

Altered patterns of soil carbon substrate usage and heterotrophic respiration in a pine forest with elevated CO₂ and N fertilization

SHARON A. BILLINGS* and SUSAN E. ZIEGLER†

*Department of Ecology and Evolutionary Biology and Kansas Biological Survey, University of Kansas, 2101 Constant Avenue, Lawrence, KS 66047, USA, †Department of Earth Science, Memorial University of Newfoundland, St John's, NL, Canada A1B 3X5

Abstract

To assess how heterotrophic microorganisms may alter their activities and thus their CO₂-C return to the atmosphere with elevated CO₂ and changing N availability, we examined soil organic matter (SOM) dynamics at the Duke Free Air Carbon Enrichment (FACE) site, after N fertilizer was applied. We measured heterotrophic respiration during early and late stages of SOM mineralization in soil incubations to capture activity on relatively labile and refractory SOM pools. We also measured $\delta^{13}\text{C}$ of respired CO₂-C and phospholipid fatty acids (PLFAs) during early mineralization stages to track the microbial groups involved in substrate use. We calculated $\Delta_{\text{PLFA-CO}_2}$, a measure of $\delta^{13}\text{C}_{\text{PLFA}}$ normalized by respired $\delta^{13}\text{C}_{\text{CO}_2}$, to assess microbial function with C substrates formed with elevated CO₂ and altered N availability, via the distinct $\delta^{13}\text{C}$ of the supplemental CO₂. We also quantified extracellular enzyme activity (EEA) during labile and recalcitrant SOM mineralization. Early in the incubations, increased N availability reduced heterotrophic CO₂-C release. By the later stages of SOM mineralization, elevated CO₂ soils with fertilization had respired 72% of the CO₂-C respired by all other soils. $\Delta_{\text{PLFA-CO}_2}$ values suggest that fungi in elevated CO₂ plots took up C substrates possessing the $\delta^{13}\text{C}$ signature of recently formed SOM, and added N promoted the activity of Gram-negative bacteria and reduced that of Gram-positive bacteria, particularly actinomycetes. Consistent with this, the enzyme responsible for the degradation of peptidoglycan and chitin, compounds produced by Gram-positive bacteria and fungi, respectively, experienced a decline in activity with N fertilization. If patterns observed in this study with N additions are reversed with progressive N limitation at this site, actinomycetes and other Gram-positive bacteria responsible for mineralizing relatively recalcitrant substrates may experience increases in their activity. Such shifts in microbial functioning may result in increased turnover of, and C release from, relatively decay-resistant material.

Keywords: $\delta^{13}\text{C}$, Duke FACE, elevated CO₂, extracellular enzyme activity, forest soil C, heterotrophic respiration, loblolly pine, PLFA, progressive N limitation, soil incubation

Received 13 September 2007 and accepted 19 November 2007

Introduction

Estimates of the soil organic carbon (C) pool range from 1456 (Schlesinger, 1977) to 2344 (Jobbagy & Jackson, 2000) Gt. This pool of organic C is of particular interest because even small changes in flux rates into or out of such a large pool could have a strong influence on

atmospheric CO₂ concentrations and associated climate change. In spite of recent advances in tracing C dynamics in terrestrial ecosystems (Tu & Dawson, 2005; Robinson, 2007), large uncertainties remain in our ability to describe C flux rates into and out of pools of soil organic matter (SOM). These uncertainties are compounded when we attempt to predict soil organic C dynamics with increasing concentrations of atmospheric CO₂. Of key concern is how the net impacts of

Correspondence: Sharon A. Billings, tel. +1 785 864 1560, fax +1 785 864 1534, e-mail: sharonb@ku.edu

rising levels of atmospheric CO₂ on ecosystem metabolism will influence the balance between net primary productivity (NPP) and soil respiration. These two processes are critical drivers of the CO₂ source or sink strength of terrestrial ecosystems (Chapin *et al.*, 2006; Lovett *et al.*, 2006).

Investigators are particularly interested in how aggrading forests in North America will function with elevated CO₂. The North American continent is currently a net source of CO₂ ($\sim 1264 \text{ Mt C yr}^{-1}$), reflecting fossil fuel emissions of $1856 \pm 464 \text{ Mt C yr}^{-1}$ and a net terrestrial C sink of $592 \pm 296 \text{ Mt C yr}^{-1}$ (US Climate Change Science Program, 2006). Forests represent the largest sink term in the North American C budget, with those regrowing after agricultural abandonment providing a sink of $\sim 269 \text{ Mt}$ over the last decade (Goodale *et al.*, 2002; US Climate Change Science Program, 2006). We are uncertain how C release via heterotrophic respiration (R_h) from soils supporting these forests will be altered with elevated CO₂. A better understanding of these soil C transformations will require studies that elucidate the possible fates of soil organic C upon entering the soil profile. Possible fates include becoming complexed with mineral soil or into soil aggregates, with possible physical protection from microbial attack; being incorporated into microbial biomass; being transformed physically or biologically into other C-containing compounds of various degrees of recalcitrance; or being respired and released as CO₂ (Paul & Clark, 1996; Schlesinger, 1997; Six *et al.*, 2006). The continued contribution of aggrading forests to the sink term in the North American C budget depends, in part, on how these complex and interacting processes function across time.

Further complicating our attempts to predict future forest C dynamics, nitrogen (N) availability can govern the response of forest productivity to elevated CO₂ (Oren *et al.*, 2001), and this productivity in turn can influence soil C processes, including R_h , via litterfall production and/or rhizosphere activity (Andrews & Schlesinger, 2001; Bernhardt *et al.*, 2006; Pregitzer *et al.*, 2006). Although increases in N availability can enhance forest response to elevated CO₂ (Oren *et al.*, 2001), such increases have also been linked to reductions in soil respiration (R_s ; Maier & Kress, 2000; Butnor *et al.*, 2003). It is unclear to what extent these reductions in respiration are governed by changes in autotrophic vs. heterotrophic activity, but incubation data suggest that at least some of the observed declines in soil respiration with added N may result from declines in heterotrophic respiration (Ågren *et al.*, 2001; Teklay *et al.*, 2007). Such alterations in microbial function would suggest that N availability could influence heterotrophic soil C transformations, which

in turn will dictate C release vs. retention within the soil profile.

Studies from the longest running elevated CO₂ experiment in an intact forest ecosystem [the Duke Free Air C Enrichment (FACE) site, NC, USA] indicate that both NPP and soil respiration can increase with supplemental CO₂ (DeLucia *et al.*, 1999; Andrews & Schlesinger, 2001; Hamilton *et al.*, 2002), and that these responses are in part governed by N availability (Oren *et al.*, 2001; Butnor *et al.*, 2003). Increases in NPP with elevated CO₂ have been relatively sustained at the site since 1999, while observed increases in soil respiration with elevated CO₂ have declined over the same time period (Bernhardt *et al.*, 2006). Some of the changes in soil respiration likely emanate from alterations in heterotrophic activity within the soil profile, because some heterotrophic microbial communities appear to have shifted in activity with elevated CO₂ (Billings & Ziegler, 2005; Finzi *et al.*, 2006b). These changes in patterns of soil microbial activity have potentially large implications for soil C storage and release.

In this study, we explore how the soil microbial processes linked to soil C transformations and CO₂-C release may function in an elevated CO₂ environment, particularly with altered N availability, in this same forest. We address two questions important for predicting how soil microbial processes will contribute to future C balances in forested ecosystems:

1. Does elevated CO₂ alter the microbially mediated processes that determine whether C is lost to the atmosphere as R_h vs. retained within the soil?
2. Given the apparent importance of N in governing R_s at this site (Butnor *et al.*, 2003), to what extent are changes in soil microbial processes governed by N availability?

In an earlier study, we suggested that elevated CO₂ may promote altered activity of several groups of bacteria (Billings & Ziegler, 2005). Here, we assess the response of soil C mineralization to elevated CO₂ during three distinct phases of SOM transformations during soil incubations, and examine the influence of field N additions on those responses. We recognize that *ex situ* soil incubations generate conditions very different from those found in an intact soil profile, but they are useful for isolating heterotrophic activity as labile and recalcitrant organic material are mineralized. During the mineralization of labile SOM, we also quantified $\delta^{13}\text{C}_{\text{PLFA}}$ and $\delta^{13}\text{C}$ of respired CO₂ to assess how soil microbial groups responsible for transforming SOM into other organic compounds vs. releasing C as CO₂ may function in the future. Because $\delta^{13}\text{C}$ of respired CO₂ closely tracks the integrated $\delta^{13}\text{C}$ of the C substrates mineralized by microorganisms (Tu & Dawson,

2005), this approach permits us to assess the relative contributions of microbial groups as specified by $\delta^{13}\text{C}_{\text{PLFA}}$ to the degradation of SOM with elevated CO₂ and N additions. By using recent N additions at the site to assess the sensitivity of heterotrophic activity to N availability, we can investigate how the opposite trend of progressive N limitation (Luo *et al.*, 2004; Finzi *et al.*, 2006a,b) may influence these processes.

Materials and methods

Site description

The Duke FACE experiment consists of eight, 30 m diameter plots in a loblolly pine (*Pinus taeda* L.) forest planted in 1983 in Orange County, NC, USA (35°58'N, 79°05'W; Hendrey *et al.*, 1999). Three of the plots have been exposed to concentrations of atmospheric CO₂ approximately 200 $\mu\text{L L}^{-1}$ above ambient concentrations since August, 1996, and one plot since June, 1994. The site is well described in multiple studies (Andrews & Schlesinger, 2001; Billings & Ziegler, 2005; Lichter *et al.*, 2005; Finzi *et al.*, 2006a,b; Schlesinger *et al.*, 2006). Briefly, supplemental CO₂ fumigates the treatment plots via pipes that extend to the top of the canopy and define the circular plots. Control plots are set up in a similar manner but receive no supplemental CO₂. Treatment CO₂ is depleted in ¹³C [$\delta^{13}\text{C}$ of $-43.1 \pm 0.6\%$ (SE)] such that the well-mixed CO₂ within the treatment plots has an average $\delta^{13}\text{C}$ of -20% , compared to $\delta^{13}\text{C}$ in control plots of -8% . As a result, foliage and fine roots produced in treatment plots exhibit $\delta^{13}\text{C}$ values of approximately -39.3% (Ellsworth, 1999; Matamala & Schlesinger, 2000). Some biomass possessing this distinct isotopic signal has been transformed into SOM, as reflected in increasingly depleted $\delta^{13}\text{C}$ signatures of SOM fractions (Schlesinger & Lichter, 2001; Lichter *et al.*, 2005; S. Billings, unpublished data). In spring 2005, half of each of the eight plots (two of the four quadrants) was fertilized with NH₄NO₃ (two applications, each of 5.6 g N m⁻² yr⁻¹). This fertilization rate is approximately one order of magnitude greater than local atmospheric N deposition, and is approximately 10% of the gross rates of N mineralization reported for the top 10 cm of the soil profile in Zak *et al.* (2003).

Soil collection and processing

We collected soil samples (mineral soil profile, 5 cm diameter, 30 cm deep) from all four quadrants of all eight plots in late October to early November 2005. Root biomass is relatively high during these months (McClagherty *et al.*, 1982; Waring & Schlesinger, 1985), and 99% of this forest's root biomass is contained within

our sampling depth (Matamala & Schlesinger, 2000). We shipped the samples in coolers to the University of Kansas for processing. Soils were sieved (2 mm) and all roots > 1 mm in diameter were removed. Care was taken to return soil clinging to roots back to the soil samples. Subsamples of each soil were finely ground for analyses of total organic C and N, and $\delta^{13}\text{C}$ on a Carlo Erba elemental analyzer (NA1500 CHN Combustion Analyzer, Carlo Erba Strumentazione, Milan, Italy) coupled to a Finnigan Delta⁺ mass spectrometer (Finnigan MAT, Bremen, Germany) via a Finnigan Conflo II Interface.

Soil samples were then subjected to two sets of incubations. For one set of incubations (hereafter referred to as the long-term incubations), we weighed 60 g of field moist soil into PVC cores (5 cm diameter, 7.5 cm tall) sealed at the bottom with glass fiber filter paper. Water was added to bring the soils to 60% water-filled pore space (WFPS). The PVC cores were placed in ~1 L incubation jars on a layer of marbles to ensure that the cores did not rest in moisture at the bottom of the jar. A few mL of water were added to the jar to keep the atmosphere within the jar relatively humid. Jars were then sealed with air-tight lids equipped with a septum for gas sampling, and two 14 mL gas samples were immediately taken from each jar to establish starting conditions in the incubation vessels. One sample was injected into a previously evacuated, air-tight vial (Teledyne Tekmar, Akron, OH, USA) for analysis of CO₂ concentration. The other sample was injected into a previously evacuated, air-tight Exetainer (Labco, Buckinghamshire, UK) for analysis of $\delta^{13}\text{C}$ of CO₂. Subsequent gas samples were taken, and jars were aerated, on days 0, 3, 6, 12, 18, and 25, and every 10 days thereafter to day 365. CO₂ concentration data were obtained for every sampling date, and isotopic data were obtained on days 0, 3, 6, and 18. CO₂ concentration data were obtained via gas chromatography (thermal conductivity detector, Varian CP3800, Varian Inc., Walnut Creek, CA, USA). We calculated rates of CO₂ production using concentration data, calculated dry weight of the soil subsamples, and the headspace of each incubation vessel. $\delta^{13}\text{C}$ of CO₂ data were obtained via analysis on a Finnigan Delta⁺ mass spectrometer (Finnigan MAT), introduced via a Finnigan GasBench.

For the second set of incubations (hereafter referred to as the short-term incubations), we weighed 30 g of field moist soil into five replicates of ~1 L incubation jars and added water to 60% WFPS. We sealed the jars with air-tight lids equipped with septa as described earlier. We took two gas samples from each of these jars, for CO₂ concentration and $\delta^{13}\text{C}$ as described earlier, at 0, 8, 18, 36, and 60 h. Immediately after gas sampling, we destructively sampled one set of soils (time 18 h)

and immediately froze them at -70°C for PLFA extraction and analyses. We analyzed PLFAs at 18 h instead of directly after field sampling, because we wanted to examine $\delta^{13}\text{C}_{\text{PLFA}}$ in conjunction with the $\delta^{13}\text{C}$ of microbially respired CO_2 (see calculations in the following text). We theorized that 18 h was early enough in the incubation to mitigate the inevitable effects of examining soil *ex situ*, while ensuring that enough C substrate had been mineralized to reveal potential differences in $\delta^{13}\text{C}$ of respired CO_2 with variation in patterns of substrate usage. Thus, the short-term incubation provided information about microbial community composition and biomass during microbial metabolism of primarily labile substrate, as well as finer resolution CO_2 and $\delta^{13}\text{C}$ data than those obtained via the long-term incubation. All incubations were conducted at 22°C .

Extracellular enzyme assays (EEA)

We quantified potential EEA (Carreiro *et al.*, 2000; Saiya-Cork *et al.*, 2002; Sinsabaugh *et al.*, 2003; Finzi *et al.*, 2006b) in additional soil subsamples obtained at 18 h in the short-term incubation, and upon completion of the long-term incubation, to represent microbial processing of labile and relatively recalcitrant SOM, respectively. We assessed EEA using fluorescently labeled substrates corresponding to the enzymes acid phosphatase, β -1,4-glucosidase, cellobiohydrolase, β -1,4-*N*-acetylglucosaminidase (NAG), β -1,4-xylosidase, α -1,4-glucosidase, and L-leucine aminopeptidase, and used colorimetric techniques to assess the enzymes urease, phenol oxidase, and peroxidase. We homogenized 1.0 g (fresh weight) of each soil sample in 125 mL of 50 mM sodium acetate buffer (pH 5.5) for 30 s with a hand blender. Black, 96-well microtiter plates were used for all assays to be subjected to fluorometric analysis; for the urease, phenol oxidase, and peroxidase assays, we used clear plates for spectrophotometric analysis. For each soil sample, we used 16 replicate wells of 200 μL soil slurry and 50 μL of substrate for the assay, eight wells of 200 μL soil slurry and 50 μL of standard [10 μM 4-methylumbelliferone (MUB) or 7-amino-4-methylcoumarin (MC) for LAP] to calculate the quench coefficient, and eight sample controls containing 200 μL soil slurry only. For each plate containing three soil samples, we used eight wells of 50 μL substrate and 200 μL buffer as negative controls and eight wells of 50 μL MUB or MC and 200 μL buffer to calculate the emission coefficient. For spectrophotometric analyses, we used L-3,4-dihydroxyphenylalanine (DOPA) as a substrate, except for urease, for which we used urea. Phenol oxidase assays were conducted by adding 50 μL of 25 mM DOPA to each sample well; peroxidase assays received an additional

10 μL of 0.3% H_2O_2 . Urease assays received 10 μL of 400 mM urea. We incubated all plates at 20°C for approximately 18 h. After incubation, urease assays received 40 μL of salicylate solution and, after waiting 3 min, 40 μL cynaurate solution. We waited for color to develop before reading these plates (~ 20 m). We raised the MUB and MC emission coefficients to readable levels in all plates via the addition of 10 μL of 0.5 M NaOH to each well. We measured fluorescence with a microtiter plate fluorometer (Molecular Devices, Sunnyvale, CA, USA) set to 365 nm excitation and emission of 460 nm, and spectrophotometric activity with a spectrophotometer (Molecular Devices) measuring absorbance for urease at 610 nm and for phenol oxidase and peroxidase at 460 nm. Results are expressed as $\text{nmol g}_{\text{soil}}^{-1} \text{h}^{-1}$.

Phospholipid fatty acid (PLFA) analyses and calculations

For PLFA analyses, frozen soil samples were transported to the University of Arkansas. Frozen samples were lyophilized and extracted within 2 weeks of collection using the modified Bligh–Dyer method (Pinkart *et al.*, 1998; White & Ringelberg, 1998). Samples were fractionated into neutral lipids, glycolipids, and phospholipids using solid phase extraction with silicic acid phase on a vacuum manifold system to facilitate elution (Dobbs & Findlay, 1993; White & Ringelberg, 1998). PLFAs were transmethylated into their corresponding fatty acid methyl esters (FAMES) using the methods described by Findlay (2007). The resulting FAMES were purified using reverse-phase solid phase extraction according to Findlay & Dobbs (1993). FAMES were quantified using a gas chromatograph with a flame ionization detector (GC-FID; Agilent 6890, Santa Clara, CA, USA) and equipped with a 70% cyanopropyl polysilphenylene-siloxane capillary column (SGE BPX-70; 50 m length, 0.22 mm i.d., and 0.25 μm film thickness). Helium was used as a carrier at 1 mL min^{-1} , and all samples were injected onto a split:split-less injector in split-less mode at 240°C . Samples were also analyzed by GCMS to identify each FAME using the same GC described but interfaced with a mass selective detector (Agilent 5973_{inert}). Identification was based on retention time and mass spectra of known standards, including individual FAMES (Sigma Aldrich Chemical Company, St Louis, MO, USA) and mixtures (Bacterial FAMES and 37 component FAME standards, Supelco Co., Rockford, IL, USA) in addition to direct comparison of mass spectra to a NIST database. PLFA recovery was 60–75% ($n = 8$) as determined from phospholipid recovery standards (phosphatidylcholine deheptadecanoyl and phosphatidylcholine nonadecanoyl; Avanti Lipids, Alabaster, AL, USA). Microbial biomass was estimated

from total PLFA concentration ($\mu\text{g PLFA g soil}^{-1}$) according to Zelles *et al.* (1992).

$\delta^{13}\text{C}$ of PLFAs was determined by GC combustion isotope ratio mass spectrometry (GC-C-IRMS). Isotopic composition of each individual FAME was determined using an Agilent 6890 GC coupled to a stable isotope ratio mass spectrometer (Thermo Finnigan Delta⁺, Bremen, Germany) via a combustion interface (Thermo Finnigan GC/CIII). Use of the transmethylation procedure described earlier (Findlay, 2007) does not methylate free fatty acids, precluding the use of free fatty acid isotopic standards. A standard mixture of four phospholipids (1,2-dimyristoyl-sn-glycero-3-phosphocholine, 1,2-diheptadecanoyl-sn-glycero-3-phosphocholine, 1,2-dinonadecanoyl-sn-glycero-3-phosphocholine, and 1,2-diheneicosanoyl-sn-glycero-3-phosphocholine; Avanti Lipids), each with fatty acid moieties that were predetermined for $\delta^{13}\text{C}$, was saponified and methylated to determine the change in $\delta^{13}\text{C}$ of the PLFAs following methylation. We used this standard mixture to correct each fatty acid for the addition of the methyl C by mass balance from the known fatty acid $\delta^{13}\text{C}$ of each of the phospholipid standards, and the measured methylated value (Silfer *et al.*, 1991; Abrajano *et al.*, 1994). $\delta^{13}\text{C}$ values were measured relative to high purity, reference gas standards expressed relative to international standard PDB (Pee Dee Belemnite).

Because elevated CO₂ plots at this site receive distinctly labeled, ¹³C-deplete CO₂ and the control plots do not, the $\delta^{13}\text{C}$ of available C substrates in the soil profile varies with CO₂ treatment. As a result, assessing $\delta^{13}\text{C}_{\text{PLFA}}$ in isolation does not yield informative data describing the potential influences of CO₂ or N additions on microbial activity. We thus calculated $\Delta_{\text{PLFA-CO}_2}$, the difference between $\delta^{13}\text{C}_{\text{PLFA}}$ and the $\delta^{13}\text{C}$ value of the CO₂ respired during the incubation. A $\Delta_{\text{PLFA-CO}_2}$ value less than zero for an individual PLFA suggests that the C used by that microbial grouping to generate its membrane was depleted in ¹³C relative to the 'average' C respired by all heterotrophs in that incubated soil sample. It is important to note that it is extremely challenging, if not impossible, to know the $\delta^{13}\text{C}$ of each individual C substrate used by the microbial groupings assessed. By calculating $\Delta_{\text{PLFA-CO}_2}$, however, we can normalize $\delta^{13}\text{C}_{\text{PLFA}}$ values by the integrated $\delta^{13}\text{C}$ of all the C mineralized during the soil incubations. We thereby incorporate a metric close in value to the $\delta^{13}\text{C}$ of SOM used by the aggregate soil microbial community into our calculations.

Because the ambient plots are not exposed to a distinct ¹³CO₂ label, any test for elevated CO₂ effects on $\Delta_{\text{PLFA-CO}_2}$ requires the assumption that the shift in $\delta^{13}\text{C}$ values between individual C substrates is the same in elevated CO₂ and ambient plots. This may be valid

for relatively labile substrates, which were likely formed recently with 'new' C (i.e. C exhibiting the ¹³C signature of the supplemental CO₂). After 18 h of incubation conditions, mineralization of labile substrates likely represents the majority of microbial activity. However, we must consider that not all C substrates being metabolized by elevated CO₂ microbial communities at the time point sampled for our PLFA data were equally labeled with the distinct $\delta^{13}\text{C-CO}_2$ signature. This is feasible given that some PLFAs assayed likely represent *in situ* microbial processing before our soil collections, and thus reflect microbial uptake of some C substrates that are relatively slow to form. In contrast, testing for differences between N levels requires no such assumption, because the $\delta^{13}\text{C}$ of photosynthates is the same between the two N levels within each CO₂ treatment.

Statistical analyses

We employed analyses of variance (ANOVA, PROC GLM, SAS, Cary, NC, USA) to assess effects of CO₂ and N treatments, and their interaction, on cumulative respired CO₂-C data from the incubations. For the long-term incubation, we performed two ANOVAs, dividing the data into early (days 3 through 35) and later (days 45 through 365) sections, because of the distinct patterns of CO₂-C accumulation during these time periods (see 'Results'). Because of the repeated nature of these sampling events, we also assessed CO₂-C production using repeated-measures ANOVA on non-cumulative respiration rates during the incubations. Differences in statistics from these tests were minimal. We performed analogous ANOVA to assess the effects of CO₂ and N treatments and their interaction on $\delta^{13}\text{C}$ of respired CO₂ during the incubations, and additional ANOVA to analyze $\Delta_{\text{PLFA-CO}_2}$, microbial biomass estimates derived from PLFAs, and EEA for each enzyme assayed. Data were transformed when necessary (natural log or negative reciprocal) to achieve normal distributions. Because of the necessarily low number of replicates for this study ($n = 4$ for most assays) and the conservative nature of the repeated measures ANOVA model used (Littell *et al.*, 1996), we report all *P* values equal to or below $\alpha = 0.10$.

Results

CO₂-C release during soil incubations

The CO₂-C data from the long-term incubations (Fig. 1a) did not fit an exponential decay model, which can be useful for estimating labile C pool sizes and turnover rates of labile and recalcitrant C pools (Bridgman *et al.*,

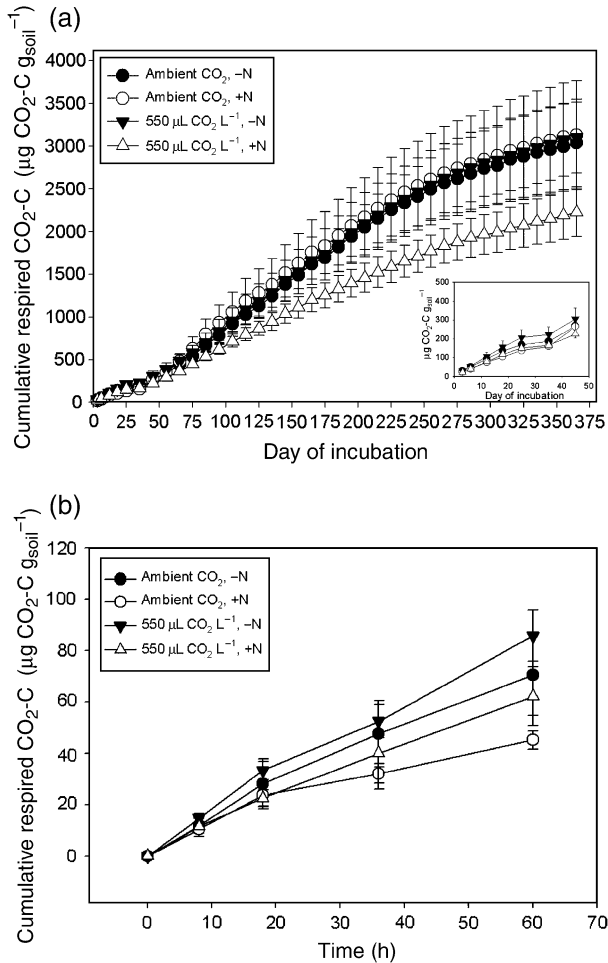


Fig. 1 Cumulative respired CO₂-C during long-term (a) and short-term (b) incubations of soil (0–30 cm) from the Duke FACE facility with elevated CO₂ and N treatments. Each point is the mean of four replicates, except for elevated CO₂, unfertilized soils in the long-term incubation, for which there were three replicates due to sampling error. Inset graph expands the first 45 days of the incubation. Error bars are one standard error of the mean.

1998; Billings *et al.*, 2006). The rates of respiration used to calculate cumulative CO₂-C produced slowed between days 3 and 35, then increased sharply and slowly declined throughout the remainder of the incubation (data not shown). Thus, we separated the cumulative CO₂-C respired data from the long-term incubations into two data sets because of the inflection point in the cumulative curves between days 35 and 45 of the incubations. ANOVA revealed that soils with N additions had respired approximately 79% of the respiration produced by soil with no N additions by day 35, regardless of CO₂ treatment (Fig. 1a; $P = 0.04$). From day 45 through day 365 of the incubation, soils from

elevated CO₂ plots with N additions respired significantly less than all other treatments ($P < 0.002$ for all dates), such that by the end of the incubation they had respired approximately 72% of the mean of all other treatments. In the short-term incubations, ANOVA revealed that fertilized soils respired 74% of the C respired by unfertilized soils, with no CO₂ treatment interaction (Fig. 1b; $P = 0.026$).

In both the short- and long-term incubations, values of $\delta^{13}\text{C}$ of the respired CO₂ reflected the ¹³C-deplete organic matter in the elevated CO₂ plots (Fig. 2). In both incubations, $\delta^{13}\text{C}$ of respired CO₂ in ambient CO₂ plots was more enriched relative to $\delta^{13}\text{C}_{\text{SOM}}$ than in elevated CO₂ plots, where $\delta^{13}\text{C}$ of respired CO₂ more closely matched $\delta^{13}\text{C}_{\text{SOM}}$ values ($P < 0.0001$). In the long-term incubations, $\delta^{13}\text{C}_{\text{CO}_2}$ was an average of 0.9‰ higher with N additions than in unfertilized soils across 18 days of sampling ($P = 0.023$; Fig. 2a), with no interaction with

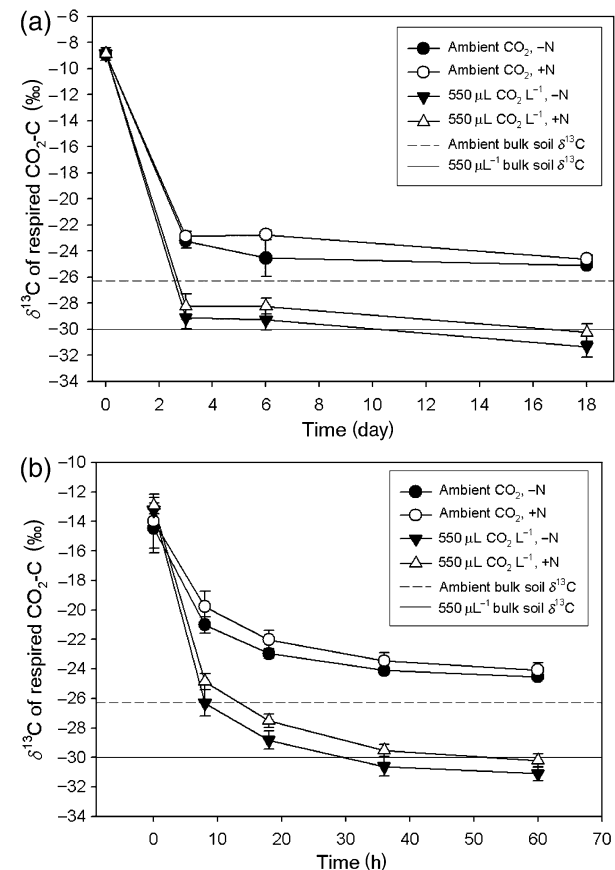


Fig. 2 $\delta^{13}\text{C}$ of soil respired CO₂-C across 18 days of the long-term incubations (a) and the 60 h, short-term incubation (b). Horizontal lines represent the mean $\delta^{13}\text{C}$ of SOM in elevated CO₂ and ambient plots. Each point is the mean of four replicates, except for elevated CO₂ soils in the long-term incubation, for which there were three replicates. Error bars are one standard error of the mean.

CO₂ treatment. The same effect was evident in the short-term incubations, with $\delta^{13}\text{C}_{\text{CO}_2}$ significantly higher with N additions by a mean of 1.0‰ ($P = 0.001$; Fig. 2b), with no interaction with CO₂ treatment.

PLFA data

Microbial biomass expressed as total PLFAs was greater with N additions, regardless of CO₂ treatment ($P = 0.038$). There was no effect of elevated CO₂ on the relative abundance of fungal vs. bacterial PLFAs. Nitrogen additions, however, resulted in reduced abundance of fungal relative to bacterial PLFAs, suggesting that nonfertilized soils had 2.8 times the fungal biomass of the fertilized soils based upon relative abundance of fungal and bacterial PLFAs ($P < 0.0001$). No other significant differences in the abundance of individual PLFAs were detected in these incubations.

$\delta^{13}\text{C}_{\text{PLFA}}$ values from elevated CO₂ plots were an average of 4.5‰ depleted in ¹³C relative to those from ambient plots, reflecting microbial uptake of the ¹³C-deplete organic inputs to the soil profile with elevated CO₂. For both fertilized and nonfertilized soils, $\delta^{13}\text{C}_{\text{PLFA}}$ ranged from -38.3 to -23.1‰ with elevated CO₂ and from -31.4 to -19.1‰ in ambient plots (Fig. 3a). The $\Delta_{\text{PLFA-CO}_2}$ values for 10me18:0, the biomarker for actinomycetes, exhibited a significant interaction between CO₂ treatment and N additions ($P = 0.01$) such that all four treatment combinations had values of $\Delta_{\text{PLFA-CO}_2}$ statistically distinct from each other, with $\Delta_{\text{PLFA-CO}_2}$ from elevated CO₂, fertilized soils being the most ¹³C-enriched and $\Delta_{\text{PLFA-CO}_2}$ from elevated CO₂, nonfertilized soils being the most ¹³C-deplete (Fig. 3b). No other $\Delta_{\text{PLFA-CO}_2}$ value exhibited a significant interaction between CO₂ and N treatments. The PLFA i17:0 and a17:0, representing other groups of Gram-positive bacteria, also exhibited an increase in $\Delta_{\text{PLFA-CO}_2}$ with elevated CO₂ ($P = 0.06$ and 0.05 , respectively). Multiple Gram-negative bacterial PLFAs exhibited an average decrease in $\Delta_{\text{PLFA-CO}_2}$ of 2.2‰ with fertilization, including cy19:0 ($P = 0.026$), one of two 16:1 ($P = 0.008$), and 18:1 ω 7 ($P = 0.009$). We observed a significant effect of CO₂ treatment in $\Delta_{\text{PLFA-CO}_2}$ for 18:2 ω 6, a biomarker for fungi, which experienced a decline of approximately 3.1‰ with elevated CO₂ ($P < 0.001$).

Extracellular enzyme data

Differences in EEA were evident only during the short-term incubation, and were not observed at the end of the long-term incubation, after 1 year of no substrate renewal. At 18 h during the short-term incubations, potential urease activity with N additions was 8.4 times

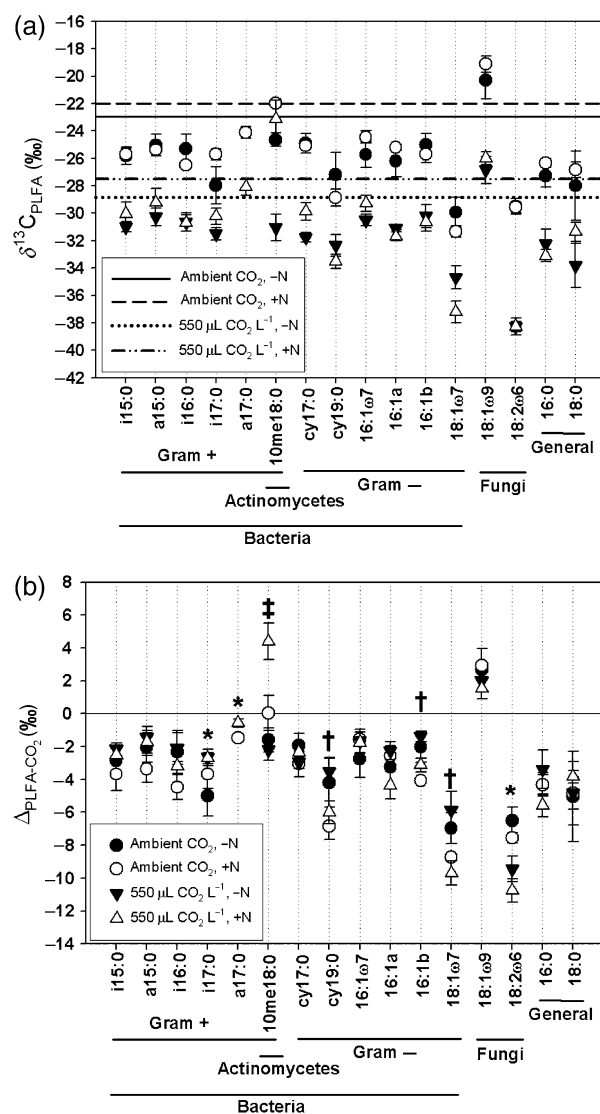


Fig. 3 The mean $\delta^{13}\text{C}$ of PLFA extracted from soils [symbols in (a)] and average $\delta^{13}\text{C}$ of soil respired CO₂ [horizontal lines in (a)], and $\Delta_{\text{PLFA-CO}_2}$ values for each PLFA (b) at 18 h in the short-term incubation. Symbol legend in part (b) also applies to part (a). 16:1a and 16:1b refer to separate 16:1 PLFA, each possessing an unsaturation point with an unidentified location. We were unable to resolve peaks for a17:0 in unfertilized soils. Refer to text for details on $\Delta_{\text{PLFA-CO}_2}$ calculations. In part (b), * indicates a significant difference with CO₂ treatment (i17:0 and a17:0 for Gram-positive bacteria, 18:2 ω 6 for fungi), † indicates a significant difference with N additions (cy19:0, 16:1b, and 18:1 ω 7 for Gram-negative bacteria), and ‡ indicates a significant CO₂ × N interaction (10me18:0 for Gram-positive actinomycetes). For 10me18:0, all values are statistically different from each other. Error bars are one standard error of the mean. For 59 of the 62 points presented, $n = 4$; $n = 3$ for the remaining three points.

that in soils with no added N ($P = 0.04$), and NAG activity with N additions was 76% of nonfertilized soils ($P = 0.08$; Fig. 4).

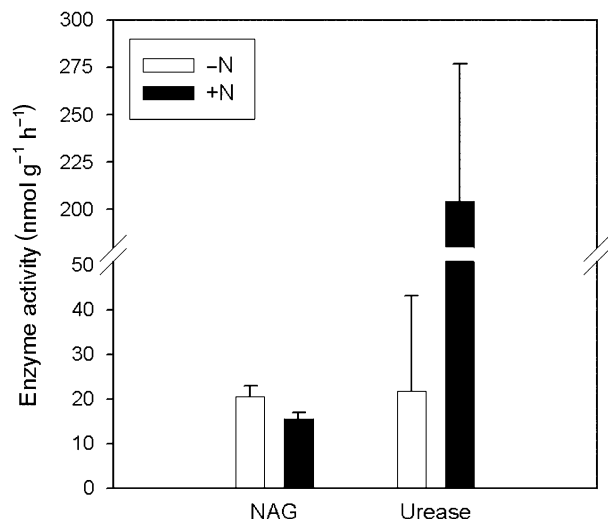


Fig. 4 Average activity of urease and β -1,4-*N*-acetylglucosaminidase (NAG), the two extracellular enzymes for which we observed statistically significant treatment effects, in soils from the Duke FACE facility (0–30 cm mineral soil) at 18 h in the short-term incubation with elevated CO_2 and N treatments. Error bars are one standard error of the mean.

Discussion

Influences of N availability on forest soil C cycling

The response of C uptake to elevated CO_2 in this forest is limited by N availability (Oren *et al.*, 2001; Schlesinger *et al.*, 2006). How will N availability influence the return of C to the atmosphere from the soil in an elevated CO_2 environment? To address this question, we must assess how heterotrophic functioning in the soil profile changes with N availability and elevated CO_2 . *Ex situ* incubations, which isolate heterotrophic activity, revealed a negative effect of N additions on respiration from these soils during the short-term and early phase of the long-term incubation, and with elevated CO_2 later in the long-term incubation. These data, in conjunction with the difference between $\delta^{13}\text{C}$ of individual PLFAs and respired CO_2 ($\Delta_{\text{PLFA}-\text{CO}_2}$) and EEA, indicate that soil microbial function changed with elevated CO_2 and altered N availability. These changes in microbial function could have significant influence over below-ground C balance in this forest.

Butnor *et al.* (2003) report a positive effect of elevated CO_2 and a negative effect of N additions, but no $\text{CO}_2 \times \text{N}$ interaction, in their study exploring *in situ* soil respiration with N additions in one elevated CO_2 plot and one ambient plot at the Duke FACE site. We report a difference of $0.55 \text{ g C m}^{-2} \text{ day}^{-1}$ (R_h) between elevated CO_2 , fertilized soils and the three other treatments; Butnor *et al.* (2003) invoke a reduction in below-ground

C allocation to explain a decline in CO_2 -C release (R_s) with N additions of $0.85 \text{ g C m}^{-2} \text{ day}^{-1}$. Because we isolated heterotrophic respiration via *ex situ* incubations, our data should have exhibited similar CO_2 -C release curves across all treatments if autotrophic respiration was the only driver of reductions in soil respiration with N additions. Instead, the CO_2 -C release patterns observed suggest that elevated CO_2 combined with N additions reduced available C supplies for mineralization and/or altered microbial functioning.

There are two possible mechanisms by which fertilization could have induced a decline in the pools of soil C mineralized in our study. Tree C allocation below-ground could have been altered by N additions, as suggested at this site by Butnor *et al.* (2003) and in other studies (Haynes & Gower, 1995; Albaugh *et al.*, 1998; Lu *et al.*, 1998). Alternatively, stimulated microbial activity in the field during the 6 months of increased N availability prior to our soil collections could have reduced the pool size of readily mineralizable C in soils collected from elevated CO_2 plots in fall. Both mechanisms are consistent with the significantly lower respiration with N additions, regardless of CO_2 treatment, during the short-term and the early phase of the long-term incubation. However, neither mechanism explains the lack of a continued decline in respiration with N additions in soils from ambient plots late in the long-term incubation, suggesting that microbial functioning was altered by elevated CO_2 in some way that was evident only with N additions. Consistent with this, Finzi *et al.* (2006b) suggest that microbial functioning in these soils is becoming increasingly more N limited with elevated CO_2 . Such changes in function may include (1) shifts in the C substrate pools that microorganisms can access, (2) altered activity levels of specific microbial groups, and (3) changes in microbial growth efficiency (Ågren *et al.*, 2001; Frey *et al.*, 2001; Thiet *et al.*, 2006; Teklay *et al.*, 2007), the proportion of substrate used to generate biomass vs. released as CO_2 -C via R_h .

Soil microbial functioning with altered N and CO_2

The $\delta^{13}\text{C}$ values of the respired CO_2 in this study indicate potential shifts in the pools of substrates soil microbes access with N additions and elevated CO_2 . We observed an increase in $\delta^{13}\text{C}$ of respired CO_2 with N additions. Although N additions did not result in significant increases in inorganic N availability at the start of our incubations (data not shown), the increase in potential urease activity with added N suggests that there were greater quantities of organic N accessed by microbes with N additions. This source of N may have supported the use of relatively N-poor substrates; such

components may include carbohydrate-rich compounds such as cellulose, which can be 4‰ enriched relative to bulk plant biomass (Benner *et al.*, 1987; Loader *et al.*, 2003). In contrast with N additions, we observed a decline in $\delta^{13}\text{C}$ of respired CO₂ relative of $\delta^{13}\text{C}_{\text{SOM}}$ in elevated CO₂ soils. These data suggest that with elevated CO₂, progressive N limitation (Luo *et al.*, 2004; Finzi *et al.*, 2006a,b) may induce the use of more ¹³C-deplete substrates; such substrates could include lignin (Benner *et al.*, 1987) and humic acids (Balesdent & Mariotti, 1996), both relatively recalcitrant materials.

To further examine how substrate usage and microbial functioning have shifted with elevated CO₂ and N additions, we employed PLFAs and their isotopic composition. For example, the relatively low values of $\Delta_{\text{PLFA-CO}_2}$ for the fungal biomarker 18:2 ω 6 with elevated CO₂ are consistent with greater use of recently formed SOM by fungi with elevated CO₂. The lack of adequate controls with equivalently ¹³C-labeled photosynthates entering the soil profile precludes this as conclusive evidence for increased fungal activity under elevated CO₂ in these soils. However, the data do indicate that fungi in elevated CO₂ plots were actively incorporating the ¹³C label into their biomass, and that the relative extent of the ¹³C depletion under elevated CO₂ represents a shift from ambient plots. In contrast, Billings & Ziegler (2005) reported no difference with elevated CO₂ in the degree to which fungi incorporated the ¹³C label of recently formed organic matter. This previous study, however, examined soils collected during the spring, when bacterial functioning can dominate in soil profiles relative to fall (Lipson *et al.*, 2002; Schadt *et al.*, 2003). Increased fungal activity with elevated CO₂, as implied by the data presented here, is consistent with a widening ecosystem C:N ratio (Finzi *et al.*, 2006a), given the ability of fungi to mineralize relatively poor quality organic material (Killham, 1994; Paul & Clark, 1996).

We also observed a strong influence of fertilization on bacterial $\Delta_{\text{PLFA-CO}_2}$. Values of $\Delta_{\text{PLFA-CO}_2}$ suggest that increased N resources can positively influence Gram-negative bacterial groups at the expense of some Gram-positive bacteria. For example, $\Delta_{\text{PLFA-CO}_2}$ values of two abundant Gram-negative bacterial biomarkers (cy19:0 and 18:1 ω 7) suggest an increased role for Gram-negative bacteria with added N in transforming recently formed, ¹³C-depleted SOM. In contrast, the relatively high $\Delta_{\text{PLFA-CO}_2}$ values for the actinomycetes biomarker with elevated CO₂ and N additions indicate that these organisms took up very little recently formed SOM. Fertilization may have induced these organisms to use older, recalcitrant material that has not yet acquired the $\delta^{13}\text{C}$ signature of recently photosynthesized organic matter; actinomycetes are well-adapted for metaboliz-

ing such material (McCarthy & Williams, 1992). However, the relatively low C:N ratios exhibited by most older SOM (Tiessen & Stewart, 1983; Billings, 2006) suggests that fertilization may not necessarily promote increased access to these pools. Alternatively, actinomycetes may have reduced their activity levels in elevated CO₂, fertilized soils, and relied on storage compounds for cell wall maintenance. Relative to most extracellular C substrates in the soil profile, C in actinomycetes storage compounds such as glycogen (Schneider *et al.*, 2000; Hoskisson *et al.*, 2004) can be ¹³C enriched (Benner *et al.*, 1987; Teece & Fogel, 2007). Consistent with this, respiration from elevated CO₂, fertilized soils declined late in the long-term incubation. This suggests that some groups of microorganisms well adapted to accessing relatively recalcitrant C substrates, such as actinomycetes, experienced a decline in metabolism.

Links between microbial functioning and SOM cycling

The N-governed competition between microbial groupings suggested by these data has large implications for soil C transformations in this forest. With increasing N limitations at this site (Luo *et al.*, 2004; Finzi *et al.*, 2006a,b), Gram-positive bacteria may increase their rates of SOM transformations relative to Gram-negative bacteria. Such a shift could influence soil organic C balances via changes in substrate usage patterns. For example, actinomycetes tend to access relatively stable soil C pools (McCarthy & Williams, 1992). A sustained increase in activity of this group of Gram-positive bacteria with elevated CO₂ (Billings & Ziegler, 2005) in an increasingly N-limited environment could result in increased turnover rates of soil C pools typically considered relatively decay-resistant. Additionally, the dependence of relative abundances of bacteria vs. fungi on N availability, and the implied increased activity of some fungal groups with elevated CO₂, suggest that progressive N limitation with elevated CO₂ may result in an increase in the dominance of fungal activity in these soils. This, in turn, would likely result in increased turnover rates of relatively recalcitrant soil C.

Increased turnover of relatively recalcitrant SOM may be partially mitigated by the formation of such material by fungi and Gram-positive bacteria. Gram-positive bacteria possess a greater proportion of peptidoglycan than Gram-negative bacteria, and fungal cell walls are composed primarily of chitin; both compounds contain significant quantities of N-acetylglucosamine, and are precursors to relatively decay-resistant SOM (Sollins *et al.*, 1996; Simpson *et al.*, 2007). We observed reduced potential NAG activity in soils with N additions, implying that increased N availability may have reduced pool sizes of chitin and peptidoglycan in these soils.

Accordingly, further N limitations induced by elevated CO₂ (Luo *et al.*, 2004; Finzi *et al.*, 2006a,b) may result in increased availability of the relatively recalcitrant compounds that comprise cell walls of Gram-positive bacteria and fungi. However, the influence of increased formation rates of chitin and peptidoglycan on stable C pool sizes would be small relative to increased turnover rates of SOC given the small amount of microbial biomass C (1–2% of total soil C; Killham, 1994) relative to fluxes of soil C in these soil (Lichter *et al.*, 2005).

The influence of microbial growth efficiency on soil C balance

Because the efficiency with which microorganisms use C substrates varies greatly (del Giorgio & Cole, 1998; Frey *et al.*, 2001; Thiet *et al.*, 2006), we would expect shifting levels of activity among microbial groupings to result in altered microbial growth efficiency as well. Consistent with this idea, we observed a decline in heterotrophic respiration with N additions during both soil incubations with no detectable change in microbial biomass. These reductions in CO₂-C release with N additions suggest an increase in the efficiency with which microorganisms can incorporate C into their biomass vs. releasing it as CO₂-C. Nutrient availability has influenced C return to the atmosphere in multiple ecosystems by altering heterotrophic metabolism (del Giorgio & Cole, 1998). If progressive N limitation is realized in this system (Finzi *et al.*, 2006a), N limitations may indirectly influence the C balance of this forest by promoting decreased microbial growth efficiencies and relatively greater releases of CO₂-C from the soil profile.

Conclusions

Although above- vs. belowground C allocation of autotrophs may induce part of the decline in soil respiration observed with N additions at this site (Butnor *et al.*, 2003), data from the *ex situ* soil incubations described in this study indicate that altered soil heterotrophic structure and functioning can also result from N additions. Microbial structure can change via decreased fungal abundance with N additions, implying that with increasing N limitations in this forest, fungal abundance may increase. Because the Duke FACE site lacks control plots exposed to a ¹³C tracer similar to that applied in the elevated CO₂ plots, we cannot state conclusively that soil fungi in this forest are more active with elevated CO₂. However, fungal biomarker $\Delta_{\text{PLFA-CO}_2}$ values are consistent with this process, as was suggested for actinomycetes in a previous study at this site (Billings & Ziegler, 2005). More conclusively, $\Delta_{\text{PLFA-CO}_2}$ data suggest a competitive interplay between Gram-

positive and Gram-negative bacteria in elevated CO₂ soils governed by N availability, with N-limited soils exhibiting greater relative activity levels of Gram-positive bacteria compared to soils receiving N additions. Given the important role of Gram-positive actinomycetes and fungi in metabolizing material such as lignin, chitin, and humic acids, we would predict that increasing N limitations in this forest (Luo *et al.*, 2004; Finzi *et al.*, 2006a,b) will result in greater turnover rates of relatively stable soil C pools. This increased 'microbial mining' of decay-resistant pools would likely result in relative increases in CO₂-C release from the soil profile, contributing to greater C losses from this forest.

Acknowledgements

This research was supported by the Office of Science (BER) Program, US Department of Energy grant number DE-FG02-95ER62083, and the US Department of Energy's National Institute of Climate Change Research (NICCR) grant number DE-FC02-03ER63613 [awarded from the National Institute for Global Environmental Change Southeast Regional Center (NIGEC SERC)]. Two anonymous reviewers provided helpful comments. We thank Jill Baird, Lindsey Conaway, Laurel Haavik, Andrea Kopecky, David Lyon, Tom Millican, Glenn Piercey, Jeff Phippen, Erik Pollock, Ryan Rastok, Alexis Reed, Vaughn Salisbury, Lisa Tiemann, and Guihong Zhang for assistance in the field and laboratory.

References

- Abrajano TA, Murphy DE, Fang J, Comet P, Brooks JM (1994) C-13 and C-12 ratios in individual fatty acids of marine mytilids with and without bacterial symbionts. *Organic Geochemistry*, **21**, 611–617.
- Ågren G, Bosatta E, Magill A (2001) Combining theory and experiment to understand effects of inorganic nitrogen on litter decomposition. *Oecologia*, **128**, 94–98.
- Albaugh TJ, Allen HL, Dougherty PM, Kress LW, King JS (1998) Leaf area and above- and belowground growth responses of loblolly pine to nutrient and water additions. *Forest Science*, **44**, 317–328.
- Andrews J, Schlesinger W (2001) Soil CO₂ dynamics, acidification, and chemical weathering in a temperate forest with experimental CO₂ enrichment. *Global Biogeochemical Cycles*, **15**, 149–162.
- Balesdent J, Mariotti A (1996) Measurement of soil organic matter turnover using ¹³C natural abundances. In: *Mass Spectrometry of Soils* (eds Boutton TW, Yamasaki SI), pp. 83–111. Marcel Dekker Inc., New York.
- Benner R, Fogel ML, Sprague EK, Hodson RE (1987) Depletion of C-13 in lignin and its implications for stable carbon isotope studies. *Nature*, **329**, 708–710.
- Bernhardt E, Barber J, Phippen J (2006) Long-term effects of free air CO₂ enrichment (FACE) on soil respiration. *Biogeochemistry*, **77**, 91–116.

- Billings SA (2006) Soil organic matter dynamics and land use change at a grassland/forest ecotone. *Soil Biology and Biochemistry*, **38**, 2934–2943.
- Billings SA, Brewer CM, Foster BL (2006) Incorporation of plant residues into soil organic matter fractions with grassland management practices in the North American Midwest. *Ecosystems*, **9**, 805–815.
- Billings SA, Ziegler SE (2005) Linking microbial activity and soil organic matter transformations in forest soils under elevated CO₂. *Global Change Biology*, **11**, 203–212.
- Bridgman SD, Updegraff K, Pastor J (1998) Carbon, nitrogen, and phosphorus mineralization in northern wetlands. *Ecology*, **79**, 1545–1561.
- Butnor J, Johnsen K, Oren R (2003) Reduction of forest floor respiration by fertilization on both carbon dioxide-enriched and reference 17-year-old loblolly pine stands. *Global Change Biology*, **9**, 849–861.
- Carreiro MM, Sinsabaugh RL, Repert DA *et al.* (2000) Microbial enzyme shifts explain litter decay responses to simulated nitrogen deposition. *Ecology*, **81**, 2359–2365.
- Chapin FS, Woodwell GM, Randerson JT *et al.* (2006) Reconciling carbon-cycle concepts, terminology, and methods. *Ecosystems*, **9**, 1041–1050.
- del Giorgio PA, Cole JJ (1998) Bacterial growth efficiency in natural aquatic systems. *Annual Review of Ecology and Systematics*, **29**, 503–541.
- DeLucia E, Hamilton JG, Naidu SL *et al.* (1999) Net primary production of a forest ecosystem with experimental CO₂ enrichment. *Science*, **284**, 1177–1179.
- Dobbs FC, Findlay RH (1993) Analysis of microbial lipids to determine biomass and to detect the response of sedimentary microorganisms to disturbance. In: *Handbook of Methods in Aquatic Microbial Ecology* (eds Kemp PF, Sherr BF, Sherr EB, Cole JJ), pp. 347–358. Lewis Publications, Boca Raton, FL.
- Ellsworth DS (1999) CO₂ enrichment in a maturing pine forest: are CO₂ exchange and water status in the canopy affected? *Plant, Cell and Environment*, **22**, 461–472.
- Findlay RH (2007) Determination of microbial community structure using phospholipid fatty acid profiles. In: *Molecular Microbial Ecology Manual*, Vol. 2 (eds Kowalchuk GA, de Bruijn FJ, Head IM, Akkermans AD, van Elsas JD), pp. 983–1003. Springer, New York.
- Findlay RH, Dobbs FD (1993) Quantitative description of microbial communities using lipid analysis. In: *Handbook of Methods in Aquatic Microbial Ecology* (eds Kemp PF, Sherr BF, Sherr EB, Cole JJ), pp. 271–284. Lewis Publications, Boca Raton, FL.
- Finzi A, Moore DJ, Delucia E (2006a) Progressive nitrogen limitation of ecosystem processes under elevated CO₂ in a warm-temperate forest. *Ecology*, **87**, 15–25.
- Finzi A, Sinsabaugh RL, Long TM, Osgood MP (2006b) Microbial community responses to atmospheric carbon dioxide enrichment in a warm-temperate forest. *Ecosystems*, **9**, 215–226.
- Frey S, Gupta V, Elliott E (2001) Protozoan grazing affects estimates of carbon utilization efficiency of the soil microbial community. *Soil Biology and Biochemistry*, **33**, 1759–1768.
- Goodale C, Apps M, Birdsey R (2002) Forest carbon sinks in the Northern Hemisphere. *Ecological Applications*, **12**, 891–899.
- Hamilton J, DeLucia E, George K, Naidu S, Finzi A, Schlesinger W (2002) Forest carbon balance under elevated CO₂. *Oecologia*, **131**, 250–260.
- Haynes BE, Gower ST (1995) Belowground carbon allocation in unfertilized and fertilized red pine plantations in northern Wisconsin. *Tree Physiology*, **15**, 317–325.
- Hendrey GR, Ellsworth DS, Lewin KF, Nagy J (1999) A free-air enrichment system for exposing tall forest vegetation to elevated atmospheric CO₂. *Global Change Biology*, **5**, 293–309.
- Hoskisson PA, England R, Sharples GP, Hobbs G (2004) Modulation of glycogen and trehalose levels in *Micromonospora echinospora* (ATCC 15837). *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology*, **86**, 225–233.
- Jobbagy E, Jackson R (2000) The vertical distribution of soil organic carbon and its relation to climate and vegetation. *Ecological Applications*, **10**, 423–436.
- Killham K (1994) *Soil Ecology*. Cambridge University Press, Cambridge.
- Lichter J, Barron SH, Bevacqua CE *et al.* (2005) Soil carbon sequestration and turnover in a pine forest after six years of atmospheric CO₂ enrichment. *Ecology*, **86**, 1835–1847.
- Lipson DA, Schadt CW, Schmidt SK (2002) Changes in soil microbial community structure and function in an alpine dry meadow following spring snow melt. *Microbial Ecology*, **43**, 307–314.
- Littell RC, Milliken GA, Stroup WW, Wolfinger RD (1996) *SAS System for Mixed Models*. SAS Institute Inc., Cary, NC.
- Loader NJ, Robertson I, McCarroll D (2003) Comparison of stable carbon isotope ratios in the whole wood, cellulose, and lignin of oak tree-rings. *Palaeogeography, Palaeoclimatology, Palaeoecology*, **196**, 395–407.
- Lovett G, Cole J, Pace M (2006) Is net ecosystem production equal to ecosystem carbon accumulation? *Ecosystems*, **9**, 152–155.
- Lu SJ, Mattson KG, Zaerr JB, Marshall JD (1998) Root respiration of Douglas-fir seedlings: effects of N concentration. *Soil Biology and Biochemistry*, **30**, 331–336.
- Luo Y, Su B, Currie WS *et al.* (2004) Progressive nitrogen limitation of ecosystem responses to rising atmospheric carbon dioxide. *Bioscience*, **54**, 731–739.
- Maier CA, Kress LW (2000) Soil CO₂ evolution and root respiration in 11 year-old loblolly pine (*Pinus taeda*) plantations as affected by moisture and nutrient availability. *Canadian Journal of Forest Research*, **30**, 347–359.
- Matamala R, Schlesinger W (2000) Effects of elevated atmospheric CO₂ on fine root production and activity in an intact temperate forest ecosystem. *Global Change Biology*, **6**, 967–979.
- McCarthy AJ, Williams ST (1992) Actinomycetes as agents of biodegradation in the environment – a review. *Gene*, **115**, 189–192.
- McLaugherty CA, Aber JD, Melillo JM (1982) The role of fine roots in the organic matter and nitrogen budgets of two forested ecosystems. *Ecology*, **63**, 1481–1490.
- Oren R, Ellsworth DS, Johnsen KH *et al.* (2001) Soil fertility limits carbon sequestration by forest ecosystems in a CO₂-enriched atmosphere. *Nature*, **411**, 469–472.
- Paul EA, Clark FE (1996) *Soil Microbiology and Biochemistry*. Academic Press, San Diego.

- Pinkart HC, Devereux R, Chapman PJ (1998) Rapid separation of microbial lipids using solid phase extraction columns. *Journal of Microbiological Methods*, **34**, 9–15.
- Pregitzer K, Loya W, Kubiske M, Zak D (2006) Soil respiration in northern forests exposed to elevated atmospheric carbon dioxide and ozone. *Oecologia*, **148**, 503–516.
- Robinson D (2007) Implications of a large global root biomass for carbon sink estimates and for soil carbon dynamics. *Proceedings of the Royal Society B*, **274**, 2753–2759.
- Saiya-Cork KR, Sinsabaugh RL, Zak DR (2002) The effects of long term nitrogen deposition on extracellular enzyme activity in an *Acer saccharum* forest soil. *Soil Biology and Biochemistry*, **34**, 1309–1315.
- Schadt CW, Martin AP, Lipson DA, Schmidt SK (2003) Seasonal dynamics of previously unknown fungal lineages in tundra soils. *Science*, **310**, 1359–1361.
- Schlesinger W (1977) Carbon balance in terrestrial detritus. *Annual Review of Ecology and Systematics*, **8**, 51–81.
- Schlesinger W (1997) *Biogeochemistry: An Analysis of Global Change*. Academic Press, Boston.
- Schlesinger W, Lichter J (2001) Limited carbon storage in soil and litter of experimental forest plots under increased atmospheric CO₂. *Nature*, **411**, 466–469.
- Schlesinger WH, Bernhardt ES, DeLucia EH *et al.* (2006) The Duke Forest FACE experiment: CO₂ enrichment of a loblolly pine forest. In: *Managed Ecosystems and CO₂: Case Studies, Processes, and Perspectives, Ecological Studies*, Vol. 187 (eds Nosberger J, Long SP, Norby RJ, Stitt M, Hendrey GR, Blum H), pp. 197–212. Springer, Berlin.
- Schneider D, Bruton CJ, Chater KF (2000) Duplicated gene clusters suggest an interplay of glycogen and trehalose metabolism during sequential stages of aerial mycelium development in *Streptomyces coelicolor* A3(2). *Molecular and General Genetics*, **263**, 543–553.
- Silfer JA, Engel MH, Macko SA, Jumeau EJ (1991) Stable carbon isotope analysis of amino acid enantiomers by conventional isotope ratio mass spectrometry and combined gas chromatography isotope ratio mass spectrometry. *Analytical Chemistry*, **63**, 370–374.
- Simpson AJ, Song G, Smith E, Lam B, Novotny EH, Hayes MB (2007) Unraveling the structural components of soil humin by use of solution-state nuclear magnetic resonance spectroscopy. *Environmental Science and Technology*, **41**, 876–883.
- Sinsabaugh RL, Saiya-Cork K, Long T, Osgood MP, Neher DA, Zak DR, Norby JR (2003) Soil microbial activity in a *Liquidambar* plantation unresponsive to CO₂-driven increases in primary production. *Applied Soil Ecology*, **24**, 263–271.
- Six J, Frey SD, Thiet RK, Batten KM (2006) Bacterial and fungal contributions to carbon sequestration in agroecosystems. *Soil Science Society of America Journal*, **70**, 555–569.
- Sollins P, Homann P, Caldwell BA (1996) Stabilization and destabilization of soil organic matter: mechanisms and controls. *Geoderma*, **74**, 65–105.
- Teece MA, Fogel ML (2007) Stable carbon isotope biogeochemistry of monosaccharides in aquatic organisms and terrestrial plants. *Organic Geochemistry*, **38**, 458–473.
- Teklay T, Nordgren A, Nyberg G, Malmer A (2007) Carbon mineralization of leaves from four Ethiopian agroforestry species under laboratory and field conditions. *Applied Soil Ecology*, **35**, 193–202.
- Thiet R, Frey S, Six J (2006) Do growth yield efficiencies differ between soil microbial communities differing in fungal: bacterial ratios? Reality check and methodological issues. *Soil Biology and Biochemistry*, **38**, 837–844.
- Tiessen H, Stewart J (1983) Particle size fractions and their use in studies of soil organic matter 2. Cultivation effects on organic matter composition in size fractions. *Soil Science Society of America Journal*, **47**, 509–514.
- Tu K, Dawson T (2005) Partitioning ecosystem respiration using stable carbon isotope analyses of CO₂. In: *Stable Isotopes and Biosphere–Atmosphere Interactions* (eds Flanagan LB, Ehleringer JR, Pataki DE), pp. 125–153. Academic Press, San Diego.
- US Climate Change Science Program (2006) *Synthesis and Assessment Product 2.2, The First State of the Carbon Cycle Report (SOCCR): The North American Carbon Budget and Implications for the Global Carbon Cycle*. Subcommittee on Global Change Research, US Carbon Cycle Science Program, Washington, DC.
- Waring RH, Schlesinger WH (1985) *Forest Ecosystems: Concepts and Management*. Academic Press, San Diego.
- White DC, Ringelberg DB (1998) Signature lipid biomarker analysis. In: *Techniques in Microbial Ecology* (eds Burlage RS, Atlas R, Stahl D, Geesey G, Saylor G), pp. 255–272. Oxford University Press, New York.
- Zak D, Holmes W, Finzi A, Norby R, Schlesinger W (2003) Soil nitrogen cycling under elevated CO₂: a synthesis of forest face experiments. *Ecological Applications*, **13**, 1508–1514.
- Zelles L, Bai QY, Beck T, Beese F (1992) Signature fatty acids in phospholipids and lipopolysaccharides as indicators of microbial biomass and community structure in agricultural soils. *Soil Biology and Biochemistry*, **24**, 317–323.