_2$ tracer stays in the leaves maybe stored as arginine or even as non-reduced nitrate. The ^{15}N of $^{15}NH_4Cl$ is much better transferred onto the classical N transport molecules in trees and, thus, ^{15}N was much better distributed over the entire tree and allocated into soil. Therefore, we suggest to use $Ca(^{15}NO_3)_2$ only for leaf litter decomposition studies. In contrast, we recommend ^{15}N from ammonium to trace N flux in stem, roots or rhizodeposits.

One great advantage of the leaf labeling approaches based on $Ca(^{15}NO_3)_2$ and $^{15}NH_4Cl$ is the ability to understand and quantify the N cycles for adult trees *in situ* under less disturbed conditions than urea labeling, causing physiological damage to the leaf, or the wick method, causing mechanical damage of the stem transport systems.

5 Conclusions

The two tree species investigated had a significant effect on the uptake of the N form [$^{15}Ca(NO_3)_2$ / $^{15}NH_4Cl$] applied *via* leaf labeling into the tree. Leaf ^{15}N labeling has advantages over the stem labeling methods because it is possible to achieve high ^{15}N enrichments of the litter, but it has also advantages for N allocation studies as a specified unidirectional transport of the label is given. Compared to the urea leaf labeling method, $Ca(NO_3)_2$ and $^{15}NH_4Cl$ do not damage the leaves, and therefore leaf labeling with $Ca(^{15}NO_3)_2$ can be used as a long-term ^{15}N labeling technique. Both leaf labeling forms allow ^{15}N detection even in slow-responding pools such as bulk soil organic matter after 8 d. Therefore, both labeling approaches are generally appropriate for targeted studies focused on the N allocation pattern of individual trees within a forest ecosystem. As the biochemistry of $^{15}NH_4^+$ allocation is better understood, more ^{15}N of the $^{15}NH_4Cl$ tracer gets allocated belowground and the distribution throughout the plant organs was more homogeneous than in case ^{15}N from $Ca(^{15}NO_3)_2$ application, the $^{15}NH_4Cl$ tracer might be the more appropriate for labeling roots (*e.g.*, for fine root turnover studies) or rhizodeposits (*e.g*., for rhizosphere microbial studies) in trees but further studies under 100% identical conditions, *i.e*., on same sized trees and same environmental conditions need to verify those result .

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