

Fungal functioning in a pine forest: evidence from a ^{15}N -labeled global change experiment

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Summary

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- We used natural and tracer nitrogen (N) isotopes in a *Pinus taeda* free air CO_2 enrichment (FACE) experiment to investigate functioning of ectomycorrhizal and saprotrophic fungi in N cycling.
- Fungal sporocarps were sampled in 2004 (natural abundance and ^{15}N tracer) and 2010 (tracer) and $\delta^{15}\text{N}$ patterns were compared against litter and soil pools.
- Ectomycorrhizal fungi with hydrophobic ectomycorrhizas (e.g. *Cortinarius* and *Tricholoma*) acquired N from the Oea horizon or deeper. Taxa with hydrophilic ectomycorrhizas acquired N from the Oi horizon (*Russula* and *Lactarius*) or deeper (*Laccaria*, *Inocybe*, and *Amanita*). ^{15}N enrichment patterns for *Cortinarius* and *Amanita* in 2010 did not correspond to any measured bulk pool, suggesting that a persistent pool of active organic N supplied these two taxa. Saprotrophic fungi could be separated into those colonizing pine cones (*Baeospora*), wood, litter (Oi), and soil (*Ramariopsis*), with $\delta^{15}\text{N}$ of taxa reflecting substrate differences. ^{15}N enrichment between sources and sporocarps varied across taxa and contributed to $\delta^{15}\text{N}$ patterns.
- Natural abundance and ^{15}N tracers proved useful for tracking N from different depths into fungal taxa, generally corresponded to literature estimates of fungal activity within soil profiles, and provided new insights into interpreting natural abundance $\delta^{15}\text{N}$ patterns.

Introduction

In most forests, fungi are key players in the below-ground cycling of nitrogen (N) and carbon (C), with saprotrophic fungi mainly decomposing surface litter and woody debris and ectomycorrhizal fungi more active in organic and mineral horizons below the litter layer (Lindahl *et al.*, 2007). Yet information on how ectomycorrhizal fungi differ in exploration for N is scarce. In one approach that may provide some insight into how ectomycorrhizal taxa differ in their N acquisition strategies, Agerer (2001) proposed that ectomycorrhizal fungi could be classified based on the extent and method of hyphal exploration of the soil, with fungi possessing hydrophilic ectomycorrhizas usually classified into contact, short-distance, or medium-distance smooth exploration types, and fungi with hydrophobic ectomycorrhizas generally classified into medium-distance fringe and long-distance exploration types. Peay *et al.* (2011) proposed that these exploration types may correlate with strategies for acquiring C from roots, with medium- and long-distance exploration types more likely to colonize roots at greater depth or further from trees than short-distance exploration types. Exploration types may be

adapted for specific N forms, with taxa with hydrophobic ectomycorrhizas focused on insoluble forms of N such as protein or chitin, and taxa with hydrophilic ectomycorrhizas focused on soluble forms of N (Trudell *et al.*, 2004; Hobbie & Agerer, 2010; Lilleskov *et al.*, 2011).

Nitrogen isotopes (^{15}N : ^{14}N , expressed as $\delta^{15}\text{N}$) have been a useful tool in exploring fungal functioning. For example, several studies have demonstrated that saprotrophic fungi are lower in $\delta^{15}\text{N}$ than ectomycorrhizal fungi, presumably for two reasons: saprotrophic fungi assimilate primarily wood-derived or litter-derived N (^{15}N -depleted) and ectomycorrhizal fungi assimilate deeper soil N (^{15}N -enriched) (Kohzu *et al.*, 1999; Hobbie, 2005); ectomycorrhizal fungi transfer ^{15}N -depleted N to their host plants, leading to ^{15}N -enriched fungal biomass (Hobbie & Colpaert, 2003). Hobbie & Agerer (2010) observed that $\delta^{15}\text{N}$ patterns in sporocarps correlated with hydrophobicity of ectomycorrhizas, with hydrophobic exploration types $c. 3\text{‰}$ higher in ^{15}N than hydrophilic exploration types. Soil $\delta^{15}\text{N}$ increases with depth (Billings & Richter, 2006; Hobbie & Ouimette, 2009), and Agerer *et al.* (2012) correlated hyphal exploration depth in *Ramaria* taxa with fungal $\delta^{15}\text{N}$. The higher $\delta^{15}\text{N}$ in hydrophobic

than in hydrophilic ectomycorrhizal fungi may accordingly indicate that hydrophobic taxa are active at deeper depths than hydrophilic taxa.

One way to assess whether source N or transfer of ^{15}N -depleted N controls fungal $\delta^{15}\text{N}$ is to use ^{15}N labeling to generate data that can be compared against natural abundance patterns. ^{15}N labeling should change the $\delta^{15}\text{N}$ of N sources but not change ^{15}N effects of internal partitioning and N transfer to host plants. Therefore, by comparing natural abundance and tracer results, differences in the source $\delta^{15}\text{N}$ of different fungal taxa can be estimated, because we can assume that the ^{15}N enrichment (ε) between sporocarps and source N is the same in both cases. This information can then be used to estimate the ^{15}N enrichment between sources and sporocarps and the probable sources for sporocarp N. Although tracer ^{15}N labeling studies have also been useful to study N dynamics in many terrestrial ecosystems (Currie *et al.*, 1996; Hofmockel *et al.*, 2011; Wang & Macko, 2011), ^{15}N labeling has yet to be examined systematically in sporocarps of different fungal taxa, despite the potential insights into both fungal functioning and the interpretation of natural abundance $\delta^{15}\text{N}$.

Here, we used information on natural abundance and tracer ^{15}N patterns in sporocarps and other ecosystem pools at the Duke free air CO_2 enrichment (FACE) study in a *Pinus taeda* forest. Although we report differences in some cases between elevated and ambient CO_2 treatments in $\delta^{15}\text{N}$ of different pools, we do not focus on treatment effects of elevated CO_2 . Instead, we use the ^{15}N -applied label to provide general insights into fungal functioning that are unavailable from studies solely at natural abundance.

At the Duke FACE site, the forest floor was labeled with tracer concentrations of ^{15}N in 2003. Ecosystem samples were collected in 2003, 2004, 2005, and 2010, allowing the ^{15}N label to be tracked over time as it was assimilated by biota and migrated from surface to deeper horizons. In soil collected from 2003 to 2005, ^{15}N labeling patterns were initially high in surface layers and then decreased over time, while increasing over time at greater depths (Hofmockel *et al.*, 2011). We suggest that fungal ^{15}N labeling patterns will reflect two factors: the $\delta^{15}\text{N}$ of the soil and litter pools from which fungi obtain N; and ^{15}N enrichment between sources and sporocarps arising from ^{15}N and ^{14}N partitioning within fungi. Therefore, measuring fungal $\delta^{15}\text{N}$ at two different time points under changing background concentrations of ^{15}N labeling allows the second factor to be accounted for and can improve estimates of the soil horizons from which fungi acquire N (Fig. 1). This can be expressed mathematically in the following equations, where $\delta^{15}\text{N}_{\text{ft}1}$ and $\delta^{15}\text{N}_{\text{ft}2}$ are the fungal signatures at time t1 and t2, $\delta^{15}\text{N}_{\text{st}1}$ and $\delta^{15}\text{N}_{\text{st}2}$ are the source signatures at time t1 and t2, and ε is the ^{15}N enrichment between sources and sporocarps.

$$\varepsilon = (\delta^{15}\text{N}_{\text{ft}1} - \delta^{15}\text{N}_{\text{st}1}) / (1 + \delta^{15}\text{N}_{\text{st}1}) \quad \text{Eqn 1}$$

Multiplying both sides by $(1 + \delta^{15}\text{N}_{\text{st}1})$ and solving for $\delta^{15}\text{N}_{\text{ft}1}$ we get:

$$\delta^{15}\text{N}_{\text{ft}1} = \varepsilon + (\varepsilon + 1)\delta^{15}\text{N}_{\text{st}1} \quad \text{Eqn 2}$$

A similar equation can be written for the fungal signature at time t2:

$$\delta^{15}\text{N}_{\text{ft}2} = \varepsilon + (\varepsilon + 1)\delta^{15}\text{N}_{\text{st}2} \quad \text{Eqn 3}$$

Subtracting Eqn 3 from Eqn 2 gives:

$$\delta^{15}\text{N}_{\text{ft}1} - \delta^{15}\text{N}_{\text{ft}2} = (\varepsilon + 1)(\delta^{15}\text{N}_{\text{st}1} - \delta^{15}\text{N}_{\text{st}2}) \quad \text{Eqn 4}$$

By measuring isotopic patterns in ecosystem pools and fungi at the Duke FACE site, we tested the following hypotheses:

- (1) Saprotrophic fungi will generally use N sources found close to the surface and ectomycorrhizal fungi generally use deeper N. Ectomycorrhizal fungi with hydrophobic ectomycorrhizas will use N from deeper depths than taxa with hydrophilic ectomycorrhizas.
- (2) Both source differences and ^{15}N enrichment relative to sources contribute to ^{15}N differences among different fungal taxa, with ^{15}N enrichment relative to sources greater for ectomycorrhizal fungi than for saprotrophic fungi.

Materials and Methods

FACE experiment

The FACE experiment at the Duke Forest (35°58'41"N, 79°05'39"W 163 m elevation, Orange County, NC, USA) comprised six 30-m-diameter plots. Three experimental plots were fumigated with CO_2 to maintain the atmospheric CO_2 concentration at 200 ppm above ambient (i.e. 565 ppm at the start of the experiment). Three control plots were fumigated with ambient air only (365 ppm at the start of the experiment). The experiment began on 27 August 1996, and was continuous during daylight hours until fumigation ended on 1 November 2010. Additional details on the FACE operation are available in Hendrey *et al.* (1999) and at http://www.bnl.gov/face/Duke_Forest_FACE_Performance.asp.

The Duke Forest originated from 3-yr-old loblolly pine (*P. taeda*) seedlings that were planted in 1983 in a 2.4 × 2.0 m spacing. In 1996, the 16-yr-old pine trees were c. 14 m tall, had a density of 1600 stems ha⁻¹, and comprised 98% of the basal area of the stand. A deciduous understory layer consisted primarily of sweetgum (*Liquidambar styraciflua*), with some red maple (*Acer rubrum*), redbud (*Cercis canadensis*), and dogwood (*Cornus florida*). Topographic relief is <1° throughout the 32 ha site. Soils are derived from mafic bedrock and classified as Enon Series (fine, mixed, active, thermic Ultic Hapludalfs). Soils are slightly acidic (pH = 5.75 in 0.1 M CaCl_2), and have well-developed soil horizons with mixed clay mineralogy. Mean annual temperature is 15.5°C and mean annual precipitation is 1140 mm. Additional site details are available in Schlesinger & Lichter (2001) and Finzi *et al.* (2001).

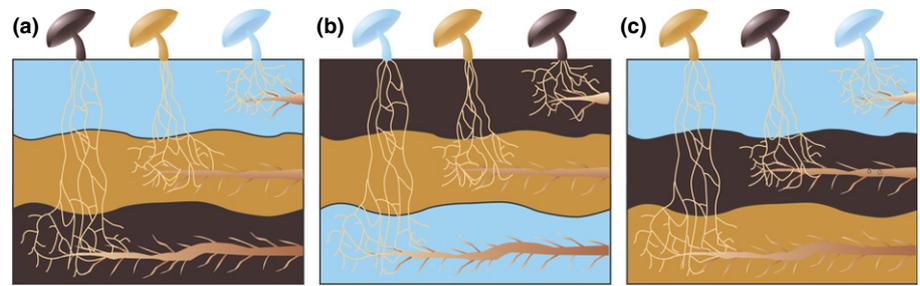


Fig. 1 Schematic of ^{15}N movement through the soil profile and into fungi at: (a) natural abundance, (b) 1 yr after ^{15}N labeling, and (c) 7 yr after ^{15}N labeling. Relative ^{15}N for soil horizons and fungi within each diagram indicated by colors, as follows: light blue, low ^{15}N ; tan, medium ^{15}N ; brown, high ^{15}N .

^{15}N labeling and fertilization experiment

In May 2003, 98 atom% ^{15}N was applied via backpack sprayers in each of the six plots, with 75% of the ^{15}N applied as NH_4Cl and 25% as KNO_3 at a rate of $15 \text{ mg } ^{15}\text{N m}^{-2}$ in $0.25 \text{ l H}_2\text{O}$. This represented 3% of the inorganic N pool (0–15 cm depth).

Starting in 2005, each of the six FACE plots was divided in two, and one-half of each was fertilized with N in a split-plot design. Here, we only used samples from the unfertilized plot sections.

Sporocarp collection

Sporocarps were collected in 2004 on 14 October, 25 October, and 27 October from ambient plots (^{15}N tracer), elevated CO_2 plots (^{15}N tracer), and from outside the experimental plots (ambient CO_2 , natural abundance ^{15}N). Sites were thoroughly surveyed and all sporocarps collected and identified. In 2010 sporocarps were collected from ambient and elevated CO_2 plots from 30 October until 3 December. The 2010 sampling was not quantitative. Fungal sporocarps were either air-dried or flash-frozen in the field and freeze-dried. Taxa were identified from macroscopic and microscopic morphological characteristics. Taxa were further classified as to whether they possessed hydrophobic or hydrophilic ectomycorrhizas (Agerer, 2006; Di Marino *et al.*, 2008). We have assumed that hydrophobicity of ectomycorrhizas is a characteristic that is conserved at the genus level (Unestam & Sun, 1995; Agerer, 2006).

Soil and other ecosystem pool collection and analysis

The Oi (forest litter), Oea, 0–15 cm, and 15–30 cm soil horizons were sampled in March 2003 (natural abundance) and in September 2003–2005 (^{15}N tracer; Hofmockel *et al.*, 2011). In addition to bulk analyses, dissolved organic N (DON), microbial biomass, and fine roots (< 2 mm) were analyzed for %N and $\delta^{15}\text{N}$. Identical sampling protocols were used in September 2010 for bulk analyses.

Samples from 2003 to 2005 were analyzed according to Hofmockel *et al.* (2011), with isotopic analyses at the UC Davis Stable Isotope Facility. Analyses of 2010 samples were treated similarly, but were analyzed at the UNH Stable Isotope Laboratory. Natural abundance $\delta^{15}\text{N}$ was measured on whole sporocarps from outside the FACE plots collected in 2004. For tracer ^{15}N measurements in FACE rings, we used whole sporocarps in 2004 and the average of sporocarp caps and stipes in 2010.

Values are reported in the standard notation ($\delta^{15}\text{N}$; ‰) relative to atmospheric N_2 for N, where $\delta^{15}\text{N} = (R_{\text{sample}}/R_{\text{standard}} - 1)$ and R is the molar ratio of $^{15}\text{N} : ^{14}\text{N}$ (Lajtha & Michener, 1994).

Statistical analysis

JMP (SAS, Cary, NC, USA) was used for statistical analysis. We used linear regression models to explore the factors regulating isotopic composition of fungal sporocarps. Data were not log-transformed, as fungal N isotopes passed a normality test for 2004 and 2010 samples (Shapiro–Wilk goodness of fit, $P = 0.071$ and 0.292 , respectively). Regression models in JMP explaining $\delta^{15}\text{N}$ values in 2004 were run separately for natural abundance and tracer data sets and for saprotrophic and ectomycorrhizal fungi. Linear regression models for $\delta^{15}\text{N}$ values in 2010 combined saprotrophic and ectomycorrhizal sporocarps, with CO_2 treatment and genus as the explanatory variables. Because %N can correlate with sporocarp $\delta^{15}\text{N}$ at natural abundance (Hobbie *et al.*, 2012), potential explanatory variables at natural abundance included genus, hydrophobicity, CO_2 treatment, and N concentration, whereas tracer ^{15}N data sets did not include N concentration. For the categorical variables of CO_2 treatment and hydrophobicity, JMP assigned a value of -1 for elevated CO_2 and hydrophobic taxa, with ambient CO_2 and hydrophilic taxa receiving a value of $+1$. Interaction terms were included in models unless they resulted in singular terms. To test for statistical differences between two pools, t -tests assuming unequal variance were used.

Linear regression models of $\delta^{15}\text{N}$ in soil profiles included horizon and CO_2 treatment as explanatory variables. For statistical comparisons of $\delta^{15}\text{N}$ in 2004 among fine roots, DON, and microbial biomass in different horizons, paired t -tests were used to compare specific soil pools, with CO_2 treatment included as an explanatory variable.

Results

Natural abundance isotopic patterns in soil and fungi

Isotopic values for bulk soil at natural abundance as sampled in March 2003 under ambient and elevated CO_2 are presented in Table 1. In a regression model that included horizon and CO_2 treatment, horizon affected $\delta^{15}\text{N}$ ($P < 0.001$), with the Oi horizon the lowest and the mineral soil at 15–30 cm the highest. CO_2 treatment did not affect soil $\delta^{15}\text{N}$ ($P = 0.284$). Complete statistical results are given in Supporting Information, Table S1.

Table 1 $\delta^{15}\text{N}$ (in ‰) in ambient and elevated CO_2 plots at the Duke Forest free air CO_2 enrichment (FACE) site for litter and soil pools, 2003–2010 (\pm SE)

Year	Oi		Oea		0–15 cm		15–30 cm	
	Ambient	Elevated	Ambient	Elevated	Ambient	Elevated	Ambient	Elevated
2003 ^a	-5.0 ± 0.3	-4.2 ± 0.2	-3.0 ± 0.3	-3.1 ± 0.4	2.6 ± 0.3	2.0 ± 0.8	6.6 ± 0.4	5.5 ± 0.2
2003 ^b	340.3 ± 31.5	362.5 ± 32.0	90.9 ± 6.8	87.6 ± 27.8	7.1 ± 0.3	6.8 ± 0.6	14.0 ± 0.5	15.6 ± 1.8
2004	51.9 ± 8.8	47.2 ± 14.4	74.4 ± 14.2	67.6 ± 9.7	5.6 ± 1.1	4.7 ± 0.5	12.4 ± 0.3	12.2 ± 1.3
2005	14.6 ± 1.5	12.5 ± 1.2	67.9 ± 9.1	59.4 ± 3.6	11.7 ± 1.8	8.2 ± 1.0	4.8 ± 0.6	6.6 ± 0.4
2010	12.2 ± 2.2	10.3 ± 1.3	18.5 ± 2.4	18.0 ± 0.8	12.3 ± 1.4	11.7 ± 1.0	9.8 ± 0.3	8.2 ± 0.3

^aNatural abundance values, sampled in March 2003, before ^{15}N labeling.

^bSeptember 2003, after ^{15}N labeling.

Fungi were sampled for natural abundance in 2004 outside the FACE plots. At natural abundance $\delta^{15}\text{N}$, ectomycorrhizal taxa were higher than saprotrophic fungi (t -test, $P < 0.001$; $2.9 \pm 0.3\text{‰}$, $n = 94$ vs $-0.5 \pm 0.2\text{‰}$, $n = 70$). Among ectomycorrhizal taxa, *Amanita* and *Russula* were the lowest and *Sistotrema* the highest. Taxa with hydrophobic ectomycorrhizas were significantly higher in ^{15}N than were taxa with hydrophilic ectomycorrhizas (t -test, $P < 0.001$; $6.9 \pm 0.3\text{‰}$, $n = 17$ vs $2.1 \pm 0.2\text{‰}$, $n = 77$). In saprotrophic fungi, sporocarp $\delta^{15}\text{N}$ varied with substrate, with the soil fungus *Ramariopsis* the highest, litter decay fungi intermediate, and the cone-colonizing fungus *Baeospora* and wood decay fungi the lowest (Fig. 2). In a regression model of sporocarp $\delta^{15}\text{N}$ that included sporocarp %N and genus, %N significantly affected $\delta^{15}\text{N}$ of saprotrophic fungi but not ectomycorrhizal fungi, whereas genus significantly affected $\delta^{15}\text{N}$ in both ectomycorrhizal and saprotrophic fungi (Table 2). In saprotrophic fungi, %N accounted for 16% and genus accounted for 84% of the explained variance. In Fig. 2, %N and $\delta^{15}\text{N}$ are plotted for different genera of saprotrophic and ectomycorrhizal fungi, showing that $\delta^{15}\text{N}$ is generally in the

order hydrophobic ectomycorrhizal taxa > hydrophilic ectomycorrhizal taxa > saprotrophic taxa, with %N generally higher in saprotrophic taxa than in ectomycorrhizal taxa.

Tracing ^{15}N labeling in soil and fungi

After tracer application in May 2003, $\delta^{15}\text{N}$ values in the Oi horizon were initially very high (*c.* 350‰), but declined dramatically over the next 2 yr (Table 1). By contrast, the high values in the Oea horizon in 2003 (*c.* 90‰) declined more slowly. The low $\delta^{15}\text{N}$ values of the shallow mineral soil horizon (0–15 cm) increased slightly from 2003 to 2005, while decreasing over this period in the 15–30 cm horizon. $\delta^{15}\text{N}$ values in fine roots, DON, and microbial biomass in 2004 were much higher in the Oea horizon than in the 0–15 cm and 15–30 cm horizons (Fig. 3). In the Oea horizon, the $\delta^{15}\text{N}$ order was roots \ll DON < microbes, with all three pools differing significantly from one another in paired t -tests (Table S3). These three pools did not differ significantly in paired t -tests at 0–15 cm, but at 15–30 cm microbes were again higher than roots (paired t -test, $n = 6$, $P = 0.001$). None of these pools differed by CO_2 treatment.

By 2010, soil $\delta^{15}\text{N}$ was lower in the Oi horizon than in the Oea horizon, and then further decreased in the mineral soil (Table 1). Depth ($P < 0.001$), but not CO_2 treatment ($P = 0.358$), affected soil $\delta^{15}\text{N}$ (Table S1).

Application of ^{15}N tracer in 2003 greatly affected the $\delta^{15}\text{N}$ of sporocarps collected in 2004. Both symbiotic status ($P = 0.002$) and CO_2 treatment ($P = 0.009$) significantly affected sporocarp $\delta^{15}\text{N}$, with no significant interaction between these two variables ($P = 0.084$). Saprotrophic fungi were significantly higher in $\delta^{15}\text{N}$ in elevated CO_2 treatments than in ambient treatments (t -test, $P = 0.008$), but ectomycorrhizal fungi were not (t -test, $P = 0.191$) (Fig. 4). In ambient CO_2 plots, ectomycorrhizal fungi averaged $69.9 \pm 7.2\text{‰}$ ($n = 33$) and saprotrophic fungi averaged $79.8 \pm 8.3\text{‰}$ ($n = 36$), whereas in elevated CO_2 plots, ectomycorrhizal fungi averaged $76.5 \pm 6.1\text{‰}$ ($n = 45$) and saprotrophic fungi averaged $112.1 \pm 7.9\text{‰}$ ($n = 32$). The $\delta^{15}\text{N}$ among saprotrophic genera did not differ significantly, whereas the ectomycorrhizal genera *Lactarius* and *Russula* were significantly higher than several other ectomycorrhizal genera, including *Inocybe*, *Laccaria*, and *Cortinarius* (complete statistical model given in Table S4). Sporocarps with hydrophilic ectomycorrhizas (e.g.

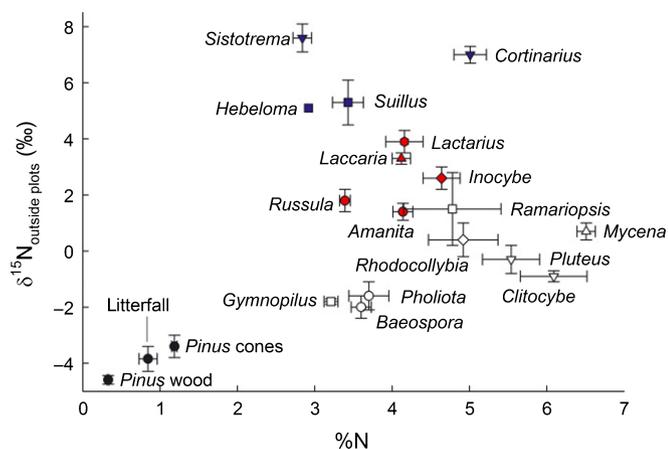


Fig. 2 Natural abundance $\delta^{15}\text{N}$ plotted against %N (\pm SE) of different genera of ectomycorrhizal and saprotrophic fungi collected outside of free air CO_2 enrichment (FACE) rings across all plots. Data are from 2004 collections as reported for %N in Table S2. Closed blue symbols, ectomycorrhizal with hydrophobic ectomycorrhizas; closed red symbols, ectomycorrhizal with hydrophilic ectomycorrhizas; open symbols, saprotrophic. Values for *Pinus* litter, *Pinus* cones, and wood are also plotted (\pm SE).

Table 2 Regression model of $\delta^{15}\text{N}$ of saprotrophic and ectomycorrhizal fungi as a function of genus and sporocarp %N at natural abundance in 2004

Saprotrophic			Ectomycorrhizal		
Parameter	Value \pm SE	<i>P</i>	Parameter	Value \pm SE	<i>P</i>
Intercept	-2.5 ± 0.8	0.004	Intercept	4.7 ± 1.1	0.004
%N	0.42 ± 0.18	0.022	%N	-0.12 ± 0.27	0.660
Genus	–	0.003	Genus	–	< 0.001
Genus-specific effects					
<i>Baeospora</i> (C)	-0.9 ± 0.5	0.098	<i>Amanita</i> (phi)	-2.8 ± 0.5	< 0.001
<i>Clitocybe</i> (L)	-0.9 ± 0.7	0.181	<i>Cortinarius</i> (pho)	2.9 ± 0.7	< 0.001
<i>Gymnopilus</i> (W)	-0.7 ± 0.4	0.067	<i>Hebeloma</i> (pho)	0.8 ± 1.8	0.602
<i>Marasmius</i> (L)	-1.1 ± 1.2	0.365	<i>Inocybe</i> (phi)	-1.5 ± 0.8	0.031
<i>Mycena</i> (L)	0.5 ± 0.5	0.322	<i>Laccaria</i> (phi)	-1.0 ± 0.9	0.191
<i>Pholiota</i> (W)	-0.6 ± 0.5	0.241	<i>Lactarius</i> (phi)	-0.3 ± 0.7	0.618
<i>Pluteus</i> (W)	0.0 ± 0.6	0.976	<i>Russula</i> (phi)	-2.5 ± 0.4	< 0.001
<i>Ramariopsis</i> (S)	2.5 ± 0.8	0.002	<i>Sistotrema</i> (pho)	3.3 ± 0.8	< 0.001
<i>Rhodocollybia</i> (L)	1.1 ± 0.5	0.033			

Only data on sporocarps from outside free air CO₂ enrichment (FACE) rings were used in the model. Adjusted *r*² was 0.529 (*n* = 70, *P* < 0.001) for saprotrophic fungi and 0.574 (*n* = 94, *P* < 0.001) for ectomycorrhizal fungi for the entire model. Values were estimated from the regression models analyzed for $\delta^{15}\text{N}$. Preferred substrates for saprotrophic fungi are indicated in parentheses after the genus name, as follows: C, pine cones; L, leaf litter; W, wood; S, soil. Hydrophobicity of ectomycorrhizas is indicated after the genus, as follows: phi, hydrophilic; pho, hydrophobic. Table S3 has complete statistical information. Statistical significance (*P* < 0.05) is indicated in bold.

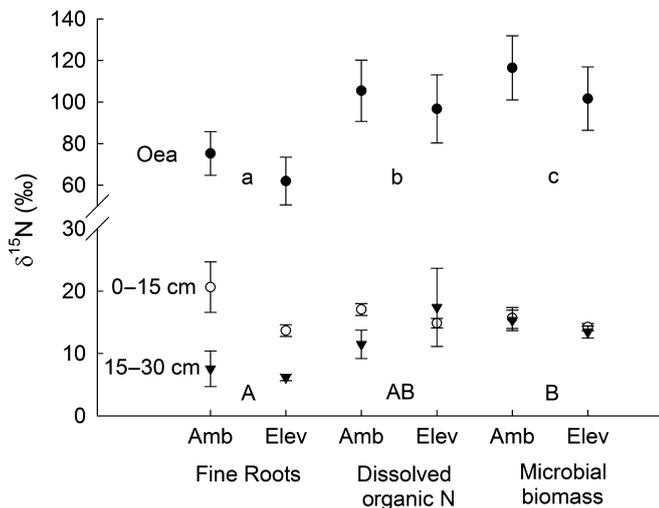


Fig. 3 Nitrogen (N) isotope values for fine roots, dissolved organic N, and microbial biomass in 2004 under ambient (amb) and elevated (elev) CO₂ treatments after ¹⁵N labeling (\pm SE). The sampling horizon is indicated in the figure by closed circles (Oea), open circles (0–15 cm), or closed triangles (15–30 cm). Statistically significant differences in paired *t*-test comparisons within a horizon are shown by lowercase letters for Oea and uppercase letters for 15–30 cm, with different letters indicating differences.

Lactarius, *Russula*) averaged 20.8‰ higher in $\delta^{15}\text{N}$ than sporocarps with hydrophobic ectomycorrhizas (e.g. *Cortinarius*) (*t*-test, *P* = 0.034).

In 2010, 30 sporocarps were collected from unfertilized plots. In a model exploring the factors influencing fungal $\delta^{15}\text{N}$ in 2010, genus affected fungal $\delta^{15}\text{N}$ (*P* = 0.041) but CO₂ treatment did not (*P* = 0.577). Some taxa with hydrophilic ectomycorrhizas were high in $\delta^{15}\text{N}$ (28‰ for *Amanita* and 27‰ for *Laccaria*) and some were low (15‰ for *Russula* and 18‰ for *Lactarius*),

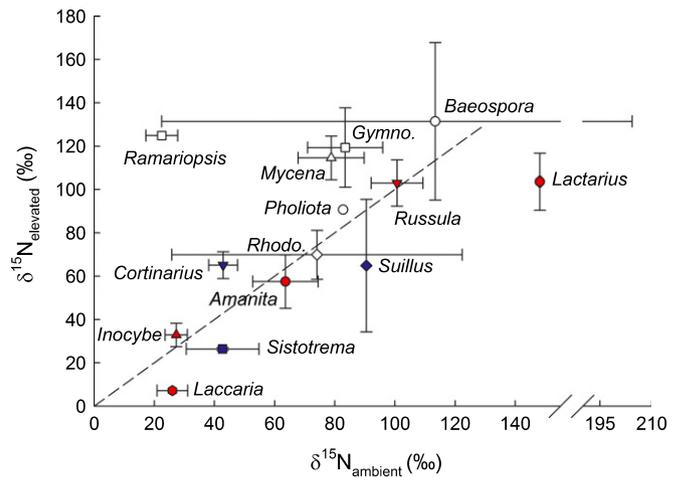


Fig. 4 Nitrogen (N) isotopes of different genera of ectomycorrhizal and saprotrophic fungi (\pm SE) in ambient and elevated CO₂ treatments in 2004. A 1 : 1 line (dashed) is also shown. Closed blue symbols, ectomycorrhizal with hydrophobic ectomycorrhizas; closed red symbols, ectomycorrhizal with hydrophilic ectomycorrhizas; open symbols, saprotrophic. *Rhodo.*, *Rhodocollybia*; *Gymno.*, *Gymnopilus*.

whereas both taxa with hydrophobic ectomycorrhizas were high in $\delta^{15}\text{N}$ (39‰ for *Cortinarius* and 24‰ for *Tricholoma*). Hydrophobic taxa averaged 10.7‰ higher than hydrophilic taxa (*t*-test, *P* = 0.004). A single *Hygrophorus* was at 17.9‰ and a single *Rhodocollybia* was at 15.8‰. Between 2004 and 2010 in ¹⁵N-labeled plots, *Lactarius* declined by 72‰, *Russula* by 60‰, and the saprotrophic *Rhodocollybia* by 56‰. *Cortinarius* declined 15‰ on average (Figs 4, 5).

When $\delta^{15}\text{N}$ values of soil pools and of sporocarps at natural abundance in 2004 and in 2010 are plotted, we can estimate the $\delta^{15}\text{N}$ of the source N if we assume that ¹⁵N enrichment from source to sporocarp is the same for 2004 and 2010 samples

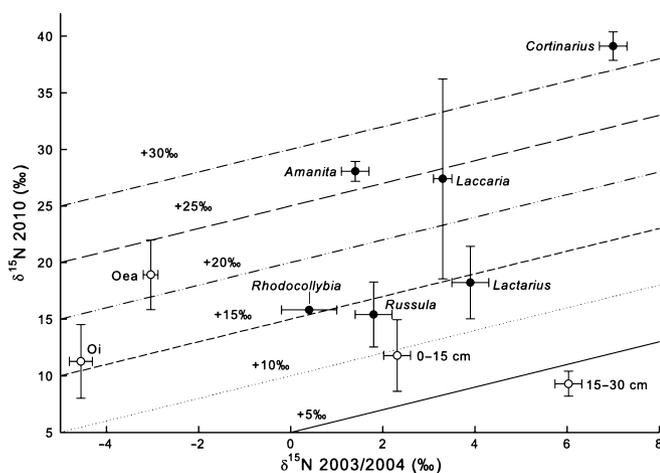


Fig. 5 Comparison of $\delta^{15}\text{N}$ values (\pm SE) of five taxa of fungi and four soil pools between natural abundance samples in 2003 (soils) and 2004 (fungi) and ^{15}N -enriched samples in 2010 (soils and fungi). Lines of constant ^{15}N enrichment between 2004 and 2010 samples are shown as dotted lines between 5 and 30‰.

(Eqns 1–4). In the resulting Fig. 5, lines of constant ^{15}N enrichment between 2004 and 2010 samples were used to suggest potential sources. The patterns indicate that, between 2004 and 2010, ^{15}N enrichment of *Rhodocollybia*, *Lactarius*, and *Russula* was similar to the Oi horizon and ^{15}N enrichment for *Laccaria* was similar to the Oea horizon, whereas ^{15}N enrichment values for *Amanita* and *Cortinarius* were greater than that of any measured bulk pool.

Discussion

Depth distribution of fungal activity

Because supplies of energy-rich C are largest in the surface litter, saprotrophic fungi are commonly found there and should obtain N from those sources (Lindahl *et al.*, 2007). By contrast, ectomycorrhizal fungi acquire labile C from plant sugars, and therefore have a relative advantage over free-living heterotrophs in nutrient acquisition as the energy content of organic matter declines at deeper depths. Ectomycorrhizal fungi in the surface litter may rely on enzymatic breakdown by free-living saprotrophic microbes (Lindahl *et al.*, 2005), whereas those active at greater depths presumably rely on their own enzymatic capabilities. Within ectomycorrhizal fungi, the enzymatic capabilities to acquire N from polymeric substances such as protein and chitin appear greater for hydrophobic than for hydrophilic taxa (Lilleskov *et al.*, 2011). Accordingly, taxa with hydrophobic ectomycorrhizas should acquire N from greater depth and, because soil $\delta^{15}\text{N}$ increases with depth, be higher in $\delta^{15}\text{N}$ than taxa with hydrophilic ectomycorrhizas.

To assess whether different fungal groups acquired soil N from different depths, we compared natural abundance $\delta^{15}\text{N}$ in fungal taxa in 2004 with soil in 2003 and compared fungi with soil from ^{15}N -labeled plots sampled in 2003–2005 and in 2010 (Table 1, Fig. 5). In apparent support of our first hypothesis, fungal $\delta^{15}\text{N}$

patterns at natural abundance were at their lowest in saprotrophic fungi, intermediate in hydrophilic ectomycorrhizal fungi, and at their highest in hydrophobic ectomycorrhizal fungi, presumably reflecting the use of N from shallow litter horizons or wood by saprotrophic fungi and two taxa of hydrophilic ectomycorrhizal fungi (*Lactarius* and *Russula*), and assimilation of deeper soil N by both hydrophilic (*Laccaria* and *Amanita*) and hydrophobic ectomycorrhizal taxa.

After plots were labeled with ^{15}N in 2003, $\delta^{15}\text{N}$ values were initially very high in the leaf litter (*c.* 350‰), but quickly declined (*c.* 50‰ by 2004) as new litter of low $\delta^{15}\text{N}$ was added, ^{15}N -labeled litter decomposed, and its N was incorporated into the Oea horizon (Table 1). In 2004, the year following application of the tracer, fungal $\delta^{15}\text{N}$ followed expected patterns, with $\delta^{15}\text{N}$ of saprotrophic fungi > hydrophilic ectomycorrhizal fungi > hydrophobic ectomycorrhizal fungi, again reflecting use of shallow soil N for saprotrophic and hydrophilic ectomycorrhizal fungi and use of deeper soil N for hydrophobic (and some hydrophilic) ectomycorrhizal fungi. However, *Russula*, *Lactarius*, and most of the saprotrophic fungi appeared to at least partially sample the 2003 litter cohort, as their $\delta^{15}\text{N}$ values in 2004 in ^{15}N -labeled plots were higher than any measured bulk pool for that year but lower than Oi values for 2003. Alternatively, DON and microbial N may have supplied N to these fungi, as they generally had similar $\delta^{15}\text{N}$ values as the DON and microbial N in the Oea horizon in 2004 (Fig. 3); unfortunately, DON and microbial biomass were not measured in the Oi horizon, so precise comparisons are not possible. In contrast to these patterns, the hydrophilic taxa *Inocybe*, *Amanita*, and *Laccaria* had lower $\delta^{15}\text{N}$ values than DON or microbial N from the Oea horizon, suggesting a possible contribution from mineral horizon N (Fig. 4, Table 1).

Finally, in 2010, 7 yr after ^{15}N tracer was applied, soil and fungal $\delta^{15}\text{N}$ patterns had changed, with the applied ^{15}N migrating deeper down the soil profile. Between 2004 and 2010, fungal $\delta^{15}\text{N}$ decreased more in the saprotrophic *Rhodocollybia* and in the hydrophilic *Lactarius* and *Russula* than in the hydrophobic *Cortinarius* and the hydrophilic *Amanita* and *Laccaria*. This indicates that these latter two taxa assimilated deeper N than *Lactarius* and *Russula* but did not increase in ^{15}N relative to sources as much as other ectomycorrhizal taxa. These patterns were consistent with saprotrophic fungi and some hydrophilic ectomycorrhizal fungi using shallow soil N. In addition, the hydrophobic *Tricholoma* in 2010 was also high in $\delta^{15}\text{N}$, suggesting that taxa with hydrophobic ectomycorrhizas generally explore deeper horizons. High $\delta^{15}\text{N}$ in *Tricholoma* have been reported before and attributed to high ^{15}N enrichment between the mycelia and sporocarps (Zeller *et al.*, 2007); here, our results indicate that acquisition of deeper, ^{15}N -enriched N partially explains the high $\delta^{15}\text{N}$ values of *Tricholoma* sporocarps.

To infer potential N sources, we paired $\delta^{15}\text{N}$ measurements in 2004 at natural abundance with 2010 measurements from the FACE rings (Fig. 5). We assumed comparable ^{15}N enrichment between sources and fungi for both sampling times. Lines of constant ^{15}N enrichment for 2010 relative to 2004 indicate the probable $\delta^{15}\text{N}$ at natural abundance of the source N, which could

then be compared against the measured bulk soil pools. Based on high ^{15}N labeling patterns in 2010 and the assumed similar ^{15}N enrichment relative to sources in 2004 (natural abundance) and 2010 (^{15}N tracer applications), *Amanita*, *Cortinarius*, and *Laccaria* acquired N from deeper in the soil profile (where ^{15}N is higher) than *Lactarius*, *Russula*, and the litter-inhabiting *Rhodocollybia*. According to Eqn 4, the $\delta^{15}\text{N}$ differences from 2004 to 2010 between sources and between sporocarps are not exactly equivalent, as they are related by the factor $(1 + \epsilon)$. However, if the estimated ^{15}N enrichment value (ϵ) is 20‰, then they would only differ by 0.2‰ in ^{15}N enrichment.

High ^{15}N enrichments (between 25 and 32‰) of ^{15}N -labeled 2010 values relative to natural abundance values in 2004 for *Amanita* and *Cortinarius* (as indicated by isolines on Fig. 5) are higher than any estimated soil pool enrichment. This implies that these two taxa access N pools that have retained more ^{15}N than any measured bulk soil pool. Although additional pools other than bulk were not measured in 2010, the $\delta^{15}\text{N}$ values in 2004 in the FACE plots for DON and microbial N were *c.* 30‰ higher than concurrently measured bulk values, indicating that pools such as these could potentially be similarly elevated relative to bulk pools in 2010. Such differences in $\delta^{15}\text{N}$ between bulk pools and other soil fractions have previously been reported from ^{15}N labeling experiments (Zeller & Dambrine, 2011), and presumably reflect variability in turnover times of different biochemical or biophysical fractions.

Resource partitioning in saprotrophic fungi

The saprotrophic taxa colonized different substrates, with *Baeospora myosura* commonly colonizing conifer cones (Rayner *et al.*, 1985), *Gymnopilus*, *Pholiota*, *Pluteus*, and *Oligoporus* commonly colonizing wood (Pouska *et al.*, 2010), and *Mycena*, *Rhodocollybia*, *Marasmius*, and *Clitocybe* commonly colonizing litter (Rayner *et al.*, 1985). *Baeospora* and the wood decay fungi were similar in natural abundance $\delta^{15}\text{N}$ (Fig. 2), suggesting that these taxa are using N of similar $\delta^{15}\text{N}$, with that N presumably relatively unprocessed and drawn from undecayed pine cones or wood. However, we point out that the litter decay fungi *Clitocybe* and *Marasmius* had similar values to *Baeospora* and the wood decay fungi in the regression model that included %N (Table 2). Some of the $\delta^{15}\text{N}$ variability among taxa should accordingly be driven by variable proportions of ^{15}N -enriched protein and ^{15}N -depleted chitin, with higher %N and $\delta^{15}\text{N}$ presumably correlating with higher relative protein content (Hobbie *et al.*, 2012).

Pine cones closely matched *Baeospora* in 2004 (natural abundance), with a measured ^{15}N enrichment in *Baeospora* relative to pine cones of 1.4‰. Higher $\delta^{15}\text{N}$ values for the litter decay fungi *Rhodocollybia* and *Mycena* than for *Baeospora*, wood decay fungi, and the other two litter decay taxa suggest some degree of ^{15}N enrichment during initial stages of decay of litter and subsequent acquisition of this N by *Rhodocollybia* and *Mycena*. The relatively high $\delta^{15}\text{N}$ in *Ramariopsis* may indicate foraging for N in deeper soil horizons than other saprotrophic taxa. Whereas the other saprotrophic taxa feed on wood or fresh litter, *Ramariopsis kunzei* is typically reported as fruiting on ground, often buried below

the surface litter (Arora, 1986), implying that it obtains N from deeper sources than fresh litter.

Depth distribution of ectomycorrhizal fungi and comparisons with other studies

Studies that have examined the depth distribution through soil profiles of ectomycorrhizal fungi can provide additional insights. In general agreement with the patterns reported here, some studies detected taxa of *Lactarius* and *Russula* in litter horizons or coarse woody debris, with *Amanita*, *Cortinarius*, *Inocybe*, and *Laccaria* found in deeper horizons (Dickie *et al.*, 2002; Landeweert *et al.*, 2003; Tedersoo *et al.*, 2003). Although patterns were not as clear in other studies (summarized in Table S5), we note that fungal presence is unlikely to correlate precisely with fungal N source. For example, the density of the N resource will generally be greater in organic horizons than in mineral horizons, and therefore N uptake per unit of fungal biomass may be greater in organic horizons.

Some indication of the greater spatial extent of exploratory hyphae in taxa with hydrophobic vs hydrophilic ectomycorrhizas can be inferred from the study of Genney *et al.* (2006), in which fungal identity of both colonized root tips and of extraradical hyphae were assessed. In this study, 88% of the *Cortinarius* observations were of extraradical hyphae, compared with only 31% of the *Lactarius rufus* observations and 34% of the *Cenococcum geophilum* observations. *Cortinarius* has hydrophobic ectomycorrhizas, whereas *Lactarius* and *Cenococcum* have hydrophilic ectomycorrhizas. Similarly, a reanalysis of data in Peay *et al.* (2011) from *Pinus*-associated communities at forest edges and interior locations (10 m inside the edge) for the genera found at Duke FACE indicates that 77% of hydrophilic ectomycorrhizas were found at interior locations, compared with only 46% of hydrophobic ectomycorrhizas, with interior locations presumably associated with less extensive extraradical hyphal development than edge locations. As suggested in Hobbie & Agerer (2010), the apparently greater spatial exploration in hydrophobic than in hydrophilic taxa may lead to higher relative sequestration of ^{15}N -depleted chitin in transport structures (extraradical hyphae) and the formation of ^{15}N -enriched N within fungi that is metabolically available for sporocarp formation, therefore contributing to ^{15}N enrichment of hydrophobic relative to hydrophilic taxa.

Conclusions

We conclude that saprotrophic fungi primarily assimilate N found in the Oi horizon (with the possible exception of *Ramariopsis*), and fungi with hydrophobic ectomycorrhizas primarily assimilate N from deeper horizons such as the Oea, supporting Hypothesis 1. However, taxa with hydrophilic ectomycorrhizas appeared to assimilate from the Oi horizon in some cases (e.g. *Russula* and *Lactarius*) and in other cases from deeper horizons (e.g. *Amanita* and *Laccaria*), which only partially supports Hypothesis 1. These differences in N acquisition largely corresponded to N isotope patterns at natural abundance.

Estimated ^{15}N enrichment relative to bulk substrates was in the order: *Lactarius* > *Russula* > *Rhodocollybia* > *Baeospora*, and *Cortinarius* > *Amanita*. It appears that ^{15}N enrichment relative to source is variable among taxa, and therefore contributes, along with source $\delta^{15}\text{N}$, to ^{15}N differences among taxa in sporocarps, which supports Hypothesis 2. Higher $\delta^{15}\text{N}$ values in some fungi than in any measured ecosystem pool suggest that fungi access N pools that are not captured in the relatively gross characterizations of litter and soil $\delta^{15}\text{N}$ distributions presented here. In previous work, ^{15}N enrichments have been analytically linked to partitioning of N between plants and fungi, within different fungal structures, such as extraradical hyphae vs sporocarps, or between protein and chitin (Hobbie *et al.*, 2005, 2012). The results here suggest one approach to generate the data needed to test these analytical approaches.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Regression model for $\delta^{15}\text{N}$ in soil profiles in May 2003 in FACE plots (natural abundance, before ^{15}N additions) and in 2010

Table S2 Average nitrogen concentration and $\delta^{15}\text{N}$ values for sporocarp taxa sampled in 2004 from outside plots (natural abundance)

Table S3 Paired *t*-tests in the Oea and 0–15 cm horizons for DON, microbial biomass, and roots

Table S4 Regression model of $\delta^{15}\text{N}$ values for saprotrophic and ectomycorrhizal fungi in ambient and elevated CO_2 treatments in 2004

Table S5 Studies of depth distribution of ectomycorrhizal fungal taxa

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