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4 **Depleted ^{15}N in hydrolysable-N of arctic soils and its implication for mycorrhizal fungi-**
5 **plant interaction**

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18 **Key Words**

19 ^{15}N ; arctic tundra; mycorrhizal fungi; decomposition; hydrolysable amino acids; plant-fungal

20 interaction

1 **Abstract**

2 Uptake of nitrogen (N) via root-mycorrhizal associations accounts for a significant portion of
3 total N supply to many vascular plants. Using stable isotope ratios ($\delta^{15}\text{N}$) and the mass balance
4 among N pools of plants, fungal tissues, and soils, a number of efforts have been made in recent
5 years to quantify the flux of N from mycorrhizal fungi to host plants. Current estimates of this
6 flux for arctic tundra ecosystems rely on the untested assumption that the $\delta^{15}\text{N}$ of labile organic
7 N taken up by the fungi is approximately the same as the $\delta^{15}\text{N}$ of bulk soil. We report here
8 hydrolysable amino acids are more depleted in ^{15}N relative to hydrolysable ammonium and
9 amino sugars in arctic tundra soils near Toolik Lake, Alaska, USA. We demonstrate, using a
10 case study, that recognizing the depletion in ^{15}N for hydrolysable amino acids ($\delta^{15}\text{N} = -5.6\text{‰}$ on
11 average) would alter recent estimates of N flux between mycorrhizal fungi and host plants in an
12 arctic tundra ecosystem.

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16 **Key Words**

17 ^{15}N ; arctic tundra; decomposition; hydrolysable amino acids; mycorrhizal fungi; nitrogen
18 transfer; plant-fungal interaction

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20 **Abbreviations**

21 C, carbon; HAA, hydrolysable amino acids; HAS, hydrolysable amino sugars; HNH_4^+ ,
22 hydrolyzable ammonium; N, nitrogen; NH_4^+ , ammonium; NO_3^- , nitrate; TDN, total dissolved N

1 **Introduction**

2 Traditional approaches to estimate available N to plants have relied on determining the
3 amount and rate of production of inorganic N (NH_4^+ and NO_3^-) in soil. In recent years, however
4 increasing evidence suggests that some plant groups directly use organic N, such as amino acids,
5 bypassing the mineralization process especially under strong N limitation (Schimel & Chapin
6 1996; Lipson & Monson 1998; McKane et al. 2002). A number of laboratory and field studies
7 indicate that mycorrhizal fungi are important in this direct uptake of organic N not only via
8 expansion of the absorptive surface area of roots but also via enzymatic breakdown of large
9 organic-N polymers into monomers (Smith & Read 1997). Schimel & Bennett (2004) discussed
10 the potential roles of mycorrhizal fungi both as an agent of organic-N depolymerization (by
11 releasing extracellular enzymes) and as a direct conduit between organic-N polymers and plants
12 by immediately capturing resulting monomers. Some ericoid mycorrhizal and ectomycorrhizal
13 fungi excrete extracellular enzymes that hydrolyze organic-N polymers (e.g., protein, chitin) and
14 directly take up the resulting monomers and oligomers, such as amino acids (Abuzinadah &
15 Read 1986a), amino sugars (Kerley & Read 1995), and oligopeptides (Hobbie & Wallander
16 2005) as N sources. Additional evidence from laboratory labeling experiments indicates that
17 amino acids taken up by mycorrhizal fungi are subsequently transferred to the host (Taylor et al.
18 2004).

19 To understand the contribution of organic N to the plant's N economy, quantifying the
20 proportion of plant N that comes via fungi is critical. Efforts to quantify N transfer between
21 fungi and plants have taken advantage of differences in stable-isotope ratios across plant, fungal,
22 and soil N. The stable isotope ratio ($\delta^{15}\text{N}$) of plant N varies consistently among species and
23 plant families in arctic, taiga, and temperate ecosystems. Generally, foliar N is strongly depleted

1 in ^{15}N in ericoid and ectomycorrhizal plants ($\delta^{15}\text{N} = -8$ to -3 ‰) relative to bulk soil and to
2 inorganic N in pore water ($\delta^{15}\text{N} = -1$ to $+1$ ‰), whereas foliar $\delta^{15}\text{N}$ of nonmycorrhizal plants (-2
3 to $+3$ ‰) is similar to that of bulk soil or inorganic N (Nadelhoffer et al. 1996; Michelsen et al.
4 1998; Hobbie et al. 2000; Hobbie & Hobbie 2006). More recently, it has been shown that
5 mycorrhizal fruiting bodies are significantly enriched in ^{15}N relative to bulk soil and
6 nonmycorrhizal plants in these ecosystems (Taylor et al. 1997; Hobbie et al. 1999; Hobbie et al.
7 2000; Hobbie & Hobbie 2006; Zeller et al. 2007) and that mycelia are more depleted in ^{15}N than
8 their fruiting bodies by as much as 10 ‰ (e.g., Clemmensen et al. 2006, Zeller et al. 2007).

9 In theory, differences in natural abundance of ^{15}N across available soil N, fungi, and plants
10 should allow identification of N sources for fungi and plants, if isotopic fractionation against ^{15}N
11 among N pools is taken into account. Hobbie et al. (2000) examined $\delta^{15}\text{N}$ variations of leaves of
12 non-N-fixing plants and extractable ammonium-N (NH_4^+ -N) across the gradient of forest
13 succession in Alaska, USA. Based on the relatively constant $\delta^{15}\text{N}$ signature of soil NH_4^+ -N at
14 approximately 0 ‰ and a mass balance of ^{15}N between plants and inorganic N, they
15 hypothesized that the variation in $\delta^{15}\text{N}$ of foliar N was due to isotopic fractionation upon transfer
16 of N from mycorrhizal fungi to host plants. Hobbie & Hobbie (2006) estimated the proportion of
17 plant N that comes from mycorrhizal fungi based on the $\delta^{15}\text{N}$ of bulk soil, exchangeable
18 inorganic N, fungi, plants, and estimated isotopic fractionation for transamination (at least 8 to
19 10 ‰; Macko et al. 1986; Werner & Schmidt 2002). These studies assume that $\delta^{15}\text{N}$ signatures
20 of labile organic N compounds in soil are relatively uniform and resemble those of bulk soil or
21 inorganic N.

22 Up until now, metabolic fractionation of N isotopes has been presumed to be the single most
23 important process that causes the observed differences in $\delta^{15}\text{N}$ signatures across plant and fungal

1 species. Thus, isotopic fractionation across fungal and plant species (Emmerton et al. 2001a, b),
2 across different groups of compounds (e.g., proteins vs. chitins; Werner & Schmidt 2002) and
3 across different parts of mycorrhizal fungi (caps vs. stems; Taylor et al. 1997) has been
4 intensively studied. These studies relied heavily on laboratory experiments under which N
5 concentrations were unrealistically high relative to natural N-limited environment. In contrast,
6 little attention has been paid to the variability in $\delta^{15}\text{N}$ across various forms of soil N, in spite of
7 the recent recognition of proteins and chitins as the sources of N for uptake by mycorrhizal fungi
8 and plants. In the present study, using a combination of acid hydrolysis and sequential diffusion
9 methods we determined pool size and $\delta^{15}\text{N}$ of labile-N fractions: ammonium, amino sugars
10 (building block of chitins), and amino acids (building block of proteins). We report here a large
11 range in $\delta^{15}\text{N}$ among these hydrolysable-N pools, bulk soil, and dissolved N in arctic tundra
12 soils. We also demonstrate, using hydrolysable amino acids and amino sugars as an index of
13 labile organic N, that using more accurate estimations of $\delta^{15}\text{N}$ of labile-N pools in soil can lead
14 to alternative interpretations of many results that have already been published. To do so, we
15 chose a recently published conceptual model for C and N flux through mycorrhizal symbiosis in
16 arctic tundra ecosystem as an example. We reanalyzed the model after dividing the model's
17 single labile-N pool into three labile-N pools with different $\delta^{15}\text{N}$ values, and discussed
18 alternative implications. The purpose of this study is to point out differences in implications
19 based on different $\delta^{15}\text{N}$ values assumed for soil labile-N pools and to provide a revised model as
20 a working tool to help us fully understand N pathways in arctic tundra ecosystems.

21

1 **Methods**

2 Samples were collected from four sites on a moist, acidic tussock tundra on a west-facing hill
3 slope of the Imnaviat Creek watershed (2.2km²; 68°37'N, 149° 18' W), near Toolik Lake, on the
4 North Slope of the Brooks Range, Alaska, USA (Hinzman et al.1996, Walker & Walker 1996).
5 The four study sites were “watertrack” and “nonwatertrack” at midslope and footslope locations
6 of the hillside. The “watertracks” are areas of greater soil water flow with greater abundance of
7 deciduous shrubs and mosses (Chapin et al. 1988). Within each site, three sampling areas were
8 established (total = 12 sampling areas).

9 At each sampling area the upper layer of organic soil beneath the live-plant/moss layer
10 (thickness =15 cm) was collected by taking two random cores, one from a tussock mound and
11 the other from inter-tussock mounds within 2 m of each other (total = 24 cores, 12 cores each
12 from midslope and footslope locations). The soil samples were immediately weighed,
13 homogenized after removal of live roots, and subsamples were stored frozen for hydrolysis.
14 Foliar samples of common plant species were collected from locations adjacent to the soil cores.
15 Because previous studies conducted near Toolik Lake indicate that $\delta^{15}\text{N}$ are similar in leaves
16 (Nadelhoffer et al. 1996) and whole plants (Hobbie & Hobbie 2006), we assumed that foliar $\delta^{15}\text{N}$
17 in this study represented that of the whole plant. The plants collected were: deciduous shrubs
18 (*Betula nana*, *Salix* spp., ectomycorrhizal), evergreen shrubs (*Vaccinium vitis-idaea*, ericoid
19 mycorrhizal), and sedges (*Carex* spp., *Eriophrum vaginatum*, nonmycorrhizal). Plant and
20 remaining soil samples were dried at 50 °C, and bulk N and $\delta^{15}\text{N}$ were determined.

21 In the laboratory, the frozen soil samples were thawed, ground immediately to a paste, and
22 hydrolyzed with 6N HCl for 12 hrs under reflux according to Mulvaney & Khan (2001).
23 Hydrolysable ammonium ($\text{H}\text{N}\text{H}_4^+$) was diffused with MgO, hydrolysable amino sugars (HAS)

1 with NaOH, and hydrolysable amino acids (HAA) with NaOH following a sequential diffusion
2 for HNH_4^+ and HAS and conversion of HAA to NH_4^+ by ninhydrin reaction (Mulvaney & Khan
3 2001). For concentration, all the hydrolysates were diffused separately using 4% H_3BO_3 , and the
4 mean concentrations were calculated for each site. For ^{15}N analysis, equal volume of
5 hydrolysates from tussock and inter-tussock were combined by sampling area prior to diffusion.
6 The hydrolysate mixture was diffused using an acidified glass fiber filter enclosed in Teflon tape,
7 and the mean concentrations were calculated for each site. The concentrations of diffused N
8 were determined by the indophenol blue method (Keeney & Nelson 1982). Average N
9 recoveries after diffusion (and ninhydrin reaction for amino acids) were: $\text{NH}_4^+ = 96.5 \pm 1.8$ (SE),
10 glucosamine = 100.2 ± 1.57 , and glycine = 101.3 ± 2.38 . Because of these high N recoveries,
11 isotopic fractionation associated with the HAA to NH_4^+ conversion was negligible.

12 Studies have shown that a significant portion of the proteinaceous N in soil may not be
13 hydrolyzed by hot 6N HCl because of a physical protection of N compounds by non-
14 hydrolysable soil components such as humic substances (Zang et al. 2000; Friedel & Scheller
15 2002). Some amino acids and amino sugars are known to decompose during acid hydrolysis,
16 while some amino bonds may not be broken. Thus, caution is necessary in interpreting the
17 results, because the incomplete hydrolysis and decomposition of amino acids or amino sugars
18 both may influence N-isotopic ratio of resulting hydrolysates. Taken these into account, it is still
19 reasonable to assume that hydrolysable amino acids determined in this study approximate the
20 fraction of proteinaceous N in soil that is susceptible to hydrolytic degradation by extracellular
21 enzymes.

22 The $\delta^{15}\text{N}$ of total dissolved N (TDN) in soil pore water was determined on samples collected
23 using microlysimeters at 10 cm depth on the tussock tundra near the soil-core sampling

1 locations; these microlysimeters were installed two yrs prior to the water sampling. TDN in
2 these samples was converted to NO_3^- by alkaline persulfate oxidation (Cabrera & Beare 1993)
3 and diffused after NaCl amendment (Holmes et al. 1998) for ^{15}N analysis following conversion
4 of NO_3^- to NH_4^+ with Devarda's alloy (Sigman et al. 1997).

5 The $\delta^{15}\text{N}$ of soil ammonium (NH_4^+) was determined by the deployment of cation-exchange
6 resins (Giblin et al. 1994). To collect enough NH_4^+ , we deployed ten resin bags (five resin bags
7 at each of two soil sampling areas) for one month in the summer of 2003 at each study site (10
8 bags x 4 sites =40 bags). Each resin bag consisted of 8mL of cation-exchange resin (IONAC C-
9 267, IONAC Chemical Company, Birmingham, NJ, USA) in nylon stocking material. Prior to
10 analysis, the five resin bags were pooled by site and extracted with 2N KCl and diffused for ^{15}N
11 analysis as described in Hobbie & Hobbie (2006).

12 The analysis of ^{15}N was conducted at the Marine Biological Laboratory, Woods Hole,
13 Massachusetts, USA, using a PDZ Europa 20-20 continuous-flow isotope ratio mass
14 spectrometer. Total N that is not detected in the hydrolysable labile N fractions (HNH_4^+ , HAS,
15 and HAA) is by definition non-labile N, which includes hydrolysable-unknown N and non-
16 hydrolysable N. Pool size and $\delta^{15}\text{N}$ signature of non-labile N was calculated by differences in
17 $\delta^{15}\text{N}$ and mass of known N pools.

18 Statistical analysis was performed using SYSTAT 11.0 (2004). We tested the effect of
19 tundra types on N pools size using analysis of variance (ANOVA), followed by multiple
20 comparisons using a least significant difference (LSD) test.

21

22 **Results and Discussion**

23 *$\delta^{15}\text{N}$ of soil and plant N*

1 Of the total hydrolysable labile N, hydrolysable amino acids (HAA) were the largest pool,
2 contributing on average >14% of total soil N and with a pool size 4 – 6 times larger than
3 hydrolysable NH_4^+ (HNH_4^+) and amino sugars (HAS, Table 1).

4 *(Table 1)*

5 The $\delta^{15}\text{N}$ of the HAA pool (-3.9 to -8.7 ‰) was depleted in ^{15}N relative to other soil N pools
6 (Tables 1 and 2). This low $\delta^{15}\text{N}$ signature for the HAA pool (average -5.6 ‰) is within the range
7 of values previously predicted (-6 to -4 ‰) for the available N source for mycorrhizal plants
8 calculated by mass balance between fungal and plant-N pools of known size and $\delta^{15}\text{N}$ signatures
9 (Taylor et al. 1997).

10 The ^{15}N depleted HAA pool may indicate that relatively intact proteins from plants rather
11 than microbes are the major source of HAA-N. Studies found that soil microbial-N is generally
12 ^{15}N enriched relative to likely sources and its host plants (if mycorrhizal). Across a wide range
13 of vegetation (grassland, shrub, forest), climate (semi-arid to sub-tropical), and soil types (sandy
14 to clay-loam), Dijkstra et al. (2006) found that chloroform-extractable fractions in the A-horizon
15 soils were enriched in ^{15}N relative to bulk soil and extractable-N by 3 to 4 ‰. Mycorrhizal
16 fungi are also found to be enriched in ^{15}N relative to their host plants by as much as 5 to 10 ‰
17 (Michelsen et al. 1998; Hobbie et al. 1999) perhaps because of high ^{15}N -enrichment of fungal
18 amino acids and proteins (Taylor et al. 1997; Zeller et al. 2007). Given the general trend of ^{15}N -
19 enrichment in soil microbial biomass, mycorrhizal plant (Table 2) is likely the only source for
20 ^{15}N -depleted proteins in the soil.

21 Because primary sources of amino sugars in soils are fungal (chitin) and bacterial
22 (peptidoglycan) cell walls (Kerley & Read 1997), the relative enrichment of the HAS pool
23 observed in this study would suggest ^{15}N enrichment in microbial biomass. This idea is

1 consistent with the general ^{15}N enrichment in soil microbial biomass (Dijkstra et al. 2006), but is
2 inconsistent with the opposite pattern (i.e., ^{15}N was enriched in proteins and depleted in chitin)
3 found for ectomycorrhizal fungal fruiting bodies in boreal forests (Taylor et al. 1997). This
4 discrepancy may be explained by strong isotopic fractionation between chitins in mycelia and
5 fruiting bodies. For example, Clemmensen et al. (2006) found that ^{15}N of mycelia in arctic
6 tussock tundra soils was depleted by about 2-10 ‰ relative to fruiting bodies. The ^{15}N -
7 enrichment of the HAS relative to HAA may also be a result of relatively fast turnover of the
8 HAS pool, resulting in greater ^{15}N enrichment of remaining HAS. One recent study found a
9 decline only in HAS on native grassland after > 80 yrs of cultivation, suggesting faster turnover
10 of the HAS pool relative to other N-compounds in the soil (Zhang et al. 1999). Fractionation
11 during amino sugar metabolism may also contribute to enrichment of ^{15}N of microbial HAS in
12 soil. Bacteria metabolize the amino sugar by first cleaving off the acetyl group, then
13 deaminating the sugar (Macko 1984). This two-step metabolic process might fractionate ^{15}N
14 further than the metabolic pathway for amino acids, which can directly enter metabolic pathway
15 or require one transamination step, contributing to ^{15}N enrichment of remaining HAS.

16 In this study, non-labile N pool explained >75 % of total N (Table 1) and was slightly ^{15}N
17 enriched relative to bulk N (by 1.3 ‰, on average, Table 1). This is consistent with findings of
18 previous studies: Knicker (2004) observed the formation of recalcitrant soil organic N through
19 microbial reworking of organic matter (i.e., humification), and Kramer et al. (2003) found that
20 humification is associated with ^{15}N enrichment of soil N.

21 The $\delta^{15}\text{N}$ range for the HAA pool relative to bulk soil observed in this study was lower than
22 previously reported for grassland and arable soils (1.9-5.9 ‰; Ostle et al. 1999, 6.5 – 8.1 ‰; Bol
23 et al. 2008). Our lower range may be specific to arctic tundra ecosystems, where decomposition

1 is extremely slow because of the ambient cold and wet conditions (Chapin et al. 1995; Shaver et
2 al. 2000). In an in situ incubation experiment of marsh plant materials, Fogel & Truoss (1999)
3 found that $\delta^{15}\text{N}$ of degraded plant material was altered in most amino acids by up to -15 ‰ and
4 that degree and direction of the changes were influenced by plant material types and
5 environmental conditions (e.g., oxic status, temperature). Thus, ^{15}N enrichment of HAA may
6 vary greatly across a wide range of ecosystems.

7 The $\delta^{15}\text{N}$ of non-mycorrhizal *Carex* and *E. vaginatum* fell within the range of $\delta^{15}\text{N}$ for NH_4^+
8 and NO_3^- in pore water (Table 2). We attribute this to high reliance of these plants on uptake of
9 inorganic N, assuming that fractionation against ^{15}N was negligible upon N uptake. Although a
10 laboratory study showed discrimination against ^{15}N when inorganic N was taken up by some
11 non-mycorrhizal plants (Emmerton et al. 2001a), this could have been caused by the high N
12 concentration (2 – 4 mmol L⁻¹ NH_4^+) and by the closed-system incubation in which the
13 availability of N in the culture media changes over time as it is taken up by mycorrhizal fungi
14 and plants (Emmerton et al. 2001a). In the field under strong N limitation such as at our study
15 site (average total inorganic N in pore water on the tussock tundra collected by lysimeters at the
16 depths of 10 and 20 cm were 1.3 $\mu\text{mol L}^{-1}$, Yano et al. unpublished data) fractionation on uptake
17 would be negligible, unless N-transport mechanisms across cell membranes are significantly
18 different between microbes and plants, because most or all of the available pool is taken up
19 (Hobbie & Hobbie 2006).

20 (Table 2)

21 Fractionation against ^{15}N upon uptake of amino acids and amino sugars into hyphae is also
22 likely to be negligible because of their larger mass compared with inorganic N (Hobbie &
23 Hobbie 2006). The laboratory study by Emmerton et al. (2001b) supported this idea, showing

1 little change in $\delta^{15}\text{N}$ between N source and fungal hyphae when amino acids were the only N
2 source. Additionally, extremely low concentrations of water-extractable amino acids in our
3 samples ($0.21 \mu\text{mol g}^{-1}$ soil, Yano et al., unpublished data) in contrast with HAA ($200 \mu\text{mol g}^{-1}$
4 soil) also suggests that amino acids do not exist in pore water in excess, but production and
5 uptake are fairly well balanced so that concentrations are maintained at low levels (also, free
6 amino acids dissolved in pore water would be a much smaller fraction than the water-extractable
7 fraction, which includes amino acids that were adsorbed on surface of soil particles). The
8 concentrations of water-extractable amino acids observed in this study site were comparable to
9 the concentrations of amino acids extracted with $0.5 \text{ M K}_2\text{SO}_4$ for a taiga ecosystem in central
10 Alaska ($0.20 - 1.72 \mu\text{mol g}^{-1}$ soil) reported by Kielland et al. (2006). Thus $\delta^{15}\text{N}$ in hyphae or
11 plants in this N-limited natural tundra ecosystem should be determined mostly by: 1) $\delta^{15}\text{N}$ of the
12 source(s) such as amino acids released during hydrolysis of proteinaceous N by extracellular
13 enzymes, 2) metabolic fractionation within hyphae, and 3) the proportion of N absorbed by fungi
14 that is transported to host plant.

15 Among the plant species collected, *V. vitis-idaea* was most depleted in ^{15}N (mean $\delta^{15}\text{N} = -6.0$
16 ‰), and its ^{15}N level fell within the range of $\delta^{15}\text{N}$ of the HAA pool. This species is not only
17 capable of using organic-N (amino acids) as a N source on its own when grown aseptically
18 without mycorrhizal fungi in the laboratory (Emmertson et al. 2001a), but under natural
19 conditions it is also associated with ericoid fungi known as “protein fungi” for their high
20 capacity to use proteins as a nitrogen source (Read & Perez-Moreno 2003) as well as chitin
21 (Kerley & Read, 1995). Some ectomycorrhizal fungi can also use proteins (Abuzinadah & Read
22 1986a, b). Because plant production in the studied watershed is strongly N-limited despite a
23 large accumulation of organic N in the soil (Shaver et al. 2001), enzymatic decomposition of

1 proteins and chitin followed by uptake of resulting monomers by fungi can be one of the major
 2 pathways for N acquisition by ericoid and ectomycorrhizal plants. Thus, the strong ^{15}N -
 3 depletion of ericoid (*V. vitis-idaea*), and ectomycorrhizal plants (*B. nana* and *Salix* sp.) relative
 4 to non-mycorrhizal plants (*Carex* and *E. vaginatum*.) can be explained not only by fractionation
 5 within hyphae during the synthesis of transfer compounds as suggested earlier (Macko et al.
 6 1986; Hobbie et al. 2000; Hobbie & Hobbie 2006), but also by extensive exploitation of ^{15}N -
 7 depleted protein-N (i.e., HAA) by mycorrhizal fungi–plant associations or direct uptake of amino
 8 acids by those plants. The higher usage of amino compounds over inorganic N (Emmerton et
 9 al. 2001a; McKane et al. 2002) of ericoid mycorrhizal plants relative to ectomycorrhizal plants
 10 is also consistent with the lower foliar $\delta^{15}\text{N}$ for the former plants.

11 *Pathways of N in arctic tundra ecosystems*

12 We have revised the conceptual model of Hobbie & Hobbie (2006) (Hobbie-Hobbie model,
 13 hereafter) to reflect the different uptake pathways of N from soil to mycorrhizal fungi and plants
 14 (Figure 1-a). In the Hobbie-Hobbie model, $\delta^{15}\text{N}$ of all available N, inorganic or organic, is
 15 considered to be at around 0 ± 2 ‰, a range commonly observed in inorganic N and bulk N of
 16 upper organic soils of temperate forests (Hobbie et al. 1999) and boreal and arctic ecosystems
 17 (Michelsen et al. 1998; Hobbie & Hobbie 2006). In contrast, in our revised model (Figure 1-b),
 18 $\delta^{15}\text{N}$ differs for various soil-N compartments.

19 *(Figure 1)*

20 In both models, plant N derives both from direct uptake of available inorganic N (N_{inorg} , NH_4^+
 21 and NO_3^-) as well as transfer of N to plants through mycorrhizal fungi. This assumption is
 22 reasonable, because rates of mycorrhizal fungal colonization reported for a nearby arctic tundra
 23 ecosystem are ~40 % of for ericoid mycorrhizal fungi (Urcelay et al. 2003) and up to 60 % for

1 ectomycorrhizal fungi (Clemmensen & Michaelsen 2006) and because colonization rates of
2 ectomycorrhizal fungi fluctuate widely across seasons (Clemmensen & Michaelsen 2006). The
3 main difference between the two models is in the pathways between soil and mycorrhizal fungi.
4 In the revised model, $\delta^{15}\text{N}$ of fungal N is determined largely by the proportion of N entering
5 fungi from the HAA pool (Figure 1-b).

6 Amino acids (and amino sugars) entering hyphae may be transferred directly to plants or may
7 go through deamination and transamination followed by biosynthesis of new compounds (e.g,
8 glutamine), most of which will be subsequently transferred to plants. Laboratory experiments
9 suggest that fractionation against ^{15}N during transamination and deamination processes is at least
10 8 to 10 ‰ (Macko et al. 1986; Werner & Schmidt 2002). There is no comprehensive
11 understanding among ecologists on how much of the amino acids (and amino sugars) that enter
12 hyphae go through these transformation processes, and on the proportion of these newly
13 synthesized compounds that are transferred to plants. Taylor et al. (1997), however, suggested
14 that most of the N taken up by plants of northern Sweden boreal forests must have passed
15 through fungi, judging from the fact that most fine root tips (98%) were mycorrhizal. In
16 contrast, Clemmensen et al. (2008) observed in their isotope tracer experiment in the field that
17 87-99 % of added ^{15}N was immediately incorporated into microbial biomass and that half of the
18 biomass turned over to form soil N during a 26-day period, whereas accumulation of ^{15}N in
19 ectomycorrhizal plant was slow (5-14 % of added ^{15}N) during the same period. Based on this
20 and a poor relationship between ^{15}N uptake by mycelia and host plants (*Betula nana*), they
21 concluded that N transfer between fungi and host plants would be determined by the strength of
22 N sinks (plant vs. fungi).

1 We demonstrate below the significant effect that separating the HAA pool from bulk N might
 2 have on the estimation of plant N that comes through fungi. The calculations based on the
 3 Hobbie-Hobbie model and detailed methods for the calculations are described in Hobbie &
 4 Hobbie (2006). For simplicity, we assumed that fractionation against ^{15}N was similar during
 5 transamination regardless of the form of N entering hyphae, and we omitted the pathway from
 6 HAS in our demonstration below. Because the $\delta^{15}\text{N}$ of HAS (average 2.3 ‰) is similar to that of
 7 inorganic N (Table 1), inclusion of the pathway from the HAS pool would not have as strong an
 8 effect as the pathway from HAA, unless metabolic fractionation against ^{15}N is significantly
 9 different between pathways from HAS versus the inorganic N pathways.

10 For direct comparison, we kept all parameters used in Hobbie-Hobbie model (Figure 1-a).
 11 These are: $\delta^{15}\text{N}$ of plant N (N_{pl} , -5 ‰); fungal N ($\delta^{15}\text{N}_{\text{fun}}$, +7 ‰); a range of $\delta^{15}\text{N}$ for
 12 exchangeable inorganic N (N_{inorg} , +1 to +2 ‰); fractionation during transamination (Δ , +8 to +10
 13 ‰). Available N (N_{av}) in the Hobbie-Hobbie model is separated into inorganic N (N_{inorg}) and
 14 hydrolysable amino acids (N_{aa}) in the revised version with $\delta^{15}\text{N}$ signatures for N_{inorg} ($\delta^{15}\text{N}_{\text{inorg}}$)
 15 ranging from +1 to +2 ‰ and for N_{aa} ($\delta^{15}\text{N}_{\text{aa}}$) from -3 to -9 ‰. Because the N entering hyphae
 16 (N_{mix}) is a mixture of fractions coming from available inorganic N (f_{inorg}) and the rest ($1-f_{\text{inorg}}$)
 17 coming from amino acids, an isotope ratio of N_{mix} (R_{mix}) is expressed as:

$$18 \quad R_{\text{mix}} = R_{\text{inorg}} * f_{\text{inorg}} + R_{\text{aa}} * (1 - f_{\text{inorg}}) \quad (1)$$

$$19 \quad \delta^{15}\text{N}_{\text{mix}} = (R_{\text{mix}}/R_{\text{std}} - 1) * 1000 \quad (2)$$

20 where R_{std} is the isotope ratio of the standard (atmospheric N_2) and R_{inorg} is the isotopic ratio of
 21 N_{inorg} . Limited information is available for the range of f_{inorg} , i.e., it is not clear how much N that
 22 enters hyphae is coming from N_{inorg} relative to N_{aa} . Recent studies suggest that mycorrhizal
 23 fungi prefer NH_4^+ and/or amino acids over NO_3^- , but the degree of preference appear to vary

1 across fungal types and environmental conditions (e.g., Emmerton et al. 2001b, Clemmensen et
 2 al. 2008). Because of strong N limitation in the study area and uncertainty in fungal preference
 3 of N forms, we assumed for simplicity that $f_{\text{inorg}} : (1-f_{\text{inorg}})$ ratio is approximated by the ratio of
 4 extractable inorganic N to extractable amino acids in the soil. For the soils studied, overall mean
 5 of $f_{\text{inorg}} : (1-f_{\text{inorg}})$ was 0.18:0.82 (inorganic N = 0.05 $\mu\text{mol g}^{-1}$ soil, amino acids = 0.23 $\mu\text{mol g}^{-1}$
 6 soil, data not shown), and the means by tundra type ranged from 0.09:0.91 to 0.29:0.71. For a
 7 watershed near our study site, the ratio of net N-mineralization to plant-uptake requirement that
 8 could not be accounted for by the net N-mineralization (i.e., N presumably derived from organic
 9 N) was approximately 1:2 for all tundra sites except those that were P-limited or had very
 10 shallow soil (Shaver et al. 1991). Thus, a most likely range for f_{inorg} would be 0.1 to 0.3. In the
 11 following example we used the range 0.1 to 0.5 for f_{inorg} to include cases of extremely high
 12 availability of inorganic N, although unlikely, to mycorrhizal plants.

13 Mass balance of ^{15}N between soil N and fungal hyphae and between fungal hyphae and plants
 14 was calculated using the following equations:

$$15 \quad 100 * f_{\text{inorg}} * \delta^{15}\text{N}_{\text{inorg}} + 100 * (1 - f_{\text{inorg}}) * \delta^{15}\text{N}_{\text{aa}} = \delta^{15}\text{N}_{\text{fun}} * (100 - T) + \delta^{15}\text{N}_{\text{tr}} * T \quad (3)$$

$$16 \quad \delta^{15}\text{N}_{\text{pl}} * 100 = \delta^{15}\text{N}_{\text{tr}} * F_f + \delta^{15}\text{N}_{\text{inorg}} * (100 - F_f) \quad (4)$$

$$17 \quad \Delta = \delta^{15}\text{N}_{\text{mix}} - \delta^{15}\text{N}_{\text{tr}}, \quad (5)$$

18 where $(100 * f_{\text{inorg}})$ is the percentage of N entering hyphae that comes from N_{inorg} and $(100 * (1 -$
 19 $f_{\text{inorg}}))$ is that coming from N_{aa} , T is the percentage of N entering hyphae that is transferred to the
 20 plants, F_f is the percentage of N entering plants that is coming from fungi, $\delta^{15}\text{N}_{\text{tr}}$ is the isotope
 21 ratio of transfer compounds synthesized within hyphae, and $\delta^{15}\text{N}_{\text{pl}}$ is the isotope ratio of plant N.
 22 In equation (3), total N entering hyphae is shown both as the sum of N coming from N_{inorg} and
 23 N_{aa} and as the sum of N transferred to plants and that remains in hyphae. Similarly, in equation

1 (4) total plant N is expressed as the sum of N coming from fungi and from N_{inorg} pool.
 2 Fractionation against ^{15}N (Δ) during biosynthesis of transfer compounds is shown in equation (5)
 3 as the difference between $\delta^{15}\text{N}$ signatures of substances and products synthesized.

4 We found several significant differences in the parameters estimated using our model relative
 5 to those estimated using the Hobbie-Hobbie model (Table 3). Our revised model suggests that,
 6 in the ecosystem studied, approximately 30-60 % of plant N comes from fungi for a wide range
 7 of inorganic N availability relative to organic N ($f_{\text{inorg}} = 0.1 - 0.5$). A fraction of N taken up by
 8 fungi that is transferred to plants (T) varied somewhat depending on f_{inorg} assumed, but it ranged
 9 approximately 40 - 65% in this system. When compared with the Hobbie-Hobbie model,
 10 estimated range for T was somewhat higher in the revised model, whereas the percentage of plant
 11 N coming from fungi (F_f) estimated by the revised model was 30 – 50 % lower (Table 3). Our
 12 model estimates (Table 3) are consistent with the hypothesis by Hobbie et al. (2000) that
 13 mycorrhizal fungi pass an increasingly larger fraction of the N they absorb to plants as
 14 availability of inorganic N decreases.

15 *(Table 3)*

16 Because the Hobbie-Hobbie model relies solely on metabolic fractionation in hyphae for the
 17 low $\delta^{15}\text{N}$ of mycorrhizal plant ($\delta^{15}\text{N}_{\text{pl}} = -5 \text{ ‰}$), a larger percentage of the plant N must come via
 18 fungi in their model. In contrast, in our revised model mycorrhizal plants maintain their low
 19 $\delta^{15}\text{N}$ with less reliance on fungal N-transfer because of the low $\delta^{15}\text{N}$ of soil proteins (-6 ‰).

20 The models presented here use some assumptions that are critical to parameter estimates.
 21 For example, the model assumes specific amino acids, whose $\delta^{15}\text{N}$ is similar to that of bulk
 22 HAA, for N transfer between fungi and host plants. This needs to be examined because $\delta^{15}\text{N}$ of
 23 individual HAA in soil and decomposing plants can vary (e.g., Ostle et al. 1999, Fogel & Truoss

1 1999). Furthermore, if a direct transport of NH_4^+ from fungi to host plant that bypasses
2 transamination process (Selle et al. 2005, Chalot et al. 2006) were significant at our study site,
3 the current estimate of $\delta^{15}\text{N}$ values for transfer compounds (N_T) would be altered. Fungal and
4 plant $\delta^{15}\text{N}$ values used here are those of fruiting bodies and leaves. However, mycelia are most
5 likely the majority of fungal biomass and are significantly depleted in ^{15}N relative to their
6 fruiting bodies (e.g., Clemmensen et al. 2006, Zeller et al. 2007). While foliar $\delta^{15}\text{N}$ was similar
7 to that of whole plants near our study watershed, a comprehensive understanding of tissue-level
8 variation in $\delta^{15}\text{N}$ for host plants does not exist. Thus, in addition to various N sources in soil, a
9 better understanding of metabolic pathways, isotopic fractionation during metabolic processes,
10 and isotopic composition of fungal and plant N are important for better understanding of N
11 transfer within fungi-plant symbiosis.

12 *Implications*

13 We found that the HAA pool was an important component of soil N and that the pool is
14 depleted in ^{15}N relative to other soil N pools in the arctic tundra soils studied. Slow
15 decomposition of plant materials that are depleted in ^{15}N (i.e., ericoid and ectomycorrhizal
16 plants) is the likely reason for the large pool size and ^{15}N depletion of this pool. Because these
17 mycorrhizal plants are the only species in the tundra currently known to be significantly ^{15}N -
18 depleted (Nadelhoffer et al. 1996; Michelsen et al. 1998), ecosystems without these mycorrhizal
19 fungal-plant associations may not produce a soil-N pool that is ^{15}N -depleted. Thus, we
20 hypothesize that the HAA pool is relatively large and its ^{15}N depleted in ecosystems where the
21 ericoid- or ectomycorrhizal plant is a significant component of the plant community and
22 decomposition is slow (e.g., arctic ecosystems). In contrast, the HAA pool may be smaller and
23 relatively rich in ^{15}N in ecosystems where decomposition is fast (e.g., temperate ecosystems). In

1 systems where there are few ericoid or ectomycorrhizal plants (e.g., grasslands), the pool size of
2 HAA may be large but relatively rich in ^{15}N . This idea is partially supported by findings by
3 Ostle et al. (1999) who found that acid-hydrolysable amino acids in grassland that had not been
4 fertilized accounted for 27 % of total N, whereas $\delta^{15}\text{N}$ of this pool was similar to that of bulk soil
5 N (+1.9 ‰).

6 We revised a recent conceptual model linking soil N, fungal N, and plant N, by separating soil
7 N into hydrolysable amino acids, amino sugars, and extractable inorganic N. The revised model
8 allows us to evaluate current understanding of linkages among soil N, fungi, and plant and helps
9 to identify critical elements necessary for better understanding of N cycling via fungal-plant
10 associates.

11

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1 **Figure Legend**

2

3 **Figure 1.** Conceptual models of fluxes of ^{15}N across soil, mycorrhizal fungi, and plants by
4 Hobbie-Hobbie model (a) and by revised Hobbie-Hobbie model in this study (b).

5 Hydrolysable amino acid N and amino sugar N that are available for uptake are shown as N_{aa} and

6 N_{as} , respectively; N_{inorg} is exchangeable NH_4^+ and NO_3^- ; f_{inorg} is a fraction of N absorbed by

7 hyphae that is coming from N_{inorg} ; T is the percentage of N absorbed by hyphae that is transferred

8 to plant; $100-T$ is the percentage of N absorbed by hyphae that remains in fungal biomass; F_f is

9 the percentage of N entered plant that is coming from N_{inorg} ; Δ is a fractionation factor against

10 ^{15}N during the formation of transfer compounds (N_{tr}).

11

1 **Table 1.** Mean pool size (molar% of total soil N) and natural $\delta^{15}\text{N}$ levels of various soil N pools on tussock tundra with and without
 2 watertrack at Imnavait Creek watershed, Alaska, USA.

Tundra type*	N pool [†] size \pm SE(%)				$\delta^{15}\text{N}$ of various N pools in soil \pm SE (‰)				
	HNH_4^+	HAS	HAA	Non-LN	HNH_4^+	HAS	HAA	Non-LN	Bulk N
Midslope_NWT	5.1 ± 2.7	$2.2 \pm 1.2^{\text{ab}}$	$17.0 \pm 4.7^{\text{ab}}$	$75.7 \pm 10^{\text{ab}}$	-1.2 ± 0.5	6.6 ± 5.3	-3.9 ± 1.0	-1.0 ± 2.5	-0.81 ± 1.1
Midslope_WT	3.5 ± 0.1	$2.8 \pm 0.9^{\text{ab}}$	$14.3 \pm 1.4^{\text{ab}}$	$79.4 \pm 6.1^{\text{ab}}$	1.0 ± 0.7	0.9 ± 0.0	-4.4 ± 0.2	2.2 ± 0.2	0.71 ± 0.7
Footslope_NWT	1.7 ± 0.4	$1.4 \pm 0.2^{\text{b}}$	$8.7 \pm 1.9^{\text{b}}$	$88.1 \pm 4.9^{\text{b}}$	-0.2 ± 0.2	1.0 ± 0.1	-8.7 ± 1.0	2.2 ± 0.1	0.62 ± 0.6
Footslope_WT	3.4 ± 0.6	$2.8 \pm 0.1^{\text{a}}$	$17.5 \pm 1.8^{\text{a}}$	$76.3 \pm 1.4^{\text{ab}}$	0.6 ± 0.4	0.8 ± 0.0	-5.6 ± 2.8	3.2 ± 0.8	0.94 ± 0.3

3
 4 Data for pool size are means of 2 samples, each was composite of 4 soil cores. Data for $\delta^{15}\text{N}$ are means of 3 soil samples. Superscript
 5 letters indicate significant difference ($p < 0.05$) across tundra types detected by ANOVA, followed by multiple comparisons using a
 6 LSD test.

7 *Tundra type: Crest= heath tundra at crest, Midslope and Footslope = tussock tundra, Riparian= wet-sedge tundra at valley bottom,
 8 NWT=non-water track, WT= water track. Water track had high density of deciduous shrub species (*B. nana* and *Salix*).

9 [†] N pools: HNH_4^+ =hydrolysable NH_4^+ , HAS= hydrolysable amino sugars, HAA= hydrolysable amino acids, non-LN= non-labile N
 10 that include hydrolysable-unknown N and non-hydrolysable N.

1 **Table 2.** Mean natural $\delta^{15}\text{N}$ of plant, soil water, and resin-exchangeable inorganic N on
 2 tussock tundra at Imnavait Creek watershed, Alaska, USA.

3

Source			$\delta^{15}\text{N} \pm \text{SE} (\text{‰})$
Plants	Ericoid mycorrhizal	<i>Vaccinium vitis-idaea</i> ,	-6.0 ± 0.32
	Ectomycorrhizal	<i>Betula nana</i>	-4.9 ± 0.47
		<i>Salix</i> spp.	-2.4 ± 0.27
	Arbuscular mycorrhizal	<i>Rubus chamaemorus</i>	1.9 ± 0.21
	Non-mycorrhizal	<i>Carex</i> spp.	1.6
		<i>Eriophrum vaginatum</i>	1.2 ± 1.09
Soil pore water*		TDN	3.0 ± 0.40
		NH_4^+	4.4 ± 0.90
		NO_3^-	1.0^\dagger

4

5 Plant $\delta^{15}\text{N}$ values are means of 6 - 8 samples, except for *Carex*, which was n=1. *TDN =
 6 total dissolved N in soil pore water collected by lysimeter at 10 cm (n=8), NH_4^+ = resin
 7 bags (n=4), NO_3^- = resin bags deployed in a moist acidic tussock tundra near Imnavait
 8 Creek watershed (source: Hobbie & Hobbie, 2006). † Sample size n=1.

1 **Table 3.** Summary of parameter ranges estimated by conventional and revised N
 2 pathway models for arctic tundra ecosystems.

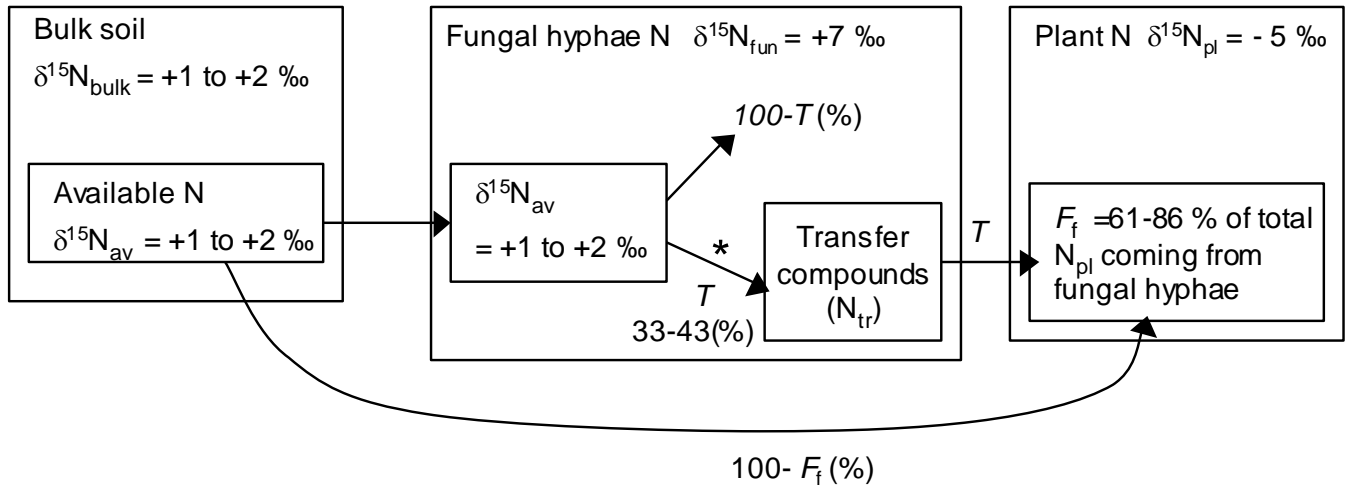
Models	Parameters*			Source
	f_{inorg}	T (%)	F_f (%)	
Hobbie-Hobbie	NA	33 – 43	61 – 86	Hobbie & Hobbie (2006)
Revised	0.1	38 - 47	31 - 55	This study
	0.3	46 - 62	35 - 61	
	0.5	49 - 65	32 - 56	

3
 4 Estimates by Hobbie-Hobbie and revised models. Both models use the following $\delta^{15}\text{N}$
 5 values: plant N (N_{pl}), -5 ‰; fungal N ($\delta^{15}\text{N}_{\text{fun}}$), +7 ‰; and fractionation during
 6 transamination (Δ) +8 to +10 ‰. In the Hobbie-Hobbie model, a range of $\delta^{15}\text{N}$ for
 7 available N ($\delta^{15}\text{N}_{\text{av}}$) is +1 to +2 ‰, whereas in the revised model, N_{av} was separated into
 8 exchangeable inorganic N ($\delta^{15}\text{N}_{\text{inorg}} = +1$ to +2 ‰) and $\delta^{15}\text{N}$ for hydrolysable amino acids
 9 ($\delta^{15}\text{N}_{\text{aa}} = -3$ to -9 ‰), and N entering hyphae is coming from N_{inorg} and N_{aa} at a ratio of
 10 $f_{\text{inorg}} : (1 - f_{\text{inorg}})$.

11 * T (%) = the percentage of N taken up by hyphae that is transferred to plants, F_f (%) =
 12 the percentage of plant N that comes from fungi. NA= not applicable in this model.

a) Hobbie-Hobbie model

* Transamination
 $\Delta = 8 - 10 \text{ ‰}$



b) Revised model

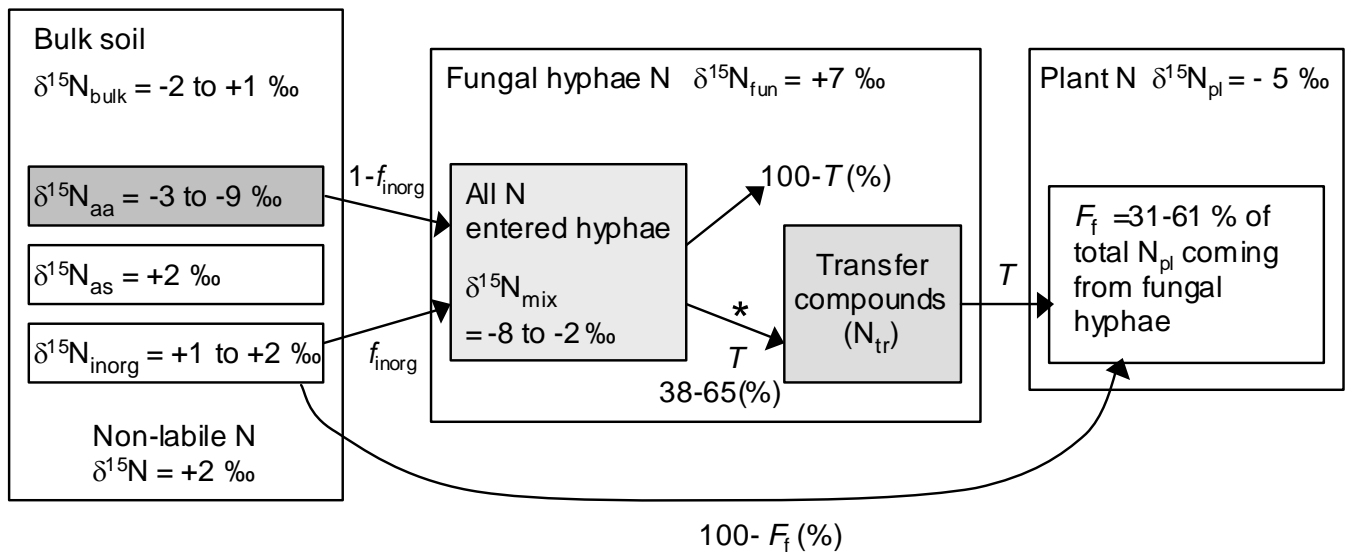


Figure 1.