Nitrogen input $^{15}$N-signatures are reflected in plant $^{15}$N natural abundances of sub-tropical forests in China

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Abstract. Natural abundance of $^{15}$N ($\delta^{15}$N) in plants and soils can provide time-integrated information related to nitrogen (N) cycling within ecosystems, but it has not been well tested in warm and humid sub-tropical forests. In this study, we used ecosystem $\delta^{15}$N to assess effects of increased N deposition on N cycling in an old-growth broad-leaved forest and a secondary pine forest in a high N deposition area in southern China. We measured $\delta^{15}$N of inorganic N in input and output fluxes under ambient N deposition, and N concentration ($\%N$) and $\delta^{15}$N of major ecosystem compartments under ambient deposition and after decadal N addition at 50 kg N ha$^{-1}$yr$^{-1}$ that has a $\delta^{15}$N of -0.7‰. Our results showed that the total inorganic N in deposition was $^{15}$N-depleted (-10 ‰) mainly due to high input of strongly $^{15}$N-depleted NH$_4^+$-N. Plant leaves in both forests were also $^{15}$N-depleted (-4 to -6 ‰). The broad-leaved forest had higher plant and soil $\%N$, and was more $^{15}$N-enriched in most ecosystem compartments relative to the pine forest. Nitrogen addition did not significantly affect $\%N$ in the broad-leaved forest, indicating that the ecosystem pools are already N-rich. However, $\%N$ was marginally increased in pine leaves and significantly in understory vegetation in the pine forest. Soil $\delta^{15}$N was not changed significantly by the N addition in either forest. However, the N addition significantly increased the $\delta^{15}$N of plants toward the $^{15}$N signature of the added N, indicating incorporation of added N into plants. Thus, plant $\delta^{15}$N was more sensitive to ecosystem N input manipulation than $\%N$ in these N-rich subtropical forests. We interpret the depleted $\delta^{15}$N of plants as an imprint from the high and $^{15}$N-depleted N deposition N that may dominate the effects of fractionation that are observed in most warm and humid forests. Fractionation during the steps of N cycling could explain the difference between negative $\delta^{15}$N in plants and positive $\delta^{15}$N in soils, and the increase in soil $\delta^{15}$N with depths. Nevertheless, interpretation of ecosystem $\delta^{15}$N from high N deposition regions needs to include data on the deposition $^{15}$N signal.

Key words: Natural $^{15}$N abundance, N addition, N deposition, sub-tropical, China
1 Introduction

Nitrogen (N) deposition onto terrestrial ecosystems has dramatically increased due to anthropogenic activities (Galloway, 2005) and since the 1980s the increase has been particularly strong in China including in the warm and humid regions (Liu et al. 2011). Nitrogen deposition that exceeds plant and microbial demand may increase nutrient leaching and soil acidification (Lu et al., 2014), and potentially causes nutritional imbalances in vegetation (Schulze, 1989). Studies of fates and process responses to increased N deposition using coordinated N addition experiments in temperate and boreal forests show that the effects of increased N deposition largely depend on the initial N status of the forests (Gundersen et al., 1998; Hyvönen et al., 2008). Accordingly, N limited forests often show a growth response to added N and retain most of the deposited N, whereas N saturated forests subjected to N deposition often lose considerable N through leaching and denitrification. Although some studies from (sub) tropical regions also suggest that N leaching from tropical forests is related to the initial N status of the forests (Chen and Mulder, 2007; Fang et al., 2009), observations thus far are not conclusive, especially in regions that are subjected to increased anthropogenic N deposition (Townsend et al., 2011).

The natural abundance of $^{15}$N ($\delta^{15}$N) in leaves and other ecosystem compartments is relatively easy to measure and may provide time-integrated information about N cycling in ecosystems (Handley and Raven, 1992; Robinson, 2001). Differences in $\delta^{15}$N between ecosystem compartments and among ecosystems result from isotopic fractionation during each of the many steps of the N cycle. In particular, N losses through leaching and denitrification lead to preferential losses of the lighter $^{14}$N forms whereas compounds with isotopically heavier $^{15}$N are retained in the N pools or further cycled in the ecosystem (Högberg, 1997). Recent advances in the interpretation of $\delta^{15}$N variation among ecosystems based on the compilation and analysis of global data on foliar and soil $\delta^{15}$N have revealed general global patterns in relation to climate and N availability (Martinelli et al., 1999; Amundsen et al., 2003; Craine et al., 2009; 2015a, b). Foliar $\delta^{15}$N values are generally elevated under N rich conditions, i.e. increasing leaf $\delta^{15}$N with increasing leaf N concentration and higher leaf $\delta^{15}$N in warmer climates (Craine et al., 2009). Tropical forests, which are often N-rich, have higher foliar $\delta^{15}$N than temperate forests (Martinelli et al., 1999). However, global analyses contain almost no data from eastern Asia, including sub-tropical regions of China now receiving high N deposition (Fang et al., 2011a).

The influence of increased N deposition on $\delta^{15}$N levels is not well known. For example, even though plant $\delta^{15}$N could increase with N deposition (Emmett et al., 1998), it may not be the case across all regions where not only ecosystem N status but also a region-specific $^{15}$N signature of deposited N may influence ecosystem $\delta^{15}$N (Fang et al., 2011b; Pardo et al., 2006). Moreover, interpretation of ecosystem $\delta^{15}$N is hampered by the uncertainties in $\delta^{15}$N of plant N sources, the magnitude of isotopic fractionations during N transformation processes, and the complex behavior of $^{15}$N in soils and plants (Robinson, 2001).

Plant leaf and soil $\delta^{15}$N are most commonly used to assess N status and changes in N cycling rates, but other ecosystem pools are neglected or rarely measured. The turnover times of N pools vary among different ecosystem compartments, and thus their $\delta^{15}$N values may respond differently to specific disturbances. For example, within plant compartments, small active N pools such as leaves reflect recent N cycling whereas the larger N pools such as wood or soil might reflect long-term changes in N cycling (Craine et al., 2015a). Nevertheless, reports of $\delta^{15}$N values in all major ecosystem pools are rare (e.g. Liu, 1995), emphasizing the need for more rigorous studies to provide complete $\delta^{15}$N patterns in the leaf-to-soil continuum, and their response to N input manipulation, especially in the tropical forests.
We evaluated δ\textsuperscript{15}N values of sub-tropical forests, and their responses to increased N deposition using long-term N addition experimental plots established in 2003 in an old-growth broad-leaved forest and a pine plantation forest in the Dinghushan Biosphere Reserve in southern China (Mo et al., 2006). The old-growth forest is more N-rich, and has less N retention capacity than the pine forest (Fang et al., 2006). Nitrogen addition studies in these forests documented that increased N input causes increased N leaching (Fang et al., 2008, 2009), N\textsubscript{2}O emission (Zhang et al., 2008) and soil acidification (Lu et al., 2014). Here, our objectives are (1) to compare δ\textsuperscript{15}N values of ecosystem compartments across the leaf-to-soil continuum in the two forests, and (2) to assess responses of δ\textsuperscript{15}N in major ecosystem pools to decadal N addition in the two forests. We hypothesized that i) δ\textsuperscript{15}N values of plants and soil in these forests would follow the global patterns predicted from climate and thus be higher in these sub-tropical forests than in those reported for temperate forests, ii) N addition would change plant and soil δ\textsuperscript{15}N towards the \textsuperscript{15}N signature of the added N due to its incorporation into ecosystem pools, and iii) response of δ\textsuperscript{15}N to N addition would differ between the two forests due to differences in their initial N status and N cycling rates.

2 Methods
2.1 Study site
The study was conducted in the Dinghushan Biosphere Reserve (DHSBR) in the Guangdong province, southern China (112°33’ E and 23°10’ N) with typical sub-tropical monsoon climate. Mean annual temperature (MAT) and mean annual precipitation (MAP) are 22.2 °C and 1927 mm, respectively. The reserve has experienced high rates of atmospheric N deposition (21-38 kg N ha\textsuperscript{-1} yr\textsuperscript{-1} as inorganic N in bulk precipitation) since 1990’s (Fang et al., 2008). In 2009 to 2010, total wet N deposition was 34.4 kg N ha\textsuperscript{-1} yr\textsuperscript{-1} (Lu et al., 2013). We used two common forest types that grow on the relatively steep slopes in the reserve; an old-growth broad-leaved forest (hereafter named as BF) and a pine plantation forest (hereafter named as PF) (Mo et al., 2006). The BF is a regional climax mixed broad-leaved forest, which has been protected for at least the last 400 years with minimum human disturbances (Shen et al., 1999). The PF was planted after a clear-cut of the original climax forest in the 1930s and has been subjected to human disturbances such as litter and shrub harvesting until the recent past (Mo et al., 2005).

Table 1. Selected characteristics of the mineral soil (0-10 cm) in the two forest types. Data on soil bulk density, total P and extractable NH\textsubscript{4}\textsuperscript{+}-N are obtained from Fang et al. (2006). Values given in parenthesis indicate SE (n = 3).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Broad-leaved forest (BF)</th>
<th>Pine forest (PF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk density (g cm\textsuperscript{-2})</td>
<td>0.9 (0.03)</td>
<td>1.3 (0.03)</td>
</tr>
<tr>
<td>pH (H\textsubscript{2}O)</td>
<td>3.8 (0.02)</td>
<td>4.0 (0.04)</td>
</tr>
<tr>
<td>C concentration (%)</td>
<td>3.8 (0.80)</td>
<td>1.8 (0.03)</td>
</tr>
<tr>
<td>N concentration (%)</td>
<td>0.3 (0.04)</td>
<td>0.1 (0.01)</td>
</tr>
<tr>
<td>C/N ratio</td>
<td>13.6 (0.9)</td>
<td>13.9 (0.7)</td>
</tr>
<tr>
<td>Total P (mg kg\textsuperscript{-1})</td>
<td>59 (3)</td>
<td>43 (3)</td>
</tr>
<tr>
<td>Extractable NH\textsubscript{4}\textsuperscript{+}-N (mg kg\textsuperscript{-1})</td>
<td>2.1</td>
<td>3.3</td>
</tr>
<tr>
<td>Extractable NO\textsubscript{3}\textsuperscript{-}-N (mg kg\textsuperscript{-1})</td>
<td>12.7</td>
<td>2.6</td>
</tr>
</tbody>
</table>

The major canopy species in the BF are Castanopsis chinensis, Machilus chinensis, Schima superba, Cryptocarya chinensis, and Syzygium rehderianum and the most common understory species is Hemigramma decurrins. Pinus massoniana and Dicranopteris dichotoma are the dominant tree and understory species in the PF, respectively.
No N-fixing tree species were found in the plots. The soil in the reserve is classified as Lateritic Red Earth (Oxisol) formed from Devonian sandstone and shale with a thin layer of forest floor litter (0.5-3.0 cm), but the soil depth is variable ranging from 30 cm in the PF to more than 60 cm in the BF. Probably due to erosion after the clear-cut and the continued human disturbance the PF had lower total soil carbon, N and phosphorus (P) content than the BF (Table 1).

2.2 Experimental design
We used an ongoing N addition experiment established in both forests in July 2003 (Mo et al., 2006). The experimental plots used for this study consist of control plots and N addition treatment at 50 kg N ha⁻¹ yr⁻¹ (hereafter named as N-plots) each with three replicates in both forests. Each plot is 10 m x 20 m with at least a 10 m wide buffer strip to the next plot. In the N-plots, NH₄NO₃ is mixed with 20 L of water, and is added monthly since July 2003 below the canopy using a backpack sprayer, whereas the control plots received equivalent 20 L water with no fertilizer. The added N has δ¹⁵N of about -3 ‰ on NH₄⁺ and about 1.8 ‰ on NO₃⁻, with δ¹⁵N of NH₄NO₃ being -0.7 ‰.

2.3 Sampling and analysis of plant and soil pools
In both forests, major ecosystem compartments including leaves, twigs, branches, bark and wood of canopy trees, leaves of understory vegetation, fine roots, and 0-30 cm mineral soil were sampled in January 2013 to determine their N concentration (%) and δ¹⁵N (%). A branch per dominant tree species per plot was cut from the height reached using a pole pruner (c. 7-8 m) taking advantage of the steep slope, and was separated into leaves, twigs and small branches. Bark samples were cutoff the dominate trees at breast height using a knife. After removing the bark, wood cores were sampled using an increment borer and separated visually into sapwood (usually the outer 2-3cm recent wood) and older wood (heartwood). Dominant understory plant species were cut with a knife and kept separate for each species. A total of seven tree species in the canopy/sub-canopy layer and five plant species in the understory layer (young trees, shrubs, herbs and liana) of the BF were sampled. In the PF, the dominant pine tree and five species in the understory layer were sampled. Mineral soil samples were taken using an auger (5.1 cm in diameter) and were divided into three layers (0-10, 10-20, 20-30 cm). Two soil cores were sampled and pooled together to form one composite sample for each depth per plot. Living fine roots were hand-sorted from the soil samples for each depth, but were combined to one composite sample for the whole profile (0-30 cm) because the amount of fine roots in each depth were too small to grind and analyze separately. Litterfall was collected monthly during July-September 2012 and was pooled together to make one composite sample per plot. The litter was sorted in the laboratory into leaf and others (branches, fruits, flowers, barks), but only leaf values are reported.

All plant and soil samples were oven-dried at 70 °C, and ground to a fine and homogeneous powder. Mineral soils were sieved (2mm mesh) to remove non-soil materials, air-dried at room temperatures and milled to fine powder. Subsamples were dried at 105 °C, and all results are reported on 105 °C basis. Based on their approximate %N, about 4-5 mg of the samples were weighed into tin capsules, and δ¹⁵N and N concentration of the samples were determined simultaneously on an isotope ratio mass spectrometer (Isoprime 100, Isoprime Ltd.) coupled to an automatic, online elemental analyzer (vario ISOTOPE cube). An internal standard needle sample from temperate forests, which has been analysed in multiple runs at several laboratories, was used to check reproducibility of the δ¹⁵N determination. We analyzed %N and δ¹⁵N separately for each dominant tree species per plot, but compartment mean values are reported. Natural abundance δ¹⁵N in samples was reported in per mil (‰) relative to the ¹⁵N content of atmospheric N₂.
2.4 Sampling and analysis of water samples

Precipitation, throughfall, surface runoff and soil solution were sampled monthly from September 2012 to February 2013 (dry December and January, where there were not enough precipitation to generate water samples) in the control plots to assess the $\delta^{15}$N of N input and output in the two forests under ambient N deposition. Bulk precipitation was collected at an open area close to the experimental site using an open glass funnel (12 cm in diameter), connected to a 5 L sampling bottle with polypropylene tubes. Throughfall was collected by PVC pipes at five random points within each plot (with a total intercept area of 0.8m$^2$) at about 1.3m above the ground in each forest. Each collector was connected to two 50L buckets with polypropylene tubes. Soil solutions from 20 cm depth (seepage water) were obtained using two zero tension tray lysimeters (755 cm$^2$ per tray) installed in each plot. Each lysimeter was connected to a 20L bottle using the steep slope of the sites to facilitate sampling. In both forests, one selected plot for each treatment was delimited hydrologically by placing stable plastic materials and low cement barriers around them. The cement barriers (covered by the plastic material) on the downslope side of these plots were constructed to enable the sampling of the surface runoff in three sections, which were then used as pseudo-replicates.

Natural $^{15}$N abundances of NH$_4^+$-N and NO$_3^-$-N in water samples were analyzed after chemical conversion to nitrous oxide (N$_2$O). The NH$_4^+$-N was initially oxidized to nitrite (NO$_2^-$) by hypobromite (BrO$^-$) and the NO$_2^-$ is then quantitatively converted into N$_2$O by hydroxylamine (NH$_2$OH) under strongly acidic conditions (Liu et al., 2014). Similarly, a series of chemical reactions of vanadium (III) chloride (VCl$_3$) and sodium azide under acidic conditions was used to convert NO$_3^-$-N into N$_2$O (Lachouani et al., 2010). The produced N$_2$O was subsequently analysed for $^{15}$N abundance by a purge-and-trap coupled with an isotope ratio mass spectrometer (PT-IRMS) (Liu et al., 2014).

2.5 Calculations and Statistics

To evaluate effects of decadal N addition on the whole ecosystem (plant and soil) %N and $\delta^{15}$N, we determined N pool weighted plot means of %N and $\delta^{15}$N using N pools for each compartment and tree species contribution quantified in Gurmesa et al. (2016). We excluded the heartwood and sapwood pools in the plant pool calculations for two reasons; first the low %N in wood samples caused larger uncertainties on the $\delta^{15}$N determinations, and secondly heartwood and a major part of the sapwood were formed prior to the initiation of the N addition treatment. We expect the later to be the explanation that particular heartwoods showed opposite effects of N addition compared to all other compartments. Differences between the two forests in plot mean %N and $\delta^{15}$N of the different ecosystem compartments and N pool weighted plot means in control plots were analysed using t-tests. The effect of N addition treatment on %N and $\delta^{15}$N of each tree compartments in the BF and understory leaf in both forests was analyzed using mixed model ANOVA with treatment as explanatory factor and plant species as a random factor because plant species differed significantly in both parameters (Gurmesa, 2016). All other tests of treatment effects on %N and $\delta^{15}$N was analysed using simple t-test on plot means.

3 Results

3.1 Concentration and $\delta^{15}$N of NH$_4^+$-N and NO$_3^-$-N in water samples

Dissolved NH$_4^+$-N in water samples in both input (precipitation and throughfall) and output fluxes (surface runoff and soil solution) were $^{15}$N-depleted (negative $\delta^{15}$N) in both forests (Table 2). The $\delta^{15}$N of NO$_3^-$-N was $^{15}$N-enriched in precipitation and throughfall, and became $^{15}$N-depleted in surface runoff and soil solution. However, for dissolved
inorganic N (DIN) the concentration weighted δ^{15}N (calculated based on data in Table 2 and concentration data in Table S1) were ^15N-depleted but slightly increased from precipitation input to soil solution. Mean δ^{15}N of both NH$_4^+$-N and NO$_3^-$-N in input and output fluxes did not significantly differ between the two forests. The temporal variation in δ^{15}N was large (-28 to 2 ‰) for NH$_4^+$-N but minor (2 to 5 ‰) for NO$_3^-$-N (Fig. 1b, d, x-axis). The δ^{15}N of NH$_4^+$-N in surface runoff and soil solution were significantly and positively related to the variation in δ^{15}N of NH$_4^+$-N in throughfall in both forests (Fig 1a, b), but the correlation was not significant for NO$_3^-$-N (Fig 1c, d).

Table 2. δ^{15}N (‰) of NH$_4^+$-N, NO$_3^-$-N and dissolved inorganic N (DIN) in bulk precipitation, throughfall, surface runoff and soil solution at 20 cm depth in control plots from September 2012 to February 2013. Numbers in parenthesis for precipitation, throughfall and soil solution indicate standard error of the mean (SE) (n = 3). For all water fluxes, no significant difference in δ^{15}N of both NH$_4^+$-N and NO$_3^-$-N was detected between the two forests.

<table>
<thead>
<tr>
<th>Fluxes</th>
<th>Broad-leaved forest (BF)</th>
<th>Pine forest (PF)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NH$_4^+$-N</td>
<td>NO$_3^-$-N</td>
</tr>
<tr>
<td>Precipitation$^a$</td>
<td>-16.6</td>
<td>4.1</td>
</tr>
<tr>
<td>Throughfall$^b$</td>
<td>-15.2 (2.3)</td>
<td>3.6 (0.2)</td>
</tr>
<tr>
<td>Surface runoff$^b$</td>
<td>-13.1 (1.7)</td>
<td>-1.9 (0.6)</td>
</tr>
<tr>
<td>Soil solution</td>
<td>-22.6 (0.9)</td>
<td>-0.9 (1.3)</td>
</tr>
</tbody>
</table>

$^a$ Precipitation was collected at open area within the reserve, and was assumed to be the same for both forests.

$^b$ The indicated SE is for pseudo-replicates within one plot.

### 3.2 Effects of forest type

As expected based on the differences in disturbance regime, the BF is more N-rich than PF. Nitrogen concentrations of plant compartments were significantly higher in the BF than in the PF, except in leaves of canopy trees, litter-fall and fine roots for which the difference was marginally significant (Table 3). Soil %N was significantly higher in the BF at all depths (Table 3).

Most plant compartments are ^15N-depleted with understory and tree leaves, twigs and branches being most ^15N-depleted (below -4 ‰) whereas bark and sapwood were less ^15N-depleted within each forest (Table 4). The δ^{15}N of all plant compartments differ significantly between the two forests with the PF being more ^15N-depleted than the BF (Table 4). Soil δ^{15}N did not show significant difference between the two forests at any depth (Table 4).
Figure 1. Correlation between δ\(^{15}\)N (‰) of NH\(_4^+\)-N in throughfall and that of NH\(_4^+\)-N in surface runoff (a), and soil solution (b), and correlation between δ\(^{15}\)N of NO\(_3^-\)-N in throughfall and that of NO\(_3^-\)-N in surface runoff (c), and soil solution (d). For throughfall and soil solution, δ\(^{15}\)N were from samples taken monthly between September and February in each of the 3 plots. For surface runoff, samples were only from one plot. No significant effect of forest type was detected; thus the regression line shown was based on data from both forests.

When compared based on N pool weighted plot mean, the two forests differed significantly in plant %N and δ\(^{15}\)N (Fig. 2a). For the soil, the two forests also differed significantly in N pool weighted plot mean %N, with the BF having the higher value, but not in N pool weighted plot mean δ\(^{15}\)N (Fig. 2b).

3.3 Effects of N addition on %N and δ\(^{15}\)N

Nitrogen concentrations in all measured plant and soil compartments were not significantly affected by N addition in the BF, except in the sapwood (Table 3). In the PF, mean %N values were greater in most plant compartments on fertilized plots, but the change was significant only in leaves of understory plants, whereas soil %N was unchanged (Table 3).

Plant δ\(^{15}\)N was negative in both control and N-plots in both forests, but N addition significantly increased the δ\(^{15}\)N of most plant compartments (Table 4). The changes were more pronounced in the small active plant pools such as leaves of trees and understory plants.
Table 3. Mean %N of different ecosystem pools in the broad-leaved (BF) and pine forests (PF). Values in parenthesis indicate SE of plot means (n = 3). Within each forest type p-values for the effect of N addition are shown. The last column shows p-values for a difference between the ambient plots of the two forests using t-test. Bolded p-values indicate significant difference.

<table>
<thead>
<tr>
<th>Compartment</th>
<th>Broad-leaved forest (BF)</th>
<th>Pine forest (PF)</th>
<th>Forest type effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>N addition</td>
<td>p-values</td>
</tr>
<tr>
<td><strong>Plants</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tree leaf</td>
<td>1.71 (0.19)</td>
<td>1.69 (0.19)</td>
<td>0.48 §</td>
</tr>
<tr>
<td>Twig</td>
<td>1.28 (0.19)</td>
<td>1.17 (0.23)</td>
<td>0.59 §</td>
</tr>
<tr>
<td>Branch</td>
<td>0.86 (0.15)</td>
<td>0.81 (0.16)</td>
<td>0.13 §</td>
</tr>
<tr>
<td>Bark</td>
<td>0.71 (0.16)</td>
<td>0.7 (0.16)</td>
<td>0.55 §</td>
</tr>
<tr>
<td>Sapwood</td>
<td>0.27 (0.07)</td>
<td>0.3 (0.07)</td>
<td>&lt;0.01 §</td>
</tr>
<tr>
<td>Heartwood</td>
<td>0.16 (0.04)</td>
<td>0.16 (0.03)</td>
<td>0.28 §</td>
</tr>
<tr>
<td><strong>Understory leaves</strong></td>
<td>2.04 (0.02)</td>
<td>1.98 (0.17)</td>
<td>0.09 §</td>
</tr>
<tr>
<td>Fine root</td>
<td>1.4 (0.16)</td>
<td>1.81 (0.17)</td>
<td>0.15</td>
</tr>
<tr>
<td>Litter-fall</td>
<td>1.56 (0.05)</td>
<td>1.48 (0.06)</td>
<td>0.45</td>
</tr>
<tr>
<td><strong>Soil</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-10 cm</td>
<td>0.27 (0.04)</td>
<td>0.28 (0.01)</td>
<td>0.83</td>
</tr>
<tr>
<td>10-20 cm</td>
<td>0.18 (0.01)</td>
<td>0.19 (0.01)</td>
<td>0.59</td>
</tr>
<tr>
<td>20-30 cm</td>
<td>0.12 (0.00)</td>
<td>0.14 (0.00)</td>
<td>0.14</td>
</tr>
</tbody>
</table>

§ Due to significant differences between the sampled tree or understory plant species the effect of N addition was tested in a mixed model ANOVA with species as random factor.

However, effect of N addition on δ¹⁵N was inconsistent in the wood parts (Table 4). For heartwood, the effects were significant, but in different directions than in other plant pools for both forests. Due to low %N and challenges in grinding of wood samples it was difficult to get reliable δ¹⁵N results for these samples. Also much of the sampled wood was formed prior to the treatment and thus, no further evaluation was done for the wood samples. Nitrogen addition did not cause significant effects on δ¹⁵N of litter-fall and fine roots. In the BF, there was no correlation between leaf %N and δ¹⁵N, but a positive correlation was found for the PF as both %N and δ¹⁵N tended to increase in parallel due to N addition (data not shown).

Nitrogen addition tended to decrease soil δ¹⁵N in the BF at all depths, but with no significant changes in any layer (Table 4). In the PF, soil δ¹⁵N was unchanged by N addition (Table 4).
Table 4. Mean δ¹⁵N (‰) of plant pools in the broad-leaved (BF) and pine forests (PF). Values in parenthesis indicate SE of plot means (n = 3). Within each forest type p-values for the effect of N addition is shown. The last column shows p-values for differences between the ambient plots of the two forests using t-test. Bolded p-values indicate significant differences.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Broad-leaved forest (BF)</th>
<th>Pine forest (PF)</th>
<th>Forest type effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>N addition</td>
<td>p-values</td>
</tr>
<tr>
<td>Tree leaf</td>
<td>-4.0 (0.5)</td>
<td>-3.4 (0.6)</td>
<td><strong>0.02</strong> §</td>
</tr>
<tr>
<td>Twigs</td>
<td>-4.3 (0.8)</td>
<td>-3.8 (0.9)</td>
<td>0.09 §</td>
</tr>
<tr>
<td>Branches</td>
<td>-4.6 (0.4)</td>
<td>-4.1 (0.3)</td>
<td><strong>&lt;0.01</strong> §</td>
</tr>
<tr>
<td>Bark</td>
<td>-2.8 (0.8)</td>
<td>-2.4 (0.6)</td>
<td>0.05 §</td>
</tr>
<tr>
<td>Sapwood</td>
<td>-1.9 (0.5)</td>
<td>-1.8 (0.3)</td>
<td>0.51 §</td>
</tr>
<tr>
<td>Heartwood</td>
<td>-1.6 (0.9)</td>
<td>-2.3 (0.9)</td>
<td><strong>0.05</strong> §</td>
</tr>
<tr>
<td>Understory leaves</td>
<td>-3.6 (0.9)</td>
<td>-2.2 (1.1)</td>
<td><strong>&lt;0.01</strong> §</td>
</tr>
<tr>
<td>Fine root</td>
<td>-2.8 (0.6)</td>
<td>-1.7 (0.8)</td>
<td>0.33</td>
</tr>
<tr>
<td>Litter-fall</td>
<td>-3.9 (0.1)</td>
<td>-3.9 (0.1)</td>
<td>0.98</td>
</tr>
</tbody>
</table>

**Soil**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>N addition</th>
<th>p-values</th>
<th>Control</th>
<th>N addition</th>
<th>p-values</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-10 cm</td>
<td>2.2 (0.4)</td>
<td>1.6 (0.6)</td>
<td>0.46</td>
<td>2.6 (0.8)</td>
<td>2.3 (0.4)</td>
<td>0.69</td>
<td>0.63</td>
</tr>
<tr>
<td>10-20 cm</td>
<td>4.0 (0.3)</td>
<td>3.2 (0.2)</td>
<td>0.09</td>
<td>4.1 (1.4)</td>
<td>4.4 (0.3)</td>
<td>0.88</td>
<td>0.93</td>
</tr>
<tr>
<td>20-30 cm</td>
<td>5.4 (0.3)</td>
<td>4.8 (0.5)</td>
<td>0.39</td>
<td>3.3 (1.4)</td>
<td>4.0 (0.2)</td>
<td>0.68</td>
<td>0.26</td>
</tr>
</tbody>
</table>

§ Due to significant differences between the sampled tree or understory plant species the effect of N addition was tested in a mixed model ANOVA with species as random factor.

In summary, the effect of added N on pool weighted plot mean plant %N was not significant in either BF (p = 0.86) or in PF (p = 0.25) more pronounced in the PF (Fig. 2a). However, weighted plot mean plant δ¹⁵N were significantly increased in both forests (p = 0.04 for BF and p = 0.03 for PF) by the N addition. In the soil, where the N pool is obviously larger than in the plants, the effect of the N addition on weighted average %N was not significant in both forests (Fig. 2b). The direction of change in soil δ¹⁵N was a decrease as expected with incorporation of the added N (δ¹⁵N = -0.7 ‰), but the change was again not significant (Fig. 2b).
Figure 2. Overall effect of N addition on plot average weighted %N and δ^{15}N of plants (a), and soil (b) for broad-leaved forest (○) and pine plantation (Δ). Error bars indicate SE of plot means (n = 3). Open symbols indicate control plots and closed symbols indicate N-plots. In (a), significant effects of N addition within forest type is indicated by different letters; lowercase for BF and uppercase for PF. The p-values shown in the upper right corners are tests for differences in %N and δ^{15}N between the two forests (ambient plots).

4 Discussions

4.1 δ^{15}N of N in deposition and soil solution

Deposition N (bulk precipitation and throughfall) was ^15N-depleted in NH$_4^+$-N and ^15N-enriched in NO$_3^-$-N (Table 2), but since NH$_4^+$-N is the dominating N form (Table S1) DIN deposition is ^15N-depleted (-10 to -8 ‰) as also previously reported in the region (Zhang et al., 2008; Koba et al., 2012). The source of the NH$_4^+$-N is likely NH$_3$ emissions from activities in the intensively used agricultural land surrounding DHSBR. Agricultural NH$_3$ emissions are usually ^15N-depleted (Bauer et al., 2000). The source of the NO$_3^-$-N contribution may originate from NO$_2$ produced by coal combustion in mega-cities in the Guangdong province.
The low δ¹⁵N of NH₄⁺-N in the soil solution of both forest resemble that in precipitation and throughfall (Table 2), and it is likely due to transport of ¹⁵N-depleted throughfall N through macrospores as supported by the positive relationship between δ¹⁵N of NH₄⁺-N in soil solution and that in throughfall (Fig. 1b). The further ¹⁵N-depletion of NH₄⁺-N (6 to 7 ‰) from throughfall to soil solution may occur by preferential retention of the heavier ¹⁵N isotope by cation exchange on soil surfaces (e.g. Karamanos and Rennie, 1978), although preferential nitrification of the lighter isotope could work in the opposite direction. This fractionation effect of nitrification (leaving the substrate ¹⁵N-enriched and the product NO₃⁻-N ¹⁵N-depleted (Högberg, 1997)) may explain the relative ¹⁵N-enrichment of NH₄⁺-N (2 to 6 ‰) from throughfall to soil solution in both forests (Table 2). A contribution of NO₃⁻-N from nitrification of ¹⁵N-depleted throughfall NH₄⁺-N during surface runoff passing through the biological active litter layer may also explain the 4 to 6 ‰ ¹⁵N-depletion of NO₃⁻-N from throughfall to surface runoff (Table 2).

While NO₃⁻-N is the dominant N-form in soil solution (Table S1) and the N leaching fluxes are almost as large as the N inputs by deposition in both forests (Fang et al., 2009), nitrification is an important process in the soils at DHSBR. However, as soil solution NO₃⁻-N was ¹⁵N-enriched (1 ‰) relative to the ¹⁵N-depleted throughfall NH₄⁺-N (15 ‰), this cannot be the main substrate for nitrification in the soil. Also the relative narrow temporal variation of δ¹⁵N for soil solution NO₃⁻-N (Fig. 1d) indicate dominance of a substrate for nitrification with stable δ¹⁵N content such as soil organic N and/or adsorbed NH₄⁺. On the other hand, gaseous losses of ¹⁴N-depleted N by denitrification would ¹⁵N-enrich soil N as well as soil solution NO₃⁻-N (Houlton et al., 2006). For the BF, denitrification N losses have been estimated to be as high as 2.6 kg N ha⁻¹ yr⁻¹ as N₂O (Zhang et al., 2008) and 30 kg N ha⁻¹ yr⁻¹ as N₂ (Fang et al., 2015). This may explain why DIN in soil solution is slightly ¹⁵N-enriched relative to the DIN input (bulk precipitation or throughfall), despite the apparent importance of fractionation via nitrification in the soils of both forest.

4.2 δ¹⁵N of plants and soil under ambient condition

Climate is important in regulating global patterns of δ¹⁵N in plants and soils (Amundson et al., 2003; Craine et al., 2009; 2015b). Based on the relationships between plant and soil δ¹⁵N and climate parameters (MAT and MAP) established by Amundson et al. (2003), the expected δ¹⁵N at DHSBR are 0.4 ‰ for plants and 5.2 ‰ for the top 10 cm soil. In a global synthesis for forests Martinelli et al. (1999) reported an average leaf δ¹⁵N at 3.7 ± 3.5 ‰ for tropical forests and a recent survey across Amazonas observed similar ¹⁵N-enriched leaf δ¹⁵N levels (3.1 ± 2.3 ‰) (Nardoto et al., 2014). For tropical forest soils Martinelli et al. (1999) reported 9.3 ± 1.8 ‰ for the top 10 cm. However, the observed leaf δ¹⁵N at DHSBR were much lower, between -4 ‰ and -6 ‰ for the two forests (Table 4). Similar low values were found in other (sub) tropical forest in eastern Asia (Fang et al., 2011a; Wang et al., 2014; Kitayama and Iwamoto, 2001). The top 10 cm soil δ¹⁵N at DHSBR (2.2 to 2.6 ‰, Table 4) were again lower than expected from local climate or observed in tropical forest. Apparently, the ecosystem δ¹⁵N values at DHSBR are more close to the values reported for temperate forests by Martinelli et al. (1999) for leaves (-2.8 ± 1.8 ‰) and for soil (1.6 ± 3.6 ‰) as well as those reported from N-saturated temperate forests (Koopmans et al., 1997; Sah & Brumme, 2003).

Thus, our results reject our first hypothesis that ecosystem δ¹⁵N at DHSBR would compare with other observations from warm and humid climates; also DHSBR forests were not more ¹⁵N-enriched than temperate forests. Martinelli et al. (1999) discussed reasons for the ¹⁵N-enrichment of tropical ecosystems (relative to temperate forest) and concluded it could result from open N cycles in tropical forests, with fractionation during microbial activities resulting in losses of isotopically light ¹⁴N forms which leave isotopically heavy N to cycle internally within tropical
ecosystems. Despite noticeable fractionation processes in the soil at DHSBR (section 4.1) and high N availability leading to considerable N losses, there is no evident ecosystem $^{15}$N-enrichment at DHSBR or in other Chinese forests with high N deposition (Fang et al., 2011a; Wang et al., 2014).

We suspect this phenomenon to be an imprint from the high and $^{15}$N-depleted N deposition (Table 2). The $^{15}$N signature of deposition N can alter plant $\delta^{15}$N by direct uptake in the canopy and by altering the signature of available N in the soil (Craine et al., 2015a) (as it is noticeable for NH$_4^+$-N in soil solution; Fig 1b). A similar mechanism involving preferential uptake of particularly $^{15}$N depleted NH$_4^+$-N could also explain the occurrence of $^{15}$N-depleted plants in tropical rainforests in southern China (Wang et al., 2014). Such influence of deposition N can be region-specific as shown for some forests in Europe that appear to follow a different trajectory for increasing leaf $\delta^{15}$N with N deposition than forests in USA (Pardo et al., 2006).

The conclusion that plant $\delta^{15}$N is influenced by the $^{15}$N-depleted N deposition is further supported by the result that tree ring $\delta^{15}$N of Pinus massoniana at DHSBR (sampled nearby the PF plots) decreased from 2‰ in the 1960s to -1‰ in the late 1990s, and that the decrease was found to coincide with the increasing deposition of $^{15}$N-depleted N over the last 50 years (Sun et al., 2010). In line with that long-lived plant compartments (bark and wood) were less $^{15}$N-depleted than short-lived compartments (leaves, twigs and branches) in both forests (Table 4).

The lower soil $\delta^{15}$N in DHSBR relative to the global average for tropical forest soils may in part also be an imprint from the $^{15}$N-depleted N deposition. However, with an N-pool at ~2400 kg N ha$^{-1}$ (equal to more than 60 years of N deposition) alone in the top 10 cm (Gurmesa et al., 2016), the influence should be minor compared to that in short-lived plant compartments that holds an N-pool an order of magnitude less.

The steep slopes at DHSBR may contribute slightly to lower the soil $\delta^{15}$N, because steeper slopes promote non-fractionating erosional losses of soil organic matter and decrease the residence time of soil N compared to forests on more gentle slopes, that on the other hand may have more fractionation from denitrification due to greater soil moisture (Amundson et al., 2003; Hilton et al., 2013; Perakis et al., 2015).

4.3 Effects of N addition on $\delta^{15}$N

Nitrogen addition increases N availability and is thus expected to increase plant $\delta^{15}$N as a result of fractionation during N uptake and cycling, as discussed above. Several N addition experiments in temperate forests indeed observed this effect (Högberg et al., 2011; Högberg et al., 2014; Korontzi et al., 2000; McNulty et al., 2005; Näsholm et al., 1997). Accordingly, plant $\delta^{15}$N in both forests at DHSBR were increased by N addition (Table 4, Fig 2a). The changes in $\delta^{15}$N occurred in small and short-lived plant compartments (e.g. leaves, roots) that are responsive to contemporary N input manipulation (Fang et al., 2006; Johannisson and Hogberg, 1994; Pardo et al., 2002) compared to the large, long-lived and less responsive compartments (e.g. bark and wood). Such changes in plant $\delta^{15}$N could be a result of fractionation processes, but alternatively it may originate from uptake and incorporation of the added N fertilizer, that had an enriched $^{15}$N signature (-0.7‰) relative to $\delta^{15}$N of the plants (e.g. -4 to -6‰ in leaves).

Assuming fractionation effects are minor, the decadal N addition with $\delta^{15}$N value of -0.7‰ can be viewed as a tracer addition, since it differs from the abundance in the major ecosystem pools. Based on a $^{15}$N mass balance calculation (Nadelhoffer and Fry, 1994), and using the control plots as reference, the fraction of added N that was incorporated into plants could be estimated (Table S2). Since the calculation relies on the difference in $\delta^{15}$N between the control and the N-plots in the target pool, it is only meaningful when this difference is significant. Thus, the fraction of
added N incorporated could only be estimated for the total plant N pool, but not for the soil (Fig. 2). The results showed that ~15 % of the total 500 kg N ha$^{-1}$ added over a decade was incorporated into plant pools in both forests. For BF this was less than the estimated fate (24 % to plants) of a stronger tracer (Gurmesa et al., 2016). Nevertheless, it indicates substantial incorporation of input N into plants in BF even though the N addition did not increase the net uptake in the forest, i.e. no change in %N in plant compartments at BF.

For soils, N addition tended to decrease $\delta^{15}$N, opposite to results in other long-term experimental N addition (Högberg, 1991; Högberg et al., 1996, 2011) where soil $\delta^{15}$N increased after addition of N. The authors explained that the increase was the result of fertilizer-induced fractionation due to increased N transformation rates. In our study, fractionation may also occur, but with the decreasing tendency of soil $\delta^{15}$N indicates incorporation of the isotopically lighter added N (relative to the soil) is likely as discussed by Högberg et al. (2014).

The result supports our second hypothesis that the added N is incorporated into the ecosystem N pools with plant (and soil) $\delta^{15}$N changing slowly toward the $^{15}$N signature of the decadal N addition. This again highlights the importance of the $^{15}$N signature of input N in controlling ecosystem $\delta^{15}$N.

### 4.4 Effects of forest type

As expected from previous studies, the BF is more N-rich than the PF as indicated by higher %N in major ecosystem pools in BF (Table 3). Accordingly, plant %N in short-lived compartments (and in the pool weighted plant pools) did not respond to the decadal N addition in BF, whereas plant %N in PF tended to increase, though only significantly in understory plants (Table 3, Fig. 2a). In the BF, the plant tissues were apparently saturated with N, while the PF still could retain part of the addition (Fang et al., 2009). Most plant compartments in BF are more $^{15}$N-enriched than the PF (Table 4) and the change in plant $\delta^{15}$N after decadal N addition was most pronounced in PF (Fig. 2a). This again could hint a difference in N status, where the larger changes in plant $\delta^{15}$N in the PF indicate larger incorporation of added N into plants in PF than in BF in agreement with our third hypothesis.

The difference under ambient conditions may in part be related to higher N cycling rates and subsequent losses of the lighter $^{14}$N in the BF through fractionating processes, and subsequent plant uptake of $^{15}$N-enriched soil N (Magill et al., 2000; Zhang et al., 2008; Nadelhoffer and Fry, 1994). On the other hand, leave $\delta^{15}$N in PF can be more affected by $^{15}$N-depleted deposition as the forest is still expanding in biomass and has lower N availability, thus it might depend more on the $^{15}$N-depleted atmospheric N input than the BF does. An additional explanation could be that the PF is dominated by *Pinus massoniana*, which has ectomycorrhizal fungi whereas majority of the plants in the BF have arbuscular mycorrhizal association (Gurmesa, 2016), and ectomycorrhizal plants are found to be more $^{15}$N-depleted than arbuscular mycorrhizal plants (Craine et al., 2009; 2015a).

Soil $\delta^{15}$N did not significantly differ between the BF and PF (Table 4; Fig. 2b), although we expected soil to be more $^{15}$N-enriched in the BF than in the PF. Soil $\delta^{15}$N are reported to increase with organic matter age (Bauer et al., 2000), and we expect soil organic matter of the top soil to be older in the BF, because this layer might have been lost by erosion in the PF as it could be noted from the lower C, N and P concentration (Table 1), and lack of depth pattern of soil $\delta^{15}$N in the PF (Fig 2b). A common feature in soil profiles is $^{15}$N-enrichment with soil depth (Bauer et al., 2000; Emmet et al., 1998; Koba et al., 2010; Boeckx et al., 2005) as observed in the undisturbed BF, but not in the disturbed PF (Table 4). The absence of a $^{15}$N-enrichment profile may again be an effect of erosion and soil mixing from human disturbances that may shape soil N and $\delta^{15}$N patterns over ecosystem succession (Perakis et al., 2015).
enrichment with depth is known to occur as a result of fractionation followed by removal of lighter $^{14}$N by plants, microbes, or through leaching following decomposition, whilst the $^{15}$N-enriched N fraction is transported and accumulated at deeper soil profile (Högberg et al., 2011; Hobbie and Högberg, 2012; Nadelhoffer et al., 1988).

5 Conclusion

We show that forests at DHSBR (and other humid tropical forests of southern China) are likely $^{15}$N-depleted due to imprints from $^{15}$N-depleted N deposition, particularly $\text{NH}_4^+$-N in the region. This effect of the input N (deposition)$^{15}$N signature was further supported by our observation that $\delta^{15}$N of plants (and soil) were changed toward the $^{15}$N signature of added fertilizer N, which also shows that fertilizer additions are incorporated into forest N pools even at high N availability. We found that broad-leaved forests and early successional forests differ in their %N and $\delta^{15}$N, and accordingly differ in their response to increased N input. The significant changes in plant $\delta^{15}$N toward the $\delta^{15}$N value of the added N observed in both forests indicate that the $^{15}$N signature of incoming N could dominate the effects from fractionation during the steps of N cycling. Thus, the $^{15}$N imprint of increased N deposition should be considered in using ecosystem $\delta^{15}$N to interpret ecosystem N cycling characteristics, particularly in regions with high N emissions.

Authors’ contribution: Gundersen P. and Mo J. conceived and designed the experiments. Gurmesa A.G., Lu X., Mao Q. and Zhou K. performed the data acquisition. Gurmesa A.G. analyzed the data. Gurmesa A.G. and Gundersen P. wrote the manuscript. Lu X., Fang Y. and Mo J. commented and edited the article.

Conflicts of interest: The authors declare that they have no conflict of interest.

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