

Effects of elevated atmospheric carbon dioxide on amino acid and NH_4^+ -N cycling in a temperate pine ecosystem

KIRSTEN S. HOFMOCKEL*†, WILLIAM H. SCHLESINGER* and ROBERT B. JACKSON‡

*Nicholas School of the Environment and Earth Sciences, Duke University, Durham, NC 27708, USA, †School of Natural Resources and Environment, University of Michigan, Dana Building, Room G540, 440 Church St., Ann Arbor, MI 48109-1041, USA,

‡Department of Biology & Nicholas School of the Environment and Earth Sciences, Duke University, Durham, NC 27708, USA

Abstract

Rising atmospheric carbon dioxide (CO_2) is expected to increase forest productivity, resulting in greater carbon (C) storage in forest ecosystems. Because elevated atmospheric CO_2 does not increase nitrogen (N) use efficiency in many forest tree species, additional N inputs will be required to sustain increased net primary productivity (NPP) under elevated atmospheric CO_2 . We investigated the importance of free amino acids (AAs) as a source for forest N uptake at the Duke Forest Free Air CO_2 Enrichment (FACE) site, comparing its importance with that of better-studied inorganic N sources. Potential proteolytic enzyme activity was monitored seasonally, and individual AA concentrations were measured in organic horizon extracts. Potential free AA production in soils ranged from 190 to 690 $\text{nmol N g}^{-1} \text{h}^{-1}$ and was greater than potential rates of soil NH_4^+ production. Because of this high potential rate of organic N production, we determined (1) whether intact AA uptake occurs by *Pinus taeda* L., the dominant tree species at the FACE site, (2) if the rate of cycling of AAs is comparable with that of ammonium (NH_4^+), and (3) if atmospheric CO_2 concentration alters the aforementioned N cycling processes. A field experiment using universally labeled ammonium ($^{15}\text{NH}_4^+$) and alanine ($^{13}\text{C}_3\text{H}_7^{15}\text{NO}_2$) demonstrated that ^{15}N is more readily taken up by plants and heterotrophic microorganisms as NH_4^+ . Pine roots and microbes take up on average 2.4 and two times as much NH_4^+ ^{15}N compared with alanine ^{15}N 1 week after tracer application. N cycling through soil pools was similar for alanine and NH_4^+ , with the greatest ^{15}N tracer recovery in soil organic matter, followed by microbial biomass, dissolved organic N, extractable NH_4^+ , and fine roots. Stoichiometric analyses of ^{13}C and ^{15}N uptake demonstrated that both plants and soil microorganisms take up alanine directly, with a $^{13}\text{C}:^{15}\text{N}$ ratio of 3.3:1 in fine roots and 1.5:1 in microbial biomass. Our results suggest that intact AA (alanine) uptake contributes substantially to plant N uptake in loblolly pine forests. However, we found no evidence supporting increased recovery of free AAs in fine roots under elevated CO_2 , suggesting plants will need to acquire additional N via other mechanisms, such as increased root exploration or increased N use efficiency.

Keywords: alanine, amino acid, ammonium, ^{13}C and ^{15}N , CO_2 , FACE, microbial biomass N, organic nitrogen uptake

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Introduction

In the absence of nutrient limitation, rising atmospheric carbon dioxide (CO_2) is expected to increase carbon (C)

storage in terrestrial ecosystems, partially mitigating anthropogenic CO_2 emissions. However, some analyses suggest that nutrient limitation is likely to constrain greater plant growth under elevated atmospheric CO_2 (Hungate *et al.*, 2003). It is unlikely that the increased nitrogen (N) demand by plants under elevated atmospheric CO_2 will be satisfied by inorganic N from microbial mineralization of soil organic matter (SOM) (Hungate *et al.*, 2003). Simple organic compounds, such

Correspondence: Kirsten S. Hofmockel, School of Natural Resources and Environment, University of Michigan, Ann Arbor MI 48109-1041, USA, tel. +1 734 763 8003, fax +1 734 936 2195, e-mail: khof@umich.edu

as free amino acids (AAs), can provide an additional source of plant-available N (Schimel & Bennett, 2004) to support increased plant growth under elevated atmospheric CO₂. It is unknown how AA availability will respond to elevated atmospheric CO₂. Because simple organic compounds also supply bioavailable C, which fuels microbial activity, we expect that the cycling and demand of free AAs will differ from processes controlling inorganic N. Moreover, because N availability is important to C sequestration (Hu *et al.*, 2001; Gill *et al.*, 2002; King *et al.*, 2004), quantifying the importance of AAs in natural ecosystems and the response of AA cycling to elevated CO₂ is needed.

During the first 4 years of the Duke Forest Free Air CO₂ Enrichment (FACE) experiment, CO₂ fumigation stimulated net primary productivity (NPP) (Hamilton *et al.*, 2002). As a result, trees under elevated CO₂ required an additional 0.86 g N m⁻² yr⁻¹ compared with trees in ambient CO₂ plots (Finzi *et al.*, 2002). Net mineralization and belowground inorganic N cycling have not measurably changed, and therefore, do not contribute to the additional N requirement under elevated CO₂ (Finzi & Schlesinger, 2003). Nor has N₂ fixation increased under elevated CO₂ (Hofmockel & Schlesinger, 2007). The source of increased N supporting this enhanced NPP remains unclear, with organic N supply as an unexplored mechanism for meeting plant N demand. Trees of loblolly pine (*Pinus taeda* L.) support ectomycorrhizae, which have been proposed to produce proteolytic enzymes and take up organic N (Leake & Read, 1989). We hypothesize that under elevated CO₂, increased production of roots and ectomycorrhizae may stimulate the availability and uptake of free AAs. We are not aware of any study that has investigated the uptake of organic N in warm temperate ecosystems, nor are we aware of any study that has investigated whether greater plant growth under elevated CO₂ will influence organic N uptake.

Our objectives were to determine: (1) if potential proteolytic enzyme activity and AA production is ecologically important in loblolly pine forests, (2) whether under field conditions loblolly pine assimilates intact AAs, a source previously unmeasured in this forest, (3) if cycling of AAs among soil pools is comparable with rates of NH₄⁺ cycling, and (4) if elevated atmospheric CO₂ changes soil AA dynamics through interactions with C availability. To address our objectives, we added trace amounts of highly enriched ¹⁵N and ¹³C alanine (ala) and ammonium (¹⁵N only) to the soil *in situ* to compare the potential importance of intact AA and inorganic N uptake in forest plots exposed to ambient and elevated CO₂ concentrations. The organic horizon was the focus of our study because this layer contains the highest density of fine roots and fungal hyphae,

making it an important zone of N transformation and uptake. To understand the movement of organic N relative to inorganic N, we measured short-term cycling (hours to 1 month), retention, and distribution of labeled alanine (¹³C₃H₇¹⁵NO₂) and ammonium (¹⁵NH₄⁺) in plant, soil, and microbial pools.

Materials and methods

Study site

N cycling experiments were conducted at the Duke FACE site, North Carolina, USA. The site consists of six 30 m diameter plots in a loblolly pine (*P. taeda* L.) forest planted in 1983. Loblolly pine comprises 98% of the basal area of this forest (DeLucia *et al.*, 1999). At the time of this experiment, the canopy was ~20 m tall. Since August 1996, the experiment has maintained three control plots receiving ambient air, and three treatment plots receiving CO₂ enriched air (ambient CO₂ +200 μL L⁻¹). For further details of the FACE experiment see Hendrey *et al.* (1999). Mean annual temperature is 15.5 °C, and mean annual precipitation is 1140 mm. The soils are deep and highly weathered clay loams, developed from igneous parent materials and are classified as Ultic Hapludalfs of the Enon series. They are moderately acidic with a pH of 5.75 (Finzi *et al.*, 2002). The soils are relatively infertile and NH₄⁺ dominates inorganic N pools. Net nitrification rates are low, but highly variable and gross rates of nitrification have not been measured (A. C. Finzi, personal communication). In this forest, N is very tightly cycled and there is minimal loss of N through leaching (Lichter *et al.*, 2000). Our research focused on the O_{ea} horizon, because it supports a dense mat of roots and fungal hyphae.

Potential production rates and pools

Potential proteolytic and amidohydrolase enzyme activities were assayed seasonally on four samples from each plot. Organic horizon samples (O_{ea}) were collected from 100 cm² areas, avoiding contamination from the mineral horizon. Samples were placed on ice and returned immediately to the lab for processing. Live roots were removed from each sample. A subsample of the root-free, organic horizon was oven-dried at 60 °C to determine gravimetric soil moisture content.

The remaining sample was used for enzyme analysis. The standard method for deamidizing enzymes is the amidohydrolase activity assay (Tabatabai, 1994). This method is used to determine NH₄⁺-N released by soil samples incubated with THAM buffer and toluene. Toluene increases the permeability of the microbial cell

membrane, allowing measurement of both intracellular and extracellular enzyme activity (Skujins, 1967). By substituting sodium acetate buffer for THAM buffer, we were able to use the proteolysis assay (Watanabe & Hayano, 1995) to concurrently measure potential gross production rates of AAs and NH_4^+ . Six subsamples (3 g) of root-free O_{ea} horizon material were measured into six 40 mL centrifuge tubes, each with 10 mL of sodium acetate buffer. Next, 0.4 mL of toluene was added to inhibit microbial consumption of AAs and NH_4^+ , and samples were shaken lengthwise during incubation periods of 1, 3, 5, 7, 24, and 48 h. At the end of each incubation period, 3 mL of trichloroacetic acid was added to arrest enzyme activity. Samples were centrifuged at 710 g for 5 min, and then filtered through a 0.5 μm glass fiber filter.

Free AA concentration was determined by the *o*-phthalaldehyde and β -mercaptoethanol (OPAME) method (Jones *et al.*, 2002). The OPAME reagent does not react appreciably with sugars, organic acids, proteins, or humic acids, providing an accurate estimate of free AAs (Jones *et al.*, 2002). Aliquots of 0.1 mL of sample and standards were added to 3 mL of buffered reagent (100 mg *o*-phthalaldehyde, 10 mL methanol, 0.1 mL 3-mercaptopropionic acid, and 200 mL borate buffer, pH 9.5). A 200 μL aliquot of each reaction mixture was pipetted into a Costar 96-well round-bottom, polystyrene, assay plate. AA concentration was determined by fluorescence with a 360 nm excitation filter and a 465 nm emission filter (35 nm bandwidth of each filter), using leucine as the AA standard. NH_4^+ concentrations were analyzed colorimetrically (Lachat QuickChem FIA1 8000 Series, Lachat Instruments, Milwaukee, WI, USA). Production rates were calculated as the slope of the relationship between AA or NH_4^+ production and time.

AA profile

Before the field tracer experiment, 100 cm^2 organic horizon samples were collected from each FACE ring and brought immediately to the lab, where live roots were removed. Approximately 150 g of fresh organic horizon material was extracted in 450 mL of 0.01 M CaCl_2 for 24 h at 25 °C. Samples were centrifuged at 1645 g for 10 min and filtered. Extracts were frozen and analyzed for individual AAs at the Duke University Medical Center using tandem mass spectrometry (Millington *et al.*, 1991).

Tracer application

Our goal was to observe N cycling under field conditions using tracer techniques. Based on proteolysis results and on the abundance of individual AAs in

the field, low levels of highly enriched $^{13}\text{C}_3\text{H}_7^{15}\text{NO}_2$ (*L*-alanine) and $^{15}\text{NH}_4\text{Cl}$ were applied to small subplots within the Duke Forest FACE plots in April 2003. A 2.25 m^2 experimental plot was established in each FACE plot. The experimental plot in each FACE ring was equally divided by a polyvinyl chloride (PVC) sheet that extended into the mineral horizon.

Litter (O_i) was carefully removed from the soil surface, and tracer was applied directly to the organic (O_{ea}) horizon with a backpack sprayer (Perakis & Hedin, 2001). Isotope application consisted of a single dose of universally labeled $^{13}\text{C}_3\text{H}_7^{15}\text{NO}_2$ (98% ^{15}N and 98% ^{13}C) to one-half of each plot and $^{15}\text{NH}_4\text{Cl}$ (98% ^{15}N) to the other half. The tracer was applied with enough water to simulate a 3.5 mm rain event typical for spring in North Carolina. Tracer N was added at a rate of 7 mg N m^{-2} (0.125 mM solution), which is <5% of the extractable N pool (160 mg N m^{-2}).

Sampling

Samples were taken from the O_{ea} , typically <5 cm deep. In each plot, 100 cm^2 samples of the O_{ea} horizon were removed before tracer application (natural abundance) and at 3 h, 1 day, 1 week, and 1 month after tracer application. Fresh samples were stored on ice and taken immediately to the lab for processing and analysis. Multiple 100 cm^2 samples were typically taken to obtain enough sample for chemical analysis. Each sample was independently analyzed for root biomass and bulk density, then composited for chemical analysis, detailed below. We traced N content and isotopic signature in the following pools: (1) exchangeable NH_4^+ , (2) exchangeable dissolved organic N (DON), (3) chloroform (CHCl_3)-labile microbial N, (4) SOM N, (5) fine root N, and (6) soluble root N. For the alanine-tracer plots, pools 2–6 were also analyzed for C content and isotopic signature to measure intact AA recovery.

Live roots (<2 mm) were removed from each sample and soaked three times in 0.5 mM CaCl_2 for 5 min to remove tracer absorbed to the root surface. Roots were then freeze-dried, weighed, and prepared for analysis of C, N, ^{15}N , and ^{13}C by elemental analysis-mass spectrometry (EAMS). Soluble C and N were extracted from dried fine roots in 10 mM phosphate buffer and also prepared for EAMS (Näsholm *et al.*, 1998; Bennett & Prescott, 2004; UC Davis Stable Isotope Facility, Davis, CA, USA).

Root-free organic soil samples were composited at the plot and N treatment level, and subdivided for elemental analysis and sequential extractions. Thirty grams of fresh soil were dried at 60 °C and measured for gravimetric soil moisture content. Dry samples were ground in a ball mill and weighed on the microbalance for

EAMS analysis (UC Davis Stable Isotope Facility). A second fresh subsample of 30 g was placed in a 120 mL bottle for sequential extractions.

Sequential extractions

A sequential extraction method was used to recover isotope tracer in SOM, microbial biomass, DON, NH₄⁺ and fine roots (Holmes *et al.*, 2003). Thirty grams of root-free organic material were extracted in 0.5 M K₂SO₄, and the extract was passed through a 0.5 µm glass fiber filter. Ammonium and DON in the extract were determined by colorimetric analysis, then diffused for ¹⁵N analysis according to (Stark & Hart, 1996). The glass fiber filter was removed from the filtration apparatus, returned to the sample bottle with the remaining moist soil, and analyzed for microbial biomass.

Microbial N was extracted via CHCl₃ fumigation extraction [adapted from Brookes *et al.* (1985)]. CHCl₃ was added directly to the soil pellet and filter remaining from the initial extraction. This adaptation has been shown not to differ significantly from vacuum fumigation (Witt *et al.*, 2000; Perakis & Hedin, 2001). Fumigated samples were extracted with 0.5 M K₂SO₄, and filtered through a 0.5 µm glass fiber filter. The microbial biomass extracts were digested by persulfate oxidation (Cabrera & Beare, 1993), analyzed for NO₃⁻ colorimetrically, and diffused for ¹⁵N analysis (Stark & Hart, 1996). Extracts were oven dried and analyzed for microbial biomass ¹³C and ¹⁵N (Colorado Plateau Stable Isotope Laboratory, Northern Arizona University). A recovery coefficient was not applied to correct for the fraction of microbial biomass that is not solubilized by the CHCl₃ extraction (Brookes *et al.*, 1985) due to the uncertainty associated with both temporal variations in the extractability of C and N from microbial biomass and the variability of incorporation efficiency into cytoplasmic vs. structural microbial components (Bremer & van Kessel, 1990). Therefore, our results represent a conservative estimate of the microbial biomass pool and isotope contents.

After completing NH₄⁺, DON, and microbial biomass extractions, the N within the remaining soil pellet was considered to represent soil organic N. The soil pellet was placed in an oven at 60 °C until dry, and then ground with a ball mill and prepared for EAMS (UC Davis Stable Isotope Facility). Diffusion samples were blank corrected (Stark & Hart, 1996), with percent recovery calculations used to follow tracer movement into soil and root components after tracer additions (Nadelhoffer *et al.*, 1999).

Calculations and statistical analysis

C and N content and ¹⁵N and ¹³C atom percentage in excess of the natural abundances (time 0) were used to

calculate µmol ¹⁵N excess and µmol ¹³C excess in samples from tracer addition plots. The percent of added label recovered in each pool was calculated by multiplying the atom percent excess of the pool by the pool size and dividing this value by the amount of label added in the field.

For statistical analysis the FACE plot was used as the experimental unit. For each date, all measurements on replicate samples within a plot were averaged for a single plot value ($n = 6$). Repeated-measures analysis of variance (ANOVA) was used to determine the effects of atmospheric CO₂ concentration (two levels: ambient, elevated), sample date (four levels: March, June, August, and November) and their interaction on NH₄⁺ and free AA production rates ($n = 12$, six FACE rings × 2 N treatments). When production rates were significantly different among seasons, linear contrasts of the means, using student's *t post-hoc* tests, were used to compare production rates of free AAs and NH₄⁺ during the four sampling dates (JMP Version 5.0.1a, SAS Institute, Cary, NC, USA).

Repeated measures ANOVA was also used to determine the influence of CO₂, sample time (3 h, 1 day, and 1 week), N form (NH₄⁺, AA), and interactions on isotope excess in fine root and microbial biomass and percent recovery of ¹⁵N in all soil fractions and ¹³C in fine roots and microbial biomass. To measure uptake of alanine molecules by both fine roots and microbial biomass, linear regressions were used to evaluate the relationship between µmol excess ¹³C and µmol excess ¹⁵N, where a slope of 3 indicates the C : N of ¹³C₃H₇¹⁵N₂, suggesting the molecule was taken up intact. A slope <3 suggests extracellular mineralization prior to uptake or intact uptake and subsequent C respiration.

Results

Proteolysis results indicate that mean annual potential AA production was significantly greater than potential NH₄⁺ production (450 ± 40, 330 ± 30 nmol g⁻¹ h⁻¹, $P = 0.04$), suggesting that free AAs were an important N pool throughout the year (Fig. 1a). Average potential NH₄⁺ production was lower than AA production, ranging from 130 to 450 nmol N g⁻¹ h⁻¹ compared with 190–690 nmol N g⁻¹ h⁻¹. Potential AA production rates were two and a half times higher than rates of NH₄⁺ production during the relatively wet seasons of spring and fall ($P < 0.001$, Fig. 1a). During the summer, the production of NH₄⁺ was 1.8 times greater than the production of AAs by proteolysis ($P < 0.001$, Fig. 1a). Time, N form (AA or NH₄⁺), and their interaction significantly affected N production ($n = 12$; $P = 0.03$, 0.009, and <0.001). We detected no main or interaction CO₂ effects on AA or NH₄⁺ mobilization. Free AAs in

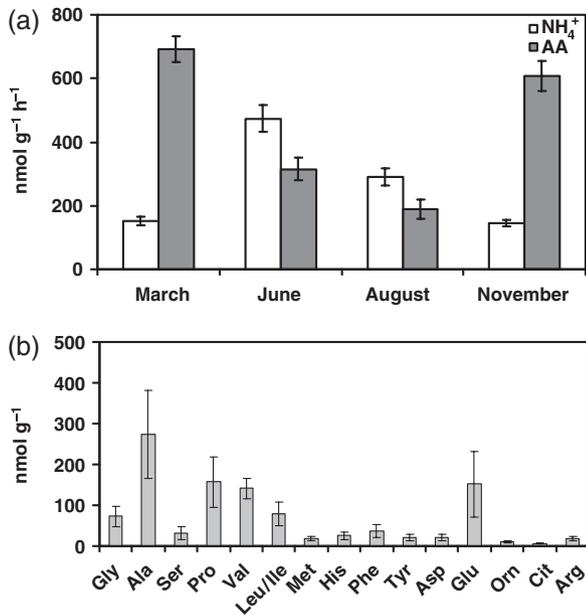


Fig. 1 Mean (\pm SE) for (a) total ammonium (open bars) and total free amino acids (solid bars) produced during proteolysis experiments in the organic horizon at the Duke Forest free air CO₂ enrichment (FACE) site in 2003 and (b) free amino acid profile for FACE organic horizon soils before tracer addition (March 2003).

soil extracts were composed predominantly of nonpolar AAs, including alanine, proline, valine, leucine, and glycine (Fig. 1b). Neutral AAs were most abundant, with alanine representing 25% of the total pool.

Both forms of ¹⁵N tracer were recovered in fine roots, but 2.7 times more ammonium than alanine was recovered during the first week of sampling ($P = 0.04$, Fig. 2a). After 1 month the ¹⁵N recoveries of NH₄⁺ and alanine were similar. Fine roots assimilated the ¹³C during the first week (3 h, 1 day, 1 week), but after 1 month no ¹³C was detected in fine root biomass (Fig. 2a). Because we have no ¹³C recovery at 1 month, we used results from the 3 h, 1 day and 1 week to measure intact alanine uptake, CO₂ effects, and ¹⁵N and ¹³C distribution among soil pools.

To determine the extent of intact alanine uptake, we examined the relationship between ¹³C and ¹⁵N recovered in the soluble fraction of fine roots (Näsholm *et al.*, 1998). After 3 h the ¹³C:¹⁵N ratio in fine roots was 3.3, similar to the ratio of these isotopes in the labeled alanine, suggesting the intact AA was taken up by roots ($r^2 = 0.91$, $P < 0.01$, $y = 3.3048x - 0.028$).

Similar to fine roots, two times more NH₄-¹⁵N than alanine-¹⁵N was recovered in microbial biomass ($P = 0.03$, Fig. 2b). Microbial biomass immobilized ¹³C during all sample dates, suggesting intact uptake of alanine (Fig. 2b). Based on the ¹³C:¹⁵N ratio of micro-

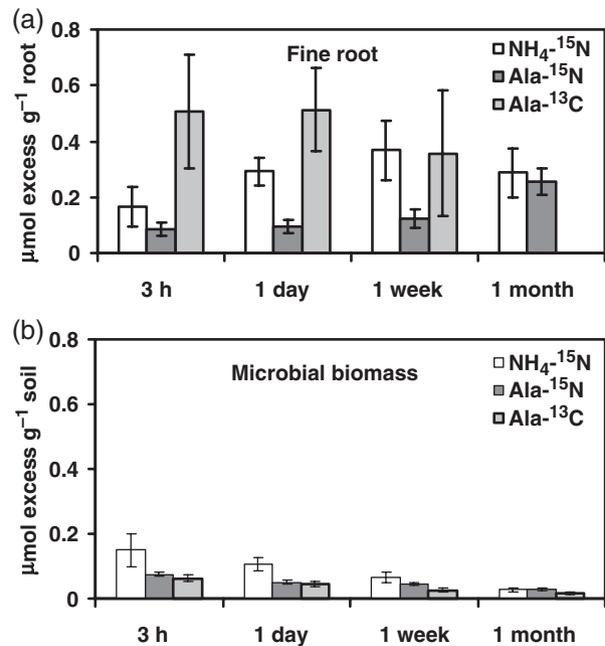


Fig. 2 Isotope excess in (a) fine root biomass and (b) microbial biomass for ¹⁵NH₄⁺ (open bars), alanine ¹⁵N (solid dark bars) and alanine ¹³C (solid light bars). Bars represent mean (\pm SE), $n = 3$.

bial biomass after 3 h, it is likely that some intact uptake of alanine by microbes occurred, but the slope of this relationship was 1.5, which is significantly less than the C:N of alanine ($r^2 = 0.68$, $P = 0.04$, $y = 1.4821x - 0.0237$).

CO₂ exposure affected alanine recovery in roots, with significantly more alanine ¹⁵N recovery under ambient CO₂ relative to elevated CO₂ ($P = 0.03$, Fig. 3a). CO₂ had no significant effect on NH₄⁺ recovery in plants ($P = 0.70$, Fig. 3b). During the first week, more alanine ¹⁵N under ambient CO₂ was recovered in microbial biomass than under elevated CO₂ ($P = 0.03$, Fig. 3c). No CO₂ effect was detected for microbial biomass NH₄⁺ recovery (Fig. 3d). Similar to ¹⁵N, more ¹³C was recovered in microbial biomass under ambient CO₂ than elevated CO₂ ($P = 0.08$, data not shown). The recovery of stable isotopes in SOM, DON, and NH₄⁺ fractions was unaffected by CO₂ treatment ($P > 0.10$, data not shown).

Both forms of N followed the same pattern of ¹⁵N distribution among ecosystem pools. Total recovery (mean \pm SE) was $105 \pm 14\%$ and $82 \pm 14\%$ for NH₄⁺ and alanine plots, respectively, and was not significantly different between N forms ($P = 0.28$). Most ¹⁵N was recovered in SOM followed by microbial biomass, DON, NH₄⁺, and roots (Fig. 4). Relative to alanine ¹⁵N, more NH₄-¹⁵N was recovered in fine roots ($P < 0.01$), microbial biomass ($P < 0.01$), and SOM ($P = 0.01$) during

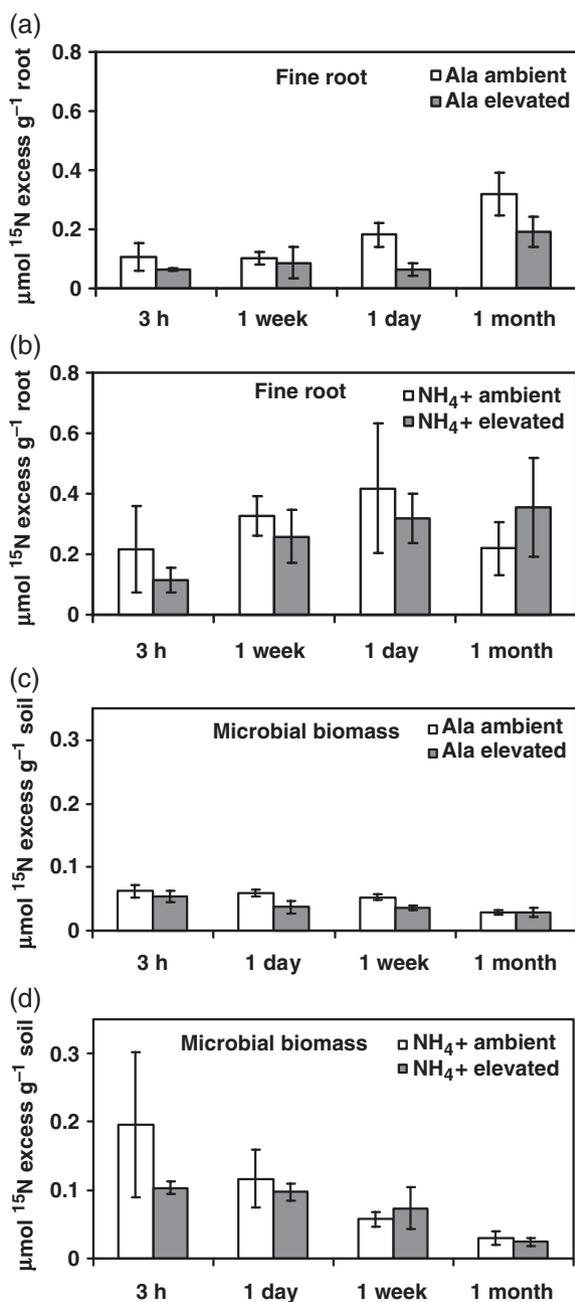


Fig. 3 Fine root ¹⁵N enrichment in (a) alanine tracer plots and (b) NH₄⁺ tracer plots and microbial biomass ¹⁵N enrichment of (c) alanine and (d) NH₄⁺ tracer plots under ambient conditions (open bars) compared with elevated carbon dioxide (CO₂) (solid bars).

the first week of the experiment (Table 1). We saw no significant differences between NH₄⁺ and alanine ¹⁵N in the pool of extractable DON or NH₄⁺. For both forms of ¹⁵N, recovery was <2% for NH₄⁺ and up to 15% for DON, indicating tight cycling and low availability of inorganic and organic N in this ecosystem. We found no significant CO₂ effect on percent recovery of ¹⁵N or ¹³C tracer for any soil fraction.

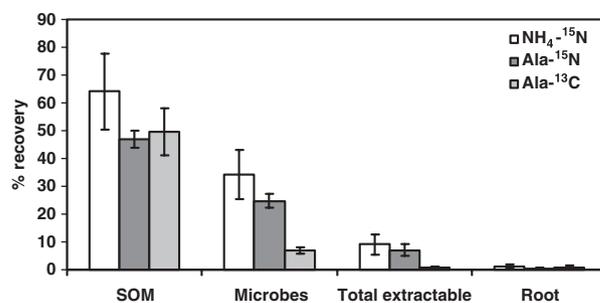


Fig. 4 Average recovery of ¹⁵NH₄⁺ (open bars), Alanine ¹⁵N (solid dark bars) and Alanine ¹³C (solid light bars) 3 h after tracer addition. Bars represent mean (±SE), *n* = 6. For both forms of tracer, recovery was greatest in pools in the following order: SOM > microbial biomass > DON > fine roots. SOM, soil organic matter; DON, dissolved organic nitrogen.

Discussion

Elevated atmospheric CO₂ experiments demonstrate that increased NPP may enhance C storage in forest ecosystems. In the absence of increased N-use efficiency by plants, additional N will be required to sustain C sequestration (Finzi *et al.*, 2002; Gill *et al.*, 2006). Elevated atmospheric CO₂ has consistently augmented NPP in the Duke FACE forest, requiring N uptake in excess of the measured inorganic N supply (Finzi *et al.*, 2002). Increased root and ectomycorrhizal growth under elevated CO₂ could enhance AA consumption, providing an additional source of bioavailable N to support increased plant growth and ecosystem C storage under elevated CO₂. By measuring inorganic and organic N production and cycling, we investigated the importance of AAs relative to NH₄⁺ under current and future atmospheric CO₂ conditions. Our results demonstrate that annual rates of potential AA production were greater than potential NH₄⁺ production, and both loblolly pine and heterotrophic microorganisms can assimilate alanine intact. However, AA uptake by overstorey trees is not stimulated by elevated atmospheric CO₂, indicating an alternative mechanism will be required to support enhanced plant N demand.

Over the course of our experiment, inorganic N was more accessible than organic N for loblolly pine roots as demonstrated by significantly greater recovery of NH₄⁺-¹⁵N than of alanine-¹⁵N in fine roots (*P* < 0.01, Fig. 2a). Despite higher potential AA production rates, 2.4 times more NH₄⁺-¹⁵N than alanine-¹⁵N was recovered in fine roots on average during the first week of our experiment. In a study of arctic plants, field uptake of inorganic ¹⁵N was at least twice that of AA-¹⁵N (Nordin *et al.*, 2004). Other field studies have shown similar root uptake of glycine and NH₄⁺ in boreal forests (Näsholm *et al.*, 1998; McFarland *et al.*, 2002) or wheat plants (Näsholm *et al.*, 2001).

Table 1 Means (\pm SE) percent recovery of $^{15}\text{NH}_4^+$ -N, Alanine- ^{15}N and Alanine- ^{13}C in soil organic matter (SOM), microbial biomass (MB), total extractable N and fine roots over the time course of the experiment

	SOM	MB	Extractable N	Root
$^{15}\text{N NH}_4^+$				
3 h	64.05 (13.73)	34.23 (8.90)	9.09 (3.56)	1.26 (0.59)
1 day	78.66 (17.17)	38.51 (9.05)	10.63 (3.42)	1.84 (0.37)
1 week	54.93 (20.11)	25.42 (7.82)	8.26 (2.92)	2.38 (0.77)
1 month	39.21 (3.82)	8.32 (0.43)	3.63 (0.56)	1.95 (0.60)
$^{15}\text{N Alanine}$				
3 h	46.90 (3.19)	22.47 (2.24)	7.02 (2.12)	0.57 (0.19)
1 day	38.09 (6.04)	14.96 (2.18)	5.66 (1.28)	0.81 (0.37)
1 week	39.77 (6.03)	13.60 (1.55)	5.38 (1.06)	0.87 (0.28)
1 month	37.64 (5.25)	8.84 (1.14)	3.82 (0.40)	1.82 (0.56)
$^{13}\text{C Alanine}$				
3 h	49.63 (8.32)	6.94 (1.17)	0.70 (0.28)	0.96 (0.42)
1 day	83.77 (28.78)	5.33 (0.96)	0.85 (0.23)	1.21 (0.45)
1 week	57.56 (18.55)	2.93 (0.67)	nd	0.66 (0.42)
1 month	8.26 (12.77)	1.88 (0.42)	nd	0.00 (0.67)

nd indicates no data.

AAs may be an important source of N for loblolly pines. Although isotope recovery in fine roots is not a true measure of plant N uptake, results from our field experiment suggest, that given equal pools, alanine recovery is at least 40% of NH_4^+ recovery. As our proteolysis data demonstrate, pools of free AAs and NH_4^+ are seldom equal. This will likely affect the relative importance of AAs in fulfilling plant N demand. During seasons when potential AA production exceeds potential NH_4^+ production, such as in the spring and fall (Fig. 1a), AAs likely provide a more important source of labile N. It is possible that colorimetric analysis underestimates NH_4^+ concentration due to interference by organic compounds (Schjoerring *et al.*, 2002) in which case potential rates of NH_4^+ production may exceed AA production throughout the year. Translating field tracer results to N budget calculations is not straight forward, because our experimental design did not include calculations of ^{15}N dilution from endogenous soil N. Greater potential AA production rates may result in dilution of the stable isotope signatures (Jones *et al.*, 2005). Consequently, our results may underestimate alanine uptake by plant roots. Even with a conservative estimate, our results demonstrate that AAs provide an important source of bioavailable N for loblolly pine.

Intact recovery of alanine in fine roots suggests N acquisition can occur independent of N mineralization. Fine root recovery of intact alanine over the short-term (<1 month) was verified by the positive relationship of excess $^{13}\text{C}:^{15}\text{N}$ in the soluble fine root fraction. The slope of this relationship was approximately 3, indicating that, on average, three ^{13}C were assimilated for each ^{15}N (i.e. the $^{13}\text{C}:^{15}\text{N}$ ratio in fine roots matches the

molecular composition of alanine tracer ($^{13}\text{C}_3\text{H}_7^{15}\text{NO}_2$), which has a $^{13}\text{C}:^{15}\text{N}$ ratio of 3:1). In fact, this relationship suggests that little ^{13}C was respired, and all alanine recovered in fine roots was indeed the intact dual labeled AA. Although some studies have reported considerable root respiration of simple AAs during tracer experiments in boreal forests (Näsholm *et al.*, 1998; Näsholm & Persson, 2001; Bardgett *et al.*, 2003; Henry & Jefferies, 2003), other studies have shown substantial intact uptake, similar to our results (Näsholm *et al.*, 1998; McFarland *et al.*, 2002). Taken together, these results clearly indicate that AA uptake is an important part of the N cycle in a variety of ecosystems, including southern pine forests.

Similar to fine roots, soil microorganisms were also able to take up alanine. There was a significant positive relationship between ^{13}C and ^{15}N recovery in microbial biomass N. The slope was 1.5, suggesting that either a portion of the ^{13}C was respired, or some of the alanine was mineralized extracellularly and recovered as ^{15}N -ammonium (Näsholm & Persson, 2001), resulting in a $^{13}\text{C}:^{15}\text{N} < 3$. Endoenzymes released from disintegrating cells may persist, resulting in extracellular mineralization (Tabatabai, 1994). If extracellular mineralization occurred, the available $^{15}\text{NH}_4^+$ would likely affect the fine root $^{13}\text{C}:^{15}\text{N}$ ratio. On average $0.5 \mu\text{mol excess } ^{13}\text{C g}^{-1}$ fine roots was recovered during the first week (Fig. 2a), indicating microbial respiration rather than extracellular mineralization. However, without direct measurements of respiration, we are unable to confirm ^{13}C respiration rates.

Although alanine was an important N source to roots and microorganisms, we did not detect a CO_2 stimula-

tion effect in alanine recovery. Significant increases in live fine root mass under elevated CO₂ have been detected in the Duke Forest soils (Matamala & Schlesinger, 2000), suggesting a greater biomass of ectomycorrhizal symbionts to exploit limiting resources from soil. However, we did not find greater potential proteolytic enzyme activity or greater AA recovery under elevated atmospheric CO₂. Results from our tracer experiment suggest that elevated atmospheric CO₂ does not enhance AA cycling through plants and microorganisms in this ecosystem. Both pine tree roots and soil microorganisms assimilated significantly more alanine-¹⁵N in ambient CO₂ than in elevated CO₂ 1 week after tracer application (Fig. 3a and c), which is surprising, considering there is greater ecosystem N demand under elevated CO₂ (Finzi *et al.*, 2006). We assumed no differences in transport of the N forms from hyphae to roots or from roots to xylem. Laboratory studies of mycorrhizal-free Scots pine (*Pinus sylvestris* L.) indicate that alanine-¹⁵N uptake and transport to needles is equal to that of ¹⁵NH₄⁺ (Persson *et al.*, 2006). Because we did not measure transport explicitly, we cannot accurately calculate total uptake rates. Instead our data provide an approximation of the potential importance of each form of N to meet loblolly pine N demand (Finzi *et al.*, 2002).

Although more alanine was recovered (μmol ¹⁵N excess g root⁻¹) in fine roots under ambient CO₂ relative to elevated CO₂, there is significantly more root biomass under elevated CO₂. Consequently, trees may be acquiring equal quantities of alanine under both CO₂ treatments. When root uptake is analyzed at the ecosystem scale, considering the pool of fine roots (% recovery ¹⁵N), we find no significant differences in alanine-¹⁵N assimilation due to the elevated CO₂ treatment. Our results indicate that root-specific responses are sensitive to C availability, however elevated CO₂ is not likely to affect AA (e.g. alanine) uptake at the ecosystem scale.

The largest biotic sink for available N throughout our experiment was microbial biomass, indicating microbial uptake was an important mechanism for retaining bioavailable N in this forest ecosystem. Three hours after application, 34 ± 8.9% (mean ± SE) of the ¹⁵NH₄⁺ and 24 ± 2.5% of the alanine-¹⁵N was recovered in microbial biomass N. These results are similar to data from an Alaskan floodplain forest, in which ¹⁵N recovery in microbial biomass was 46% and 31% under NH₄⁺ and glycine treatments, respectively (McFarland *et al.*, 2002). Other studies have demonstrated that microbial biomass is a stronger sink than roots in ¹⁵NH₄ field experiments (Zak *et al.*, 1990; Schimel & Chapin, 1996; Perakis & Hedin, 2001) and pot studies (Henry & Jefferies, 2003). However, interpreting microbial biomass measurements in ectomycorrhizal systems is com-

plicated, because fungal hyphae comprise a large portion of the microbial biomass N, but functionally may represent a fraction of root N. Symbiotic fungi may initiate nutrient transport to the root system, with N initially retained in the hyphal network and passed on to the plant over longer time periods (Näsholm & Persson, 2001). The decreasing ¹⁵N microbial signal (Fig. 2b) and increasing fine root ¹⁵N signal (Fig. 2a) during the course of our experiment may be due to transport of N from hyphae to fine roots or from turnover of microbial biomass ¹⁵N. Both would contribute to the low ¹³C: ¹⁵N signal in fine roots of alanine tracer treatment after 1 month.

Although AAs are an important source of N to loblolly pine, we found no evidence to suggest that elevated atmospheric CO₂ increases the uptake of AAs by the fine roots of these trees. Therefore, AA uptake is not likely to supply plants with the additional N required under elevated CO₂. Our research clearly demonstrates that AAs are a substantial pool of plant available N in this temperate pine forests. Annual potential AA production was 1.4 times greater than NH₄⁺ production, providing an important source of labile soil N. Our data suggest that fine roots of loblolly pine, as well as heterotrophic microorganisms can take up alanine intact. In the absence of pool dilution calculations and transport rates of various N forms, it is not possible to calculate specific uptake rates of NH₄⁺ and alanine in plants and microorganisms. Nonetheless our data indicate that alanine is potentially an important source of bioavailable N, contributing to the N economy of temperate pine forests. Our study contributes to the growing body of evidence suggesting that free AAs are a significant component of terrestrial N budgets. For the CO₂ treatment, we found no evidence supporting increased recovery of free AAs in fine roots under elevated CO₂. As a result, loblolly pine trees need to acquire additional N by other mechanisms, such as increased N use efficiency or increased root exploration.

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