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REGULAR ARTICLE

# The natural abundance of $^{15}N$ in litter and soil profiles under six temperate tree species: N cycling depends on tree species traits and site fertility

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**Abstract**
*Aims* We investigated the influence of tree species on the natural $^{15}N$ abundance in forest stands under elevated ambient N deposition.
*Methods* We analysed $\delta^{15}N$ in litter, the forest floor and three mineral soil horizons along with ecosystem N status variables at six sites planted three decades ago with five European broadleaved tree species and Norway spruce.
*Results* Litter $\delta^{15}N$ and $^{15}N$ enrichment factor ($\delta^{15}N_{litter}$–$\delta^{15}N_{soil}$) were positively correlated with N status based on soil and litter N pools, nitrification, subsoil nitrate concentration and forest growth. Tree species differences were also significant for these N variables and for the litter $\delta^{15}N$ and enrichment factor. Litter from ash and sycamore maple with high N status and low fungal mycelia activity was enriched in $^{15}N$ (+0.9 delta units) relative to other tree species (European beech, pedunculate oak, lime and Norway spruce) even though the latter species leached more nitrate.
*Conclusions* The $\delta^{15}N$ pattern reflected tree species related traits affecting the N cycling as well as site fertility and former land use, and possibly differences

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in N leaching. The tree species $\delta^{15}N$ patterns reflected fractionation caused by uptake of N through mycorrhiza rather than due to nitrate leaching or other N transformation processes.

**Keywords** Tree species trial · $^{15}N$ natural abundance · Land use · Mycorrhiza · Nitrogen · Isotopic fractionation

## Introduction

Although broadleaf forest is the potential natural vegetation in much of western and central Europe, we have limited experimentally based and systematic knowledge about effects of N deposition on N cycling in different broadleaved tree species from common garden experiments. Tree species related differences in N uptake and cycling between indigenous tree species is well-known, e.g. regarding preferences for nitrate (Gebauer and Schulze 1997), but most of the knowledge and data on responses to elevated N input are related to coniferous forests (Dise et al. 2009). However, N loads to broadleaved forests are significant and may cause nitrate leaching (Gundersen et al. 2009; Kristensen et al. 2004). The question remains whether different autecological, inherent Ncycle features among broadleaves can be traced in an environment with high N deposition?

Processes in the Ncycle are complex and involve numerous reduction and oxidation steps. Studies of variations in the ratio between the stable heavy isotope $^{15}N$ and the lighter, most abundant isotope, $^{14}N$, in ecosystems, can give information about N transformations and fluxes within terrestrial ecosystems, and their N balance (Högberg 1997; Compton et al. 2007). The $^{15}N/^{14}N$ ratios in ecosystem components vary because of isotope fractionation during kinetic or equilibrium processes. In the latter case, the heavier isotope is more strongly bound in the strongest bond than is the lighter isotope. In kinetic processes, the substrate becomes more enriched in $^{15}N$ than the product, unless the reaction goes to completion, i.e. when all N in the substrate is transferred to the product in which case no fractionation occurs (Högberg 1997). Nitrogen isotope fractionation may occur during mineralization, nitrification followed by nitrate leaching, denitrification, plant N uptake, transfer of N from soil through mycorrhizal fungi to plants, and during redistribution of N within trees (Hobbie and Ouimette 2009). External inputs of $NH_y$ and $NO_x$ may also vary in their N isotope signature (Bauer et al. 2000).

Nitrogen cycling in broadleaved forests may vary for natural reasons as trees and soils interact. For example growth of ash (*Fraxinus excelsior* L.) requires, or responds positively to high base saturation and available N in the form of nitrate (Weber-Blaschke et al. 2008). Forest floor turnover is fast in, e.g. ash and decreases corresponding with increasing litter C–N ratio for other broadleaved tree species (Vesterdal et al. 2008). Biomass production increases along soil fertility gradients characterized by decreasing C–N ratios and increasing mineral soil nutrient pools (Callesen et al. 2006; Vesterdal et al. 2008). Higher biomass production with increasing soil fertility is also associated with a more open Ncycle characterized by larger N pools and fluxes, which can result in lack of complete N retention, i.e. leaching of N in the form of nitrate under high atmospheric N load conditions (Callesen et al. 1999; Callesen 2003).

These N cycling processes may influence the natural abundance of $^{15}N$ in the tree and soil compartments. Trees and other plants in forests subjected to high N loads or naturally rich in N become enriched in $^{15}N$ for two major reasons. One reason is that they take up sources of N that are enriched in $^{15}N$ because N leaving the system (by leaching after nitrification, or as gaseous N through denitrification) is depleted in $^{15}N$, leaving the remaining available N enriched in $^{15}N$ (Högberg and Johannisson 1993). The second reason is that the importance of mycorrhiza is diminished in N-rich ecosystems, which means that the N taken up will be richer in $^{15}N$ (Högberg et al. 2011). Under N-limited conditions mycorrhizal fungi retain $^{15}N$ more strongly than $^{14}N$, and consequently transfer $^{15}N$-depleted N to their tree hosts (Hobbie and Hobbie 2006; Högberg et al. 2011; Hobbie and Högberg 2012). Thus, in N-limited systems a typical soil profile starts with comparatively low $\delta^{15}N$ in the litter layer, but the $\delta^{15}N$ increases with depth. In N saturated systems, from which N losses are large and the role of mycorrhiza in N uptake is reduced, the change in $\delta^{15}N$ with soil depth is less (Högberg et al. 1996, 2011; Emmett et al. 1998; Hobbie and Ouimette 2009). Typically, the $\delta^{15}N$ increase is 10‰ with depth in soils under N-limited forests. However, in the case of pine planted on former agricultural land a difference of 13‰ between the surface soil and the sub-soil developed over 40 years (Billings and Richter 2006).

This study reports, to our knowledge for the first time, a direct comparison of natural $^{15}N$ abundance in several tree species grown under the same conditions at multiple sites. We used a unique series of common garden experiments to compare five mature broadleaved and a conifer tree species at six sites. The aim was to explore the N status patterns of tree species across sites. We tested if five different broadleaves and one conifer tree species develop different $\delta^{15}N$ patterns in litter and soil pools. Further, we tested whether this relates to their biomass production, the cycled quantities of N, losses of nitrate or the production of mycorrhiza. Based on established knowledge (e.g. Högberg et al. 2011), we hypothesized that (a) fractionation through mycorrhizal N uptake would cause depletion of $^{15}N$ in litter and enrichment of soil $^{15}N$ pools while (b) tree species with an open N cycle in an environment with high atmospheric N inputs and possibly nitrate leaching would have $^{15}N$-enriched N pools. We wanted to investigate which of these tree species related processes would dominate the $\delta^{15}N$ profiles in litter and soil across six sites under elevated ambient N load.

## Materials and methods

### Tree species, sites and general soil descriptions

Two common garden experiments were studied. In the first experiment, five broadleaved tree species and Norway spruce were planted in 1973 by *Forest & Landscape Denmark* at five sites in a common garden block design. The six tree species were beech (*Fagus sylvatica* L.), pedunculate oak (*Quercus robur* L.), ash (*Fraxinus excelsior* L.), sycamore maple (*Acer pseudoplatanus* L.), lime (*Tilia cordata* L.) and Norway spruce (*Picea abies* (L.) Karst.). Ash and sycamore maple generally form arbuscular mycorrhiza (AM), whereas ectomychorrizal (EcM) symbiosis prevails with the other tree species (Harley and Harley 1987). The canopies of oak, ash and sycamore maple transmit more light to the forest floor (4–6 % light) and allows more ground vegetation biomass throughout the vegetation period than do beech and lime (1–2 % light after flushing), whereas Norway spruce (2 % light) has no ground vegetation (Hoffmann 2007). Based on mychorrizal association, light climate and ground vegetation biomass, we identified two groups of species based on common traits—ash and sycamore maple versus beech, lime oak and spruce. There was no replication of tree species plots within each site, and each plot was about 0.25 ha. Thinning operations were carried out from 1990 and onwards every 3–4 years by the local forest management, but synchronized across sites. All sites were located on relatively nutrient rich sandy loam and loamy soils (Table 1). The second common garden experiment at site Kragelund was established in 1961 by *Silkeborg Plantation Association* and included the same six tree species but with smaller plots (0.05–0.1 ha). This slightly older experiment was selected in order to include a less fertile (i.e. more sandy) site. In total, 35 plots were investigated, since the establishment of the ash stand failed at one site (Vallø). Two sites (Mattrup and Vallø) were selected for more intensive nutrient cycling studies including monthly sampling of precipitation, throughfall, litterfall and soil water over 2 years (2004–2006) in 11 stands, as reported by Christiansen et al. (2010).

The six sites located in eastern Jutland and southeastern Denmark were quite similar in terms of climate with mean annual temperature (MAT) of 7.5–8.1 °C and mean annual precipitation (MAP) ranging from about 600 to 800 mm $year^{-1}$. The average climate during the period 1964–1998 was interpolated for each site by the Danish Institute of Agricultural Sciences from daily observations in a grid of nearby meteorological stations operated by the Danish Meteorological Institute (Finn Plauborg, Aarhus University, pers. comm.). The sites had relatively uniform soils developed on glacial till with sandy loam texture deposited during the Weichsel glacial period (Table 1). They were classified according to USDA Soil Taxonomy (Soil Survey Staff et al. 1998) as Alfisols with high subsoil base saturation, one Hapludult (site Kragelund, due to low subsoil base saturation) or Argiudoll (Wedellsborg, due to a 30-cm deep, dark A horizon and high base saturation). The sites were well-drained or moderately well-drained with signs of imperfect internal drainage. At the Wedellsborg site ditches facilitated drainage of the soil. Soil phosphorus (P) availability measured by three consecutive extractions in 0.1 M $HNO_3$ (unburned sample free of $CaCO_3$) ranged from 30–260 g $m^{-2}$ to 100 cm soil depth and generally indicated inherent P levels that are characteristic of fertile forest soils in Denmark (Callesen and Rasmussen 2004).

**Table 1** Sites characteristics including average climate 1964–1998 as mean annual temperature (MAT) and mean annual precipitation (MAP), and soil classification according to USDA Soil taxonomy (Soil Survey Staff 1998)

| Site | Lat/Lon | Location | Former land use | MAT, °C | MAP, mm | Soil Taxonomy | [a]Clay %, subsoil | [b]Subsoil pH ($CaCl_2$) | [c]Soil P g $m^{-2}$ (0–100 cm soil depth) |
|---|---|---|---|---|---|---|---|---|---|
| KRA | 56°10′N, 9°25′E | Kragelund | Agriculture | 7.5 | 703 | Arenic Hapludult | 8 % | 4.5 | 30 |
| MAT | 55°57′N, 9°38′E | Mattrup | Agriculture | 7.5 | 783 | Lamellic Hapludalf | 17 % | 4.6 | 47 |
| ODS | 55°50′N 11°42′E | Odsherred | Beech forest >200 years | 8.0 | 579 | Oxyaquic Hapludalf | 11 % | 4.2 | 261 |
| VAL | 55°25′N, 12°03′E | Vallø | Beech forest >200 years | 7.7 | 625 | Oxyaquic Hapludalf | 13 % | 5.5 | 175 |
| VIE | 55°01′N, 12°09′E | Viemose | Beech forest >200 years | 8.1 | 603 | Typic Hapludalf | 25 % | 4.6 | 39 |
| WED | 55°24′N, 9°52′E | Wedells-borg | Beech forest >200 years | 7.8 | 658 | Oxyaquic Argiudoll | 30 % | 6.9 | 94 |

[a] Average clay% of 50–100 cm soil depth

[b] Highest pH in 50–100 cm depth. All profiles $CaCO_3$ free to soil depth 100 cm

[c] P determined by consecutive extractions in 0.1 M $HNO_3$ for 2, 48 and 168 h on samples from genetic soil horizons to soil depth 100 cm followed by FIA analysis (Callesen and Rasmussen 2004)

## Soil and litter sampling for isotope determination

Within each stand, the forest floor was sampled in the fall of 2004 and spring of 2005 in 15 randomly placed 25×25 cm plots prior to mineral soil sampling in the same 25×25 cm plot. Mineral soils were sampled with a 50-cm soil auger (core diameter 5 cm), 15 subsamples per plot, that were separated into depth segments 0–5, 5–15 and 15–30 cm and pooled by segment. The forest floor samples were air-dried and sorted into foliar and non-foliar litter, and ground to powder. The pooled mineral soil samples were air-dried, crushed and sieved through a 2-mm sieve thereby removing gravel.

Fresh litter was sampled monthly at the two intensively monitored sites by litter traps ($N$=10 per plot). In the remaining sites, the 2004 litter fall was collected in similar litter traps ($N$=10 per plot) in the broadleaved stands only, thus excluding the Norway spruce stands at these sites. Litter was hand-sorted into non-foliar and foliar fractions and dried at 55 °C. The four cases of missing Norway spruce litter samples were replaced by analyses of fresh foliar samples (collected in Aug/Sep 2006, dried at 40 °C and ground to powder). The approximation of using fresh foliar rather than fresh litter samples is supported by the similarity in $\delta^{15}N$ of current year and all-year needles (Pardo et al. 2007) and is justified by the fact that the $\delta^{15}N$ values in the fresh foliar samples in general differ little (e.g. Näsholm 1994). Here, the two available pairs of foliar and litter samples deviated by only 0.57‰ (Mattrup) and 0.08‰ (Vallø) for Norway spruce justifying the approximation in the comparison with broadleaved species.

Each sample type was pooled within plot and ground in a ball mill. About 30–100 mg of mineral soil and ~5 mg of forest floor and litter material were sub-sampled from the milled 10 g sample for C, N and $\delta^{15}N$ determination on a mass spectrometer. Further details on forest floor, soil and litterfall may be found in Vesterdal et al. (2008).

## Isotope determinations

Leaf litter, forest floor and soil samples were analysed at the Swedish Agricultural University, Umeå on a Europa Scientific, ANCA-NT system (Solids/Liquids Preparation Module), coupled with an isotope mass spectrometer (IRMS; Europa Scientific, Crewe, UK, Europa 20–20). Standards were wheat flour and EDTA measured

with a precision of ±0.3 delta units (SD). The four fresh foliar samples were analysed at the University of Copenhagen on an IRMS system (Micromass-GV Instruments, Manchester, UK) coupled to a Eurovector CN elemental analyser (Milan, Italy).

The delta values were calculated as follows:

$$\delta^{15}N_{sample}(‰)=(R_{sample}/R_{atmos}-1)*1000, \quad (1)$$

where $R_{sample}$ is the $^{15}N/^{14}N$ ratio of the sample and $R_{atmos}$ is the $^{15}N/^{14}N$ ratio for atmospheric $N_2$.

## Net N mineralization and net nitrification

In each stand and site, four mineral soil samples from the 0–5-cm depth were taken with a soil auger with an internal diameter of 4 cm and pooled into one composite sample directly in the field. O-horizon material was removed to expose the mineral soil surface prior to sampling. In the laboratory, samples were hand-sorted and visible roots and stones of all sizes were removed.

Three replicates of 10 g fresh sample from each pooled stand sample were weighed into plastic tubes and frozen. Another three 10 g replicates of the same sample from each stand were weighed in plastic containers, incubated at 15 °C for 22 days and thereafter frozen. All samples were extracted with 20 mL 1 M KCl for determinations of $NO_3$–N and $NH_4$–N by flow injection analysis (FIA) using standards with KCl. The difference in total mineral N and $NO_3$–N concentration between non-incubated and incubated samples represented the net N mineralization ($N_{min}$) and net nitrification ($N_{nit}$), respectively. Gravimetric water content was determined by oven drying (55 °C) 20 to 40 g field moist soil samples to constant weight; a subsample was used to estimate the amount of soil organic matter (SOM) as loss on ignition at 550 °C. Rates of net mineralization and nitrification were normalised to the amount of SOM and expressed in the unit $\mu g N g^{-1}$ SOM $day^{-1}$. Net nitrification ratio was expressed as a percentage of N mineralization. $N_{min}$ and $N_{nit}$ were averaged for the three subsamples as one observation per plot, i.e. experimental unit.

## Fungal mycelia production

Five mesh bags with mesh size 50 μm filled with sand were buried c. 5 cm below the forest floor at each plot in May 2006. They were harvested 6 months later and thereafter analysed for fungal biomarker content. The PLFA 18:2ω6,9 was used here to estimate the production of fungal, mainly EcM, mycelia in the in-growth mesh bags (Yarwood et al. 2009; Högberg et al. 2011). In four plots, the bags could not be recovered and data are thus missing. For more detailed information about this technique, see Wallander et al. (2001) and Nilsson et al. (2007).

## N input–output estimates at stand level

Atmospheric N inputs were estimated from an empirical model (Gundersen 2008). The model uses vegetation type at site level (both conifer and broadleaved vegetation were considered), exposure to edge effects, and regional back ground deposition as inputs. For the two intensive sites Mattrup and Vallø input–output N budgets were based on flux measurements and hydrological modeling described in Christiansen et al. (2010).

A survey of soil nitrate was carried out to screen for presence of nitrate below the root zone. Presence of nitrate would indicate potential nitrate losses with seepage water, while absence of nitrate in subsoil would indicate low leaching, since Gundersen et al. (2009) found a strong correlation between subsoil nitrate concentrations and the N fluxes estimated by use of a calibrated hydrological model. One measurement would not be a general proof of the stand being prone to nitrate leaching. The measurement does, however, provide information in a cross-plot comparison at that given point in time. Pooled soil auger samples from 70 to 90 cm soil depth ($N$=15 subsamples per plot) were sampled from the plots in January 2003 (site Kragelund and Mattrup), and in April/May 2005 from the sites Kragelund and Mattrup again, and Odsherred, Viemose and Wedellsborg. The samples were kept cool during transport and stored in a freezer until analysis. Extraction was carried out on 20 g subsample as explained for the mineralization samples (20 g sieved wet soil extracted by 40 ml 1 M KCl), followed by FIA. Gravimetric water content was determined by weighing of the sample (~200 g) before and after drying to constant weight at 105 °C. The nitrate content was expressed on the basis of soil water content as mg extractable $NO_3$–N $dm^{-3}$ soil water.

## Biomass production

The total aboveground volume production was assessed based on tree diameter (all trees) and tree

height measurements (a stratum of trees) prior to thinning operations (B. Bilde-Jørgensen and V. Kvist-Johannsen, Forest & Landscape Denmark, pers. comm.). The amount of wood removed in thinning operations was also measured. The biometric data were used in a dynamic growth model estimating the total aboveground volume production (Johannsen 1999; Callesen et al. 2006). The estimated volume production included branches and twigs (total woody aboveground biomass) in the case of broadleaves, or excluded branches representing only stem biomass in case of Norway spruce. Based on total aboveground production and year of planting, the average biomass production per year (mean annual increment), $i_{v(1973–2004 \text{ or } 1961–2004)}$, was calculated on a dry matter basis using a basic density (g dry matter per $cm^{-3}$ fresh wood) of 0.58 $gcm^{-3}$ for beech, and 0.57 $gcm^{-3}$ for ash and oak, 0.50 $gcm^{-3}$ for sycamore maple, 0.42 $gcm^{-3}$ for lime and 0.37 $gcm^{-3}$ for Norway spruce (Danish Forest and Landscape Research Institute 1990).

### Calculations and statistical analyses

We calculated the isotopic enrichment factor, ε, which here describes the fresh leaf litter $\delta^{15}N$ minus the weighted average soil $\delta^{15}N$ (weighted by soil N% to take into account variations in N concentrations) in respective soil segment (0–5, 5–15 and 30–15 cm), Eq. 2. Also the enrichment factor, ε between fresh litter and the uppermost mineral soil horizon (0–5 cm) was calculated for each stand (experimental unit).

$$\varepsilon_{L-S(i,j)} = \delta^{15}N_{litter(i,j)} - \delta^{15}N_{soil(i,j)}, \quad (2)$$

$$\text{where } \delta^{15}N_{soil(i,j)}$$

$$\sum_{ij} \delta^{15}N_{(i,j,k)} * \left(\text{soil N\%}_{(i,j,k)} \Big/ \sum \text{soil N\%}_{(i,j)}\right),$$

where $i$ ~ site ($n$=6), $j$ ~ species ($n$=6) and $k$ ~ soil section ($n$=3).

The experiment was laid out as a block design without replicates within block (i.e. the site). The correlations between the measured variables were calculated as Spearman rank correlations ($N$=35 plots). The correlation matrix of N and production related variables were summarized to two dimensions by principal component analysis (PCA) after standardization. To execute the multivariate analysis, 3 % of the values was gap filled using site or species means (fungal mycelia production on four plots and litterfall $\delta^{15}N$ on four spruce plots). The $^{15}N$ variables were not included in the PCA and could thus later be analysed for their response to the principal components.

The effect of tree species and site on ecosystem characteristics (Y) were tested by two-way MANOVA on the variables used in the PCA with tree species and site as fixed effects, $Y_{1ij},..,Y_{nij}$ or $Y_{ij}=\alpha(\text{tree species})_i + \beta(\text{site})_j + \varepsilon_{Ij}$, where $\varepsilon_i=1,\ldots,35$ is a Gauss distributed $(0, \sigma^2_{ij})$ residual error. Univariate ANOVAs on each variable and the two first principal components from the PCA on N variables were performed with the same model. Species or site differences were assessed using Tukey tests adjusted for multiple comparisons ($P<0.05$). Contrasts between groups of sites according to former land use (agriculture versus forest) within the design were tested and estimated. Significant contrasts in $^{15}N$ variables were analysed for covariance with components from the PCA. For three sites with variable N leaching, an ANOVA compared N leaching (subsoil solution concentrations >3 mg $NO_3$–N $dm^{-3}$) versus N retaining (<3 mg $NO_3$–N $dm^{-3}$) plots. The concentration of 3 mg $NO_3$–N $dm^{-3}$ was used as a conservative estimate for elevated N leaching (Gundersen 2008). All analyses were carried out using the statistical software package SAS v12 (r) using the glm, princomp and univariate procedures. The distributions of residuals were checked by visual inspection of residual plots and by Shapiro–Wilk tests for normality.

## Results

### Differences in N cycling among sites

The empirical N input model indicated that all sites received elevated N inputs, Table 2. The throughfall N inputs to the sites were 13–19 kgN $ha^{-1}year^{-1}$ for the broadleaf forest stands and 18–26 kgN $ha^{-1}year^{-1}$ for Norway spruce forest stands. Nitrate in deep percolating soil water during winter or early spring was detected at all sites, but not in all stands within each site (Table 2). Some sites (MAT, VAL, ODS and WED) had 4 or more plots per site with elevated soil nitrate (up to 42 mg $NO_3^-$–N $dm^{-3}$), whereas the KRA site had concentrations barely over 1 mg $NO_3^-$–N $dm^{-3}$. Detectable net nitrification occurred in soils from all tree species at all sites, except for Norway spruce stands at the sites KRA and VAL. Litter turnover was rather fast at all sites with $K_{CT}$ values of 30–60 %$year^{-1}$, Table 2.

**Table 2** Nitrogen inputs, outputs and internal nitrogen cycling at site level for six sites planted with five different broadleaved tree species and Norway spruce

| Site | [a]N-deposition in throughfall Broadleaf/N.spr. kg ha$^{-1}$ year$^{-1}$ | [c]Nitrate in soil water N$^{o}$ plots>det.limit /max concentration $NO_3^-$–N mg dm$^{-3}$ | Mineral soil, tot N pool 0–30 cm t, ha$^{-1}$ | [d]Litter turnover $K_{CT}$,% year$^{-1}$ | Net N mineralisation ($N_{min}$), µg N g SOM$^{-1}$ d$^{-1}$ | Net nitrification % of $N_{min}$ | Biomass production $i_{v(1973(1961)\ to\ 2004)}$ t d.m. ha$^{-1}$ year$^{-1}$ | $\varepsilon_{L\text{-}S}$ ‰ |
|---|---|---|---|---|---|---|---|---|
| KRA | 19/26 | 4/0.8 | 2.3 | 40 | 15 | 27 | 2.9 | −8.1 |
| MAT | [b]8–13/30 | 6/42.5 | 5.2 | 30 | 12 | 68 | 5.7 | −7.0 |
| ODS | 13/17 | 6/29.0 | 6.0 | 49 | 14 | 70 | 6.5 | −4.3 |
| VAL | [b]10–18/25 | 5/16.0 | 3.7 | 33 | 6 | 31 | 5.4 | −5.4 |
| VIE | 13/18 | 2/9.2 | 4.8 | 35 | 11 | 66 | 6.8 | −4.4 |
| WED | 17/24 | 4/27.0 | 6.9 | 62 | 9 | 70 | 6.6 | −4.9 |
| *P* value | na | 0.02* | <0.001*** | <0.001*** | 0.17$^{NS}$ | 0.03* | <0.0001 | <0.0001 |

*$P$<0.05; ** $P$<0.01; ***$P$<0.001***; $P$ value and level of significance from $F$ test in ANOVA

[a] Estimates based on an empirical model using forest type (coniferous/broadleaved), edge exposure, background bulk deposition and normal precipitation as inputs (Gundersen 2008)

[b] Measured ranges of N input–output across the stands at the sites Mattrup and Vallø based on Christiansen et al. (2010)

[c] Nitrate in soil water in 70–90 cm soil samples (pooled sample of 15 augured samples), extracted with 1 M KCl or pooled monthly samples of 5 tension lysimeters (MAT and VAL 2004–2006). The values indicate the number of plots with concentrations above detection limit (here ~0.5 mg N dm$^{-3}$ for 1 M KCl extracts due to 10× dilution and 0.05 mg N dm$^{-3}$ for tension lysimeter soil water. Maximum concentration measured at the site across 6 plots

[d] $K_{CT}$ is fractional annual loss of total forest floor litter carbon. Litter C/(litter C + forest floor C). Vesterdal et al. (2008)

The sites were fairly similar in average above-ground total volume production, $V_{tot}$. Between the years 1973 and spring 2004 $V_{tot}$ ranged from 338 to 410 $m^3 ha^{-1}$, except at the older site Kragelund, which on average produced 253 $m^3 ha^{-1}$ between 1961 and spring 2004 across species (data not shown).

The measured N cycling variables and the biomass production were interrelated (Table 3) and 38 % of the variation across the 35 plots was captured in the first principal component, PC1, and 21 % in the second component PC2 (Fig. 1a). The lower components all had eigen values <1. With the highest loadings from the mineral soil 0–30 cmN pool (Total soil N, being almost identical to PC1), net nitrification (%$NO_3^-$), subsoil $NO_3^-$ and litter N flux, PC1 may be interpreted as a 'N status' axis, where biomass production also increased with soil N pool and $NO_3^-$ availability (Fig. 1a). The interpretation of PC2 is less clear, but could be related to cycling rates with loadings from N mineralization (−0.78), nitrification (−0.40) and fungal mycelia (0.47) activity (measured as nmol of PLFA 18:2ω6,9 per dry weight in ingrowth meshbags) (Fig. 1a). The '−'or '+' sign indicates that high $N_{min}$ and %$NO_3^-$ values yields a low PC2 score, while a high level of fungal mycelia yields a high PC2 score. Sites clearly differed in 'N status'—PC1, but not in 'cycling rates'—PC2 (Fig. 1b; Table 4). The lowest 'N status' was found at KRA which was also reflected by the relatively lower inherent site fertility judged by the sandy soil texture (8 % clay, Table 1). The highest N status was found at the fine textured site WED (Fig. 1b), which also had the highest litter turnover rate (Table 2).

Two sites with former agricultural land use (KRA and MAT) had significantly lower *average* N status (PC1) than the sites with a long forest history (VAL, ODS, VIE, WED; $P<0.0001$). The two groups also differed on PC2 ($P=0.012$). The dominant effects were related to lower inherent soil fertility with a more sandy texture, yielding lower 'N status' especially at the KRA site.

## Differences in N cycling developed among tree species

The suite of measured variables in each plot could be characterized as belonging to the same population of tree species ($P=0.0002$) and site ($P=0.0007$), respectively in MANOVA. Whereas the multivariate MANOVA test on tree species was significant, the ANOVA on PC1 ($P=0.18$) was not significant, which implies that tree species effects were not limited to the 'N status' variables alone (Table 4, Fig. 1c). Taking a univariate view into each N variable, total soil N, net nitrification ($NO_3^-$ pct), subsoil $NO_3^-$ and litterfall N as well as biomass production were significantly different among tree species, but different species pairs were involved (Table 4). Total soil N was significantly higher under ash than under beech and spruce. Net nitrification was higher under sycamore maple than under spruce. In contrast, subsoil $NO_3^-$ was highest under spruce and significantly higher than under sycamore maple and lime, which may in part be related to the higher N input in spruce (Table 2). Litterfall N was significantly higher in sycamore maple than in beech, and beech had significantly higher biomass production than oak (Table 4).

N mineralization and fungal mycelia production were not related to tree species (Table 4); however, the ANOVA on PC2 indicated an effect of tree species on 'cycling rates' and spruce differed from sycamore maple on PC2 (Fig. 1c, Table 4). This will be further investigated in the following (Fig. 6).

## $^{15}N$ natural abundance in litter, forest floor and soil

Fresh litter and O horizons were mostly depleted in $^{15}N$ with a $\delta^{15}N$ ranging from −6‰ to 0‰ (Fig. 2). In the mineral soil, $\delta^{15}N$ was generally positive and increased up to +3‰ to +7‰ at 15–30 cm soil depth (Fig. 2). In general, the sites had positive $\delta^{15}N$ in the uppermost mineral soil layer (maximum value of $\delta^{15}N$ was +4‰), only Vallø had negative $\delta^{15}N$ (−1.4). There were highly significant differences among sites in the natural abundance of $^{15}N$ in both litter and soil pools ($P<0.0001$; Fig. 2, Table 4) as well as in the calculated enrichment factors ($\varepsilon_{L\text{-}S}$, $\varepsilon_{L\text{-}S\ 0\text{-}5cm}$) i.e. the difference between plant and soil $\delta^{15}N$ ($P<0.0001$, Table 4).

Despite the major significant site differences, fresh litter $\delta^{15}N$ differed between species ($P<0.006$), whereas no effects attributable to tree species were observed in the O horizon and mineral soil layers (Fig. 3). Litter from ash and sycamore maple both had higher $\delta^{15}N$ than Norway spruce and ash higher than oak (Table 4). The weighted average $\delta^{15}N$ of 0–30 cm soil depth and $\varepsilon_{L\text{-}S}$ had $P$ values=0.05 indicating differences that were, however, not significant for any species pair in the two-way ANOVA (Table 4).

Across the 35 plots, all measures of $\delta^{15}N$ natural abundance and enrichment factors are interrelated (Table 3). In the following, we thus mainly focus

**Table 3** Upper part: $^{15}N$ variables and their correlation with N cycle variables

| *r* (sample correlation) | $\delta^{15}N$ litter | $\delta^{15}N_{0–5cm}$ | $\delta^{15}N_{0–30cm}$ | $\varepsilon_{L\text{-}S\ 0–5cm}$ | $\varepsilon_{L\text{-}S\ 0–30cm}$ | Fung. mycelia, PLFA | $N_{min}$ | $\%NO_3^-$ | Subsoil $NO_3^-$ | Soil $N_{0–30\ cm}$ | Litter fall N[a] | Biomass prod |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $\delta^{15}N$ litter | – | 0.81*** | 0.73 | 0.52*** | 0.67*** | –0.32 (0.08) | 0.09 | 0.71 *** | 0.40* | 0.87 *** | 0.40* | 0.61*** |
| $\delta^{15}N_{0–5cm}$ | | – | 0.98*** | –0.08 | 0.13 | –0.46** | 0.09 | 0.66*** | 0.39* | 0.73*** | 0.26 | 0.47** |
| $\delta^{15}N_{0–30cm}$ | | | – | –0.19 | –0.01 | –0.42* | 0.14 | 0.60*** | 0.34* | 0.63*** | 0.21 | 0.35* |
| $\varepsilon_{L\text{-}S\ 0–5cm}$ | | | | – | 0.96*** | 0.12 | 0.02 | 0.25* | 0.11 | 0.42*** | 0.30 | 0.35* |
| $\varepsilon_{L\text{-}S}$ | | | | | – | 0.0 | –0.02 | 0.40* | 0.22 | 0.60*** | 0.38* | 0.51** |
| PC1[b] | 0.86*** | 0.75*** | 0.65*** | 0.36* | 0.56*** | –0.45*** | 0.02 | 0.76*** | 0.54** | 0.88*** | 0.51** | 0.74** |
| Fungal mycelia, PLFA | | | | | | – | –0.29 | –0.58*** | –0.14 | –0.27 | –0.05 | –0.19 |
| $N_{min}$ | | | | | | | – | 0.21 | –0.08 | –0.09 | 0.06 | –0.20 |
| $\%NO_3^-$ | | | | | | | – | 0.12 | 0.62*** | 0.31 (0.10) | 0.41* | |
| Subsoil $NO_3^-$ | | | | | | | | – | 0.53** | 0.10 | 0.30 | |
| Soil $N_{0-30\ cm}$ | | | | | | | | | | – | 0.40* | 0.58*** |
| Litterfall N, $kgNha^{-1}$ | | | | | | | | | | | – | 0.39* |
| Biomass prod., t d.m. $ha^{-1}year^{-1}$ | | | | | | | | | | | | – |

Lower part: Correlation between PC1 ('N status') and variables related with the N cycle. Spearman sample correlation, $N$=35. $\varepsilon_{L\text{-}S}$ (weighted average of 0–30 cm $\delta^{15}N$), $\varepsilon_{L\text{-}S\ 0–5cm}$ uses the $\delta^{15}N$ of the 0–5 cm mineral soil

*$P$<0.05; **$P$<0.01; ***$P$<0.001; the asterisks indicate significance of the correlations with different $P$ levels

[a] $N$=31

[b] First principal component from PCA on N cycling variables in the lower part of the table

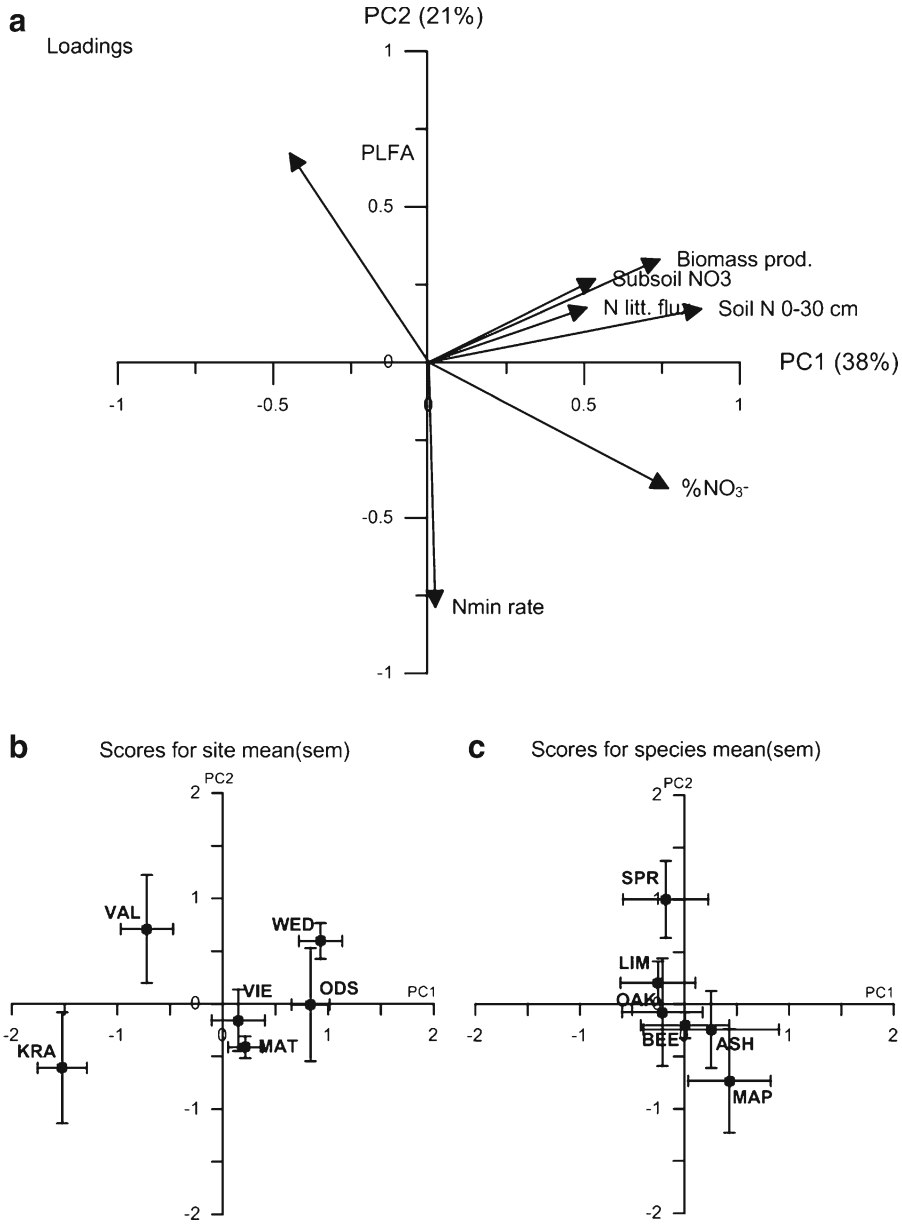


**Fig. 1** Results of the principal component analysis (PCA) using seven nitrogen and biomass production variables measured on 35 plots: **a** PC2 versus PC1 loadings for the included variables; **b** estimates of site scores and **c** tree species scores of PC1 and PC2. *Points* and *bars* indicate mean and SEM (std/sqr(N))

on the responses in $\delta^{15}N$ in litter and weighted soil $\delta^{15}N$ as well as the difference between them, $\varepsilon_{L\text{-}S}$, to simplify the analysis. Across all species and sites these three $^{15}N$ variables were positively correlated to PC1 and thus also to total soil N, biomass production and net nitrification, but not to PC2 (Table 3).

## Effects of former land use on $\delta^{15}N$ natural abundance

Land use contrasts were significant for all $^{15}N$ variables, except for $\delta^{15}N_{0\text{–}5\ cm}$ (Table 4). Fresh litters were more depleted on former agriculture than forest land use (Fig. 4), whereas soils were more enriched in $\delta^{15}N$. Therefore, the enrichment factor $\varepsilon_{L\text{-}S}$ was wide at the two former agricultural sites (−7.5‰) but more narrow at previously forested sites (−4.7‰), and the difference between the land uses −2.8‰ was significant ($P<0.0001$), Table 4, Fig. 4. The land use differences should be interpreted bearing in mind the concurrent lower average N status at the former agricultural sites (Fig. 4). The KRA site in particular contributed to the low average N status (Fig. 1b).

**Table 4** Test results from two-way univariate analysis of variance and multivariate ANOVA (significant *P* values in bold)

| Two-way ANOVA (SSIII, parallel *F* test) | | | Main factor | | Pairwise species differences | Contrast estimates | |
|---|---|---|---|---|---|---|---|
| | | | Site (*N*=6) | Species (*N*=6) | Tukey test (*P*<0.05) | Land use, Agr–For | |
| Variable | Unit | N | *P* | *P* | | Diff (SE) | *P* |
| PC1* | | 35 | **<0.0001** | 0.18 | | −1.0 (0.2) | **<0.0001** |
| Soil N 0–30 cm | $t\,ha^{-1}$ | 35 | **<0.0001** | **0.02** | ash > beech and spruce | −1.64 (0.02) | **<0.0001** |
| $\%NO_3^-$ | Ratio | 35 | **0.005** | **0.03** | maple > spruce | −0.12 (0.08) | 0.16 |
| Subsoil $NO_3^-$ | $g\,dm^{-3}$ | 35 | **<0.0004** | **0.02** | spruce > lime, syc. maple | −4.8 (1.9) | **0.02** |
| N litter flux | $kg\,N\,ha^{-1}\,year^{-1}$ | 31 | 0.12 | **0.02** | syc. maple > beech | −10.6 (5.5) | 0.07 |
| Biomass prod. | $t\,ha^{-1}$ | 35 | **<0.0001** | **0.03** | beech > oak | −1.9 (0.4) | **<0.0001** |
| PC2* | | 35 | 0.07 | **0.03** | spruce > syc. maple | −0.8 (0.3) | **0.012** |
| Nmin | $\mu g\,N\,g^{-1}\,d^{-1}$ | 35 | 0.14 | 0.20 | | 3.5 (2.0) | 0.09 |
| Fung. myc. (PLFA) | | 31 | 0.44 | 0.26 | | – | 0.89 |
| $\delta^{15}N_{\ litter}$ | ‰ | 35 | **<0.0001** | **0.006** | ash > oak, spruce; syc. maple > spruce | −2.1 (0.3) | **<0.0001** |
| $\delta^{15}N_{\ 0–5cm}$ | ‰ | 35 | **<0.0001** | 0.16 | | 0.21 (0.24) | 0.39 |
| Soil $\delta^{15}N$, weighted $_{0–30\ cm}$ | ‰ | 35 | **<0.0001** | **0.05** | | 0.8 (0.2) | **0.0009** |
| $\varepsilon_{L\text{-}S}$ | ‰ | 35 | **<0.0001** | **0.05** | | −2.8 (0.3) | **<0.0001** |
| $\varepsilon_{L\text{-}S\ 0–5\ cm}$ | ‰ | 35 | **<0.0001** | 0.09 | | −2.3 (0.3) | **<0.0001** |
| MANOVA* | | | | | | | |
| Char. Root 1 | | | 83 % | 67 % | | | |
| Pillai's Trace, *F* | | | 2.26 | 2.48 | | | |
| *P* value | | | **0.0007** | **0.0002** | | | |

Estimates of land use differences between groups of sites (KRA and MAT versus VAL, ODS, VIE and WED) with standard error in brackets

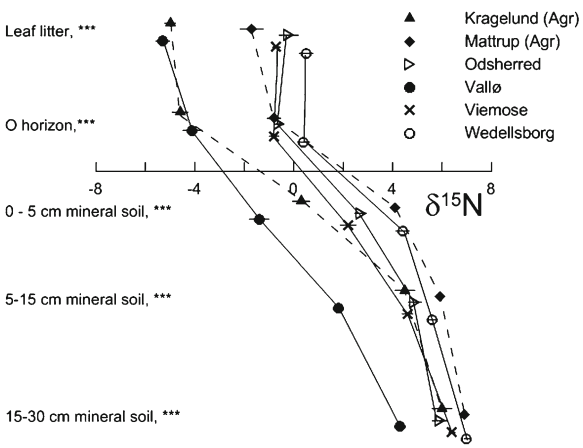


**Fig. 2** Differences in $\delta^{15}N$ soil profiles among six Danish sites. Sites Kragelund and Mattrup were under agriculture up to 1961 and 1973, respectively; the other sites have been forested for >200 years. The *asterisks* indicate the level of significance (***$P<0.001$) for the effect of site in one-way ANOVA carried out for each soil section. *Points* and *bars* indicate mean and standard deviation

## Effects of tree species on $\delta^{15}N$ natural abundance

Tree species had a significant effect on fresh litter $\delta^{15}N$ (Fig. 3) and the litter $\delta^{15}N$ is tightly related to PC1 (Fig. 5). Thus the tree species effects arise from the underlying significant changes in mineral soil N pool, presence of nitrate, litterfall N and biomass production among tree species (Table 4). Aboveground biomass production in beech was 6.6 t d.m. $ha^{-1}\,year^{-1}$ significantly higher than ash 4.9 t d.m. $ha^{-1}\,year^{-1}$ ($P$= 0.05) with remaining species in between. The significant differences between species pairs were between the two ash and sycamore maple and spruce (Table 4, Fig. 5). When N status (PC1) was included as covariate it was significant for the $\delta^{15}N$ 0–5 cm soil pool, but not for the fresh litter $\delta^{15}N$ pool. The inclusion of PC1 (N status) as a covariate diminished the variance captured by tree species. Thus, the N status variable PC1 could successfully replace the tree species factor for $\delta^{15}N$ 0–5 cm showing that tree species differences

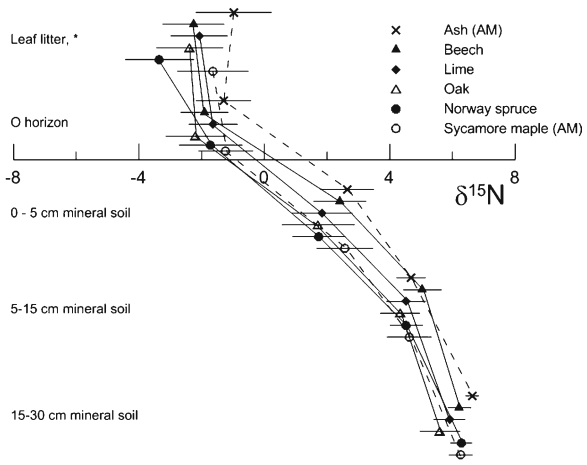


**Fig. 3** The $\delta^{15}N$ soil profiles under six tree species. The *asterisk* indicates the effect of tree species for the different horizons in a mixed ANOVA with tree species as fixed effect and site as random factor. Only in the leaf litter horizon significant differences were seen (*$P<0.05$). Note that the mean for fresh litter in Norway spruce was −4.4‰ ($N=2$), inclusion of the four fresh foliar samples values decreased the mean to −3.3‰. *Points* and *bars* indicate mean and standard deviation

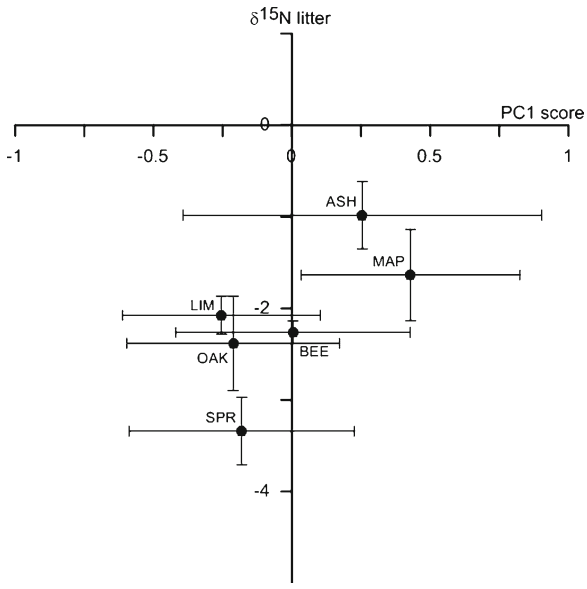


**Fig. 5** Litter $\delta^{15}N$ versus N status (PC1 is first principal component from PCA on N variables) for each tree species. *Points* and *bars* indicate mean and SEM

in soil indeed originate in tree species dependent N cycling traits.

An inverse relationship between fungal mycelia production (PLFA) and net nitrification ($\%NO_3^-$) was found, Fig. 6 ($P<0.0001$, adj $R^2$ 0.32). Slopes, but not intercepts ($P=0.054$), for each species were significantly different in a covariate model of species and $\%NO_3^-$ and their interaction (adj $R^2$ 0.52). The general F-test for model reduction proved the simple regression model without tree species effects, PLFA = $\alpha + \beta(\%NO_3^-)$ to be adequate for this dataset, when compared with models including species or tree species group (ash and sycamore versus beech, lime oak and spruce).

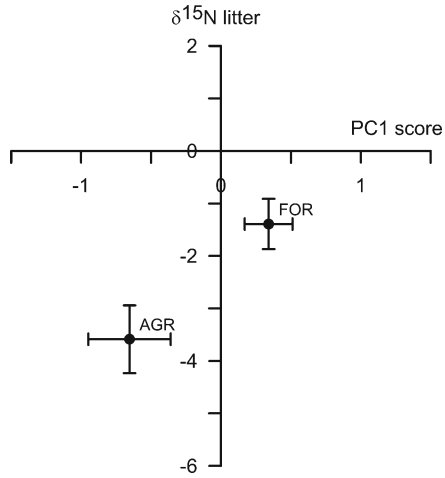


**Fig. 4** Litter $\delta^{15}N$ versus N status (PC1 is first principal component from PCA on N variables) for former land use, agriculture or forest. *Points* and *bars* indicate mean and SEM

## Effects of nitrate leaching on natural $^{15}N$ abundance in litter

Strong site effects on deep soil $NO_3^-$–N led us to carry out a within-site relative comparison of stands with negligible and stands with significant soil nitrate concentrations at 70–90 cm soil depth. Doing so, average litter $\delta^{15}N$ was *more* depleted (−0.9 delta units) in nitrate leaching stands than in stands retaining nitrate ($P=0.04$) at three sites with high N status (VIE, MAT, WED, Fig. 1b). The effect was, however, strongly confounded with the effect of tree species group ($^{15}N$ depletion in the species beech, lime, oak and spruce), and thus smaller and not significant (−0.4 delta units, $P=0.35$), when tree species group was included in the model. In addition, the effect of tree species group (−0.8 delta units, $P=0.01$) was not sensitive to first including N leaching in the ANOVA model. At site Vallø (low PC1 value, Fig. 1b), all stands had, as expected, rather $^{15}N$ depleted litter and soil compared to sites and stands with higher N status (Fig. 2).

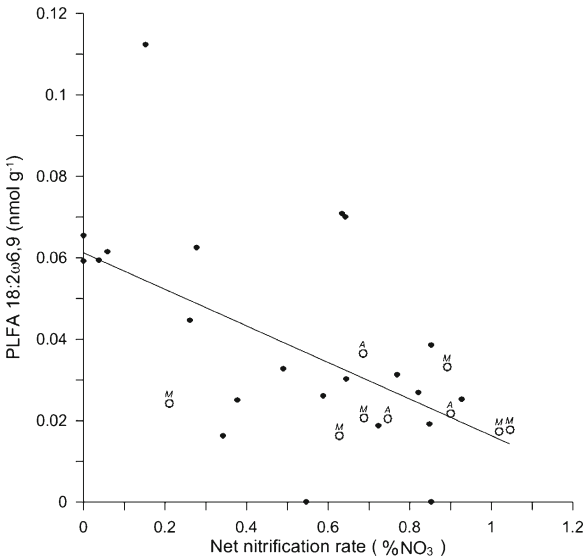


**Fig. 6** Fungal mycelia production (PLFA) versus net nitrification rate in lab incubations (net nitrification/net N mineralization). Parameter estimates (standard errors in *brackets*): PLFA= 0.0612(0.007)–0.045(0.01)×%$NO_3^-$. The tree species are marked as: *M* sycamore maple, *A* ash

## Discussion

### The role of site including former land use and N status

The natural abundances of $^{15}N$ in different pools in forest ecosystems reflect on-going processes as well as a legacy of processes in the past. For example, the isotopic signature of plant foliage reflects current processes acting on and producing the small and dynamic pools of available N, as well as fractionations related to, e.g. the physiology of mycorrhizal symbiosis (Högberg 1997; Hobbie and Hobbie 2006; Högberg et al. 2011). The $\delta^{15}N$ of the large and slowly cycling pool of N in humus to a large extent reflects processes, which occurred a long time ago. An important question is how fast observable differences in $\delta^{15}N$ can occur? An extreme change was reported by Billings and Richter (2006), who found that the enrichment factor (in our definition, Eq. 2) in the soil profile was widened by 13 ‰ from only −2‰ to −15‰ during a period of 40 years since former cropland had been planted with pine trees. After slightly shorter time, we consistently observed wide enrichment factors (range −11‰ to −3‰) also at the former cropland sites (MAT and KRA). These sites, like the site studied by Billings and Richter (2006), probably had a very narrow enrichment factor when the experiment was initiated. At the low N status KRA site, the litter became more depleted than at MAT and thus responded more strongly to afforestation, Fig. 1b. The additional 12 years of N cycling at the older KRA site could also contribute to this, Table 1.

Possible effects of tree species on abundance of $^{15}N$ have had time to evolve only since the establishment of the experiment in 1973 (or 1961 for site Kragelund). However, these two former cropland sites indeed differed in $\delta^{15}N$ profiles from those that had been forested for a longer time (Fig. 4), and the former cropland sites also had the widest enrichment factors, $\varepsilon_{L\text{-}S}$ ($P<0.0001$, Table 4). The depletion in the upper layer was most

**Table 5** Nitrogen input–output budget at Mattrup and Vallø 2004–2006 based on water sampling

| Site | Species | N input (avg 04–06) kgNha$^{-1}$year$^{-1}$ | N output (avg 04–06) kgNha$^{-1}$year$^{-1}$ | N balance kgNha$^{-1}$year$^{-1}$ | Litter $\delta^{15}N$, ‰ | Soil $\delta^{15}N$ 0–30 cm‰ | $\varepsilon_{L\text{-}S}$ |
|---|---|---|---|---|---|---|---|
| Mattrup | Ash | 9 | 2 | 7 | −0.7 | 5.2 | −5.9 |
| Mattrup | Beech | 9 | 39 | −30 | −2.5 | 5.0 | −7.5 |
| Vallø | Beech | 12 | 11 | 1 | −4.6 | 1.0 | −5.6 |
| Mattrup | Lime | 13 | 26 | −13 | −1.1 | 5.7 | −6.8 |
| Vallø | Lime | 18 | 2 | 16 | −4.9 | 0.3 | −5.3 |
| Mattrup | Oak | 8 | 31 | −23 | −1.2 | 5.4 | −6.6 |
| Vallø | Oak | 10 | 1 | 9 | −6.2 | −0.9 | −5.2 |
| Mattrup | N. spruce | 30 | 29 | 1 | −3.8 | 5.3 | −9.0 |
| Vallø | N. spruce | 25 | 0.4 | 25 | −5.0 | −0.2 | −4.8 |
| Mattrup | Sycamore maple | 12 | 3 | 8 | −1.1 | 5.4 | −6.5 |
| Vallø | Sycamore maple | 13 | 0.3 | 12 | −5.9 | 0.4 | −6.3 |

pronounced in the Norway spruce and beech plots (data not shown). The enrichment, $\varepsilon_{L\text{-}S0\text{–}5\ cm}$, of the cropland soils by 2.3‰ relative to the forested soils may be due to fertilizer additions with different signature and losses of (depleted) N during the agriculture period. This would also be reflected after the homogenizing effect of tillage on the distribution of N and N isotopes in the Ap horizon.

Across all sites, narrow enrichment factors were associated with high biomass production and with high soil N accretion (Table 3). The widest enrichment factors were associated with the lowest biomass production and lowest total soil N pools. N limitation may thus have contributed to the development of this pattern of depletion. These observations suggest that the establishment of N limited forest ecosystems with tight N cycling drives the development of increasing $\delta^{15}N$ with increasing soil depth through continued addition of $^{15}N$ depleted litter material to the soil surface, most strongly expressed at the KRA site that had relatively low production, a low soil N status (Table 2) and high litter C–N ratios (Vesterdal et al. 2008).

Differences in $\delta^{15}N$ among sites could also relate to small differences in site characteristics such as climate, soil clay content, soil phosphorus release capability, N deposition and leaching (Table 1, 2 and 3), which, in addition to early successional $N_2$ fixation (Perakis et al. 2011), may influence N transformation processes, e.g. N uptake by mycorrhizal plants, mineralization and nitrification in the mineral soil, and hence the distribution of N isotopes. Unfortunately, unlike Billings and Richter (2006) we do not have soil samples from the beginning of the experiment, and cannot, therefore, fully evaluate the role of differences in edaphic conditions. The sites were selected to represent rather uniform and suitable growth environments for the broadleaved tree species, but nevertheless the $^{15}N$ analyses, biomass production and soil characteristics revealed discernible site differences in N cycling and N leaching, as expressed by the N status principal component, that could contribute to observed fractionation of natural $^{15}N$ in litter and soil pools.

## The role of species

We found effects of the different tree species on the $^{15}N$ isotope ratios in the litter pools and on enrichment factors between litter and soil after soil development with those species over the last ~33 years. Since soil samples from the initiation of the experiment were unavailable, only relative differences between species can be evaluated, and the question remains whether they will become even more pronounced over time.

Only the $\delta^{15}N$ in litter from ash and sycamore maple differed significantly from that of Norway spruce and oak (in case of ash). This coincides with the significantly higher C–N ratios of the litter from spruce (37) than that of ash (25) and sycamore maple (27) (Vesterdal et al. 2008), and with the higher litter N cycling (and tendency for higher net nitrification, $P = 0.09$) of ash and sycamore maple in contrast to Norway spruce. The canopy light transmission allowing for ground vegetation in ash and sycamore in comparison with spruce (Hoffmann 2007) further increases the litter N cycling in these stands.

## The role of mycorrhiza

Our study enables a unique evaluation of the importance of fungal mycelia on the $\delta^{15}N$ of litter and soil in a tree species experiment with limited environmental variation (hypothesis 1). Here, we found evidence for higher $\delta^{15}N$ in litter from ash and sycamore maple than from Norway spruce (and oak), Fig. 5. That was not an effect of the N status (PC1) observed among those species (revealed by the non-significant ANOVA of PC1, Table 4), but rather indicates other differing traits of the species. Recently, Nilsson et al. (2012) observed a negative relationship between subsoil nitrate concentration and fungal mycelia production in a spruce dataset. The relation between fungal mycelia production and net nitrification ($\%NO_3^-$) may be a distinct trait for each species, or tree species group, Fig. 6. However, the simple regression model does not reveal the multiple tree species traits that may contribute to the relationship, e.g. the ash and sycamore maple stands with AM mycorrhiza and 4–6 % light transmission that permits lush ground vegetation had significantly lower PLFA values and higher $\%NO_3^-$ values than the tree species group beech, lime oak and spruce. We conclude that an inverse relationship between fungal mycelia production and net nitrification related to tree species traits involved in N cycling exists. Weber-Blaschke et al. (2008) noted that the growth of ash and sycamore responds positively to nitrate availability and high soil base status. Our data showed that ash and sycamore do not produce mycelia that may contribute to tree N uptake at low net nitrification rates.

In this case, a baseline level of fungal mycelia production from, e.g. ground vegetation may account for the measured 2–3 nmol $g^{-1}$, since a low fungal mycelia production was also found in the ash and sycamore maple stands.

Is there evidence of changes in $\delta^{15}N$ due to nitrate leaching?

The species specific $^{15}N$ profiles in litter and soil across sites in this study may be an effect of different magnitude and mode of N cycling. Abundant N supply that typically promotes less tight nitrogen cycling and risk of nitrate leaching losses (Cole and Rapp 1981) is accompanied by soil $^{15}N$ enrichment and narrow litter-soil enrichment factors (Garten and van Miegroet 1994). We measured a number of pools and fluxes, but did not have quantitative N data on, e.g. forest ground vegetation, which may also contribute to ecosystem N dynamics by taking up nitrate during spring and summer (Ellenberg 1996). Such ground vegetation N dynamics may explain that despite high N status in, e.g. ash and sycamore maple, nitrate leaching did not occur in all stands. But, in contrast to our expectations based on, e.g. Högberg et al. (1996), N leaching stands had $^{15}N$ depleted litter relative to non-leaching stands within the same site. With the common garden design, we could show that this effect was confounded with the more significant effect of tree species group, indicating that N leaching was smaller in ash and sycamore maple. The $^{15}N$ enrichment in these species may, however, be caused by a range of N loss processes including previous N leaching that we did not detect in this investigation.

The potential importance of the nitrogen input–output balance was studied more closely at Mattrup (MAT) and Vallø (VAL), Table 5, based on data from Christiansen et al. (2010). These two sites had contrasting previous land uses with agriculture at MAT and forest for more than 200 years at VAL. The N deposition in throughfall in broadleaved forest was in the range 10–18 kgN $ha^{-1}year^{-1}$ at VAL and 8–13 kgN $ha^{-1}year^{-1}$ at MAT (Table 5). Net nitrification was 68 % at MAT and 31 % of net N mineralization at VAL (Table 2). The estimated N losses by leaching from broadleaved stands were 0.3–11 kg $NO_3^-$–N at VAL and 3–39 kg $NO_3^-$–N at MAT, thus reflecting a large within-site variation between the broadleaved species. The Norway spruce stands received inputs of 25 and 30 kgN $ha^{-1}year^{-1}$ at VAL and MAT, respectively, with a high leaching at MAT, but little N leaching at VAL (Table 5). Measured N leaching and soil $^{15}N$ enrichment at these sites did not show a clear pattern. However, the enrichment factor for sycamore maple was similar for the two sites, but $\varepsilon_{L\text{-}S}$ was much wider at MAT for the spruce and beech species that were also leaching nitrate.

Any possible effect of enhanced nitrate leaching causing $^{15}N$ enrichment in soil and litter seems to be masked by the opposite fractionating (depleting) effect of fungal mycelia (hypothesis 1). In the soil N pool, the expected effect would in both cases be $^{15}N$ enrichment. Our study cannot, however, separate these two effects as discussed above. The observation that nitrate leaching effects on soil and litter enrichment cannot be detected corresponds with the significantly higher soil nitrate concentrations in spruce than in lime and sycamore maple stands (Table 4, *P=0.02*), i.e. spruce stands tend to leach nitrate at sites with high N status, N deposition and downward soil water percolation. However, fungal mycelia production (Fig. 6) simultaneously coincides with $^{15}N$ depletion of litter. Based on this evidence, we conclude that stands with an open Ncycle (large pools and fluxes) seem to cause soil $^{15}N$ enrichment. The sycamore maple stands cycled significantly more N in litter than beech, and this litter was also $^{15}N$ enriched although leaching was variable, possibly owing to abundant ground vegetation. The result fails to support the open Ncycle *with N leaching*—$^{15}N$ enrichment hypothesis (hypothesis 2) for this tree species, but the observed litter $^{15}N$ enrichment may be a redistribution of pre-existing system internal $^{15}N$ pools originating from other fractionation processes.

In general, the use of foliar or fresh litter $\delta^{15}N$ as an indicator of ecosystem N status works well in this common garden experiment (Fig. 5a) with limited variation in soils and climate. This is not generally supported by observational studies across N deposition gradients. Both positive correlations (Emmett et al. 1998; Pardo et al. 2006), and negative correlations with N deposition (Fang et al. 2011) have been found, and these papers stress the need for a soil based N cycling index, e.g., the nitrification ratio, and a measure of mycorrhizal productivity to supplement the plant $\delta^{15}N$ signal as done in the present study. Nitrogen status (pools and fluxes) may, next to N deposition, be a result of N transformations occurring during the previous land use, and the enrichment factor thus

seems to be a better integrator of ecosystem N status than foliar $\delta^{15}N$ rated against N deposition.

Losses of N may be preceded by N isotope fractionation during nitrification and denitrification, which causes $^{15}N$-depleted losses by $NO_3^-$ leaching and gaseous losses of N. High rates of losses may only occur after canopy closure (approx. at 20 years), when the strong plant demand for inclusion of soil N in the forest stand N cycle ceases in afforested plantations (Hansen et al. 2007). Thus, the N accumulation at Vallø may indicate that the $^{15}N$ depletion observed in litter and O horizon (negative $\delta^{15}N$ in 0–5 cm) was caused by internal fractionation processes, most likely retention of $^{15}N$ in mycorrhizal mycelium and export of $^{15}N$-depleted N from the mycorrhizal fungi to the trees (Hobbie and Ouimette 2009; Högberg et al. 2011).

### Other possible causes of fractionation

A high clay content protects organic matter in organo-mineral aggregates from degradation increasing the age of the organic matter (Christensen 2000), and is associated with low C–N ratios (Vejre et al. 2003; Callesen et al. 2007). Low porosity reduces internal drainage causing more frequent water logging and high reduction potentials. In other words, soils with a high clay content are wet, nutrient rich (Callesen and Rasmussen 2004) and may have changing redox potential, all of which stimulate N transforming processes that can cause fractionation. The sites with loamy subsoil (>15 % clay: WED, MAT, VIE) all showed an enriched or only slightly depleted litter $\delta^{15}N$ status (Table 1, Fig. 2). A shallow groundwater table (Jungkunst et al. 2008) and low C–N ratio of the soil (Pilegaard et al. 2006) may facilitate denitrification. This may partly explain the strong $^{15}N$ enrichment observed at site WED, where high clay content (30 %) gives rise to imperfect drainage and probably frequent stagnating soil water due to slow internal drainage. The C–N ratios observed in the mineral soil at this site are lowest among all six sites (~11±0.5) and significantly lower than the other previously forested sites (C–N site means 13–16± 0.6, $P<0.05$). In addition to this, the $\delta^{15}N$ soil enrichment may be enforced by the elevated nitrate leaching observed for most species at site WED (Table 2).

## Summary and conclusion

Tree species influenced N cycling and $^{15}N$ patterns through multiple species-specific traits. The type of mycorrhiza association (AM or EcM), light climate and ground vegetation differed between the species ash and sycamore and the species beech, lime, oak and Norway spruce. Any potential soil $^{15}N$ enrichment owing to nitrate leaching appeared masked by fractionation attributed to high levels of fungal mycelium. In stands with both traits, fractionation caused by mycorrhizal N uptake seemed to dominate the $^{15}N$ pattern.

We observed that different enrichment patterns can be established quite fast, especially when contrasting land use legacy affects the N cycling. We found limited differences between tree species in their $\delta^{15}N$ soil profiles but fresh litter of ash and sycamore maple had more enriched $^{15}N$ values than that of Norway spruce. It is noteworthy that former cropland soils differed in $\delta^{15}N$ from those in old forests, but still had soil profiles with profound increases in $\delta^{15}N$ with increasing soil depth.

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