Atmospheric CO_2 and soil extracellular enzyme activity: a meta-analysis and CO_2 gradient experiment

Alexia M. Kelley,[1,5,†] Philip A. Fay,[2] H. Wayne Polley,[2] Richard A. Gill,[3] and Robert B. Jackson[1,4]

[1]Department of Biology, Duke University, Durham, North Carolina 27708 USA
[2]Grassland, Soil & Water Research Laboratory, USDA-ARS, Temple, Texas 76502 USA
[3]Department of Biology, Brigham Young University, Provo, Utah 84602 USA
[4]Nicholas School of the Environment and Center on Global Change, Duke University, Durham, North Carolina 27708 USA

Abstract.   Rising atmospheric CO_2 concentrations can alter carbon and nutrient cycling and microbial processes in terrestrial ecosystems. One of the primary ways microbes interact with soil organic matter is through the production of extracellular enzymes, which break down complex organic molecules and release nutrients into the soil. We conducted a meta-analysis of 34 studies that examined responses in microbial enzyme activity to elevated CO_2. We also conducted a field study of soil enzyme activity in a tallgrass-prairie ecosystem growing in sandy loam (lower organic matter content) and clayey soils (higher organic matter content) exposed to a continuous gradient of 250 to 500 ppm CO_2. Of the 10 enzyme groups examined in the meta-analysis, including those degrading starch, β-glucan, cellulose, xylan/hemicellulose, lignin, organic P, and organic N, only the activity of one enzyme that degrades the C- and N-containing building blocks of chitin (N-acetyl-glucosaminidase) increased consistently at elevated CO_2 by an average of 12.6% (p < 0.05), especially in field studies and in woody ecosystems. In our field study, increasing CO_2 from subambient to elevated concentrations reduced the activity of leucine aminopeptidase by 32% in the black clay soil during the peak of the growing season, while β −1,4-N-acetyl-glucosaminidase increased by 44% near the end of the season, indicating increased N limitation with increasing CO_2. In the sandy loam soil, alkaline phosphatase activity increased by 42% with CO_2 enrichment at the end of the growing season, suggesting CO_2-induced phosphorus limitation in these soils. Additionally, a 53–83% decrease in the carbon cycling enzymes cellobiohydrolase, α-glucosidase, and xylosidase activity with increased CO_2 was found in July. Our field study shows that soil type can strongly influence how microbial functioning may change with rising CO_2 concentrations and that microbial responses associated with C-, N-, and P-cycling are likely to change—and may already have changed—with increasing CO_2 under some soil types and conditions. Our meta-analysis revealed that, despite variable enzyme activities with CO_2, chitinase activity increased consistently with CO_2 across ecosystems.

[5] Present address: Department of Forestry and Environmental Resources, North Carolina State University, Raleigh, North Carolina 27695 USA.
† E-mail: alexia.kelley@gmail.com

Introduction

Numerous studies have examined how net primary production (NPP) and soil carbon stores respond to rising atmospheric CO_2, with considerable recent emphasis on the role of soil microorganisms in these systems (Zak et al. 2000, Sinsabaugh et al. 2003, Billings and Ziegler 2005, Chung et al. 2006, Finzi et al. 2006b, Castro et al. 2010). Microbes are largely responsible for the cycling of plant-available soil nutrients and for the decomposition of organic matter and, hence, soil carbon sequestration (Bardgett et al. 2003, Allison 2006, De Deyn et al. 2009, Billings et al. 2010). Increased CO_2 can increase primary production and carbon inputs to the soil, but the question of whether soils will act as a long-term carbon sink remains unanswered, in part because of uncertainties in how microbial communities will respond to increases in CO_2 and subsequent changes in the quantity and quality of soil organic matter (SOM) (Gill et al. 2006).

Microbial physiology is unlikely to be directly affected by elevated atmospheric CO_2, but many indirect changes that accompany higher CO_2 concentrations, such as changes in net primary production (NPP), litter chemistry, soil moisture, and nutrient availability, can strongly affect microbial functions (Fitter et al. 1996, Randlett et al. 1996, Hungate et al. 1997, Norby et al. 2001, Gill et al. 2002, Polley et al. 2002, Hobbie et al. 2004, Henry et al. 2005, Jackson et al. 2009, Phillips et al. 2009). The primary mechanism by which soil microbes affect nutrient cycling is through the production of extracellular enzymes, which catalyze the breakdown of organic molecules in SOM. Extracellular enzyme production is believed to be regulated primarily by microbial "economics" because enzyme synthesis is energetically costly and requires N (Koch 1985, Allison and Vitousek 2005). Enzyme production thus reflects the balance of microbial nutrient availability and substrate abundance, constrained by the biochemical requirements of the microbes and the quality of the available organic substrate in soil organic matter (SOM) (Allison and Vitousek 2005, Geisseler and Horwath 2009, Manzoni et al. 2010, Sinsabaugh and Follstad Shah 2010).

Given that changing atmospheric CO_2 concentrations have been shown to alter decomposition rates and SOM content and quality, extracellular enzyme activity in soils is also likely to change in ecosystems exposed to altered CO_2. To understand the specific mechanisms that might cause such changes, a better understanding of microbial functioning in terrestrial ecosystems is needed. For example, is an observed decrease in net nutrient mineralization associated with increased uptake of the nutrient or decreased activity of the enzymes involved in releasing these nutrients from the SOM? Are changes in SOM dynamics associated with shifts in the production of different enzymes responsible for the degradation of different forms of organic matter?

Both the quantity and the quality of SOM often change in response to elevated CO_2, which can lead to changes in microbial functioning, including extracellular enzyme production. For example, SOM has been shown to become more recalcitrant in ecosystems exposed to elevated CO_2 (Gill et al. 2002, Feng et al. 2010). Because microbes still need to decompose SOM for C, they may increase the production of enzymes, such as phenol oxidase and peroxidase, which can degrade these molecules (Chung et al. 2006, Carney et al. 2007). Carbon and nutrient cycling are closely linked within these systems due to the stoichiometry of organic matter decomposition; therefore any changes in the production of enzymes that catalyze C-cycling-related biochemical reactions, such as the degradation of lignocellulose molecules, are likely to be associated with changes in those associated with the decomposition of nutrient-containing organic matter (Manzoni et al. 2008, Manzoni et al. 2010, Sinsabaugh and Follstad Shah 2010). Many studies have shown that terrestrial ecosystems exposed to elevated CO_2 experience progressive N limitation, as plants take up and retain N (Luo et al. 2004, Gill et al. 2006). As a result, microbes also become limited by N availability and may put more resources into producing enzymes, such as aminopetidases and chitinases, that aid in acquiring N (Mayr et al. 1999, Ebersberger et al. 2003, Kang et al. 2005). However, not all systems exposed to elevated CO_2 exhibit the same responses in soil extracellular enzyme activity.

Because microbes and the enzymes they produce regulate so many important ecosystem

Table 1. The extracellular enzymes and substrates examined in this study. The first two columns describe the enzymes examined in the meta-analysis. The last three columns describe the enzymes in the field experiment.

Meta-analysis enzyme degradation group	Description	Field experiment		
		Enzymes	Abbreviation	Target substrate
Simple Sugar	Hydrolysis of sugars to glucose	—	—	—
Starch	Starch hydrolysis	α-1,4-Glucosidase	αG	4-MUB-α-D-Glucopyranoside
β-Glucan	Hydrolysis of cellobioside to glucose	—	—	—
Cellulose	Hydrolysis of cellobioside dimers	α-D-1,4-Cellobiosidase	CBH	4-MUB-β-D-Cellobioside
Hemicellulose/Xylan	Hemicellulose hydrolysis	β-1,4-Xylosidase	βX	4-MUB-β-D-Xylopyranoside
Lignin	Oxidation lignin	—	—	—
Chitin	Hydrolysis of chitooligosaccharides into N-acetylglucosamine	β-1,4-N-Acetyl-Glucosaminidase	NAG	4-MUB-N-Acetyl-β-D-Glucosaminide
Protein	Cleaving of peptide bonds in proteins	Leucine Aminopeptidase	LAP	L-Leu-7-Amino-4-Methylcoumarin HCl
Organic P	Cleaving of PO_4 from P-containing OM	Alkaline Phosphatase	AP	4-MUB-Phosphate
Organic S	Cleaving of SO_4 from S-containing OM	Sulfatase	SUL	4-MUB-Sulfate

Notes: Dashes indicate that the enzyme group was not measured in the field experiment. OM, organic matter.

processes, we tested how enzyme activity changes in response to CO_2 concentrations in two ways. First, we conducted a meta-analysis of soil enzyme activity, incorporating data from 34 terrestrial studies to determine which, if any, common extracellular enzymes showed altered activity in response to elevated CO_2. We hypothesized that the activity of soil enzymes associated with N and P availability would increase in elevated CO_2 because high CO_2 can lead to decreased soil nutrient availability through increased plant uptake of nutrients. We also expected that the activity of enzymes associated with the degradation of labile substrates would decrease at elevated CO_2 and those associated with the degradation of recalcitrant substrates would increase at elevated CO_2. Inputs of C compounds from plant litter and root exudation are each likely to change in both quantity and quality with elevated CO_2, shifting the substrates available for soil microbes.

Second, we measured enzyme activity in two soil types sampled along a continuous field CO_2 gradient (250–500 ppm). This gradient allowed us to determine if soil enzyme activity responded nonlinearly to CO_2, and how enzyme activity may have responded in the past to subambient CO_2 concentrations. Additionally, the field experiment allowed us to examine more specific controls on enzyme activity than in the meta-

analysis. Similar to our hypotheses in the meta-analysis, we expected the activity of enzymes associated with nutrient release from SOM, such as phosphatase and leucine aminopeptidase, to increase with CO_2, and the activity of enzymes associated with the hydrolysis of simple carbon substrates to decrease.

MATERIALS AND METHODS

Meta-analysis

We performed a meta-analysis on data gathered from 34 papers (see Supplement) that examined the effects of elevated CO_2 on extracellular enzyme activity. These papers were collected using the ISI Web of Science Database, searching for papers that contained the keywords "elevated CO_2," "soil," and "enzyme." We were interested in enzymes involved in soil biogeochemical cycling (Table 1) grouped into the following broad categories: (1) simple sugar degradation (including trehalase and invertase), (2) starch degradation (α-glucosidase), (3) β-glucan degradation, (4) cellulose degradation (and other non-β-glucosidase, cellulose-degrading enzymes), (5) xylan (or hemicellulose) degradation, (6) lignin degradation (phenol oxidase and peroxidase), (7) chitin degradation (predominately N-acetyl-glucosaminidase), (8) protein degradation (including leucine and tyrosine

aminopeptidase and arginine deiminase), (9) organic phosphorus degradation (alkaline and acid phosphatase), and (10) organic sulfur degradation. We divided cellulase enzymes into two categories, β-glucosidase and cellobiohydrolase; if the type of cellulase measured in a study was unclear, we placed the enzyme within the "cellobiohydrolase and other cellulase category". We obtained data directly from tables where possible or estimated values based on digitized graphs.

We applied several filters to the dataset to ensure data consistency. We limited our dataset to soil sampled from the top 5 cm. If more than one soil depth was given for a study, we used the 0–5 cm or 0–10 cm profile. The concentration for ambient CO_2 treatments ranged from 350 to 400 ppm (the highest level being from a growth chamber study), while the elevated CO_2 treatments ranged from 540 to 760 ppm CO_2. The data were not corrected for the difference in the CO_2 concentration among the elevated treatments in the different studies because there was no correlation between the response ratio and the difference in CO_2 concentration between the treatments (data not shown). If a single study included more than one sampling site, we treated data from each site as a separate sampling point. If more than one sampling date was included, we used the average of all the sampling dates for our analysis, so as to not over-emphasize results from studies that sampled enzyme activity multiple times. All experimental treatments other than CO_2 (e.g., precipitation or nutrient manipulations) were not evaluated. A variety of methods were used to measure enzyme activity across the studies examined. All values were presented per g of soil.

To determine the effect of elevated CO_2 on enzyme activity, we applied bootstrapping techniques to the response ratios defined by Curtis and Wang (1998) and Hedges et al. (1999), using a computer program developed by J. Starmer and A. Kelley (unpublished manuscript). First, we calculated a response ratio, the natural log of the mean activity of a particular enzyme in the elevated CO_2 treatment divided by the mean activity of that same enzyme in the ambient treatment (Hedges et al. 1999). We weighted each response ratio based on the number of samples taken within its respective study (Hedges and

Olkin 1985, Adams et al. 1997) rather than by the standard deviation (Curtis and Wang 1998, Hedges et al. 1999) because several studies did not report variances. The data were resampled 2000 times, and the effect of elevated CO_2 was determined to be significant if the $1 - \alpha = 95\%$ bootstrap-bias-corrected-and-accelerated (BCa) confidence interval (CI) did not include zero. P-values were estimated by an iterative process where BCa CIs were calculated using decreasing values for α, starting at $\alpha = 0.05$ and subtracting 0.001 after each iteration. When a BCa CI contained zero, the p-value was defined as being less than the previous value for α. For example, if the BCa CI calculated for $\alpha = 0.028$ did not contain zero, and the BCa CI calculated for $\alpha = 0.027$ did, then the p-value was defined as being <0.028. We also conducted the same analysis on subsets of the data, analyzing whether vegetation type (i.e., woody vs. herbaceous vegetation) or the method of CO_2 treatment (i.e., lab- vs. field-based studies) affected the response ratio. We present data as the percent change at elevated CO_2, calculated as the exponential of the response ratio minus one, then multiplied by 100.

Study site and experimental design

Our field study took place at the USDA-ARS Lysimeter CO_2 Gradient (LYCOG) experiment in Temple, TX. This experiment exposes intact monoliths of tallgrass prairie soil and vegetation to a continuous gradient of CO_2 from 250 to 500 ppm (Fay et al. 2009). Initiated in May of 2006, the CO_2 experiment consists of two parallel, 50-m long chambers each divided into 10 5-m connected sections. One chamber provides the gradient from ambient to subambient CO_2 and the other from elevated to ambient CO_2. Pure CO_2 is injected into air introduced to the elevated chamber to increase the initial CO_2 concentration to 500 ppm. Ambient air is introduced at the beginning of the second chamber. Photosynthesis reduces the CO_2 concentration of air as it is blown through remaining sections of each chamber to produce a 500–370 ppm CO_2 gradient (elevated length) and 370–250 ppm gradient (subambient length). For a detailed description of the experimental methods, see Fay et al. (2009) and Johnson et al. (2000).

Intact monoliths of three soil types, Bastrop series, Houston series, and Austin series, are

arrayed along the two chambers. Existing vegetation was removed for each of the $1 \times 1 \times 1.5$-m-deep monoliths at collection. Each monolith was planted with the same tallgrass prairie vegetation consisting of 4 C_4 grasses and 3 C_3 forbs, including one legume species (Fay et al. 2009). We focused measurements on the two soils that differed the most in texture, the Houston and Bastrop series. The Houston series is a black clay vertisol (Udic Haplustert) comprised of 49–55% clay and 36–39% silt in the top meter. The soil contains 3.1% organic C and 0.28% total N in the top 5 cm. The Bastrop series is an alluvial sandy loam alfisol (Udic Paleustalf) comprised of 72% sand and 7–14% clay in the top 5 cm. The soil contains 1.17% organic C and 0.11% organic N in the top 5 cm (Fay et al. 2009). We hereafter refer to the Houston soils as the black clay soils and the Bastrop soils as the sandy loam soils.

Soil collection and extracellular enzyme assays

Soil samples were collected from monoliths in May, July, and September of 2009, the fourth year of CO_2 treatment. At each CO_2 level, we collected four 3-cm-deep soil cores in each of two 1-m^2 monoliths of each soil type. Samples from the two monoliths per CO_2 treatment and soil type were composited and refrigerated until analysis.

Extracellular enzyme activity was measured within 48 hours of soil sampling using fluorescing methylumbelliferyl- or methylcoumarin-labeled substrates (Table 1; Sinsabaugh et al. 2003). We measured potential activity of the following enzymes during three sampling periods: α-1,4-glucosidase, β-D-1,4-cellobiosidase, β-1,4-xylosidase, β-1,4-N-acetyl-glucosaminidase, leucine aminopeptidase, alkaline phosphatase, and sulfatase (Table 1). Although these enzymes may not be solely responsible for C, N, P, and S cycling, they do catalyze biochemical reactions that play important roles in the biogeochemical cycling of C, N, P, and S. Therefore we will use the term "C cycling-associated enzymes" to refer to enzymes such as α-1,4-glucosidase, β-D-1,4-cellobiosidase, and β-1,4-xylosidase (i.e., enzymes whose products do not contain N, P, or S). Additionally we will use the term "N cycling-associated enzymes" to refer to enzymes such as β-1,4-N-acetyl-glucosaminidase and leucine aminopeptidase, "P cycling-associated enzymes" to refer to phosphatase, and "S cycling-associated

enzymes" to refer to sulfatase.

A soil-slurry was made of each composited soil sample by mixing 1 g of soil with 100 mL of 50-mM $NaHCO_3$ buffer and shaking each slurry for 30 minutes. The slurry was then kept suspended with a magnetic stir bar, 200 μL aliquots were pipetted into a black polypropylene 96-well black plate, and 50 μL of the fluorescing substrates was added to each individual soil sample. The plates were then incubated at 20°C for 3 hr and analyzed for fluorescence using a BMG Labtech Fluostar Optima plate reader with 365 nm excitation and 450 nm emission filters. Enzyme activities are presented in μmol g^{-1} organic C h^{-1} because of the large differences in organic matter content between the two soils and were corrected for quenching and controls. Additionally, a subset of each soil sample was dried at 65°C to a constant weight to determine gravimetric soil moisture content.

Nutrient availability

During the growing season of 2009, anion and cation exchange resin probes (Plant Root Simulator probes, Western Ag, Saskatoon, Saskatchewan, Canada) were used to determine nutrient availability of each soil along the CO_2 gradient. Probes were left in soil for one month, after which they were removed, brushed free of soil, and stored in a refrigerator until extraction. Probes were extracted with 1 M KCl and analyzed for a series of plant-available nutrients with a Technicon Autoanalyzer (Hangs et al. 2004). Here, we report the data for inorganic N and P that correspond with the months preceding our analysis of enzyme activity.

Data analysis for the field experiment

Data of extracellular enzyme activities were analyzed with JMP version 7 (SAS Institute, Cary, NC) using a repeated-measures ANCOVA, with CO_2 as the covariate, soil type as a fixed effect, and sampling period as the repeated factor. Specifically, we were interested in any overall CO_2 effect and if the interaction between CO_2 and soil type affected rates of enzyme activity. We also performed a regression analysis on the activity of each enzyme to compare CO_2 concentration in each soil type and sampling date. Additionally, we examined the ratios of C cycling-associated enzymes to N and P cycling-
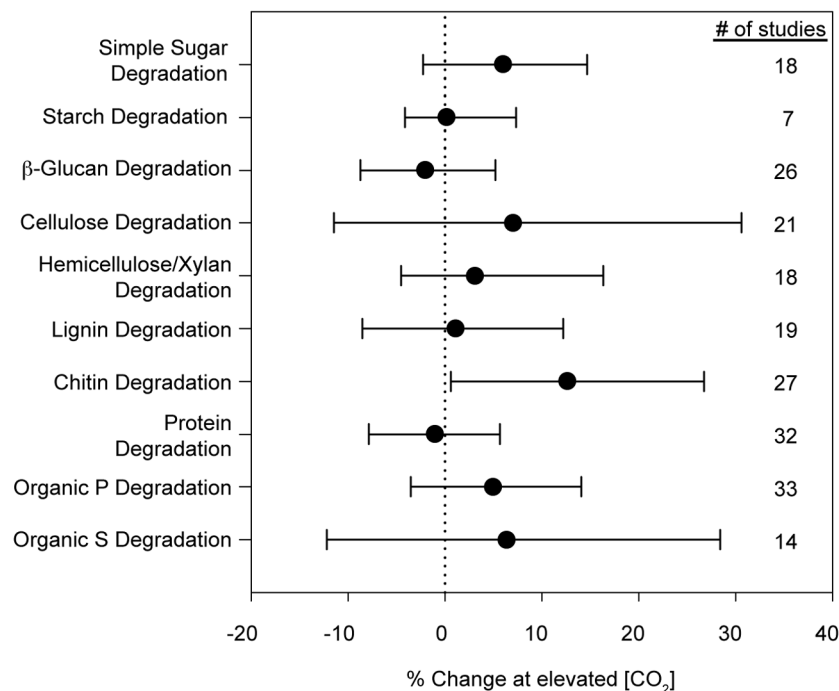
Fig. 1. The effect of elevated CO_2 on extracellular enzyme activity in soils from a meta-analysis of 34 studies (mean ± 95% confidence intervals).

associated enzymes. To do this, we first summed the activities of C cycling-associated enzymes (E_C), α-1,4-glucosidase, β-D-1,4-cellobiosidase, and β-1,4-xylosidase. We also summed the activities of N cycling-associated enzymes (E_N), leucine amino peptidase and β-1,4-N-acetyl-glucosaminidase. Because alkaline phosphatase was the only enzyme examined that was involved in P cycling, we used the activity of this enzyme to represent P cycling-associated enzymes (E_P). We then calculated the E_C:E_N and E_C:E_P ratio of enzyme activities for each CO_2 concentration, sampling date, and soil type. Similar to the analysis of the extracellular enzyme activities, we analyzed the ratios of enzyme activities using a repeated-measures ANCOVA, and regressed each on CO_2 for each soil type and sampling date.

RESULTS

Meta-analysis

Of the enzymes examined, chitin-degrading enzymes (predominately N-acetyl-glucosaminidase) showed a consistent and significant increase of 12.6% on average in elevated CO_2 (p < 0.05; Fig. 1). Other soil extracellular enzymes responded more variably to CO_2. Although several other enzymes showed trends in elevated CO_2, the 95% confidence intervals were too wide for these responses to be statistically conclusive.

Because of the wide variety of ecosystems and experimental designs used across studies, we examined the response of N-acetyl-glucosaminidase in more detail, focusing on subsets of data by vegetation and experiment methods (Fig. 2). The mean response to elevated CO_2 was positive across all of the subsets, with significant responses for studies conducted in the field, including Free-Air CO_2 Experiment and Open Top Chamber experiments (mean increase in enzyme activity of 12.9%; p = 0.009; Fig. 2A), and in systems dominated by woody vegetation, which exhibited a 13.7% increase in enzyme activity (p < 0.001). No significant responses were found in laboratory studies or in field studies of grass/forb communities.

Although other enzymes did not show an overall response to elevated CO_2 treatment, some enzymes responded significantly to elevated CO_2
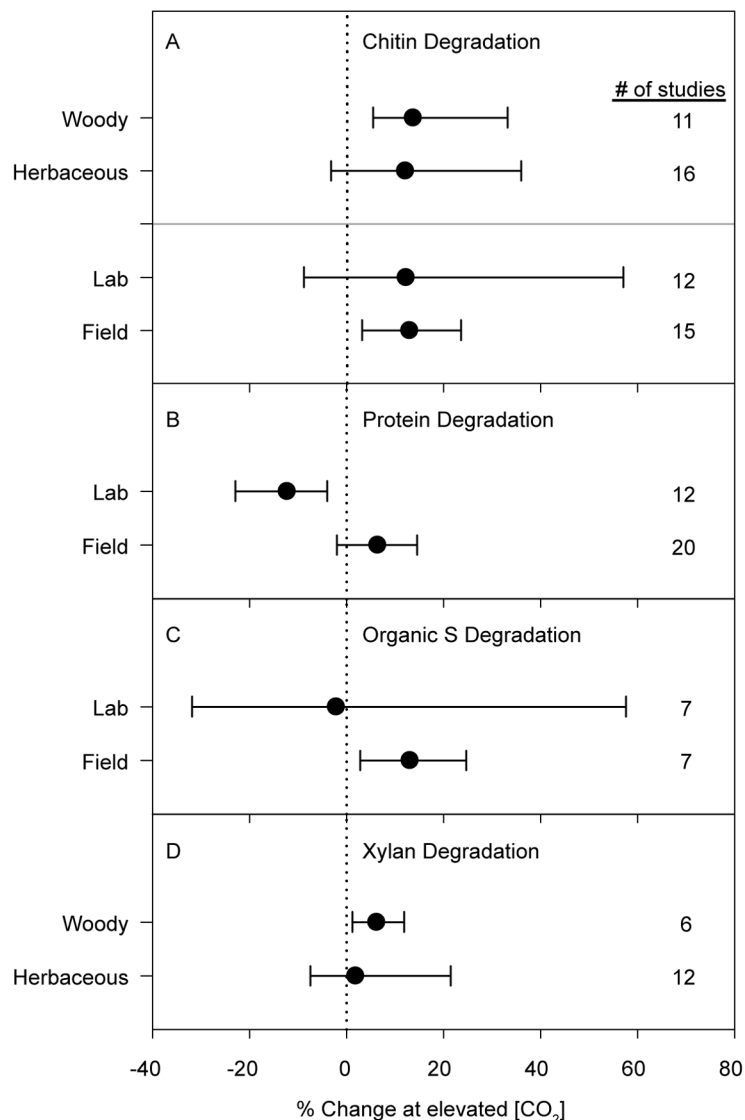
Fig. 2. The effect of elevated CO_2 on (A) *N*-acetyl-glucosaminidase in different vegetation types and experimental manipulations, (B) protein-degrading enzymes, (C) organic-S-degrading enzymes in different experimental manipulations, and (D) xylan-degrading enzymes in different vegetation types from a meta-analysis of 34 studies (mean ± 95% confidence intervals).

in the field or laboratory alone or dominant vegetation. Specifically, protein-degrading enzymes decreased 12.3% with elevated CO_2 in lab-based studies ($p < 0.001$; Fig. 2B), and organic S-degrading enzymes increased 13.0% in field CO_2 studies ($p < 0.012$; Fig. 2C). Xylan-degrading enzymes increased 6.1% in woody ecosystems ($p < 0.001$; Fig. 2D).

Extracellular enzyme activity along the field CO_2 gradient

Responses in enzyme activities differed along the field CO_2 gradient. The activity rates of C cycling-associated enzymes, specifically α-1,4-glucosidase, β-D-1,4-cellobiosidase, and β-1,4-xylosidase, decreased linearly by between 53 and 83% along the entire CO_2 gradient in the sandy loam soil in July (Fig. 3). Similarly, α-1,4-
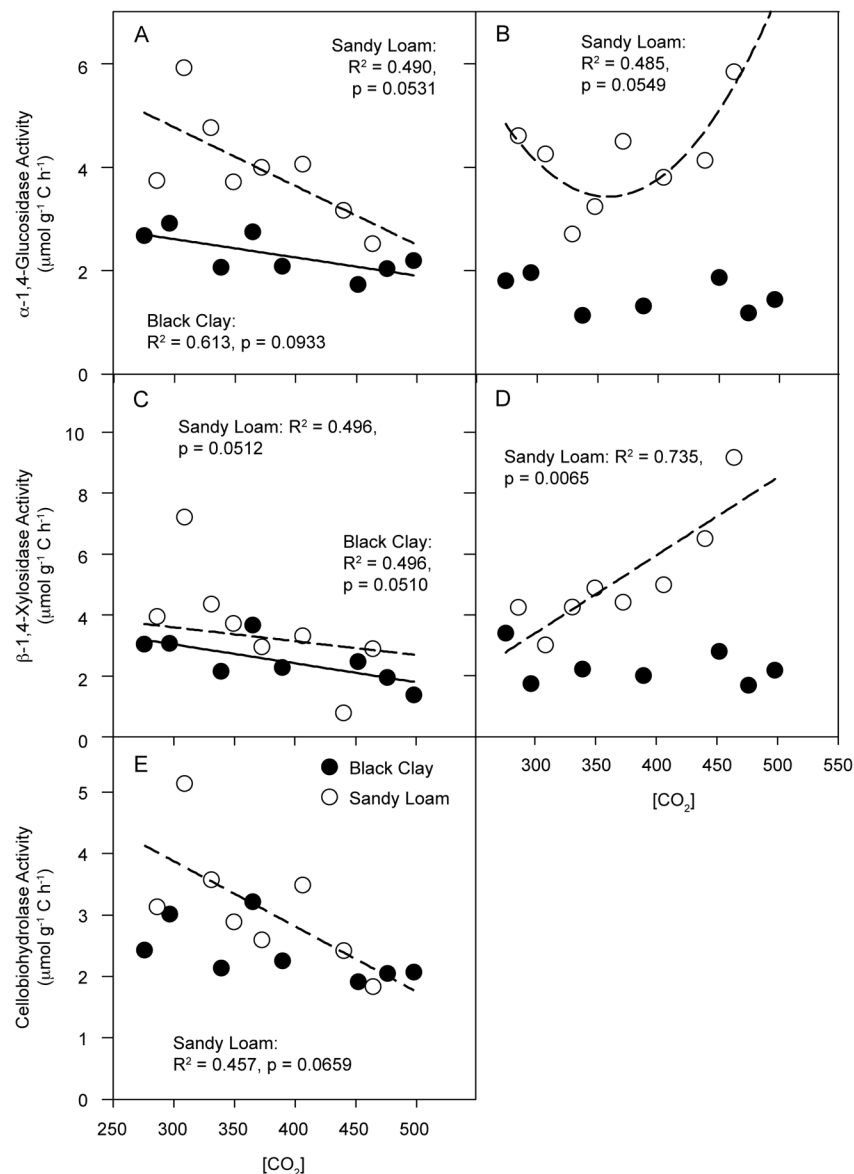
Fig. 3. The enzyme activity response of α-1,4-glucosidase in (A) July 2009 and (B) September 2009; β-1,4-xylosidase in (C) July 2009 and (D) September 2009; and (E) β-D-1,4-cellobiosidase in July 2009 in two soil types exposed to a field gradient of atmospheric CO_2. Lines represent a significant relationship ($p < 0.10$) between enzyme activity and CO_2.

glucosidase and β-1,4-xylosidase decrease linearly by 32% and 46%, respectively, along the gradient in the black clay soil during the same time period. In September, however, α-1,4-glucosidase exhibited a non-linear, second order polynomial trend, while β-1,4-xylosidase increased linearly by 302% in the sandy loam along the gradient.

The activities of N and P cycling-associated enzymes also varied with CO_2. In July, leucine aminopeptidase activity decreased linearly by 32% along the CO_2 gradient in the black clay soils. In September, CO_2 enrichment increased the activity of β-1,4-N-acetyl-glucosaminidase by 44% in the black clay soil and alkaline phosphatase by 42% in the sandy loam soil (Fig. 4).
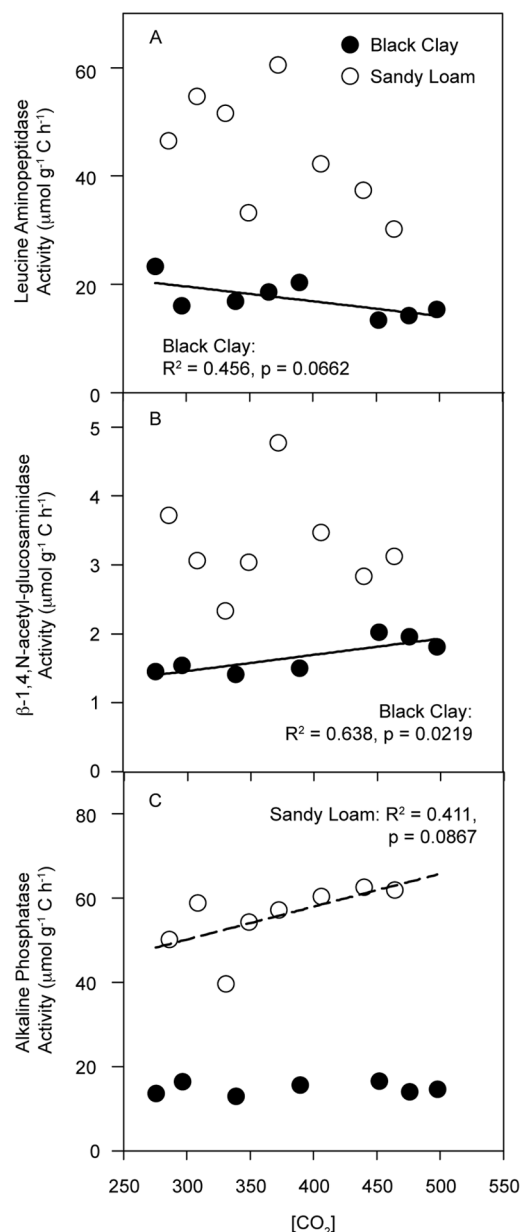
Fig. 4. Enzyme activity responses of (A) leucine aminopeptidase in July 2009, (B) β-1,4-N-acetyl-gluco-saminidase in September 2009, and (C) alkaline phosphatase in September 2009 in two soil types exposed to a field gradient of atmospheric CO_2. Lines represent a significant relationship ($p < 0.10$) between enzyme activity and CO_2.

Although we observed significant responses of some enzyme activities to CO_2 concentration, we did not see a significant response of microbial

biomass to CO_2. Similar to enzyme activity, average microbial biomass C was approximately twice as large in the sandy loam (653.6 µg microbial C g^{-1} C) as black clay soil (325.8 µg microbial C g^{-1} C).

The response of enzyme activity to CO_2 concentration and soil type depended strongly on the type of enzyme examined. For example, the response of alkaline phosphatase and β-1,4-xylosidase activities to CO_2 varied significantly with soil type and sampling time (Table 2). Although the other enzymes did not show a similar significant interaction between CO_2, soil type, and sampling date, there was a significant effect of CO_2 on α-1,4-glucosidase, β-D-1,4-cellobiosidase, and leucine aminopeptidase. Enzyme activities along the field CO_2 gradient were consistently and significantly higher in the sandy loam than black clay soil (Fig. 3, Table 2).

The ratio of C cycling-associated enzyme activity to P cycling-associated enzyme activity (E_C and E_P, respectively) was significantly affected by CO_2 concentration (Fig. 5; $p = 0.0345$). E_C:E_P decreased linearly with increasing CO_2 concentrations in the sandy loam soil ($R^2 = 0.565$, $p = 0.0314$). However, there was no significant effect of CO_2 on E_C:E_N ($p = 0.7123$). Overall, both ratios were significantly higher in the black clay than sandy loam soil ($p = 0.0004$ and <0.0001 for E_C:E_N and E_C:E_P, respectively).

Inorganic nitrogen and phosphorus availability

Inorganic N availability decreased with CO_2 in both July and September in the black clay soil (Fig. 6). In July, inorganic N availability in the black clay soil linearly decreased 17.6% along the entire CO_2 gradient, whereas the trend in September was more closely fit using an exponential decay function. P availability increased linearly 112.2% with CO_2 in the sandy loam soil in September. We did not see any other significant trends in nutrient availability with CO_2 concentrations for the other monthly sampling time points during this growing season (R. Gill et al., unpublished manuscript).

DISCUSSION

Microbial responses to increasing atmospheric CO_2 concentrations often vary across ecosystems (Zak et al. 2000, Freeman et al. 2004, de Graaff et

Table 2. Summary of p-values from repeated-measures ANCOVA for each enzyme (see Table 1 for explanation of abbreviations). Bold text indicates significant responses ($\alpha \leq 0.05$), and italics indicates a response at $\alpha \leq 0.10$.

| Source of variation	CBH	βX	αG	LAP	NAG	AP	SUL
Soil	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**0.001**	**<0.0001**	**0.0001**
CO_2	**0.0180**	0.4442	**0.0269**	**0.0373**	0.3014	0.5801	0.1844
Soil × CO_2	0.2476	0.3430	0.2525	0.1407	0.4820	0.5651	0.1613
Time	0.1785	**0.0045**	*0.0926*	*0.0835*	0.3944	**0.0011**	0.4805
Time × Soil	**0.0464**	**0.0213**	*0.0925*	0.7061	**0.0134**	**0.0169**	0.5142
Time × CO_2	0.1714	**0.0013**	0.1050	0.2299	0.3179	**0.0013**	0.4070
Time × Soil × CO_2	0.1367	**0.0019**	0.1352	0.5090	0.9428	**0.0010**	0.5161
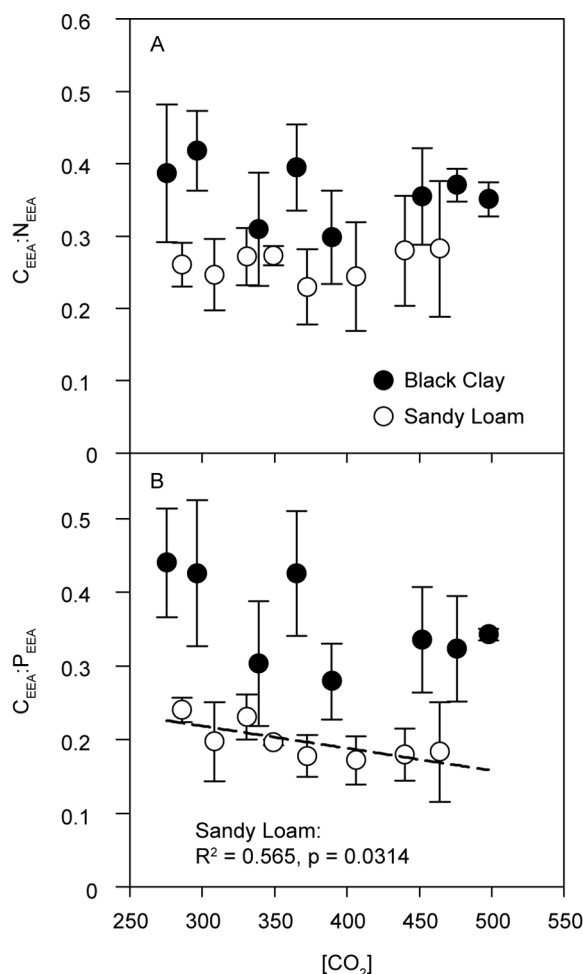
Fig. 5. Ratios of (A) the activity of enzymes associated with C cycling to those associated with N cycling enzyme activity ($E_C:E_N$) and (B) the activity of enzymes associated with C cycling to those associated with P cycling ($E_C:E_P$) in two soil types exposed to a field gradient of atmospheric CO_2 averaged across three sampling dates (mean ± SE). Lines represent a significant relationship ($p < 0.10$) between enzyme activity and CO_2.

al. 2006). Although our meta-analysis revealed considerable variation in the overall responses of extracellular enzyme activity to elevated CO_2 across ecosystems, we found increased activity at high CO_2 of a chitin-degrading enzyme important in N cycling (Fig. 1). Additionally, enzymes that catalyze the degradation of proteins, organic S, and xylan responded to elevated CO_2 within specific experimental conditions or for particular vegetation types (Fig. 2). Our results from the field experiment also support this observation in that many of the observed trends in enzyme response to the CO_2 gradient were observed for a specific soil type (Figs. 3 and 4). These results highlight the importance of ecosystem characteristics in understanding ecosystem responses to rising atmospheric CO_2.

When exposed to the conditions produced by elevated CO_2, soil microbial communities can change from being C limited to nutrient limited, as nutrients become sequestered in plant biomass and SOM (Oren et al. 2001, Gill et al. 2002, Luo et al. 2004, Finzi et al. 2006a, Gill et al. 2006). Increased nutrient limitation may lead soil microbes to increase the activity of enzymes that release nutrients such as N and P from SOM. However, our meta-analysis did not show a consistent effect of elevated CO_2 on the activity of protein-degrading enzymes, phosphatase, or sulfatase activity (Fig. 1), possibly because of the wide variety of ecosystems and soils of different nutrient status examined within the meta-analysis.

One enzyme, N-acetyl-glucosaminidase, did show increased activity at elevated CO_2 across variable field and experimental conditions (Fig. 1). This enzyme degrades chitin by hydrolyzing the β-1,4-glycosidic bonds of chitooligosaccharides into N-acetylglucosamine. Chitin is found primarily in fungal walls and also in exoskele-
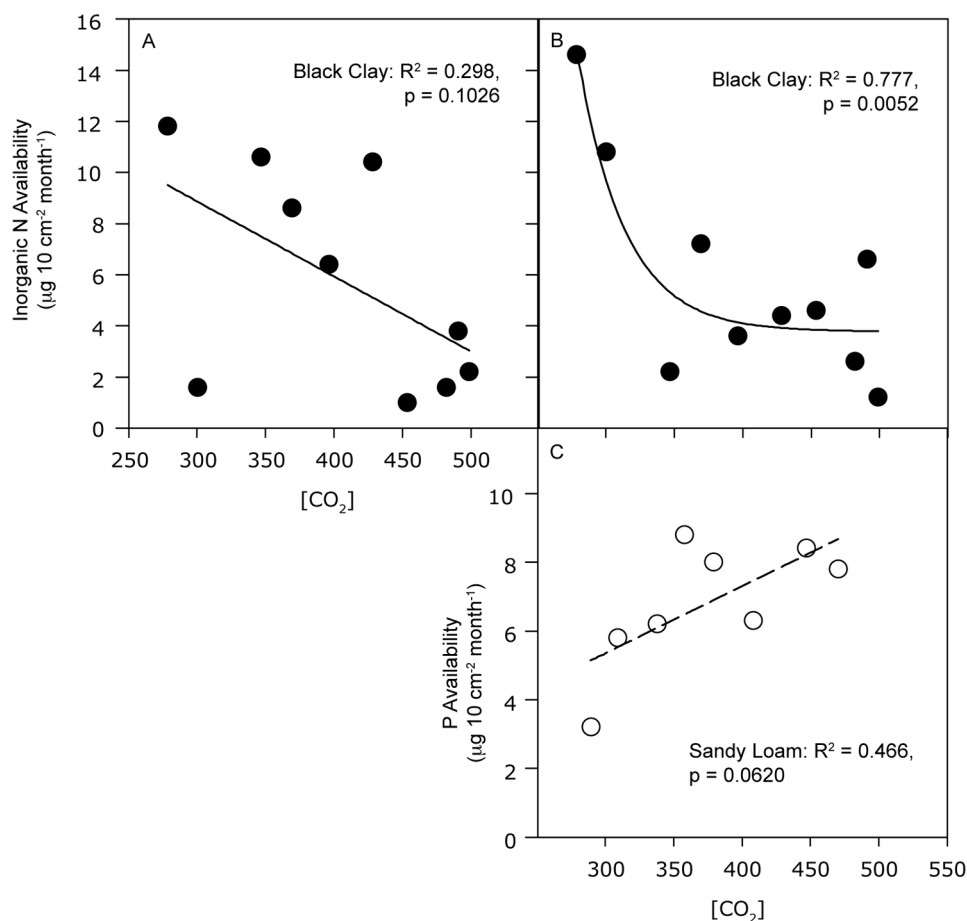
Fig. 6. (A) Nitrogen availability in a black clay soil in July 2009, (B) N availability in a black clay soil in September 2009, and (C) P availability in a sandy loam soil in September 2009 along the CO_2 gradient, as measured by Plant Root Simulator anion and cation exchange probes (see Methods).

tons of some arthropods. Chitin contains ~6% N (by weight) in a relatively recalcitrant form and is relatively abundant in soils, making it an important source of organic N in terrestrial ecosystems (Ueno et al. 1991, Ekenler and Tabatabai 2002). In N-limited systems, such as those induced by elevated CO_2, microbes are likely to degrade more labile forms of N-containing organic matter first because these molecules require less energy to degrade. After these labile forms are degraded, microbes are thought to produce enzymes responsible for degrading more recalcitrant forms of N, such as β-1,4-N-acetyl-glucosamine, to meet metabolic N demands (Ekenler and Tabatabai 2002, Gill et al. 2006).

This transition is, in fact, what we observed in the black clay soil in the field CO_2-gradient experiment. We found that the activity of leucine aminopeptidase decreased with increasing CO_2 in July, whereas CO_2 enrichment increased the activity of β-1,4-N-acetyl-glucosaminidase activity in September. These results indicate a transition towards microbes producing enzymes that can access more recalcitrant forms of N with increasing CO_2, as expected in soils that are becoming increasingly N-limited. Measurements of decreasing N availability, as determined by anion-cation exchange resins, also showed increased N limitation with increasing CO_2 in the black clay soil in both the July and September sampling periods (Fig. 6).

Results from the meta-analysis indicated that elevated CO_2 concentrations significantly in-

creased the activity of *N*-acetyl-glucosaminidase only in field studies, such as in FACE and OTC experiments. These experiments often run for longer periods of time than do greenhouse or growth-chamber experiments (see Supplement). Therefore, field systems may experience more nutrient limitation over the course of the experiments. Similarly, we saw an increase in the response of sulfatase to elevated CO_2 only in the field (Fig. 2C). Sulfur (S) is an essential component of some amino acids, such as cystine, cysteine, and methionine, found in plant proteins. This result suggests a possible S-limitation as a result of elevated CO_2. However, few CO_2 experiments have examined the interaction between CO_2 and S limitation in terrestrial systems. We also saw a decrease in the response of protein-degrading enzymes to elevated CO_2 in lab-based studies. This indicates that in shorter experiments, such as those conducted in greenhouses or growth chambers, N limitation may be less common, likely reflecting N release in the soils due to disturbance from the initial preparation or planting rather than a response to the elevated CO_2 treatment itself.

Despite the lack of an overall phosphatase response in the meta-analysis, we observed an increase in alkaline phosphatase activity in the sandy loam soils along the CO_2 gradient at the final sampling period (Fig. 3). Microbial production of phosphatase is induced when P limits growth (Spiers and McGill 1978, Dhillion et al. 1996, Moscatelli et al. 2005). This observed increase in phosphatase activity within our field experiment could suggest that these soils are more P-limited with increasing CO_2, though P availability was greater at high CO_2 in the sandy loam soil in the month before the enzyme measurements (Fig. 6C). Indeed, plant tissue P concentrations decreased with increasing CO_2 in the sandy loam soil (Polley et al. in press).

Microbial-mediated nutrient cycling in soils is constrained by both the quality of the SOM (nutrient content and recalcitrance) and the stoichiometry of the microbes associated with these processes (Sinsabaugh et al. 2009, Manzoni et al. 2010, Sinsabaugh and Follstad Shah 2010). Sinsabaugh et al. (2008) showed that the stoichiometry of enzyme activity, specifically $E_C{:}E_N$ and $E_C{:}E_P$ across multiple ecosystems varied with environmental variables such as soil pH, tem-

perature, and precipitation. We found that $E_C{:}E_P$ varied with CO_2 concentration along the gradient in the sandy loam soil (Fig. 5). These changes in enzyme ratios seem to be driven not only by the increase in alkaline phosphatase with CO_2 concentrations (Fig. 4), but also by the decrease in activity of the C cycling-associated enzymes with CO_2 in some sampling periods (Fig. 3), a response that has been seen in other elevated CO_2 experiments (Moscatelli et al. 2005, Kandeler et al. 2006).

Our field study showed that soil characteristics could strongly influence nutrient cycling responses to increasing CO_2 concentrations. In particular, increasing CO_2 induced apparent changes in N cycling in the black clay soil and P cycling in the sandy loam soil, as indicated by the responses of enzyme activity, nutrient availability, and plant nutrient dynamics to the CO_2 gradient. These soils differ in soil texture and organic matter quality and quantity, both of which can influence enzyme activity and nutrient cycling (Marx et al. 2005, Grandy et al. 2009). The black clay soil has higher clay content and organic matter content (Fay et al. 2009; R. Gill et al., unpublished manuscript). C particles can adsorb soil enzymes, thereby stabilizing them from degradation and allowing them to remain active longer. Enzyme activity was consistently higher per g of soil dry weight in the black clay than sandy soil, as expected (see Appendix). To compare these soil types more completely, we calculated enzyme activity per gram of soil organic C, which resulted in the highest enzyme activity in the sandy loam soil. The organic matter in the sandy loam soil may therefore be more easily degraded by extracellular enzymes or is more accessible to degradation.

Microbes can be tightly coupled to the plant community through competition between plants and microbes for resources or through plant-induced changes to soils that may change seasonally (Dhillion et al. 1996, Ebersberger et al. 2003, Moscatelli et al. 2005, Jin and Evans 2007). Therefore, the timing of soil sampling can influence the results of enzyme activity to changing CO_2 concentrations, both within growing seasons and across years (Larson et al. 2002, Finzi et al. 2006*b*, Jin and Evans 2007). Although we did not find a direct correlation between enzyme activity and changing plant productivity

throughout the growing season (assessed by Leaf Area Index; data not shown), we did find that enzyme activity response to the CO_2 gradient did vary during the different sampling dates (Figs. 3 and 4, Table 2). These differences were likely due to a variety of aspects associated with plant response to changing CO_2, such as productivity and soil water availability.

To our knowledge, ours is the first meta-analysis to examine the response of soil extracellular enzyme activities in ecosystems exposed to elevated CO_2. Although analyzing 34 studies is useful for providing a summary of enzyme responses to CO_2, we were unable to determine whether enzyme activities varied among data subsets, such as ecosystems, climatic conditions, or other variables. Enzyme activity assays have become a more commonly used technique to assess soil microbial functioning based on the development of faster and easier techniques for estimating enzyme activity and increased scientific interest in this topic (Wallenstein and Weintraub 2008). As more studies begin to use these techniques, we can expect that larger datasets should allow more powerful statistical analyses for microbial responses to CO_2 and other variables.

Changing CO_2 concentrations can alter soils through shifts in organic matter quantity and quality, which can in turn affect how microbes function. Across studies in different ecosystems, activity of N-acetyl-glucosaminidase, an enzyme responsible for degrading N-containing chitin, increased significantly in response to elevated CO_2. Our field study in turn showed that soil type and the timing of sampling, can strongly influence CO_2 effects on enzyme activity. Specifically, soil characteristics play an important role in determining CO_2 effects on soil C dynamics and nutrient availability. Understanding how microbial functioning, such as soil enzyme activity, responds to changing CO_2 should help researchers better predict how nutrient and C cycling will respond to changing environmental conditions in the future.

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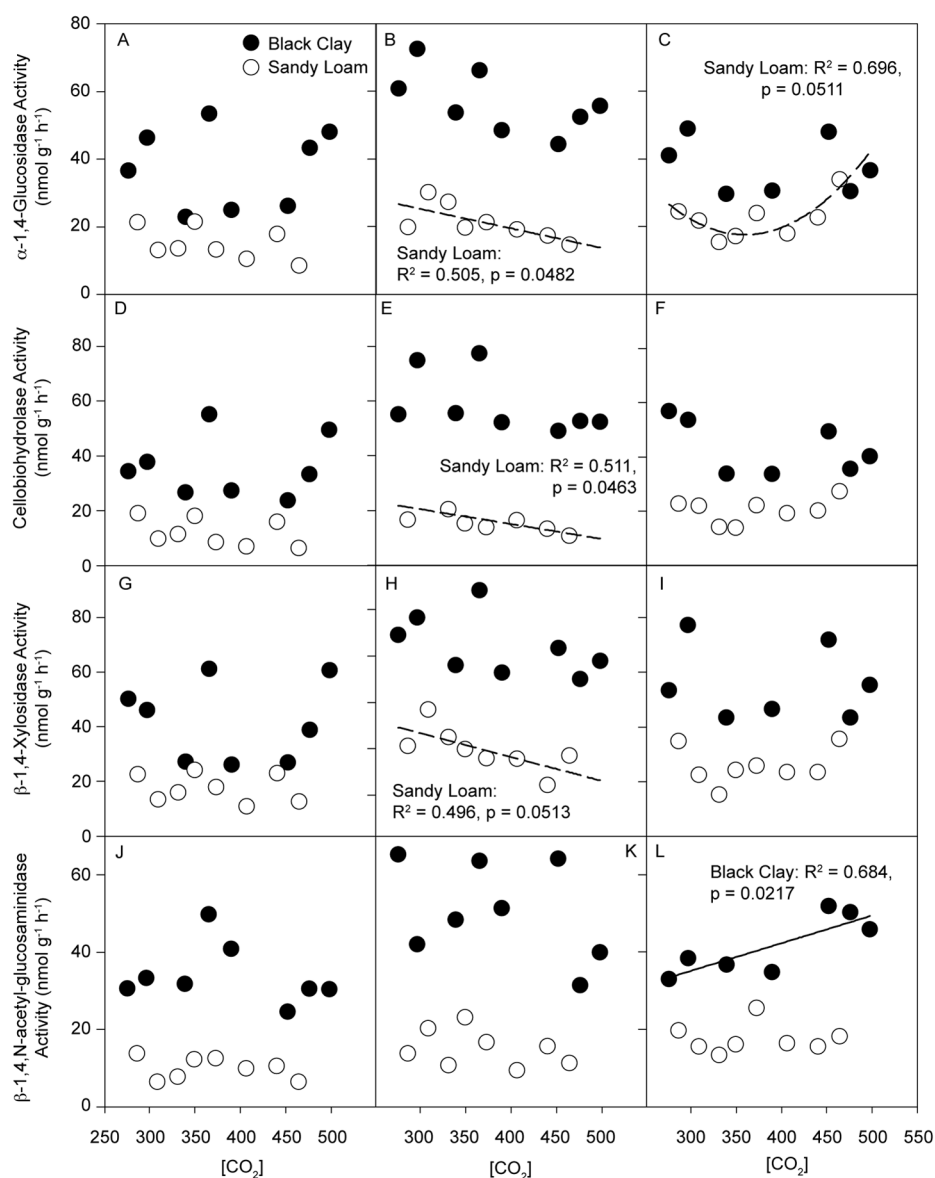
APPENDIX

Fig. A1. Response of (A) May α-1,4-glucosidase, (B) July α-1,4-glucosidase, (C) September α-1,4-glucosidase, (D) May β-D-1,4-cellobiosidase, (E) July β-D-1,4-cellobiosidase, (F) September β-D-1,4-cellobiosidase, (G) May β-1,4-xylosidase, (H) July β-1,4-xylosidase, (I) September β-1,4-xylosidase, (J) May β-1,4-N-acetyl-glucosaminidase, (K) July β-1,4-N-acetyl-glucosaminidase, and (L) September β-1,4-N-acetyl-glucosaminidase enzyme activity in two soil types exposed to a field gradient of atmospheric CO_2. Here the results are shown on a per g soil basis. Black lines represent a significant relationship ($p < 0.10$) between enzyme activity and CO_2.
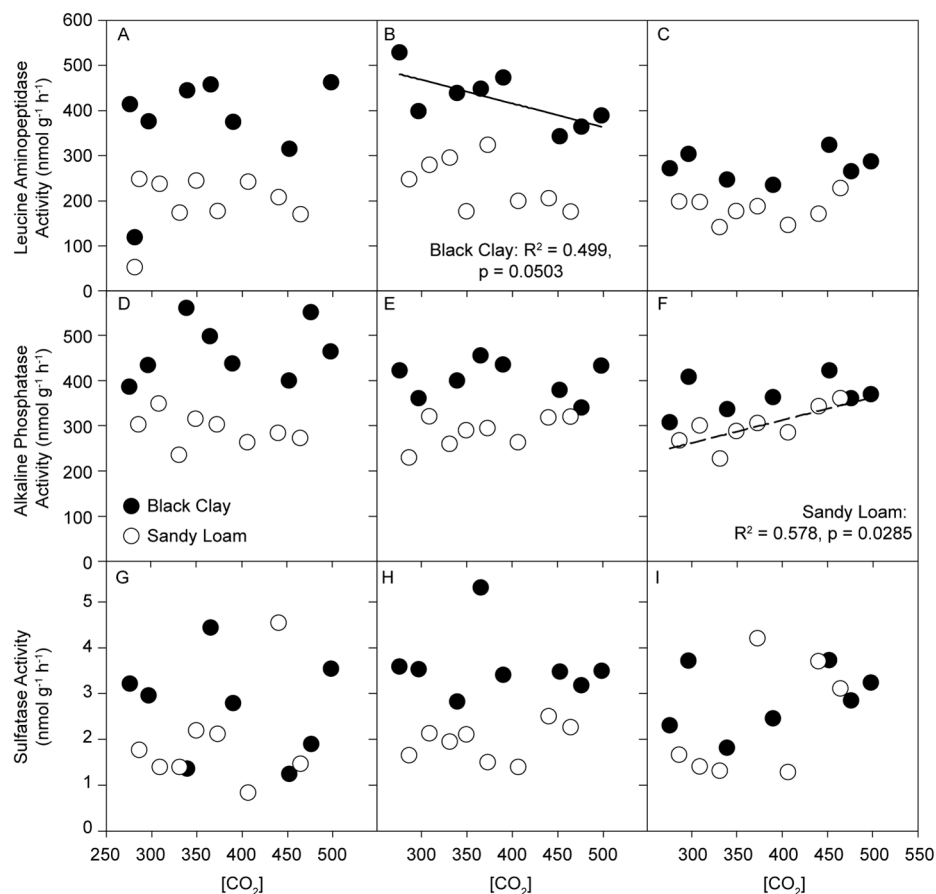
Fig. A2. Response of (A) May leucine amino peptidase, (B) July leucine amino peptidase, (C) September leucine amino peptidase, (D) May alkaline phosphatase, (E) July alkaline phosphatase, (F) September alkaline phosphatase, (G) May sulfatase, (H) July sulfatase, and (I) September sulfatase enzyme activity during three sampling dates (2009) in two soil types exposed to a field gradient of atmospheric CO_2. Here the results are shown on a per g soil basis. Black lines represent a significant relationship ($p < 0.10$) between enzyme activity and CO_2.
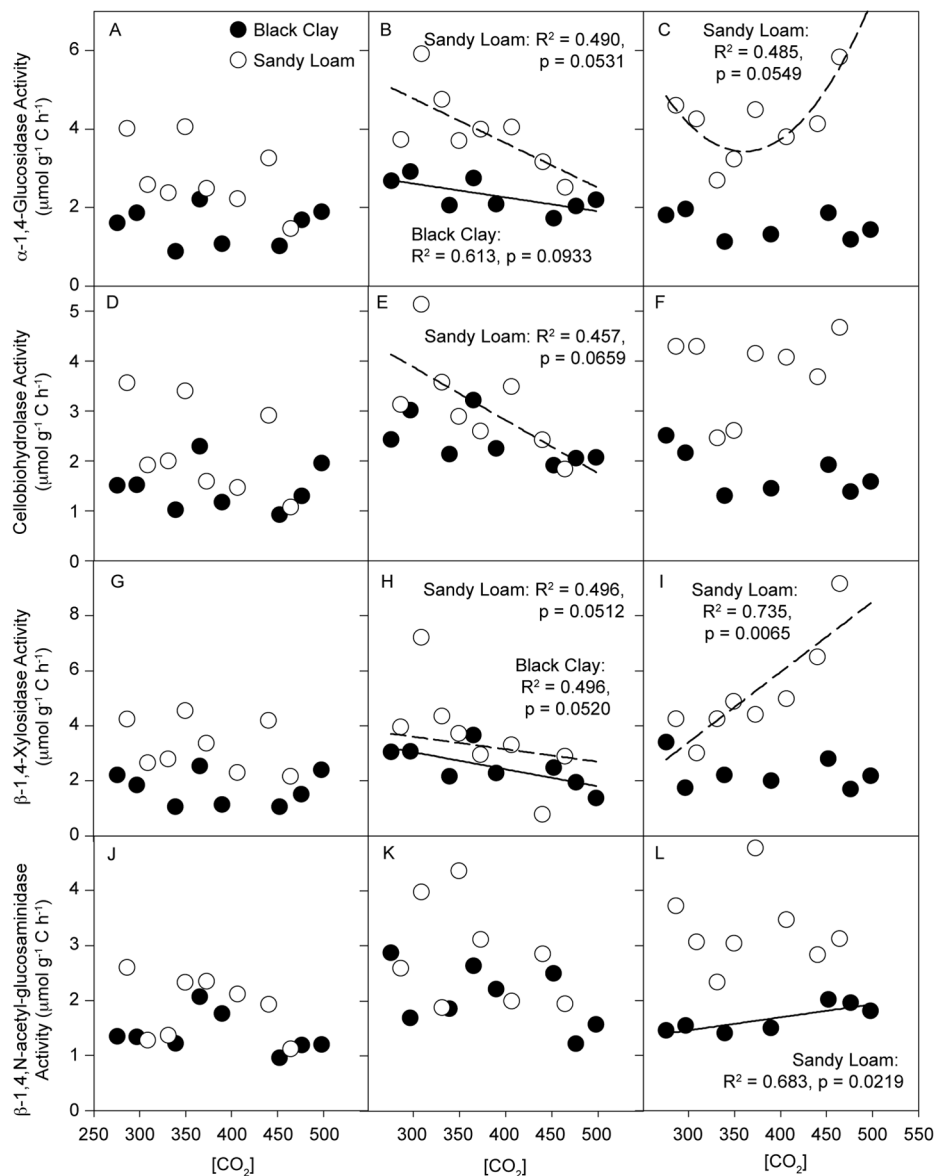
Fig. A3. Response of (A) May α-1,4-glucosidase, (B) July α-1,4-glucosidase, (C) September α-1,4-glucosidase, (D) May β-D-1,4-cellobiosidase, (E) July β-D-1,4-cellobiosidase, (F) September β-D-1,4-cellobiosidase, (G) May β-1,4-xylosidase, (H) July β-1,4-xylosidase, (I) September β-1,4-xylosidase, (J) May β-1,4-N-acetyl-glucosaminidase, (K) July β-1,4-N-acetyl-glucosaminidase, and (L) September β-1,4-N-acetyl-glucosaminidase enzyme activity in two soil types exposed to a field gradient of atmospheric CO_2. Here the results are shown on a per g C basis. Black lines represent a significant relationship ($p < 0.10$) between enzyme activity and CO_2.
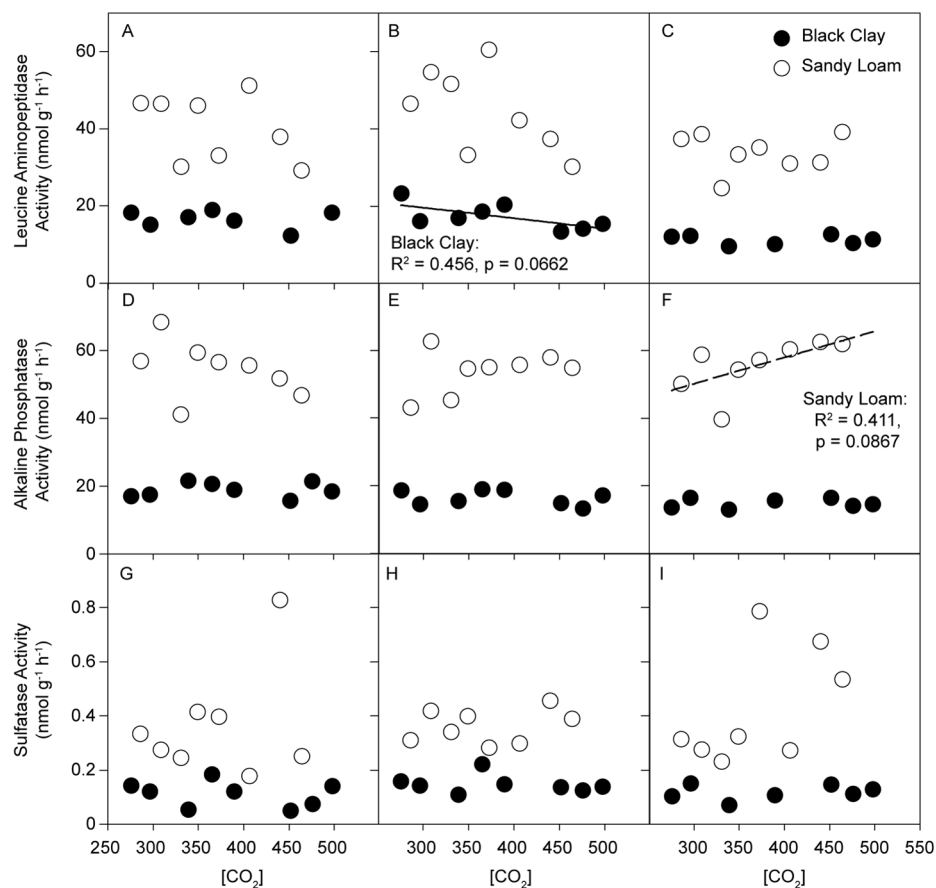
Fig. A4. Response of (A) May leucine amino peptidase, (B) July leucine amino peptidase, (C) September leucine amino peptidase, (D) May alkaline phosphatase, (E) July alkaline phosphatase, (F) September alkaline phosphatase, (G) May sulfatase, (H) July sulfatase, and (I) September sulfatase enzyme activity during three sampling dates (2009) in two soil types exposed to a field gradient of atmospheric CO_2. Here the results are shown on a per g C basis. Black lines represent a significant relationship ($p < 0.10$) between enzyme activity and CO_2.

Table A1. The mean enzyme activities, slope, intercept, R^2, and p-values of enzymes measure in the field CO_2 experiment (based on per g C calculations).

Enzyme	Sampling date	Bastrop					Houston				
		Mean	Slope	Intercept	R^2	p-value	Mean	Slope	Intercept	R^2	p-value
αG	May	2.81					1.53				
	July	**3.98**	**−0.01**	**8.18**	**0.49**	**0.05**	**2.31**	**0.003**	**3.67**	**0.49**	**0.05**
	September	**4.13**	†	**2.04**	**0.61**	**0.09**	1.53				
CBH	May	2.24					1.46				
	July	**3.13**	**−0.01**	**7.06**	**0.46**	**0.07**	2.38				
	September	3.78					1.76				
βX	May	3.28					1.72				
	July	**3.65**	**−0.02**	**11.08**	**0.50**	**0.05**	**2.50**	**−0.01**	**4.91**	**0.50**	**0.05**
	September	**5.18**	**0.03**	**−4.27**	**0.73**	**0.01**	2.29				
NAG	May	1.89					1.39				
	July	2.84					2.07				
	September	3.29					**1.67**	**0.002**	**0.75**	**0.68**	**0.02**
LAP	May	40.05					16.58				
	July	44.51					**17.24**	**−0.030**	**27.790**	**0.46**	**0.0700**
	September	33.77					11.15				
AP	May	54.60					18.86				
	July	53.65					16.40				
	September	**55.60**	**0.08**	**26.82**	**0.04**	**0.09**	14.81				
SUL	May	0.36					0.11				
	July	0.36					0.15				
	September	0.43					0.12				

Notes: Empty cells indicate non-significant regressions (p-value > 0.10). See Table 1 for explanations of enzyme abbreviations.
† Slope of α-1,4-glucosidase in Sept = $0.004 \times CO_2 + 0.0002(CO_2 − 369.791)^2$

SUPPLEMENT

A list of studies used in the meta-analysis, including type of CO_2 treatment, dominant vegetation, and values for mean ambient and mean elevated activity for the various enzyme groups (*Ecological Archives* C002-009-S1).