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Soil microbial activity in a *Liquidambar* plantation unresponsive to CO_2-driven increases in primary production

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Abstract

The indirect responses of soil microbiota to changes in plant physiology effected by elevated atmospheric carbon dioxide have the potential to alter nutrient availability and soil carbon storage. We measured fine root density, microbial biomass nitrogen, rates of nitrogen mineralization and nitrification, substrate utilization by soil bacteria and extracellular enzyme activities (EEA) associated with bulk soil and fine root rhizoplanes within a 3-year period at the Oak Ridge National Laboratory (ORNL) Free Air Carbon Enrichment (FACE) experiment, situated in a *Liquidambar styraciflua* plantation. Rhizoplane EEA was similar to that of bulk soil. Prior studies have reported a 21% increase in net primary production (NPP) in the enrichment plots and evidence that additional carbon is reaching the soil system, however we observed no response in any of the variables we measured. These results, which contrast with those from other temperate forest FACE sites, suggest that soil characteristics can influence the magnitude and timing of belowground responses.
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Keywords: Carbon dioxide enrichment; Soil; Extracellular enzyme activity; Bacterial substrate utilization; Nitrogen mineralization; Global change

1. Introduction

Rising concentrations of atmospheric carbon dioxide can alter global patterns of organic matter production and decomposition through direct effects of CO_2 on primary producers and CO_2-driven climatic changes. Forest effects are of particular concern because they account for a large fraction of global net primary production (NPP). Numerous studies have shown that tree growth is stimulated by elevated atmospheric CO_2. However, the response of forest ecosystems cannot be predicted from short-term changes in the production dynamics of individual plants. The magnitude and sustainability of the production response, as well as the capacity of the ecosystem to sequester carbon, are ultimately determined by a variety of environmental factors including soil microbial processes.

Plant responses to elevated CO_2 affect soil processes through changes in C allocation to roots and foliage. Increases in root exudation and fine root turnover have been observed in many studies (Zak et al., 2000).

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These carbon inputs have been shown to stimulate microbial activities in the rhizosphere, potentially increasing mineral nutrient availabilities, a phenomenon that has been termed "carbon priming". Over time, labile reservoirs of N and P in the rhizosphere could be depleted unless the decomposition of litter, roots and soil organic matter (SOM) increases. Thus, soil processes may limit the responses of plant communities to CO_2 enrichment (Oren et al., 2001).

CO_2 enrichment has been shown, in some cases, to increase the production of secondary compounds in foliage; reduced N concentration and increased C:N ratio have also been observed. These changes, if maintained in the litter, may slow decomposition rates, though empirical evidence for this is mixed (Norby et al., 2001a).

Clearly, the effects of CO_2 enrichment on decomposition and nutrient cycling processes in the soil are complex. Reported responses vary in direction and magnitude (Zak et al., 2000; Norby et al., 2001a). Because soil systems are affected by changes in the composition and magnitude of foliar and root carbon inputs, the net effect of elevated atmospheric CO_2 on soil carbon storage and nutrient regeneration is difficult to predict and likely to vary with soil type and plant community composition. Progress toward a general understanding requires mechanistically linking plant and soil characteristics to microbial responses.

For the past 3 years, we have been investigating microbial activity in soil at the Oak Ridge National Laboratory (ORNL) Free Air Carbon Enrichment (FACE) site, near Oak Ridge, TN, to determine the extent and mechanisms by which primary production responses influence soil processes. The site is a sweetgum (*Liquidambar styraciflua*) stand planted in 1988. In this report, we compare microbial activity in treatment plots exposed to elevated atmospheric CO_2 to that of control plots.

2. Methods and materials

2.1. Study site and sample collection

Soil samples were collected from the Oak Ridge National Laboratory Free Air Carbon Enrichment experimental site near Oak Ridge, TN. The site is a sweetgum (*L. styraciflua* L.) plantation established in 1988 on a floodplain terrace. The soil is an alluvial aquic hapludult with an organic carbon content of about 1% and a silty clay loam texture (Van Miegroet et al., 1994). The FACE array consists of five 25 m diameter rings (plots) distributed within the contiguous forest. The design and operation of the FACE apparatus for forest systems has been described by Hendrey et al. (1999). Site conditions and the experimental design of the ORNL FACE have been presented by Norby et al. (2001b). Three of the rings receive ambient air (control plots) and two (enrichment plots) receive ambient air amended with CO_2 at a target concentration is 565 ppm. Fumigation began April 1998 and has been applied each year during the growing season (mid-April through early November).

In 1999 and 2000, soil samples were collected three times per year: May, July, and September. At each sampling date, 8–12 soil cores (2 cm (diameter) × 10 cm (deep)) were randomly collected across the soil sampling zones within each ring. The soil cores collected within each ring were pooled. The pooled samples were homogenized by passing soil through a 2 mm sieve. Subsamples were removed for measurements of moisture content, enzyme activity, substrate utilization, nitrogen mineralization and nitrification.

2.1.1. Enzyme assays

Enzyme assays were performed at ORNL and began within 2–3 h of sample collection. Sample suspensions were prepared by adding 1.0 g soil to 125 ml of 50 mM, pH 5.0, acetate buffer and homogenizing for 1 min with a Brinkmann Polytron. The resulting suspensions were continuously stirred using a magnetic stir plate while 200 μl aliquots were dispensed into 96-well microplates: 16 replicate wells per sample per assay. The soil suspensions were assayed for the activity of ten enzymes involved in the degradation of plant litter or cycling of organic nitrogen and phosphorus (Table 1).

All the assays except phenol oxidase, peroxidase and amidohydrolase were fluorimetric. Fifty microliters of 200 μM substrate solution (Table 1) were added to each sample well. Blank wells received 50 μl of acetate buffer plus 200 μl of sample suspension. Negative control wells received 50 μl substrate solution plus 200 μl of acetate buffer. Quench standard wells received 50 μl of standard (10 μM 4-methylumbelliferone, or 7-amino-4-methylcoumarin in the case

Table 1
Enzyme assays conducted on soil and root samples collected from the FACE experimental plots at Oak Ridge National Laboratory

Enzyme	EC	Substrate
Urease	3.5.1.5	Urea
Phenol oxidase	1.10.3.2	L-3,4-Dihydroxyphenylalanine
Peroxidase	1.11.1.7	L-3,4-Dihydroxyphenylalanine
Alkaline phosphatase	3.1.3.1	4-MUB-phosphate
Acid phosphatase	3.1.3.2	4-MUB-phosphate
β-1,4-Glucosidase	3.2.1.21	4-MUB-β-D-glucoside
Cellobiohydrolase	3.2.1.91	4-MUB-β-D-cellobioside
β-1,4-*N*-Acetylglucosaminidase	3.1.6.1	4-MUB-*N*-acetyl-β-D-glucosaminide
β-1,4-Xylosidase	3.2.1.37	4-MUB-β-D-xyloside
α-1,4-Glucosidase	3.2.1.20	4-MUB-a-D-glucoside
L-Leucine aminopeptidase	3.4.11.1	L-Leucine-7-amino-4-methylcoumarin
Fatty acid esterase	3.1.1.1	4-MUB-acetate

The enzyme names are followed by their enzyme commission number (EC) and the substrate used in our assays (MUB: 4-methylumbelliferyl).

leucine aminopeptidase) plus 200 μl sample suspension. Reference standard wells received 50 μl of standard plus 200 μl acetate buffer. There were eight replicate wells for each blank, negative control, and quench standard. The microplates were incubated in the dark at 20 °C for 1–4 h. To stop the reaction, a 10 μl aliquot of 1.0 M NaOH was added to each well. Fluorescence was measured using a microplate fluorometer with 365 nm excitation and 450 nm emission filters. After correcting for controls and quenching, activities was expressed in units of nmol/(h g).

Phenol oxidase and peroxidase activities were measured spectrophotometrically using L-3,4-dihydroxyphenylalanine (DOPA) as the substrate. For phenol oxidase, 50 μl of 25 mM DOPA was added to each sample well. Peroxidase assays received 50 μl of 25 mM DOPA plus 10 μl of 0.3% H_2O_2. Negative control wells for phenol oxidase contained 200 μl of acetate buffer and 50 μl of DOPA solution; blank wells contained 200 μl of sample suspension and 50 μl of acetate buffer. For peroxidase, negative control and blank wells also contained 10 μl of H_2O_2. There were 16 replicate sample wells for each assay and eight replicate wells for blanks and controls. The microplates are incubated in the dark at 20 °C, approximately 4 h for peroxidase and 18 h for phenol oxidase. Activity was quantified by measuring absorbance at 450 nm using a microplate spectrophotometer and expressed in units of nmol/(h g).

Amidohydrolase (urease) activity was measured spectrophotometrically (610 nm) using urea as a substrate. The microplate configuration was similar to that described for the phenol oxidase assay. Microplates were incubated for 18 h at 20 °C. The concentration of urea in the assay wells was 20 mM. Ammonium released by the reaction was quantified using colorimetric reagent packets from Hach (Sinsabaugh et al., 2003). Activity was expressed as nmol of ammonium released per hour per gram of soil (nmol/(h g)).

2.2. Substrate utilization

In 1999 and 2000, substrate utilization by soil bacteria was profiled using BIOLOG GN and ECO plates (BIOLOG Inc., Haywood, CA). The BIOLOG GN plate is a 96-well microtiter plate containing 95 different carbon substrates plus one negative control with no carbon substrate. The GN plates were originally developed to facilitate identification of gram negative bacteria isolated from clinical samples. The ECO plates are intended for environmental samples and contain 31 different substrates in a triplicated pattern. Using both types of plates, bacterial response to 120 different substrates was surveyed. Bacterial growth within the wells was detected by reduction of a colorigenic tetrazolium dye.

Sample suspensions were produced by homogenizing 1.0 g soil in 100 ml of deionized water; 150 μl of suspension was dispensed into each well. The plates were inoculated at ORNL within 3–4 h after sample collection. The microplates were incubated in the dark at 20 °C for 48 h. Absorbance in each

well was measured at 590 nm using a microplate spectrophotometer.

2.3. Rhizoplane assays

In 2001, soil cores (eight/ring) were collected in May, July and October by ORNL personnel and shipped overnight with cold packs to University of Toledo. Upon arrival, roots were extracted by washing soil material through sieves. The collected roots from each ring were subsampled and assayed for the activity of five extracellular enzymes: acid phosphatase, alkaline phosphatase, β-glucosidase and fatty acid esterase.

For each assay, eight 50–100 mg (dry mass equivalent) subsamples (typically several roots per subsample) of fine roots (<2 mm diameter) were placed intact into the wells of a 12-well microplate. The roots were covered with 2.0 ml of buffer: 50 mM bicarbonate, pH 8, for alkaline phosphatase assays, 50 mM acetate, pH 5, for others. Substrate solution, 0.5 ml, was added to eight replicate wells (Table 1). Two wells on each plate were negative controls, roots plus 2.5 ml buffer; two wells were used to measure quenching effects, roots plus 2.0 ml buffer, plus 0.5 ml of 10 μM 4-methylumbelliferone. The plates were incubated at 20 °C for 1–4 h. After incubation, 250 μl aliquots were withdrawn and pipetted into 96-well microplates. After adding 10 μl of 1.0 M NaOH to each well fluorescence was measured as described for the bulk soil assays. The root material from each plate was rinsed, dried and weighed so that activity in each well could be expressed as nmol of substrate hydrolyzed per gram dry mass per hour.

2.4. Nitrogen dynamics

We calculated microbial biomass N and gross rates of N mineralization and nitrification from samples collected in July 1999 and 2000 (Davidson et al., 1992; Hart et al., 1994). Samples were stored at 4 °C until analyzed; the measurements were done at the University of Michigan. We also used samples collected in 1999 to trace the transfer of ^{15}N from soil solution into microbial biomass and soil organic matter. Microbial biomass N was determined by direct extraction following chloroform fumigation (Brookes et al., 1985).

To measure transformation rates, 12.0 g from each soil sample was weighed into five glass vials. Two vials were enriched with 1.00 ml (±0.005 ml) ^{15}N-NH_4Cl solution, and two were enriched with the same volume of ^{15}N-KNO_3 solution. The ^{15}N solutions were prepared using a mixture of ^{15}N-labeled (99.5%) and unlabeled N, which produced similar target concentrations of N (2–5 mg N/g soil) and ^{15}N (2–4 at.% excess ^{15}N) in both $^{15}NH_4^+$ and $^{15}NO_3^-$-labeled soil. Labeling solutions were added by pipetting the liquid (1 ml) evenly over the soil surface, which brought the soil samples to field capacity. This volume was adequate to disperse the label evenly throughout each 12-g sample, evidenced by a uniform wetting of the entire soil volume. Within 1 h after isotope addition, one $^{15}NH_4^+$-enriched sample and one $^{15}NO_3^-$-enriched sample from each treatment combination was extracted with 2 M KCl. The remaining two ^{15}N-enriched samples from each treatment combination were incubated at 20 °C for 2 days, after which, we extracted inorganic N with 2 M KCl. Ammonium and NO_3^- concentrations in soil extracts were analyzed on an OI Analytical Flow Solution 3000 continuous flow analyzer (OI Analytical, College Station, TX).

Ammonium-N and NO_3^--N were sequentially diffused from each KCl extract onto acid traps in preparation for ^{15}N analysis by isotope ratio mass spectrometry. Diffusions were conducted in 120 ml specimen cups, which contained an acidified cellulose disk (10 ml of 2.5 mol/l $KHSO_4$) suspended above the liquid on a stainless steel wire. We added MgO to each solution and collected NH_3 on the acidified cellulose disk. After 6 days, we removed the disk and wire, added Devarda's alloy to convert NO_3^- to NH_4^+, and collected the resulting NH_4^+ on new acidified cellulose disk. The cellulose disks containing ^{15}N were dried in a desiccator, encapsulated in Sn, and analyzed for at.% ^{15}N using a Finnigan Delta Plus isotope ratio mass spectrometer with a Conflo II interface (Thermo Finnigan, San Jose, CA).

2.5. Root density

The soil samples collected in 1999 and 2000 were also analyzed for root density and nematode abundance (data not presented here). To quantify root density, soil was gently washed through a 810 μm-mesh

sieve. Roots and organic matter retained on the sieve were spread uniformly in 15 cm diameter glass petri dishes on a light table and digital images captured by a Sony CCD video camera with a macro lens. Root length was quantified by the line-intercept method (Newman, 1966; Harris and Campbell, 1989) calculated from the number of root intercepts along parallel scan lines using KS-300 video imaging software (Axiovision 2.0).

3. Results

Over the 2-year sampling period, soil extracellular enzyme activities (EEA) was relatively stable. Spatial variation among the plots was low; there were no significant temporal patterns and no significant treatment effects (Fig. 1). Six of the 10 enzymes assayed had coefficients of variation (CV) <30%. For leucine aminopeptidase, the CV was 45%; the values for phenol oxidase, peroxidase and amidohydrolase ranged from 50 to 70%. Principal components analysis showed a strong pattern of correlation among the hydrolases whereas peroxidase and phenol oxidase activities loaded on separate axes (results not shown). Peroxidase activity dominated soil EEA, exceeding most other activities by two orders of magnitude.

Substrate utilization responses were also relatively stable. The CV for both GN and ECO plates were approximately 30%. Activity was lower in July than in May and September/October. Because there were no differences among treatments in respiratory response to the various substrate classes, only summary data, obtained by adding absorbance values for all wells across the microplates is presented (Fig. 2).

Rhizoplane EEA was more variable than the bulk soil parameters; CV ranged from 50 to 100% for individual enzymes. Activities were generally highest in May and lowest in October (Fig. 3). There were no apparent rhizoplane responses to the CO_2 enrichment. Because enzyme activities for roots and soil were expressed differently (gram root versus gram soil), the values are not directly comparable. However, activity ratios suggested that the distribution of EEA on root surfaces was similar to that of bulk soil. The ratio of acid phosphatase:β-glucosidase activity was 1.8 for root surfaces and 2.0 for bulk soil; the ratio

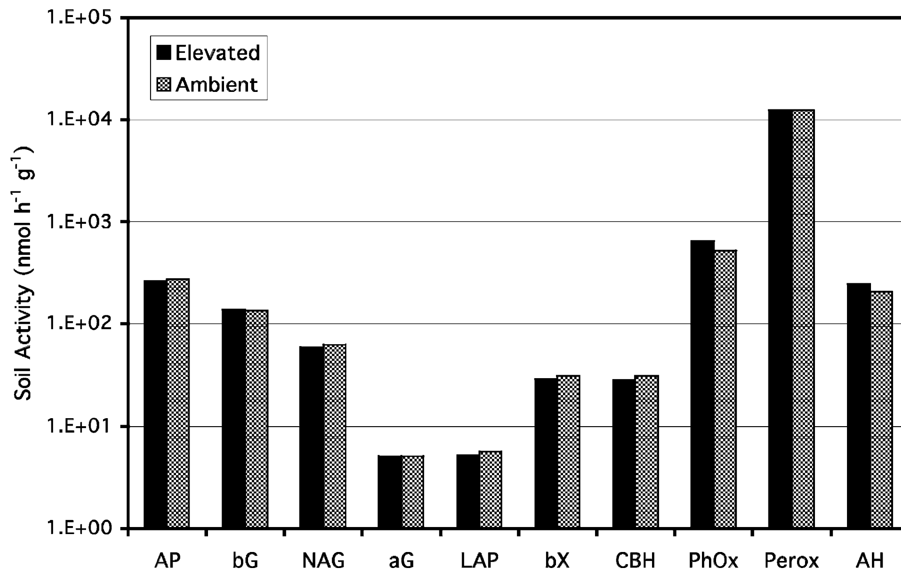

Fig. 1. Extracellular enzyme activity in bulk soil from the ORNL FACE experiment. The bars represent mean activity over the 1999 and 2000 growing seasons ($n = 12$ for enrichment plots, $n = 18$ for ambient plots). Enzyme abbreviations: AP, acid phosphatase; bG, β-glucosidase; NAG, β-*N*-acetylglucosaminidase; aG, α-glucosidase; LAP, leucine aminopeptidase; bX, β-xylosidase; CBH, cellobiohydrolase; PhOx, phenol oxidase; Perox, peroxidase; AH, amidohydrolase.

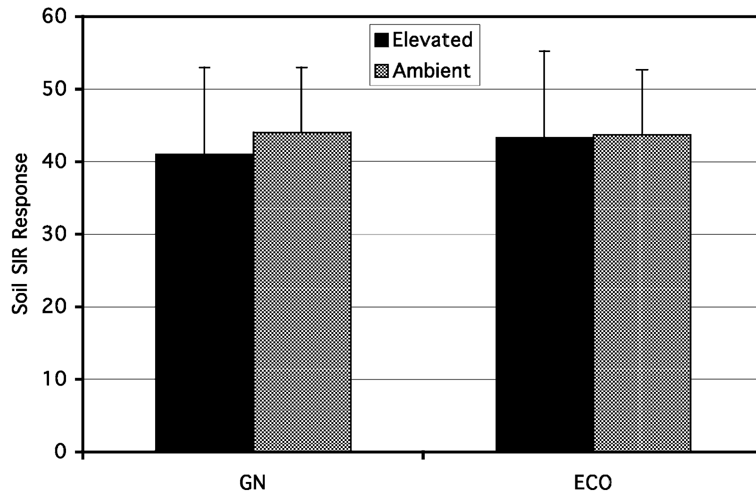

Fig. 2. Substrate utilization responses of soil bacteria, measured using BIOLOG GN and ECO plates over the 2000 growing season. The bars represent mean response ± S.D., measured as absorbance and summed across the microplate, after 48 h incubations at 20 °C ($n = 12$ for enrichment plots, $n = 18$ for ambient plots).

of β-*N*-acetylglucosaminidase:β-glucosidase was 0.49 for fine roots and 0.45 for bulk soil.

Estimates of fine root density averaged about 1 cm/g for both ambient and enrichment plots (Fig. 4). Extreme high values from ambient treatment rings sampled in May 2000 were responsible to the large apparent increase in root density from 1999 to 2000.

Rates of gross N mineralization and denitrification were similar (Table 2). Neither process showed a consistent response to CO_2 enrichment.

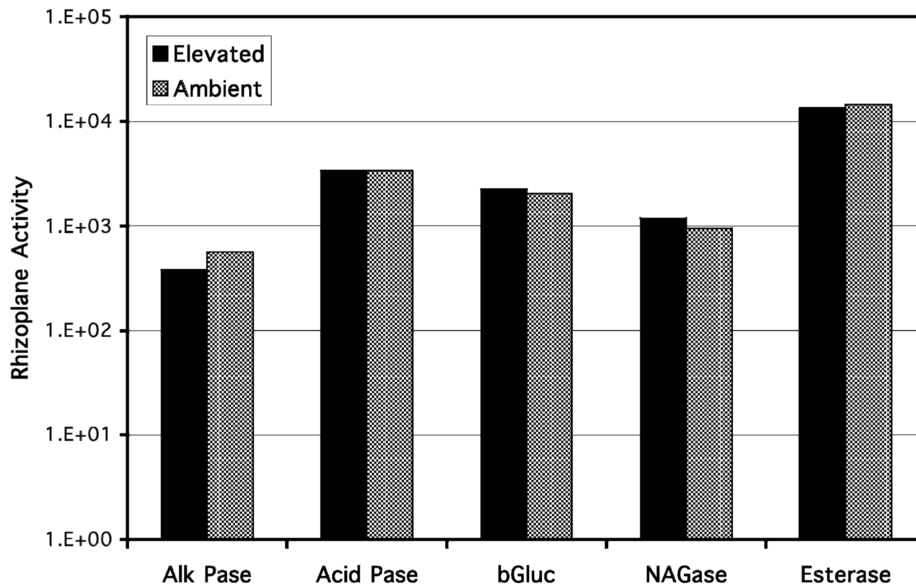

Fig. 3. Rhizoplane enzyme activity for fine roots collected from the ORNL FACE experiment. The bars represent mean activity over the 2001 growing season ($n = 12$ for enrichment plots, $n = 18$ for ambient plots). Enzyme abbreviations: Alk Pase, alkaline phosphatase; Acid Pase, acid phosphatase; bGluc, β-glucosidase; NAGase, β-*N*-acetylglucosaminidase; Esterase, fatty acid esterase.

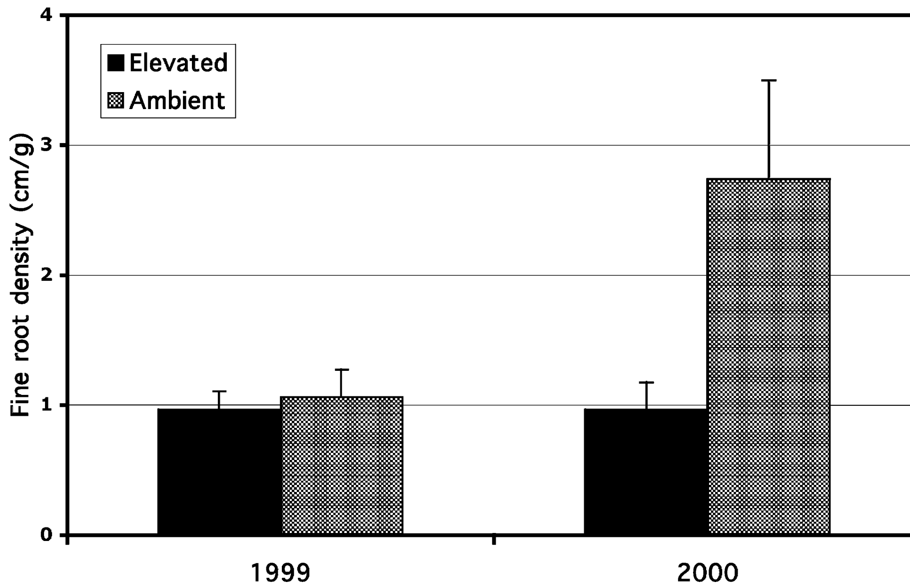

Fig. 4. Fine root density (mean ± S.D.) in bulk soil from the ORNL FACE experiment during the 1999 and 2000 growing seasons. The high value for the ambient plots in 2000 resulted from an unusually high abundance of roots in the May samples.

4. Discussion

Soil EEA at the ORNL FACE site was less dynamic than that of other temperate forest sites where we have done similar monitoring: Duke Forest FACE experiment, NC; Rhinelander FACE experiment, WI; Manistee National Forest, MI (Saiya-Cork et al., 2001; Larsen et al., 2003; Sinsabaugh et al., 2003). At other locations, coefficients of variation for individual enzymes often exceed 100% and seasonal patterns in activity are evident. The comparative stability of the ORNL FACE site may be linked to soil properties. The fine texture and low organic matter content suggest that a large portion of the enzyme activity in bulk soil may be associated with stable organoclay complexes rather than active biomass (Quiquampoix et al., 2002).

Table 2
Microbial biomass N and N transformation rates for 1999 and 2000 growing seasons for soil samples from the FACE experimental plots at Oak Ridge National Laboratory

	Year	Ambient	Enrichment
Gross N mineralization	2000	0.49 (0.06)	0.63 (0.10)
	1999	0.88 (0.21)	0.69 (0.07)
Gross nitrification	2000	0.39 (0.08)	0.71 (0.14)
	1999	0.34 (0.26)	0.32 (0.30)
Microbial biomass N	2000	34.3 (6.4)	40.9 (4.8)

Unit for transformation rate: μg N/g soil per day. Unit for biomass: μg N/g soil. Values are shown as: mean (standard deviation).

The phenology of the vegetation may also contribute to the stability of soil EEA. Sweetgum litter decomposes relatively fast, disappearing before the next cohort falls the following autumn. This rapid turnover reduces its contribution to the SOM pool and soil microbial activity. The high peroxidase activity relative to that of glycosidases and peptidases suggests that the quality of soil organic matter is very low. On average, peroxidase activity at the ORNL site is 88 times larger than β-glucosidase activity; at the Duke Forest FACE site, a *Pinus taeda* plantation, the ratio is 51, for an *Acer saccharum* dominated stand in northern Michigan the ratio is 14 (Saiya-Cork et al., 2001), in the *Betula*/*Populus* plots at the Rhinelander FACE site the ratio is 8 (Larsen et al., 2003).

During the growing season, the principal source of labile carbon for soil microbiota is probably fine root exudation and mortality. If so, it is reasonable to expect increased microbial activity in proximity

to root surfaces. On a mass basis, EEA associated with root surfaces is more than an order of magnitude higher than that of the bulk soil. However, if both are compared on an organic matter basis, this increment disappears. It is perhaps more surprising that there were no differences in the ratios of acid phosphatase, β-*N*-acetylglucosaminidase and β-glucosidase activities between root surfaces and bulk soil. Sweetgum roots are colonized by arbuscular mycorrhizae, so they lack a fungal mantle. But arbuscular mycorrhizae are known to express cellulases, chitinases and phosphatases and it is likely that substrate and nutrient availabilities in the vicinity of active roots differ from those of the bulk soil. On the other hand, attempts to alter enzyme expression by arbuscular mycorrhizae through various treatments have produced mixed results (reviewed by García-Garrido et al., 2002). The only indication that we found for a root effect on EEA is that rhizoplane activities were generally highest in the spring and declined through summer and autumn.

During the first 3 years of CO_2 enrichment (1998–2000), the increment in net primary production relative to the ambient plots averaged 21% (Norby et al., 2002). In the first year, most of the NPP increment was directed to stem growth. In subsequent years, the stem growth increment declined and the additional carbon was increasingly allocated to the production of foliage and fine roots. Fine root production, relative to the control plots, increased by 19% (38 g C/m^2), 30% (76 g C/m^2) and 112% (293 g C/m^2) during the 1998–2000 growing seasons. During the same period, the increment in heterotrophic respiration, relative to control plots, increased from 58 g C/m^2 in the first year to 78 g C/m^2 in the third.

Although the mini-rhizotron data show that root production has increased (Norby et al., 2002), we did not find any significant difference in fine root density within our sample cores. We expected that higher inputs of labile carbon into the rhizosphere would alter EEA and bacterial substrate utilization potential. These measures are generally considered to be highly responsive to changes in environment. In the temperate forest FACE systems at Duke and Rhinelander, primary production responses of similar magnitude produced readily measured responses in both types of parameters: bacterial respiratory activity on BIOLOG plates increased, as did the activities of enzymes involved in the degradation of plant and microbial cell walls (Larsen et al., 2003; Sinsabaugh et al., 2003). At the Duke Forest FACE site, for which we have a 5-year record of EEA, it is possible to distinguish responses associated with rhizosphere inputs and those associated with increased litterfall.

Microbial biomass N and rates of N transformation also showed no treatment response. However, significant shifts in microbial function or process rates in soil systems are often effected without changes in biomass (e.g. Holmes and Zak, 1994) and the lack of change in soil N transformation rates is consistent with observations from other FACE sites. At least in the early stages of response to step increases in CO_2, N availability from existing reservoirs is sufficient to support higher rates of primary production.

Given the comparative stability of soil EEA, the lack of a pronounced rhizoplane effect, and the minimal impact of litter inputs on soil microbes, it is perhaps not surprising that we found no microbial responses to CO_2 enrichment. Our findings suggest that changes in the composition and magnitude of soil inputs and outputs have either been functionally neutral, or they have not “reached” the bulk of the microbial community. Belowground metabolism, as measured by CO_2 efflux (Norby et al., 2002), has increased, but the relative availability of existing nutrient pools within the soil apparently has not shifted. In the long term, this condition is likely to change if the enrichment plots continue to accrete organic matter at a higher rate than the control plots. Our results, considered with those from other temperate forest FACE experiments, indicate that microbial responses to CO_2 enrichment reflect both soil and vegetation characteristics, and may be difficult to generalize.

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