

Microbial Community Responses to Atmospheric Carbon Dioxide Enrichment in a Warm-Temperate Forest

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ABSTRACT

Forest productivity depends on nutrient supply, and sustained increases in forest productivity under elevated carbon dioxide (CO₂) may ultimately depend on the response of microbial communities to changes in the quantity and chemistry of plant-derived substrates. We investigated microbial responses to elevated CO₂ in a warm-temperate forest under free-air CO₂ enrichment for 5 years (1997–2001). The experiment was conducted on three 30 m diameter plots under ambient CO₂ and three plots under elevated CO₂ (200 ppm above ambient). To understand how microbial processes changed under elevated CO₂, we assayed the activity of nine extracellular enzymes responsible for the decomposition of labile and recalcitrant carbon (C) substrates and the release of nitrogen (N) and phosphorus (P) from soil organic matter. Enzyme activities were measured three times per year in a surface organic horizon and in the top 15 cm of mineral soil. Initially, we found significant increases in the decomposition of labile C substrates in the mineral soil horizon under elevated CO₂; this overall pattern was present but much

weaker in the O horizon. Beginning in the 4th year of this study, enzyme activities in the O horizon declined under elevated CO₂, whereas they continued to be stimulated in the mineral soil horizon. By year 5, the degradation of recalcitrant C substrates in mineral soils was significantly higher under elevated CO₂. Although there was little direct effect of elevated CO₂ on the activity of N- and P-releasing enzymes, the activity of nutrient-releasing enzymes relative to those responsible for C metabolism suggest that nutrient limitation is increasingly regulating microbial activity in the O horizon. Our results show that the metabolism of microbial communities is significantly altered by the response of primary producers to elevated CO₂. We hypothesize that ecosystem responses to elevated CO₂ are shifting from primary production to decomposition as a result of increasing nutrient limitation.

Key words: extracellular enzyme activity; elevated carbon dioxide; microbial activity; global change; decomposition.

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INTRODUCTION

Rising concentrations of atmospheric carbon dioxide (CO₂) increase terrestrial productivity and the rate at which carbon (C) and nutrients are delivered to

the soil system through the turnover of plant tissues (Zak and others 2000). Under elevated CO₂, changes in the quantity and chemistry of organic matter can alter the composition and the function of soil microbial communities (Hu and others 2001; Phillips and others 2002). Soil microbes regulate the decomposition of soil organic matter (SOM) through the production of extracellular enzymes, thereby controlling the flow of nutrients to plant-available forms. Under elevated CO₂, changes in microbial function are likely to impact the rate of primary production.

There is active debate as to whether forest productivity can be stimulated by elevated CO₂ in the long term (Luo and others 2004). In the absence of exogenous inputs of nitrogen (N), biogeochemical models predict a negative feedback of enhanced primary production under elevated CO₂ on soil nutrient availability because of increases in the immobilization of plant N and microbial N (Comins and McMurtrie 1993; Rastetter and others 1997; Luo and Reynolds 1999). In contrast to biogeochemical models, empirical studies have not found any significant change in gross or net rates of nutrient transformations by microbes under elevated CO₂, at least over short time scales (less than 5 years), (Johnson and others 2001; Finzi and Schlesinger 2003; Holmes and others 2003; Sinsabaugh and others 2003). Only a single study has identified a reduction in biomass production under elevated CO₂ due to nutrient limitation (Oren and others 2001); other studies have found no such reduction (Norby and others 2002; Karnosky and others 2003; D. J. Moore and others, submitted). The disparity between modeling and empirical studies suggests that we still have an incomplete understanding of the processes regulating microbial function and nutrient cycling in response to enhanced inputs of plant-derived substrates under elevated CO₂.

Elevated CO₂ commonly increases the rate of surface soil CO₂ efflux as a result of fine-root turnover and exudation (Hungate and others 1997; Andrews and others 1999; Norby and others 2002; King and others 2004). Belowground C budgets for these systems attribute a fraction of the increase in surface CO₂ efflux to the stimulation of microbial activity (Hamilton and others 2002; Norby and others 2002). If the cycles of C and N are tightly coupled then the increase in microbial-C availability and metabolism should increase microbial-N demand and N immobilization. Available studies using extracellular enzymes—enzymes that are produced by the microbial community and responsible for the decomposition of organic mat-

ter—have found little support for a close coupling of C and N cycling by microbial communities under elevated CO₂. For example, Phillips and others (2001) and Larsen and others (2002) linked the increase in CO₂ efflux from surface soil to an increase in the metabolism of labile C substrates in young trembling aspen, sugar maple, and paper birch stands growing under free-air CO₂ enrichment (FACE). However, there was no evidence for an increase in the activity of the enzymes responsible for the depolymerization of organic N or phosphorus (P) or rates of N cycling in soil (Holmes and others 2003). Sinsabaugh and others (2003) found that elevated CO₂ had no effect on the metabolism of labile C substrates in a closed-canopy deciduous forest exposed to FACE in Oak Ridge, Tennessee, USA. Notably, forest production under elevated CO₂ at Oak Ridge is dominated by fine-root production and turnover (Norby and others 2002), processes that are typically assumed to deliver a rapid and large pulse of labile organic matter that is easily metabolized by the microbial community (Zak and others 2000).

We have studied the response of an aggrading southern pine-hardwood forest to elevated concentrations of atmospheric CO₂ since 1997. During the first 5 years of this experiment, net primary production (NPP) was stimulated between 18 and 24% (DeLucia and others 1999; Finzi and others 2006). Elevated CO₂ significantly increased the annual input of C and nutrients to the soil system through plant tissue turnover, with aboveground litter inputs approximately fourfold larger than belowground inputs (Matamala and Schlesinger 2000; Pritchard and others 2001; Finzi and others 2002). The ratio of C to N to P in these plant tissues has not changed under elevated CO₂ (Finzi and Schlesinger 2002; Finzi and others 2004). Plant tissue turnover has increased the mass of a surface organic horizon (Schlesinger and Lichter 2001) and the rate of surface soil CO₂ efflux, implying a significant increase in the metabolism of microbial C (Hamilton and others 2002). However, there were no significant differences in gross or net soil N transformations under elevated CO₂ (Finzi and Schlesinger 2003). This result is surprising given that an increase in the C metabolism of soil microbial communities should stimulate an additional demand for nutrients by the microbial community. To gain insights into the metabolism of C and nutrients by soil microbes at the Duke Forest FACE site, we analyzed the activity of nine extracellular enzymes responsible for the decomposition of labile and recalcitrant C substrates, as well as the release of soil nutrients (N and P) from SOM during

Table 1. Extracellular Enzymes and Their Function in Microbial Carbon or Nutrient Metabolism

Enzyme	Abbreviation	Substrates Decomposed
Cellobiohydrolase	CBH	Cellulose hydrolysis
α -1,4-Glucosidase	aG	Starch hydrolysis
β -1,4-Glucosidase	bG	Glucose hydrolysis
β -1,4-Xylosidase	bX	Xylan hydrolysis
Phenol Oxidase	PhOx	Lignin hydrolysis
Peroxidase	PerOx	Lignin hydrolysis
β -1,4-N-Acetylglucosaminidase	NAG	Organic nitrogen and microbial cell wall hydrolysis
Leucine-aminopeptidase	LAP	Organic nitrogen polypeptide hydrolysis
Acid phosphatase	AP	Organic phosphorus hydrolysis

the first 5 years of CO₂ exposure. We tested the following three hypotheses for the plots under elevated CO₂ relative to those under ambient CO₂:

1. Higher rates of aboveground litter inputs increase the activity of enzymes responsible for C metabolism by microbes in the O horizon.
2. Higher rates of aboveground litter inputs increase the demand for nutrients by microbes stimulating the activity of nutrient-releasing enzymes in the O horizon.
3. Low rates of fine-root production and turnover in mineral soil result in no significant stimulation in the activity of C- or nutrient-metabolizing enzymes.

METHODS

Study Site and Sample Collection

This research was conducted at the Duke Forest FACE site, Orange County, North Carolina, USA. The site is dominated by loblolly pine (*Pinus taeda* L.) in the overstory which was planted in a 2.4 × 2.4-m spacing in 1983. Subsequent to establishment a deciduous understory recruited from nearby hardwood forests and stump sprouts. The most abundant understory tree species is sweet gum (*Liquidambar styraciflua*), with admixtures of winged elm (*Ulmus alata*), red maple (*Acer rubrum*), red bud (*Cercis canadensis*), and dogwood (*Cornus florida*). The soil is an Ultic Alfisol of the Enon Series, pH 5, with an organic matter content of 0.5% (Allen and others 2000). The FACE array consists of six 30-m-diameter plots distributed within the contiguous forest. Additional site description is presented in Hendrey and others (1999). Three of the six plots receive ambient air (control plots); the other three plots receive ambient air amended with CO₂ at a target concentration 200 ppm above ambient, or approximately 570 ppm. Fumigation began on 27 August 1996 and is ongoing.

In May, July, and September of 1997–2001, we collected replicate organic horizon (O horizon) and surface mineral soil horizon samples to a depth of 15 cm. At each sampling date, we collected 12 samples of each soil horizon from three randomly selected sample points within the four “soil sectors” of each plot. The O horizon samples excluded the Oi horizon. The Oe and Oa layers were collected as a single 10 × 10 cm² monolith. The mineral soil sample (2 × 15 cm) was extracted directly below the surface O horizon sample. The 12 replicate samples were combined into a single sample per horizon and stored on ice. In the laboratory, the O horizon was cut into 1 cm fragments using scissors and homogenized by hand. The mineral soil samples were sieved through 2 mm mesh to remove large stones and fine-root fragments. Five homogenized subsamples of each horizon were dried at 105°C for 5 days to determine moisture content.

Enzyme Function and Assay Techniques

The O horizon and mineral soil samples were assayed for the activity of nine extracellular enzymes involved in the decomposition of C-, N-, or P-containing compounds. The names and specific functions of each enzyme are listed in Table 1. The nine extracellular enzymes can be grouped into three functional groups based on their ability (a) to decompose relatively labile C constituents (cellobiohydrolase, α -1, 4-glucosidase, β -1, 4-glucosidase, β -1, 4-xylosidase); (b) to decompose recalcitrant C constituents [mostly lignin (phenol oxidase, peroxidase)]; or (c) to depolymerize organic N (β -1, 4-N-acetylglucosaminidase, leucine-aminopeptidase) and P (acid phosphatase).

Enzyme assays began within 2–3 h of sample collection. Sample suspensions were prepared by adding 1 g of soil or 0.5 g of O horizon material 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 16 replicate 200- μ L aliquots were dispensed into 96-well microplates for each enzyme assay.

All enzyme assays, with the exception of phenol oxidase and peroxidase, were analyzed fluorimetrically. First, 50 μ L of 200 μ M substrate solution was added to each sample well. Blank wells received 5 μ L of acetate buffer plus 200 μ L of sample suspension. Negative control wells received 50 μ L substrate solution plus 200 μ L of acetate buffer. Quench standard wells received 50 μ L of standard (10 μ M 4-methylumbelliferone, or 7-amino-4-methyl coumarin in the case of leucine aminopeptidase) +200 μ L sample suspension. Reference standard wells received 50 μ L of standard plus 200 μ L 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. To stop the reaction, a 10 μ L 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 correction for controls and quenching, enzyme activity was expressed in units of nmol substrate cleaved $g^{-1} h^{-1}$.

Phenol oxidase and peroxidase activities were measured spectrophotometrically using L^{-1} 3,4-dihydroxyphenylalanine (DOPA) as the substrate. For phenol oxidase, 50 μ L of 25 mM DOPA was added to each sample well. Peroxidase assays received 50 μ L of 25 mM DOPA plus 10 μ L of 0.3% H_2O_2 . Negative control wells for phenol oxidase contained 200 μ L of acetate buffer and 50 μ L of DOPA solution; blank wells contained 200 μ L of sample suspension and 50 μ L of acetate buffer. For peroxidase, negative control and blank wells also contained 10 μ L of H_2O_2 . There were 16 replicate sample wells for each assay and 8 replicate wells for blanks and controls. The microplates were incubated in the dark at 20°C. Activity was quantified by measuring absorbance at 450 nm using a microplate spectrophotometer and expressed in units of nmol $g^{-1} h^{-1}$.

Data Analysis

We used repeated-measures analysis of variance (ANOVA) to test for the effect of elevated CO_2 (370 and 570 μ L L^{-1}), sample month (May, July, September), and sample year (time) on the activity of the nine extracellular enzymes. We analyzed the data for the O horizon and mineral soil horizons separately. For analyses in which the year \times CO_2 interaction term was statistically significant, we used one-way ANOVA to test for significant dif-

ferences between CO_2 treatments on a year-by-year basis. We log-transformed the data when variances were heterogeneous or nonnormally distributed. We used Tukey's test for post hoc comparisons among means.

Our data analysis showed significant changes in extracellular enzyme activity across the 5 years of this experiment (effect of year $P < 0.0001$ for all enzymes). To test whether elevated CO_2 affected the activity of extracellular enzymes differently in the O and mineral soil horizons, we calculated an enhancement ratio for each of the nine extracellular enzymes (that is, the average activity of an enzyme under elevated CO_2 divided by its activity under ambient CO_2). The enhancement ratio enables us to assess whether enzyme activity was stimulated (E/A greater than 1) or repressed (E/A less than 1) by elevated CO_2 independent of the difference in absolute rates, which were always higher in the O horizon (see Results, section). To help clarify emergent trends, we combined the nine extracellular enzymes into the three functional groups based on their ability to degrade labile C substrates, to degrade recalcitrant C substrates, or to depolymerize N and P from SOM. We then analyzed the data as a one-sample t -test testing the hypothesis that the enhancement ratio was not significantly different from 1 within each soil horizon. Each enzyme from each horizon contributed a data point to the analysis. Thus, one-sample t -tests were based on an $n = 4$, $n = 2$, and $n = 3$ for the labile C, recalcitrant C, and nutrient-releasing functional groups, respectively, in each horizon. We analyzed the data separately for each year.

RESULTS

Extracellular enzyme activity in the O horizon was, on average, one order of magnitude greater than that in the mineral soil horizon (Figure 1). The mean activity of the nine extracellular enzymes over the 5 years for all sample dates differed significantly ($P < 0.0001$) from one another (Figure 1). Enzyme activity in the O horizon decreased from highest to lowest in the following order: AP > NAG \geq bG > CBH > bX > aG > LAP > PhOx \geq PerOx (Figure 1). Enzyme activity in the mineral soil horizon decreased from highest to lowest in a very similar order: AP > bG \geq NAG > CBH > bX > PerOx \geq aG \geq LAP > PhOx.

In the O horizon, elevated CO_2 significantly altered the activity of cellobiohydrolase, a cellulose-degrading enzyme, and phenol oxidase, a lignin-degrading enzyme (Table 2). Cellobiohydrolase activity was significantly higher under ele-

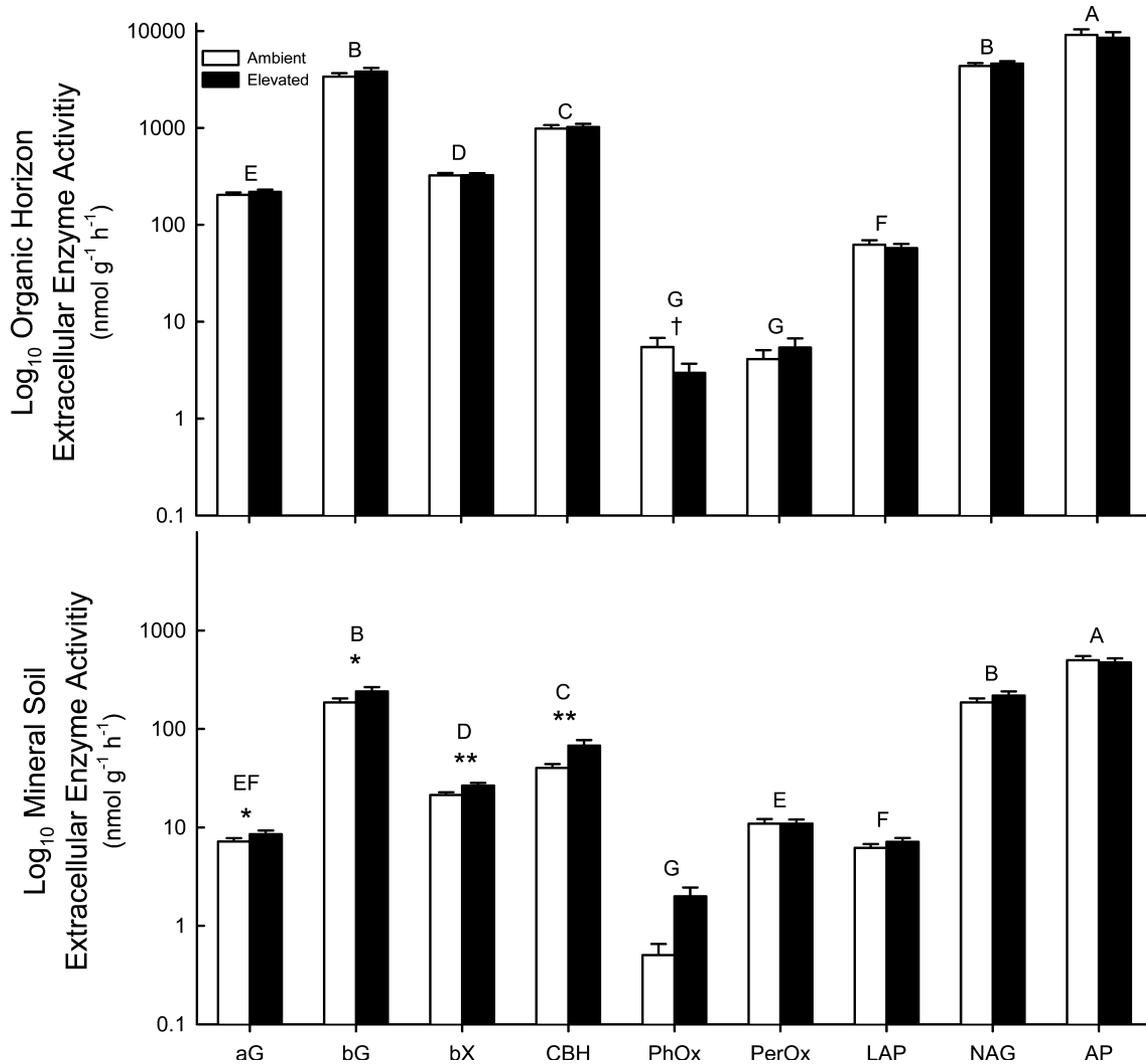


Figure 1. The mean activity (± 1 SE) of nine different extracellular enzymes in the organic horizon (A) and top 15 cm of the mineral soil horizon (B) under ambient (*open bars*) and elevated carbon dioxide (CO₂) (*filled bars*) over the 5 years for all sampling dates. *Symbols above bar pairs* indicate a significant effect of elevated CO₂ on extracellular enzyme activity at $^{\dagger}P < 0.10$, $*P < 0.05$, and $**P < 0.01$. *Capital letters* indicate significant differences among the activities of the nine extracellular enzymes. The *y-axis* was log₁₀-transformed to help visualize the data. Enzyme abbreviations are listed in Table 1.

vated CO₂ in the 1st year of this study (Figure 2A). Thereafter, cellobiohydrolase activity increased under ambient CO₂; by the 5th year of the experiment, it was significantly higher than under elevated CO₂ (Figure 2A). Overall, phenol oxidase activity was marginally lower under elevated CO₂ (Table 2); however, it was significantly lower in the 4th and 5th years (Figure 2B). None of the other extracellular enzymes' activity was affected by elevated CO₂ in the O horizon (Table 2).

In the mineral soil horizon, elevated CO₂ significantly stimulated the activity of all four of the labile C-degrading enzymes (Table 2). The activity of α -1, 4-glucosidase was stimulated by 18% relative to

ambient CO₂, β -1, 4-glucosidase was stimulated by 29%, β -1, 4-xylosidase by 24%, and cellobiohydrolase by 69% (Table 2). There was a significant time \times CO₂ interaction for cellobiohydrolase activity (Table 2) resulting from a decline in activity under elevated CO₂ in the 3rd year of CO₂ fumigation (Figure 2C). There was also a significant time \times CO₂ interaction for phenol oxidase activity (Table 2). Phenol oxidase activity under elevated CO₂ increased through time relative to that under ambient CO₂ and was significantly higher in the 4th and 5th years of the study (Figure 2D). There were also significant time \times month \times CO₂ interactions for all four labile C-degrading enzymes (Table 2).

Table 2. Results of the Repeated-Measures Analyses of Variance for the Activities of Nine Extracellular Enzymes Responsible for Carbon (C) and Nutrient Metabolism by Soil Microbial Communities in the O Horizon and top 15 cm of Mineral Soil in Response to Elevated Carbon Dioxide (CO₂) and Sample Month

Source of Variation	Labile C				Recalcitrant C		Nitrogen		Phosphorus AP
	aG	bG	bX	CBH	PhOx	PerOx	NAG	LAP	
Organic horizon									
Between subjects									
CO ₂	0.179	0.338	0.959	0.492	0.084	0.130	0.452	0.426	0.680
Month	0.001	0.513	0.018	0.191	0.174	0.001	0.001	0.100	0.017
CO ₂ × month	0.521	0.846	0.781	0.837	0.017	0.174	0.638	0.481	0.865
Within subjects									
Time	0.153	0.001	0.378	0.002	0.001	0.001	0.182	0.004	0.001
Time × CO ₂	0.226	0.105	0.379	0.026	0.150	0.337	0.463	0.488	0.909
Time × month	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Time × month × CO ₂	0.385	0.348	0.468	0.424	0.001	0.299	0.785	0.726	0.615
Mineral soil horizon									
Between subjects									
CO ₂	0.033	0.011	0.004	0.001	0.811	0.993	0.125	0.197	0.531
Month	0.001	0.001	0.001	0.003	0.005	0.583	0.001	0.171	0.001
CO ₂ × month	0.910	0.134	0.555	0.091	0.500	0.896	0.569	0.691	0.823
Within subjects									
Time	0.001	0.001	0.001	0.001	0.018	0.001	0.001	0.001	0.001
Time × CO ₂	0.747	0.417	0.299	0.001	0.036	0.933	0.816	0.332	0.701
Time × month	0.001	0.001	0.002	0.001	0.026	0.001	0.001	0.001	0.001
Time × month × CO ₂	0.001	0.001	0.001	0.001	0.478	0.997	0.668	0.039	0.379

See Table 1 for definitions of abbreviations.

Listed are the probability values for the significance of each F-test. For clarity, boldface values indicate statistically significant results ($P < 0.05$). "Month" refers to May, July, or September samplings. "Time" refers to the calendar years from 1997 through 2001.

In the O and mineral soil horizons, the enhancement ratio for the average activity of all extracellular enzymes showed no significant stimulation by elevated CO₂ during the first 3 years of this experiment (Figure 3A). Thereafter, significant stimulation in overall enzyme activity was observed in the mineral soil horizon but not in the O horizon. In the mineral soil horizon, the strong increase in overall enzymatic activity under elevated CO₂ was due to sustained stimulation of labile C metabolism (Figure 3B) and substantial stimulation in the activity of phenol oxidase (Table 2, Figure 3C). Despite a lack of stimulation in overall enzyme activity in the O horizon, labile C enzymes were significantly stimulated by elevated CO₂ in the first 2 years of the experiment, followed by a decline and significant repression in their activity in the 5th year (Figure 3B). There was no direct effect of elevated CO₂ on nutrient metabolism through time in either horizon, with the exception that nutrient metabolism was marginally stimulated by elevated CO₂ in the mineral soil horizon in the 4th year of the study (Figure 3D).

DISCUSSION

Elevated CO₂ significantly increases plant biomass and plant tissue turnover, thereby increasing the flux of energy and nutrients available for microbial biosynthesis (Zak and others 2000). At the Duke Forest FACE site, aboveground litterfall delivers approximately fourfold more organic matter to the soil system than do litter inputs via fine-root turnover (Matamala and Schlesinger 2000; Finzi and others 2002; Matamala and others 2003). Based on this observation, we hypothesized that microbial function would be significantly stimulated in the O horizon of plots under elevated CO₂ relative to those under ambient CO₂. We reasoned that the increase in C availability would stimulate the release of labile C-degrading enzymes (hypothesis 1) and create a biosynthetic demand for nutrients that would stimulate the activity of N- and P-releasing enzymes (hypothesis 2). In contrast, we hypothesized that extracellular enzyme activity in the mineral soil horizon would not be strongly stimulated by elevated CO₂, given the

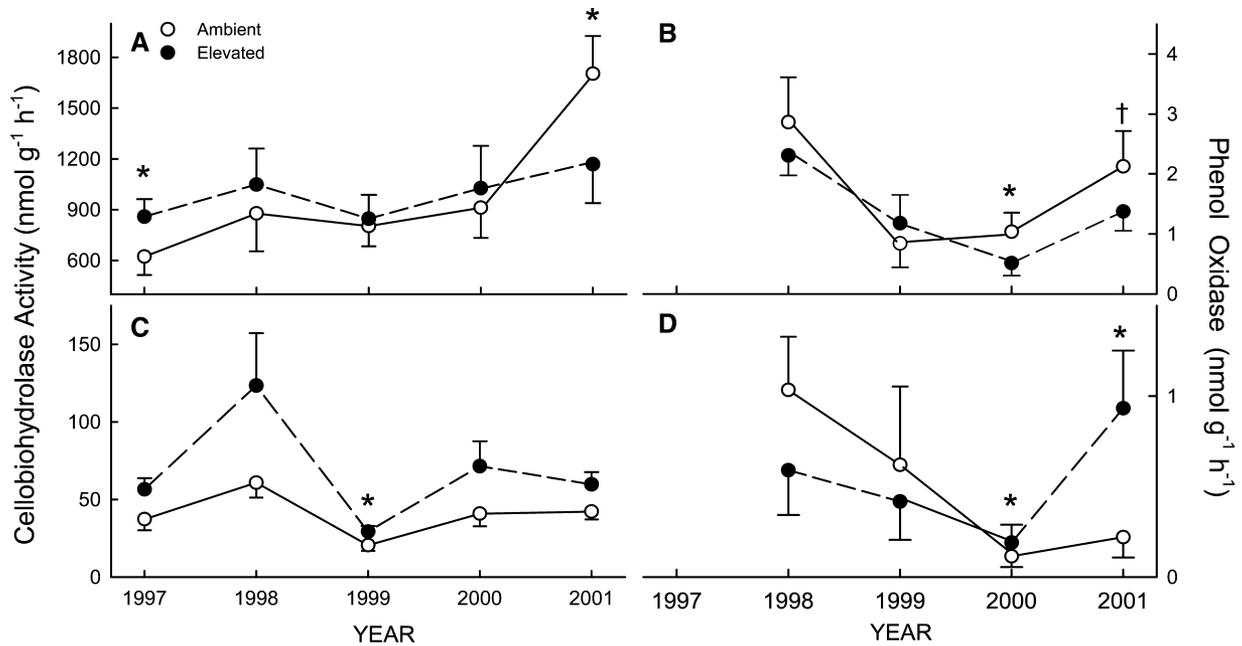


Figure 2. Yearly variation in the mean activity of cellobiohydrolase and phenol oxidase (± 1 SE) under ambient (*open symbols*) and elevated CO₂ (*filled symbols*) averaged across three sample dates. Cellobiohydrolase activity in the O horizon (**A**). Phenol oxidase activity in the O horizon (**B**). Cellobiohydrolase activity in the mineral soil horizon (**C**). Phenol oxidase activity in the mineral soil horizon (**D**). Levels of significance are given in Figure 1.

low rate of litter input under elevated CO₂ (hypothesis 3). On an enzyme-by-enzyme basis, we found the strongest modulation of microbial activity in the mineral soil horizon, not the O horizon (Table 2). Based on this observation, we largely reject our initial hypotheses. However, there were significant temporal variations in the activity of many enzymes in both the mineral soil and O horizons, suggesting a complex response of soil microbes to changes in the timing, quantity, and chemistry of plant-derived substrates under elevated CO₂.

Extracellular Enzyme Activities in Mineral Soil

Our original hypothesis for the responses of the mineral soil horizon to elevated CO₂ was based on the low rate of additional C input to the soil system from fine-root turnover. In the 2nd year of this study, Matamala and Schlesinger (2000), using the sequential coring technique, found a small but significant increase in fine-root turnover under elevated CO₂ (21 g C m⁻² y⁻¹) compared to ambient CO₂ (14 g C m⁻² y⁻¹). In the 3rd year, Pritchard and others (2001) used mini-rhizotrons to estimate similar rates of fine-root turnover (17 and 25 g C m⁻² y⁻¹ under ambient and elevated CO₂, respectively). Further, Matamala and others (2003)

used isotopic methods to conclude that fine-root NPP and turnover are very low in this ecosystem throughout the first 5 years of CO₂ enrichment. Therefore, we postulated that there should be little initial (under 2 years) microbial response to elevated CO₂; the additional 7 g C m⁻² y⁻¹ available for microbial assimilation under elevated CO₂ is small relative to the pool of C in the top 15 cm of mineral soil (2,208 g C m⁻² y⁻¹) (Schlesinger and Lichter 2001) and is not likely to elicit a strong microbial response.

The response of extracellular enzyme activities in the mineral soil horizon was consistent with an increase in the availability and metabolism of labile organic materials. The enzymes that showed a significant response to CO₂-enrichment were those involved in the breakdown of cellulosic material (cellobiohydrolase) and storage carbohydrates (α -1, 4-glucosidase, β -1, 4-glucosidase, and β -1, 4-xylosidase), (Figure 2B). The stimulation of this specific set of extracellular enzymes implies that elevated CO₂ enhanced the input of root-derived organic substrates, an inference consistent with significant increases in CO₂ efflux from surface soil (Andrews and Schlesinger 2001). The quantity of C released as root exudates has not been quantified in this ecosystem. However, our results imply a large input of C via root exudation under elevated CO₂.

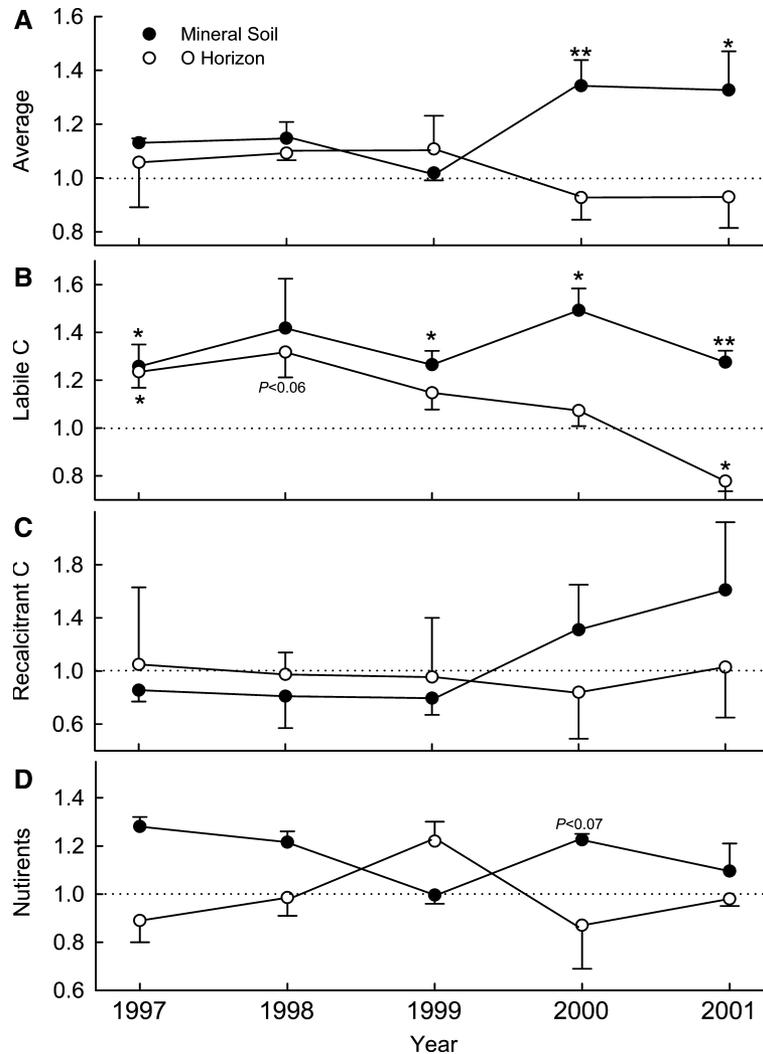


Figure 3. Yearly variation in the enhancement ratio (± 1 SE) of enzyme activity in the O and mineral soil horizons. The average enhancement ratio of all nine extracellular enzymes (**A**). The average enhancement ratio of labile C-degrading enzymes (aG, bG, bX, CBH) (**B**). The average enhancement ratio of recalcitrant C-degrading enzymes (PhOx, PerOx) (**C**). The average enhancement ratio of nutrient-releasing enzymes (NAG, LAP, AP) (**D**). Enzyme abbreviations are listed in Table 1.

In the mineral soil horizon, we also observed significant temporal variation in the activity of phenol oxidase under elevated CO_2 (Figure 2D). Phenol oxidase is an enzyme associated with the degradation of humified C, mostly lignin (Freeman and others 2001; Sinsabaugh and others 2002; Saiya-Cork and others 2002). Humified C is recalcitrant to the decomposition process and yields little net energy for microbial biosynthesis (Zak and others 2000). During the first 3 years of this study, phenol oxidase activity was lower under elevated CO_2 (Figure 2D), consistent with a large initial pulse in labile C availability. However by year 5, phenol oxidase activity increased significantly under elevated CO_2 , implying an increased demand for C by the microbial community under elevated CO_2 ,

possibly as a consequence of C priming (Fu and Cheng 2002; Hoosbeek and others 2004).

Despite increased forest productivity and shifts in microbial C metabolism, the activities of enzymes associated with the release of N (β -1, 4-N-acetyl glucosaminidase, leucine-aminopeptidase) and P (acid phosphatase) showed little response to CO_2 treatment (Table 2, and Figure 3D). Similarly, there were no significant changes in the ratio of C-acquiring to N-acquiring or P-acquiring enzyme activities under elevated CO_2 through time (data not shown). Allen and Schlesinger (2004) found that additions of labile C (cellobiose, vanillic acid, pectin, sucrose, and mannose) to mineral soil from the Duke Forest FACE site stimulated microbial activity and biomass to a much greater extent

than additions of N or P alone. Combined, these results suggest that microbial function is principally C limited and that microbial nutrient demand is met by the current allocation of resources to the production of nutrient-releasing enzymes. These data provide the clearest explanation for our prior observations showing that there is no change in gross or net rates of N mineralization under elevated CO₂ (Alien and others 2000; Finzi and Schlesinger 2003).

Extracellular Enzyme Activities in the O Horizon

A simple budget for the surface organic horizon shows a large additional influx of C under elevated CO₂ that should have increased microbial C metabolism. On average, aboveground litterfall delivered approximately 280 g C m⁻² to the surface of the O horizon through 2001. Elevated CO₂ increased the flux of C by about 16%, or +46 g C m⁻² y⁻¹ (Finzi and Schlesinger 2003). This additional C flux is six times greater than that in the mineral soil horizon, and the O horizon contains one-third the amount of C found in mineral soils (Schlesinger and Lichter 2001). Despite this large influx of C, the response of C-degrading enzymes was relatively weak. Initially, only cellobiohydrolase activity was significantly higher under elevated CO₂ (Figure 2A). And only when averaged was there a significant stimulation in labile C-degrading enzyme activity under elevated CO₂ (Figure 3B). The chemistry of litter inputs provides the best explanation for this result. The largest single fraction of dry matter delivered to the O horizon is in the form of lignin (approximately 23% of dry matter), with much smaller contributions by total nonstructural fractions (approximately 10% of dry matter) (Finzi and Schlesinger 2002). Thus, despite the significant increase in total C delivery to the O horizon, much of this C is delivered in a form that is not easily decomposed by soil microbes (Zak and others 2000).

A striking response to elevated CO₂ in the O horizon was the decline in labile C- and recalcitrant C-degrading enzymes through time (Figures 2A, B, and 3B, C). We hypothesize that the decline in microbial function was due to nutrient limitation. The production of extracellular enzymes requires a large quantity of nutrients. By the 5th year of this experiment, the steady input of litter under elevated CO₂ increased the mass of the O horizon by 33% (3,041 and 2,276 g dry matter m⁻² under elevated and ambient CO₂, respectively) and the storage of N in this horizon by 30% (24.3 and 18.8 g N m⁻² under elevated and ambient CO₂, respec-

tively; J. Lichter and others 2005). The consistency in the activity of nutrient-releasing enzymes relative to the decline in the metabolism of labile C by year 5 of this study (Figure 3B, D) suggests a shift in the allocation of resources from the acquisition of C to the acquisition of nutrients (Sinsabaugh and Moorehead 1994; Tank and others 1998). This idea can be amplified by expressing enzyme activity data on a per-unit-area basis. On a per-unit-area basis, the metabolism of labile C substrates under elevated CO₂ remained constant through time (for example, area-based, year-5 enhancement ratio = 0.97) even though activity on a per-gram litter basis declined (for example, E/A = 0.78) (Figure 3D). Similarly, the activity of nutrient-releasing enzymes increased on a per-unit-area basis under elevated CO₂ (for example, year 5 area-based E/A = 1.20), even though on a per-gram litter basis activity remained unchanged (for example, E/A = 0.98) (Figure 3D). Notably, the decline in C metabolism by soil microbes should be correlated with a decrease in the rate of O horizon decomposition. Consistent with our enzyme data, (J. Lichter and others 2005) found a significant decrease in the rate at which the O horizon is decomposing and a significant increase in the mean residence time of the O horizon under elevated CO₂.

Given our hypothesis that microbial function was increasingly N limited under elevated CO₂, we would have predicted a decline in the ratio of C-acquiring to nutrient-acquiring enzyme activities in the O horizon. These ratios did not decline through time under elevated CO₂ (either for C:N, C:P, or C:N+P) (data not shown). However, these enzyme-activity ratios were highly sensitive to the number of enzymes measured within each functional group (6 for carbon, 2 for N and 1 for P) and the vast difference (>3 orders of magnitude) in absolute enzyme activity within and between functional groups (Figure 1). Nevertheless the two indices of increasing nutrient limitation to microbial function under elevated CO₂ provided contrasting points of view. Thus, we regard increasing nutrient limitation in the O horizon only as a working hypothesis.

CONCLUSIONS

Our overall findings are consistent with a general trend toward the stimulation of microbial activity after short-term increases in atmospheric CO₂ (Körner and Arnone 1992; Dhillon and others 1996; Larsen and others 2002; Phillips and others 2002; Sinsabaugh and others 2003). Our results, however, show a very dynamic response of the

microbial community to plant-mediated changes in the quantity and chemistry of organic matter inputs stimulated by 5 years of forest growth under elevated CO₂. On the basis of extracellular enzyme activities and other supporting information, we can divide this ecosystem's response to elevated CO₂ into two stages.

The initial stage extended through the first 2 years of this experiment. During this period, microbial responses were driven by higher rates of NPP (DeLucia and others 1999; Lichter and others 2005), much of which was allocated to foliage and fine-root production (Finzi and others 2001; Matamala and others 2003). The expansion of the fine-root network enabled plant nutrient uptake to keep pace with increased C fixation, so nutrient ratios in newly produced foliage and roots remained constant (Matamala and Schlesinger 2000; Finzi and others 2001). The rhizosphere expansion also enabled greater microbial activity in both the mineral soil and O horizons with no indication of nutrient scarcity (Table 2, Figure 3). A stimulation of the enzyme activity of labile C and higher rates of soil CO₂ efflux indicated a substantial increment in rhizosphere C allocation that was consumed and respired.

The second stage began in the 3rd year of fumigation, when foliar inputs began to uncouple microbial activity in the O horizon from that of the mineral soil horizon. By the 5th year of fumigation, overall enzyme activity in the mineral soil was stimulated by 33%, whereas it was repressed by 7% in the O horizon (Figure 3A). The mass of the O horizon increased as a result of increased rates of litterfall dominated by lignin (Schlesinger and Lichter 2001). Microbial activity began to decline (Figure 3A), and enzyme activity in the O horizon began to shift from C acquisition to N and P acquisition, as implied by the decline in the activity of labile C-decomposing enzymes relative to nutrient-acquiring enzymes (Figure 3). In the mineral soil horizon, microbial activity remained stimulated by labile rhizosphere inputs through time. However in the 4th year of this experiment, phenol oxidase activity in the mineral soil horizon increased under elevated CO₂ (Figure 2D). Phenol oxidase activity is not only associated with the decomposition of humified C but also low N availability, suggesting that nutrient limitation may be increasing in the mineral soil horizon (Freeman and others 2001; Sinsabaugh and others 2002; Saiya-Cork and others 2002).

Collectively, our results provide some insights into microbial function and nutrient cycling in response to forest growth under elevated CO₂.

Biogeochemical models suggest that the initial stimulation in forest productivity under elevated should decline through time as a result of plant- and microbial-N immobilization (Rastetter and others 1997; Luo and Reynolds 1999; Lou and others 2004). At the Duke forest FACE site, microbes respond primarily to the increase in C availability under elevated CO₂, a result corroborated by laboratory studies of microbial biomass and activity (Allen and Schlesinger 2004). As a result, there was little change in the rate of soil N cycling during the first 5 years of CO₂ fumigation (Finzi and Schlesinger 2003); response that has also been observed at other forest FACE sites (Zak and others 2003). Through time, however, there is increasing evidence that microbial function is progressively N limited under elevated CO₂. Thus, we hypothesize that a third stage of development is now emerging whereby ecosystem response to CO₂ enrichment is shifting from primary production to decomposition as a direct result of initial plant C allocation strategies under elevated CO₂. Only direct observations will confirm or reject our hypothesis concerning a third stage of ecosystem development. If a microbe-driven nutrient-cycling feedback does control forest responses to elevated CO₂, this feedback will likely operate over the time scale of a decade (or more) rather than a few years.

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