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LETTER

Metagenomic analysis reveals a marked divergence in the structure of belowground microbial communities at elevated CO_2

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Abstract

Understanding the responses of biological communities to elevated CO_2 (eCO_2) is a central issue in ecology, but little is known about the influence of eCO_2 on the structure and functioning (and consequent feedbacks to plant productivity) of the belowground microbial community. Here, using metagenomic technologies, we showed that 10 years of field exposure of a grassland ecosystem to eCO_2 dramatically altered the structure and functional potential of soil microbial communities. Total microbial and bacterial biomass were significantly increased at eCO_2, but fungal biomass was unaffected. The structure of microbial communities was markedly different between ambient CO_2 (aCO_2) and eCO_2 as indicated by detrended correspondence analysis (DCA) of gene-based pyrosequencing data and functional gene array data. While the abundance of genes involved in decomposing recalcitrant C remained unchanged, those involved in labile C degradation and C and N fixation were significantly increased under eCO_2. Changes in microbial structure were significantly correlated with soil C and N contents and plant productivity. This study provides insights into potential activity of microbial community and associated feedback responses of terrestrial ecosystems to eCO_2.

Keywords

Ecosystem process, elevated CO_2, feedback, free air CO_2 enrichment, GeoChip, global climate change, metagenomics, phospholipid fatty acid, pyrosequencing, soil microbial community.

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INTRODUCTION

The global atmospheric concentration of CO_2 has increased by >30% as the industrial revolution as a consequence of fossil fuel combustion and land-use changes (Houghton *et al.* 2001; Keeling & Whorf 2004). Such an increase will be much more rapid if fossil fuel emissions continue unabated and the terrestrial or oceanic carbon sinks weaken in the future (IPCC 2007). However, a robust prediction of future atmospheric CO_2 concentrations is hampered by uncertainties regarding the responses of the biosphere to eCO_2, especially belowground microbial communities, and the soil C and N cycling processes they mediate (Luo *et al.* 2006; Reich *et al.* 2006; van Groenigen *et al.* 2006; Carney *et al.* 2007; Gruber & Galloway 2008; Heimann & Reichstein 2008). Although the stimulating effects of eCO_2 on plant growth and primary productivity are well established (Reich *et al.* 2001; Ainsworth & Long 2005; Luo *et al.* 2006), its influences on belowground microbial communities are poorly understood and controversial (Walther *et al.* 2002; Parmesan & Yohe 2003; Heath *et al.* 2005; Carney *et al.* 2007; Gruber & Galloway 2008; Heimann & Reichstein 2008; Lesaulnier *et al.* 2008; Austin *et al.* 2009). There is an active debate on whether eCO_2 leads to soil C loss (i.e. positive feedback to eCO_2) or sequestration (i.e. negative feedback) (Luo *et al.* 2006; Carney *et al.* 2007; Heimann & Reichstein 2008). In addition, it is uncertain whether the magnitude of eCO_2 fertilization is generally constrained by co-limitation by N supply (Reich *et al.* 2006) and/or whether

the stimulation of plant growth and productivity by eCO_2 can be sustained (Zak *et al.* 2003; Reich *et al.* 2006) because of progressively increasing N limitation at eCO_2.

As microorganisms mediate important biogeochemical cycles of C, N, phosphorus (P) and sulphur (S), and various metals, a robust prediction of future atmospheric CO_2 requires mechanistic understanding of how eCO_2 affects microbial community composition (van Groenigen *et al.* 2006; Carney *et al.* 2007). However, the responses of soil microbial communities to eCO_2 are poorly understood (Gruber & Galloway 2008) and controversial (Carney *et al.* 2007; Lesaulnier *et al.* 2008; Austin *et al.* 2009) because of their extreme complexity and limitations of conventional molecular microbial ecology approaches for characterizing them. Using conventional molecular biology approaches, the diversity and activity of the microbial community in response to eCO_2 has been shown to be increased (Sonnemann & Wolters 2005; Jossi *et al.* 2006; Lesaulnier *et al.* 2008), decreased (Horz *et al.* 2004; Carney *et al.* 2007), or unchanged (Austin *et al.* 2009; Loy *et al.* 2004; Chung *et al.* 2006; Gruter *et al.* 2006). The apparent disparity of microbial responses to eCO_2 could be caused partially by real differences among and complexity of various ecosystems, but likely also by differences among the methodologies used, such as terminal restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE), 16S rRNA gene-based sequencing, enzyme activities, and phospholipid fatty acid (PLFA), which may resolve differences in the soil community caused by eCO_2 to differing degrees.

With the recent development and application of large-scale high throughput pyrosequencing-based (Sogin *et al.* 2006; Huber *et al.* 2007) and microarray-based (Brodie *et al.* 2006; He *et al.* 2007; Zhou *et al.* 2008; Wang *et al.* 2009) metagenomics technologies, community-wide spatial and temporal information on microbial community functional structure and potential activity can be rapidly obtained. Although the pyrosequencing-based approach is able to identify new sequences, it suffers from very high sensitivity to random sampling errors, dominant populations and contaminated non-target DNA, whereas the microarray-based approach does not suffer from these limitations (Zhou *et al.* 2008). Therefore, it will be ideal if both pyrosequencing- and array-based technologies are used in a complementary way. In addition, compared to phylogenetic gene arrays and 16S rRNA gene-based pyrosequencing approaches, functional gene arrays (e.g. GeoChip) are advantageous for detecting specific metabolic functions with quantitative and high-resolution characteristics (He *et al.* 2007; Zhou *et al.* 2008). For example, currently available GeoChip 2.0 contains >24 000 probes and covers >10 000 genes in 150 gene families involved in biogeochemical cycling of C, N, P and sulphur, and bioremediation of metals and organic contaminants (He *et al.* 2007).

In this study, we hypothesize: (1) that microbial community composition and structure will be altered because of both the increased inputs of C to soil and altered chemistry of those inputs under eCO_2 (Dijkstra *et al.* 2005; Adair *et al.* 2009), and (2) that various microbial functional groups (e.g. decomposers of recalcitrant C, C fixers, N fixers, denitrifiers) will respond differentially to eCO_2, and in particular we expect that genes related to C and N fixation and labile C degradation will be increased due to changed belowground availabilities of labile C and other resources. To test these hypotheses, integrated metagenomic approaches were used in concert with traditional microbiological approaches, which included GeoChip (He *et al.* 2007), pyrosequencing (Margulies *et al.* 2005; Hamady *et al.* 2008), EcoPlate, and PLFA approaches. This study was conducted in a multifactor free air CO_2 enrichment (FACE) experimental facility, BioCON (Biodiversity, CO_2 and Nitrogen deposition), at the Cedar Creek Ecosystem Science Reserve area in Minnesota (http://www.biocon.umn.edu/). Our analyses indicated that eCO_2 significantly altered the functional structure of soil microbial communities with a significantly increased abundance of genes involved in labile carbon degradation, carbon fixation, nitrogen fixation and phosphorus release, but without a significant change in the abundance of genes involved in recalcitrant C degradation and methane metabolism, and such changes may have significant impacts on soil C and N dynamics. These results have important implications for feedback responses of ecosystems to atmospheric CO_2 and global climate change.

MATERIALS AND METHODS

The following is the summary of methods used in this study. More detailed information is provided in the Data S1.

Site and sampling

This study was conducted within the BioCON experiment site located at the Cedar Creek Ecosystem Science Reserve, MN, USA. The main BioCON field experiment has a total of 296 plots with three treatments: CO_2 (ambient, 368 μmol^{-1} vs. elevated, 560 μmol^{-1}), N (ambient vs. 4 g N m^{-2} per year) and plant diversity (1, 4, 9 or 16 species) (Reich *et al.* 2001). In this study, soil samples from 24 plots (12 from ambient CO_2, 12 from elevated CO_2, and all with 16 species and ambient N supply) collected in July 2005 and 2007 were analysed.

Plant, soil and microbial biomass analyses

The aboveground and belowground biomass, plant C and N concentrations, soil pH, volumetric soil moisture, total soil C and N concentrations, and *in situ* net N mineralization and net nitrification were measured as previously described (Reich

et al. 2001, 2006). Microbial biomass (e.g. total, bacterial, fungal) was estimated by PLFA analysis (Chung *et al.* 2007).

DNA extraction, purification and quantification

Soil DNA was extracted by freeze-grinding mechanical lysis as described previously (Zhou *et al.* 1996). DNA quality was assessed by the ratios of 260 nm/280 nm and 260/230 nm, and final DNA concentrations were quantified with a PicoGreen method.

454 pyrosequencing and data analysis

Pyrosequencing of PCR amplicons targeting V4-V5 hypervariable regions of the 16S rRNA was performed with the 454 FLX Systems (454 Life Sciences, Branford, CT) with a sample tagging approach (Hamady *et al.* 2008). Details of amplicon preparations, sequencing and data analysis (e.g. classification, OTU identification) are described in the Data S1.

GeoChip analysis

Two versions of GeoChips were used for this study with GeoChip 2.0 for 22 (11 for each CO_2 condition) samples taken in 2005, and GeoChip 3.0 for 24 samples taken in 2007. GeoChip 2.0 contains >24 000 probes covering *c.* 10 000 gene sequences in 150 gene families (He *et al.* 2007), while the new version, GeoChip 3.0, contains >27 000 probes and covers *c.* 57 000 gene sequences in >292 gene families (He *et al.* 2010). Details for template amplification, labelling and hybridization, image processing and GeoChip data pre-processing are described in the Data S1.

Statistical analysis

Pre-processed data (e.g. GeoChip, 454 pyrosequencing, PLFA) were further analysed with different statistical methods: (1) microbial diversity index and response ratio (Luo *et al.* 2006), (2) DCA of microbial community structure and composition, (3) ANOSIM, adonis, and MRPP analysis of differences of microbial communities, (4) Mantel test and canonical correspondence analysis (CCA) for linking the functional structure of microbial communities to plant or soil variables (Zhou *et al.* 2008), and (5) partial Mantel test and partial CCA for co-variation analysis of soil and plant variables.

RESULTS

Effects of eCO_2 on plant and microbial biomass and soil C and N

Similar to previous observations in this experiment (Reich *et al.* 2001, 2004), during the 2005–2007 period (the 9–11th years of this FACE experiment), plant biomass (total, aboveground, belowground) increased significantly ($P < 0.05$) at eCO_2 (Fig. 1a). The total ingrowth root production, soil pH, soil moisture and total plant biomass N pool also significantly increased, whereas total plant biomass N concentration significantly decreased (Fig. S1). However, no significant changes were observed for net nitrification, net N mineralization, or the total soil C or N (Fig. S2). Both total microbial and bacterial biomass were significantly increased at eCO_2, whereas fungal biomass was unaffected (Fig. 1b), consistent with previous observations (Chung *et al.* 2007). The results suggest that soil bacterial communities may be stimulated in response to eCO_2.

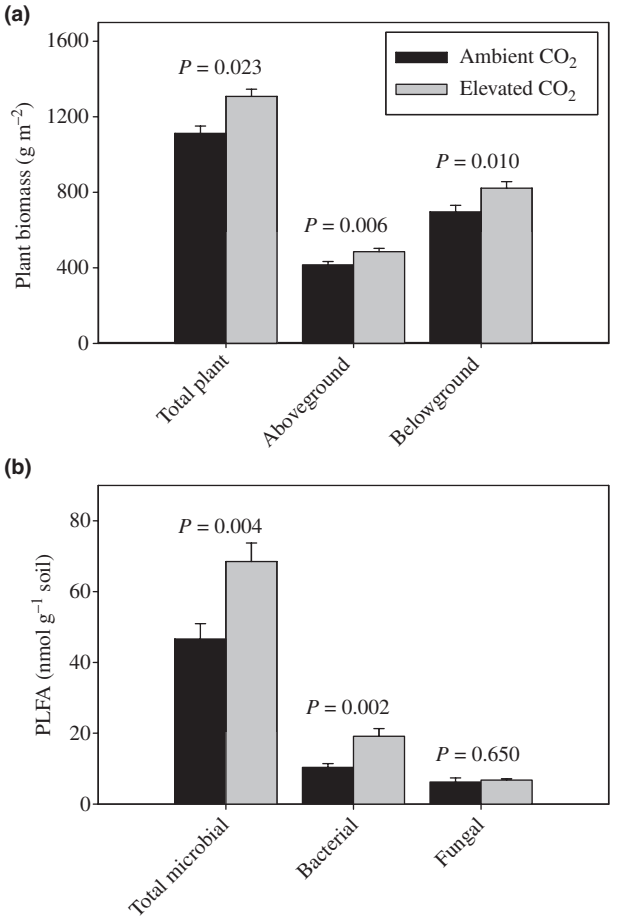

Figure 1 Effects of eCO_2 on plant (a) and microbial (b) biomass. Plant aboveground and root (0–20 cm) biomass was the average of six harvests in both June and August of 3 years (2005–2007). Total microbial, bacterial or fungal biomass was the sum of the signature phospholipid fatty acid (PLFA) from 5.0 g soil samples taken in July 2007. All data are presented with mean ± SE (error bars), and the significance of eCO_2 on plant and microbial biomass is shown by *P* values.

Overall responses of soil microbial community to eCO_2

To determine the overall response of soil microbial communities to eCO_2, the microbial communities at both aCO_2 and eCO_2 were analysed with (1) functional gene arrays (i.e. GeoChip) (He *et al.* 2007; He *et al.* 2010), which measure the functional structure and composition of microbial communities, (2) 16S rRNA gene-based pyrosequencing (Margulies *et al.* 2005; Hamady *et al.* 2008), which assesses the phylogenetic composition of microbial communities, and (3) PLFA, which provides information on the abundance and composition of microbial communities (Chung *et al.* 2007). Although no significant differences were detected in the overall microbial diversity, measured as the number of functional genes or OTUs, Shannon diversity, evenness and dominance (Table S1a), the structure of microbial communities was markedly different between aCO_2 and eCO_2 as indicated by DCA of GeoChip 3.0 data (Fig. 2a), and 454 pyrosequencing data (Fig. 2b) from 24 soil samples taken in July 2007. GeoChip 2.0 data for 22 samples taken in July 2005 also showed similar results (Fig. S3a), although those two sets of samples were collected in different years and examined with different versions of GeoChip. The gene copy number measured by quantitative real-time PCR was well correlated with the signal intensity detected by GeoChip 2.0 ($r = 0.530$, $P < 0.0001$, $n = 85$) or GeoChip 3.0 ($r = 0.724$, $P = 0.0001$, $n = 91$), indicating that GeoChip hybridization-based detection is quantitative. In addition, DCA of PLFA data showed that most of the samples under eCO_2 were separated from those under aCO_2, although no clear boundaries could be identified (Fig. S3b). Further analysis of 16S rRNA gene sequences showed the abundance of two phyla significantly changed at eCO_2 with one (Crenarchaeota) decreased and one (Verrucomicrobia) increased although the dominant phyla (e.g. Actinobacteria, Proteobacteria, Acidobacteria) did not show altered abundance (Fig. S4a); at the genus level, however, 31 genera from dominant phyla had altered abundances at eCO_2 with 18 increasing and 13 decreasing (Fig. S4b).

Three non-parametric multivariate statistical tests, ANOSIM, adonis and MRPP showed significant effects of eCO_2 based on the GeoChip 3.0, 454 pyrosequencing (at the species and genus levels), and PLFA analyses of 2007 soil samples, and based on GeoChip 2.0 analysis of 2005 samples (Table 1). Thus, all results indicated that the structure, composition and potential functional activity of microbial communities under eCO_2 were significantly different from those under aCO_2 at this FACE site. To our knowledge, this is the first comprehensive study at the whole community level to clearly demonstrate the changes in functional structure of microbial communities in response to eCO_2.

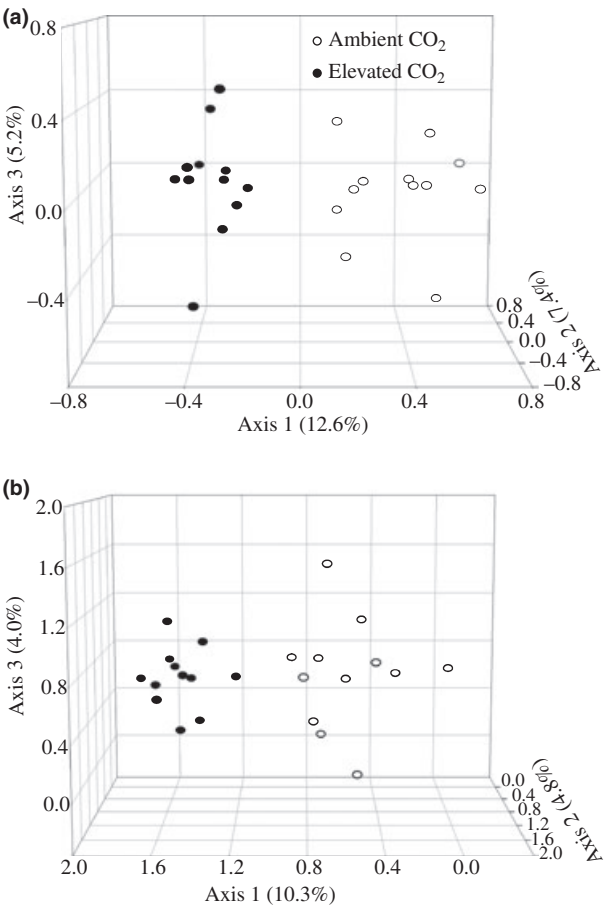

Figure 2 Detrended correspondence analysis (DCA) of GeoChip 3.0 data (a) and 454. pyrosequencing data (b) showing that eCO_2 significantly affected the soil microbial community composition and functional structure. The normalized signal intensity data for 5001 detected functional gene sequences or the relative abundance of all detected OTUs (3777) in at least three of 12 samples were used for DCA. Non-filled circles are for aCO_2 samples, and filled circles for eCO_2 samples. Details for GeoChip 3.0, 454 pyrosequencing and associated analyses were described in Data S1. For both datasets, the effects of eCO_2 on the soil microbial community composition and structure appeared to be well separated by the first axis.

Effects of eCO_2 on functional genes

To obtain more mechanistic insights into how eCO_2 affects functional processes of microbial communities, GeoChip 3.0 (He *et al.* 2010) data from 2007 samples were further examined by focusing on important biogeochemical processes, especially genes involved in C, N, P and S cycling. Among a total of 1503 detected functional genes involved in C, N, P and S cycling, a considerable portion (39%) of them were only detected under either aCO_2 (14%) or eCO_2

Table 1 Significance tests of the effects of CO_2 on the overall microbial community structure with three different statistical approaches

Statistical approaches		GeoChip		454 pyro-sequencing		
		2005	2007	Genus (0.95)	Species (0.97)	PLFA
N		1212	5001	15847	23184	34
ANOSIM*	*R*	0.514	0.141	0.081	0.148	0.209
	P	**<0.001**	**0.023**	0.072	**0.019**	**0.003**
Adonis†	*F*	7.132	1.753	1.312	1.537	6.712
	P	**<0.001**	**0.028**	**0.017**	**0.002**	**0.009**
Mrpp‡	δ	27.1	0.507	0.617	0.602	0.223
	P	**<0.001**	**0.030**	**0.022**	**0.003**	**0.009**

*Analysis of similarities ANOSIM.

†Non-parametric multivariate analysis of variance (MANOVA) with the adonis function.

‡A nonparametric procedure that does not depend on assumptions such as normally distributed data or homogeneous variances, but rather depends on the internal variability of the data.

All three tests are non-parametric multivariate analyses based on dissimilarities among samples. More detailed information is available in Data S1.

(25%) (Table S1b), indicating that the functional characteristics of the microbial community were significantly altered by eCO_2. Several biogeochemically important functional genes were substantially changed. For example, five pathways for autotrophic CO_2 fixation have been identified so far (Berg *et al.* 2007), and GeoChip 3.0 contains probes for the genes encoding CO_2 fixation enzymes from four pathways: ribulose-l, 5-bisphosphate carboxylase/oxygenase (Rubisco) for the Calvin cycle, carbon monoxide dehydrogenase (CODH) for the reductive acetyl-CoA pathway, propionyl-CoA/acetyl-CoA carboxylase (PCC/ACC) for the 3-hydroxypropionate/malyl-CoA cycle and ATP citrate lyase (AclB) for the reductive acetyl-CoA pathway. The PCC/ACC and Rubisco pathways were identified to be dominant in the BioCON grassland ecosystems, whereas the AclB pathway was not detected. A total of 79, 46 and 17 probes were detected for Pcc/Acc, Rubisco and CODH pathways, respectively, and they had significantly higher signal intensities under eCO_2 than aCO_2 (Fig. 3a). All four forms of Rubisco genes were detected, but most of them belonged to Form I, a major form for CO_2 fixation. Although the significant increase in the abundance of three C fixation genes under eCO_2 may potentially lead to more C fixation in soil, further studies are needed to determine the rates and extent of C fixation stimulated, and the impacts of the fixed C on the overall soil C dynamics in this ecosystem.

Elevated CO_2 either increased or had no effect on C degradation genes. Most C degradation genes whose abundance significantly ($P < 0.05$) increased under eCO_2 were those involved in the degradation of relatively labile C (e.g. starch, hemicelluloses, cellulose and simple aromatics), including those encoding amylase, glucoamylase, pullulanase, arabinofuranosidase and endoglucanase. The abundance of genes involved in the degradation of recalcitrant C (e.g. lignin) was largely unchanged by eCO_2, including those encoding lignin peroxidase, manganese peroxidase, glyoxyl oxidase, and phenol oxidase (Fig. 3b). The results suggest that eCO_2 might not significantly stimulate recalcitrant C degradation.

A substantial number (147) of genes involved in N_2 fixation (*nifH*) were detected, and the abundance of the detected *nifH* genes was significantly higher ($P < 0.05$) under eCO_2 than aCO_2 (Fig. 4). Among four defined clusters of *nifH* genes, only Cluster I showed a significant ($P < 0.05$) difference between aCO_2 and eCO_2, and most of the detected Cluster I genes were closely related to known organisms, such as *Rhizobium*, *Azospirillum* and *Bradyrhizobium* species. Cluster I contains *nifH* sequences from both free-living and symbiotic N_2-fixing microorganisms. In addition, most abundant *nifH* genes detected were from uncultured microorganisms (Fig. 4 and Fig. S5). The results indicate that eCO_2 may stimulate microbial N_2-fixation, but our understanding of N_2-fixing microorganisms and microbial N_2 fixation mechanisms may be very limited. No significant differences in the total signal intensity were observed for other N cycling genes except for *nirS* (Fig. 4 and Fig. S5).

GeoChip 3.0 targets three enzymes involved in P utilization, exopolyphosphatase (Ppx) for inorganic polyphosphate degradation, polyphosphate kinase (Ppk) for polyphosphate biosynthesis in prokaryotes, and phytase for phytate degradation. While no significant differences of signal intensity were observed for Ppk and phytase genes, the total signal intensity of Ppx genes was significantly increased at eCO_2 at $P < 0.1$ (Fig. S6a), suggesting a possible increase in the degradation of polyphosphates and the availability of inorganic P under eCO_2.

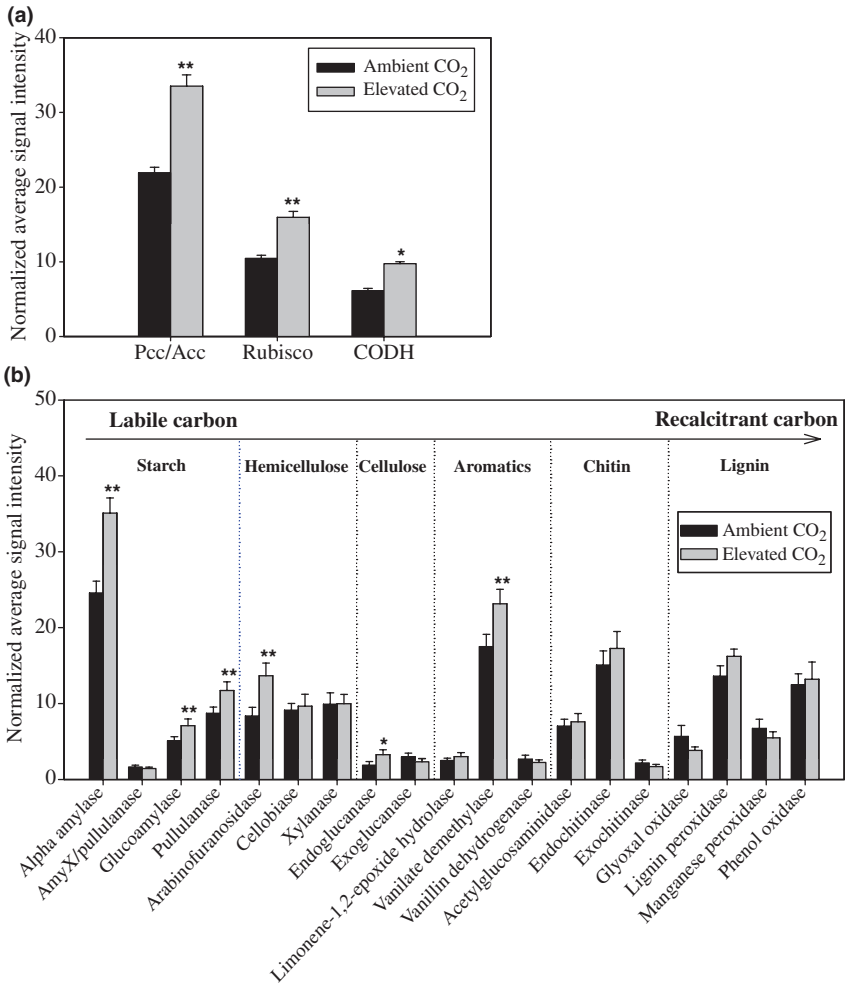

Figure 3 The normalized signal intensity of the detected key gene families involved in CO_2 fixation (a) and carbon degradation (b) under both aCO_2 and eCO_2. The signal intensities were the sum of detected individual gene sequences for each functional gene, averaged among 12 soil samples taken in July 2007. Rubisco: ribulose-l,5-bisphosphate carboxylase/oxygenase; CODH: carbon monoxide dehydrogenase; Pcc/Acc: propionyl-CoA/acetyl-CoA carboxylase. The complexity of carbon is presented in order from labile to recalcitrant. All data are presented as mean ± SE. $^{**}P < 0.05$, $^{*}P < 0.10$.

GeoChip 3.0 also has three enzymes involved in methane cycling, the alpha-subunit of methyl coenzyme M reductase (*mcrA*) for methane production, and particulate methane monooxygenase (*pmoA*) and methane monooxygenase (*mmoX*) for methane consumption. No significant differences in signal intensities were observed for *mcrA* or *pmoA*, but no probes for *mmoX* were detected at aCO_2 and very weak signals at eCO_2 (Fig. S6b), suggesting that eCO_2 may have little impact on methane cycling processes at this site.

Linking microbial community structure to soil properties and plant variables

Mantel tests and CCA were performed to assess the relationships between microbial community structure and soil properties and plant variables. As using many unrelated individual variables could mask the signature of any significant variables in Mantel tests and redundant variables would generate inefficient CCA models, BIO-ENV and CCA-based variance inflation factor (VIF) were performed to identify common sets of soil and plant variables important to the microbial community structure (Table 2 and Table S2). Both simple and partial Mantel tests revealed that the selected soil variables were positively correlated with the microbial community structure based on all detected genes, or subsets of the genes involved in C fixation, labile C degradation, or N_2 fixation ($P < 0.05$ or 0.01) (Table 2). Significant correlations at $P = 0.1$ were also observed between these soil variables and the genes involved in recalcitrant C degradation, dissimilatory nitrate reduction to ammonium, phosphorus release and denitrification (Table 2). The correlations between the selected soil variables and N_2 fixing microorganisms were also supported by CCA (Table S2). In addition, significant correlations at $P = 0.1$ were observed between the selected plant biomass variables and subsets of functional genes, especially the genes involved in the N cycling (Table 2). These results suggested that the microbial community functional struc-

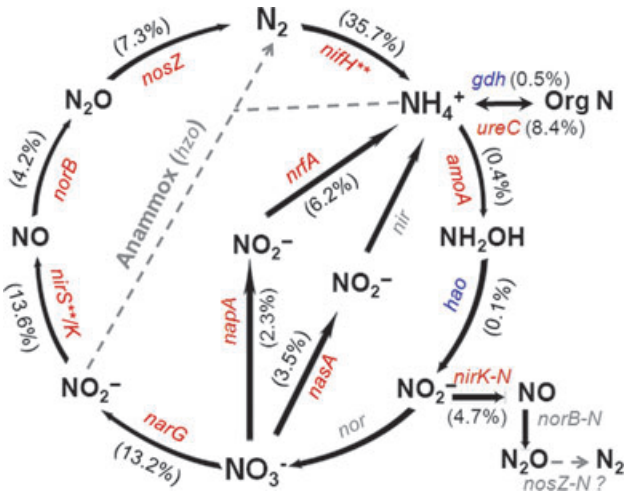

Figure 4 The relative changes of the detected genes involved in N cycling at eCO_2. The signal intensity for each gene detected was normalized by all detected gene sequences using the mean. The percentage of a functional gene in a bracket was the sum of the signal intensity of all detected sequences of this gene divided by the grand sum of the signal intensity of the detected N cycling genes, and weighted by the fold change of the signal intensity of this gene at eCO_2 to that at aCO_2. For each functional gene, colours mean that this gene had a higher (red) or lower (blue) signal intensity at eCO_2 than at aCO_2 with significance at $P < 0.05$ (**). Gray-coloured genes were not targeted by this GeoChip, or not detected in those samples. It remains unknown if *nosZ* homologues exist in nitrifiers. Details for each gene and its involved functional process are shown in Fig. S6.

ture could have significant relationships with soil C and N dynamics and plant productivity.

DISCUSSION

Although eCO_2 significantly affects plant growth and primary productivity via an increase in photosynthesis, little is known about the influence of eCO_2 on the structure and potential activity of the belowground microbial community. As microorganisms mediate important biogeochemical processes, such as C, N, P and S cycling, they are expected to play important roles in influencing future atmospheric CO_2, so understanding their response to elevated atmospheric CO_2 is critical. In this study, using functional gene-based GeoChip, 16S rRNA gene-based pyrosequenicng, and PLFA analysis we showed that 10 years of field exposure of a grassland ecosystem to eCO_2 dramatically altered the structure and potential function of soil microbial communities. At eCO_2, an increase in plant biomass (Reich *et al.* 2006) and belowground carbon inputs (Adair *et al.* 2009), and associated microenvironmental changes, such as greater soil moisture (Reich 2009), stimulated microbial, especially bacterial growth, which led to significant changes in the structure and activity of soil microbial communities. Statistical analyses showed that the changes in the microbial community structure were significantly correlated with soil C and N contents and weakly with plant productivity. These results will be important for understanding the responses of microbial communities to elevated CO_2 and their potential consequences.

Chronic eCO_2-induced increases in plant biomass, root exudation and soil moisture may stimulate soil microbial biomass. Previous studies at the BioCON site showed that both aboveground and belowground plant biomass significantly increased at eCO_2 (Reich *et al.* 2001; Reich 2009), and were associated with increased carbon inputs into soil (Adair *et al.* 2009). Under long-term eCO_2 soil moisture is also consistently increased (Reich 2009). Collectively, these shifts in resources and microenvironment enhance the microbial biomass and activity, as shown both in the current paper and previously (Dijkstra *et al.* 2005; Chung *et al.* 2007). Another study conducted in a FACE experiment at Rhinelander, WI, USA suggested no significant changes in the relative abundance or composition of fungi measured by PLFA and DGGE, respectively (Chung *et al.* 2006). Consistent with those previous studies, this study showed that bacterial biomass significantly increased at eCO_2, but fungal biomass was unchanged. However, fungal biomass significantly increased in a chaparral ecosystem at eCO_2 (Lipson *et al.* 2005), and the relative abundance of fungi was higher in a scrub-oak ecosystem at eCO_2 than at aCO_2 (Carney *et al.* 2007). This disparity among studies may be caused by the different ecosystems studied, although no patterns emerge that would allow us to speculate on what might cause such differences.

An issue relevant to our study and many other FACE studies is whether the initial abrupt increase in atmospheric CO_2 influences microbial communities. Klironomos *et al.* (2005) compared the effects of both abrupt and gradual increase in atmospheric CO_2 on a mycorrhizal fungal community, and showed that an abrupt CO_2 rise resulted in an immediate decline in fungal species richness and a significant increase in mycorrhizal functional activity with stronger effects than a gradual CO_2 rise (Klironomos *et al.* 2005). Whether such effects translate to our study or other field studies is unknown.

Although it is impossible to fully answer, it will be important to understand whether it is the stimulation of microbial biomass at eCO_2 that leads to significant alterations in the structure of microbial communities. With increased organic matter inputs into soil, soil microbes are expected to respond rapidly to the greater availability of substrates. Such increased organic matter inputs may activate the growth of previously dormant microorganisms that are able to degrade such substrates, or increase the

Table 2 The relationships of microbial community functional structure to soil C and N dynamics and aboveground plant characteristics revealed by partial Mantel test

In association with: Controlling for: Functional category	Gene no.	Soil*		Plant†	
		Plant†		Soil*	
		r_M	P	r_M	P
All detected	5001	0.312	**0.027**	0.146	0.109
C cycle	576	0.351	**0.014**	0.134	0.144
C fixation	147	0.480	**<0.001**	0.184	0.072
Labile C degradation	259	0.296	**0.005**	0.126	0.150
Recalcitrant C degradation	127	0.193	0.068	0.020	0.443
N cycle	548	0.239	0.063	0.149	0.097
N_2 fixation	147	0.320	**0.005**	0.166	0.070
Nitrification	7	0.036	0.343	0.104	0.125
Denitrification	277	0.173	0.119	0.148	0.139
N reduction to NH_4^+	55	0.202	0.064	0.068	0.256
N mineralization	62	0.095	0.176	0.069	0.234
Phosphorous utilization	74	0.197	0.069	0.008	0.448

*Selected soil variables: percentages of soil C and N at the depth of 0–10 and/or 10–20 cm and soil pH.
†Selected plant variables: biomass of *Andropogon gerardi* (C4), *Bouteloua gracilis* (C4) and *Lupinus perennis* (Legume), belowground plant C and N (%), and species count.
Soil and plant variables were selected by the BIO-ENV procedure.

growth rate of active microorganisms that were previously at a low abundance like r-strategists, which grow quickly and specifically degrade freshly input organic matter, and disappear after it is consumed (Fontaine *et al.* 2004). However, K-strategists, which are soil organic matter decomposers, may compete with r-strategists for the freshly input organic matter by increasing their growth and decomposition rates (Fontaine *et al.* 2004). The dynamics and competition of r- and K-strategists are expected to affect the structure and function of soil microbial communities. Indeed, the soil microbial community structure significantly changed at eCO_2. This was demonstrated by DCA of the abundance of functional genes, 16S rRNA genes and microbial abundance and composition measured by GeoChip, 454 pyrosequencing and PLFA, respectively. In addition, changes in the structure and potential activity of soil microbial communities at eCO_2 were reflected in a significant increase in the abundance of many functional genes, such as those involved in labile C degradation, *rbcL* for CO_2 fixation and *nifH* for N_2 fixation. Those changes may have occurred because of changes in the dynamics of active microbial populations stimulated by increased organic matter input at eCO_2. For example, the soil microbial community may change from oligotrophic regimes to copiotrophic regimes at eCO_2. All results suggest that eCO_2 drives a marked divergence in the structure and functional activity of soil microbial communities, and which microbial populations are stimulated by eCO_2 may be important to further understand if such changes lead to soil C sequestration or C loss.

Another question is whether the change in the microbial compositional structure affects community-level functional processes, especially soil C and N dynamics. Previous studies have shown inconsistent responses of soil C to eCO_2. For example, one meta-analysis showed a 5.6% increase in soil C over 2–9 years of exposure to eCO_2 (Jastrow *et al.* 2005), which may lead to a negative feedback to increased global C emissions (Houghton *et al.* 1999). In contrast, other studies showed a significant C loss at eCO_2, suggesting a positive feedback to globally increased CO_2 (Fontaine *et al.* 2004; Carney *et al.* 2007; Heimann & Reichstein 2008). A recent meta-analysis showed that elevated CO_2 generally increased net soil C accumulation when N fertilizer was added, but not under low N conditions (Hungate *et al.* 2009), consistent with findings in BioCON (Adair *et al.* 2009). Our GeoChip data showed a significant increase in the abundance of genes involved in degradation of labile substrates with rapid turnover times. Thus, eCO_2 may lead to an increase in soil microbial respiration such that elevated inputs of C are readily consumed by stimulated microbial populations (e.g. r-strategists) (Adair *et al.* 2009), resulting in little significant impact on soil C stocks. More importantly, the abundance of genes involved in recalcitrant C degradation did not significantly change at eCO_2, indicating that the soil C storage may remain unaffected in the long term. Thus, our

results are consistent with recent conclusions that eCO_2 has little effect on soil C storage (Hungate *et al.* 2009).

Whether the change observed in the microbial community structure has significant effects on N dynamics is also a central issue for the long-term sustainability of eCO_2 stimulation due to both the progressive nitrogen limitation effect and to co-limitation by CO_2 and N that can constrain the eCO_2 response when N availability is low (Reich *et al.* 2006). A previous BioCON study showed that N_2-fixing legume species responded to a greater extent than non-fixing forbs to eCO_2, and that eCO_2 stimulated symbiotic N_2 fixation, resulting in an increased amount of N derived from the atmosphere (Lee *et al.* 2003), and other studies also observed that microbial N_2 fixation increased under eCO_2 (Luo *et al.* 2006; van Groenigen *et al.* 2006). Consistently, a significant increase in the abundance of *nifH* genes for N_2 fixation, and *nirS* genes for denitrification was observed at eCO_2 in this study although net nitrification, net N mineralization, and the total soil N content were not significantly changed. Therefore, eCO_2 may affect the overall soil N budget via increased N fixation or denitrification in this grassland ecosystem, although the linkage between increased gene abundances and system-level process rates requires further study.

There are several additional related reasons why we might not see a difference in total soil C and N, net nitrification, or net N mineralization at eCO_2 despite significant changes in potential microbial function. The pool size of total soil C and N may be too large and heterogeneous relative to the eCO_2 effect to be detectable at present. Although a significant increase in C and N metabolic processes may occur, the net accumulation or consumption of soil C or N may be still very small relative to the total soil C and N pool, making it difficult to detect a difference until more time has passed (Smith 2004). With respect to net N mineralization, it is possible that impacts on mineralization of changes in stoichiometry (C:N ratios of roots and soil solution) may be offset by increased soil moisture in eCO_2, which was observed for the same plots in this study and a previous study (Reich 2009).

As suggested above, the effects of microbial structure and potential activity on ecosystem functions are in part controlled by environmental factors, such as soil moisture and pH. Indeed, multivariate statistical analyses showed that many functional genes were significantly correlated with soil variables. For example, the abundance of all detected genes, and genes involved in C fixation, labile C degradation, or/and N_2 fixation was significantly ($P < 0.05$), positively correlated with soil variables (e.g. moisture, pH), which indicates that environmental variables other than the amount and stoichiometry of plant inputs, could be important in shaping the microbial community. In addition, significant correlations among different functional genes were observed. The results indicate that soil characteristics, such as moisture and pH (which themselves are influenced by eCO_2) may mediate the effects of eCO_2 on the structure and function of microbial communities.

This study combines metagenomic technologies (e.g. GeoChip, Pyrosequencing) with traditional methods (e.g. PLFA, EcoPlate) to provide an integrative study of soil microbial communities exposed to eCO_2. GeoChip-based data especially provide large scale quantitative information on various biogeochemically important microbial functional groups, thus making it possible to link the functional structure of microbial communities with ecosystem processes. In addition, 16S rRNA gene-based pyrosequencing data provide phylogenetic information about the phylogenetic structure and composition of microbial communities. Such datasets provide an integrative approach for reliable detection of microbial structure, composition and functional activity and linking those microbial properties to ecosystem functioning, such as soil C and N dynamics.

The issues addressed in our study are important to the collective understanding of the feedback responses of terrestrial ecosystems to eCO_2 and to modelling-based projections of future atmospheric CO_2 concentrations. It is obvious that the impacts of eCO_2 on soil C and N dynamics and the feedbacks of ecosystems to eCO_2 will depend on which groups of microorganisms, and what activities and interactions, are stimulated by the increased C influx to soil. We found that eCO_2 significantly altered microbial community structure and composition and elicited the up-regulation of functional genes involved in labile C decomposition, C and N fixation and phosphorus utilization, whereas those involved in decomposing recalcitrant C were unchanged. Such shifts in microbial community structure and function could potentially modify the direction and magnitude of ecosystem regulation of the rate of increase in atmospheric CO_2 concentrations. In addition, current ecosystem modelling efforts largely treat the soil microbial component of the terrestrial biosphere as a single pool (Allison & Martiny 2008) – that is, they ignore the details of microbial communities and typically assume that changes in biogeochemical responses can be predicted from simple assumptions about system behaviour regardless of changes in the identity and abundance of the microbial community. This 'black box' assumption may be valid only if microbial composition is resistant, resilient and/or functionally redundant to disturbance (Allison & Martiny 2008). Our study revealed major shifts in the overall structure of soil microbial communities under eCO_2, indicating that microbial community structure is not resistant to disturbance in general (Allison & Martiny 2008). Although the current state of global C, N and climate model science and of soil microbiology science are not

sufficiently advanced to incorporate soil communities as anything but a 'black box' in elemental and climate modelling, more realistically linked global C, N and climate models must be developed to holistically incorporate microbial community structure and composition, at least at the levels of microbial groups with distinct functions, for more accurate and reliable predictions.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1 Effects of elevated CO_2 on plant biomass, total plant N pool, total plant biomass N concentration, and soil pH and moisture.

Figure S2 Effects of elevated CO_2 on soil C and N dynamics.

Figure S3 DCA analyses of GeoChip 2.0 and PLFA data.

Figure S4 The abundance of 16S rRNA gene sequences at the phylum level at aCO_2 and eCO_2 and the significantly changed genera under eCO_2.

Figure S5 Normalized average signal intensity of the significantly changed *nifH* genes and other top 10 abundant *nifH* sequences detected by GeoChip 3.0.

Figure S6 Normalized average signal intensity of detected key gene families involved in the N cycling under both CO_2 conditions.

Figure S7 The normalized average signal intensity of the detected key functional genes involved in P cycling (A), and methane production and oxidation (B) under ambient CO_2 and elevated CO_2.

Table S1 Overall microbial community diversity and the number of detected genes involved in carbon, nitrogen, phosphorus, and sulfur cycling under ambient CO_2 and elevated CO_2.

Table S2 Simple Mantel tests and partial CCA analyses of correlations between key functional gene categories and environmental and plant variables.

Data S1 Materials and methods.

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