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ORIGINAL ARTICLE

The phylogenetic composition and structure of soil microbial communities shifts in response to elevated carbon dioxide

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One of the major factors associated with global change is the ever-increasing concentration of atmospheric CO_2. Although the stimulating effects of elevated CO_2 (eCO_2) on plant growth and primary productivity have been established, its impacts on the diversity and function of soil microbial communities are poorly understood. In this study, phylogenetic microarrays (PhyloChip) were used to comprehensively survey the richness, composition and structure of soil microbial communities in a grassland experiment subjected to two CO_2 conditions (ambient, 368 p.p.m., versus elevated, 560 p.p.m.) for 10 years. The richness based on the detected number of operational taxonomic units (OTUs) significantly decreased under eCO_2. PhyloChip detected 2269 OTUs derived from 45 phyla (including two from Archaea), 55 classes, 99 orders, 164 families and 190 subfamilies. Also, the signal intensity of five phyla (Crenarchaeota, Chloroflexi, OP10, OP9/JS1, Verrucomicrobia) significantly decreased at eCO_2, and such significant effects of eCO_2 on microbial composition were also observed at the class or lower taxonomic levels for most abundant phyla, such as Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes and Acidobacteria, suggesting a shift in microbial community composition at eCO_2. Additionally, statistical analyses showed that the overall taxonomic structure of soil microbial communities was altered at eCO_2. Mantel tests indicated that such changes in species richness, composition and structure of soil microbial communities were closely correlated with soil and plant properties. This study provides insights into our understanding of shifts in the richness, composition and structure of soil microbial communities under eCO_2 and environmental factors shaping the microbial community structure.

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Introduction

The concentration of atmospheric CO_2 has risen by approximately 36% since the mid-19th century, largely because of human activities, such as fossil fuel combustion and land use. With the current rate of increase of 1.9 p.p.m./year, it is projected to reach 700 p.p.m. by the end of this century, which may have major consequences on carbon cycling and the functioning of terrestrial ecosystems (IPCC, 2007). The stimulating effects of elevated CO_2 (eCO_2) on plant growth and primary productivity are well-established (Reich *et al.*, 2001; Ainsworth and Long, 2005; Luo *et al.*, 2006). For example, eCO_2 has been found to increase plant growth (Curtis and Wang, 1998), enhance fine root production (Hungate *et al.*, 1997) and augment soil carbon allocation (Zak *et al.*, 1993; Hu *et al.*, 2001). However, the influence of eCO_2 on soil microbial communities remains poorly understood and controversial (Walther *et al.*, 2002; Parmesan and Yohe, 2003; Heath *et al.*, 2005; Carney *et al.*, 2007; Drigo *et al.*, 2007, 2009, 2010; Gruber and Galloway, 2008; Heimann and Reichstein, 2008; Lesaulnier *et al.*, 2008; Austin *et al.*, 2009; Ge *et al.*, 2010; He *et al.*, 2010b). Also, the plant growth stimulation observed under eCO_2 may be transient (Drake *et al.*, 1997; DeLucia *et al.*, 1999) possibly because of the depletion of available nitrogen (N)

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(Luo *et al.*, 2004; Reich *et al.*, 2006). In addition, multiple global change factors, such as eCO_2, elevated O_3, warming and/or precipitation, may interact to alter soil microbial community diversity, composition, structure and function (Chung *et al.*, 2006; Castro *et al.*, 2010). Therefore, understanding the diversity, composition and structure of soil microbial communities is necessary for us to assess how eCO_2 modifies ecosystem properties and functional processes.

Soil may be the most complex of all microbial communities with extremely high diversity. For example, 1 g of soil contains thousands to millions of different bacterial, archaeal and eukaryotic species (Torsvik *et al.*, 2002; Gans *et al.*, 2005) interwoven in extremely complex food webs. Furthermore, most (>99%) of those microbes are as-yet uncultured (Whitman *et al.*, 1998). Thus, characterizing the phylogenetic diversity of soil microbial communities and their responses to global change (for example, eCO_2) will make a significant contribution to understanding soil ecosystems.

Conventional molecular biology approaches have demonstrated that soil microbial diversity generally increased (Mitchell *et al.*, 2003; Janus *et al.*, 2005; Sonnemann and Wolters, 2005; Jossi *et al.*, 2006; Lesaulnier *et al.*, 2008), decreased (Horz *et al.*, 2004) or remained unchanged (Barnard *et al.*, 2004; Ebersberger *et al.*, 2004; Loy *et al.*, 2004; Chung *et al.*, 2006; Gruter *et al.*, 2006; Lipson *et al.*, 2006; Drigo *et al.*, 2007, 2009; Austin *et al.*, 2009; Ge *et al.*, 2010) in response to eCO_2. The apparent discrepancy of microbial responses to eCO_2 could be partially due to real differences among various ecosystems, but could also be due to differences in the methodologies used, such as terminal restriction-fragment length polymorphism, denaturing gradient gel electrophoresis, 16S rRNA-based sequencing, enzyme activities and phospholipid fatty acids. For example, it is possible that some methods may not be sensitive enough to resolve the differences caused by eCO_2 at the community level.

Recently, 16S rRNA gene-based microarray technologies have been used to obtain more comprehensive information on microbial community diversity, composition, structure and dynamics. PhyloChip (G2) consists of 506 944 probe features, and of these features, 297 851 are oligonucleotide perfect match (PM) or mismatch match (MM) probes for 16S rRNA genes (Brodie *et al.*, 2006, 2007). PhyloChip has been used to detect microorganisms in a variety of environments, such as contaminated sites (Brodie *et al.*, 2006; Rastogi *et al.*, 2010), air (Brodie *et al.*, 2007), water (Hery *et al.*, 2010), soil (Cruz-Martinez *et al.*, 2009; DeAngelis *et al.*, 2009; Yergeau *et al.*, 2009; Teixeira *et al.*, 2010), microbial fuel cell (Wrighton *et al.*, 2008) and Huanglongbing pathogen-infected citrus (Sagaram *et al.*, 2009). In addition, several studies demonstrated that PhyloChip could detect many more bacterial taxa as compared with the 16S rRNA gene-based clone library approach (DeSantis *et al.*, 2007; La Duc *et al.*, 2009; Rastogi *et al.*, 2010), suggesting that PhyloChip provides more comprehensive surveys of microbial diversity, composition and structure.

The objectives of this study were to: (i) survey the richness and composition of soil microbial communities; (ii) examine the effects of eCO_2 on the richness, composition and structure of soil microbial communities and (iii) link soil geochemistry and plant properties with the microbial community composition and structure using PhyloChip (Brodie *et al.*, 2006, 2007). For these purposes, this study was conducted in a constructed grassland ecosystem subjected to CO_2 manipulation for 10 years by using the free-air CO_2 enrichment (FACE) technology. The results showed that eCO_2 significantly altered the richness, composition and structure of soil microbial communities, especially for particular microbial populations, at the operational taxonomic unit (OTU) level. Such microbial population changes were closely correlated with soil and plant properties.

Materials and methods

The following is a summary of the methods used in this study. More detailed information is provided in Supplementary Data-A.

Site and sampling

This study was conducted within the BioCON (Biodiversity, CO_2 and Nitrogen) experiment site (http://www.biocon.umn.edu/) located at the Cedar Creek Ecosystem Science Reserve in Minnesota, USA. The main BioCON field experiment has a total of 296 plots with three treatments: CO_2 (ambient (aCO_2), 368 p.p.m. versus elevated, 560 p.p.m.), N (ambient versus 4 g N per 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 replicates from aCO_2, 12 replicates from eCO_2 and all with 16 species and ambient N supply) were collected in July 2007 when they had been exposed to aCO_2 or eCO_2 for 10 years, and each sample was composited from five soil cores at a depth of 0–15 cm for analysis of soil properties or DNA extraction. Additional information about the BioCON experimental site, and plant groups and species, is provided in Supplementary Data-A.

Plant and soil 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 described previously (Reich *et al.*, 2001, 2006; He *et al.*, 2010b).

DNA extraction, purification and quantitation

Soil DNA was extracted by freeze-grinding mechanical lysis as described previously (Zhou *et al.*, 1996).

DNA quality was assessed by the ratios of 260/280 and 260/230 nm and final DNA concentrations were quantified by the PicoGreen method (Ahn *et al.*, 1996).

PhyloChip analysis

The second generation of the PhyloChip (PhyloTech, San Francisco, CA, USA), which has 8741 OTUs and 842 subfamilies with 297 851 probes, was used for this study (Brodie *et al.*, 2006). PhyloChip analysis included three major steps: (i) Amplification of 24 soil genomic DNAs using universal 16S rRNA primers (27F/1492R for bacteria and 4Fa/1492R for archaea); (ii) 500 ng of bacterial and 30 ng of archaeal PCR products were hybridized to each PhyloChip (Brodie *et al.*, 2006, 2007) and (iii) hybridization data were preprocessed prior to statistical analysis as detailed in Supplementary Data-A. For eight of 12 eCO_2 samples with less than 30 ng of archaeal PCR products, 10 μL of concentrated archaeal amplicons were used. A mixture of amplicons at known concentrations was added to each sample prior to fragmentation, which allows for standardization/normalization of PhyloChip data. Data obtained from the CEL files (produced from GeneChip Microarray Analysis Suite, version 5.1) were scaled by setting the mixture of internal standards (spike mix) mean intensity to 2500 to compensate for slight differences in probe responses on different chips. OTU reports were generated as described in Supplementary Data-A. Because setting a positive fraction (pf) cut-off can vary the number of passing OTUs (and hence affect the reported number of OTUs for each sample), several pf cut-off values (0.86, 0.88, 0.90, 0.92, 0.94) were evaluated by using the PhyloChip data analysis pipeline PhyloTrac (http://www.phylotrac.org/Home.html) and statistical methods as described under Materials and methods and Supplementary Data-A. A pf cut-off of 0.9 was determined to be a reasonable choice and used to generate the final OTU report used in this study.

Statistical analysis

Pre-processed PhyloChip data were further analyzed by different statistical methods: (i) Response ratio (Luo *et al.*, 2006); (ii) detrended correspondence analysis of the microbial community structure; (iii) analysis of similarities (Clarke, 1993), non-parametric multivariate analysis of variance (ADONIS) (Anderson, 2001) and multi-response permutation procedure (Mielke and Berry, 2001; McCune and Grace, 2002) were used to analyze differences of microbial communities by using the Binomial index (Anderson and Millar, 2004); (iv) Mantel test and canonical correspondence analysis for linking the functional structure of microbial communities to plant or soil variables; and (v) partial Mantel test and partial canonical correspondence analysis for co-variation analysis of soil and plant variables (Zhou *et al.*, 2008; He *et al.*, 2010b).

Results

Effects of eCO_2 on plant and soil properties

The plant productivity measured by biomass of aboveground, roots and fine roots was significantly ($P<0.05$) stimulated by eCO_2 (Supplementary Table S1), which is consistent with previous studies in this site (Reich *et al.*, 2001; Adair *et al.*, 2009). Also, the whole-plot total N ($g\,m^{-2}$) and legume biomass significantly ($P<0.05$) increased at eCO_2, but the percentages of nitrogen (N) in the whole-plot plant biomass, aboveground biomass and belowground biomass significantly ($P<0.05$) decreased (Supplementary Table S1). In addition, the aboveground carbon/nitrogen (C/N) ratio significantly ($P<0.05$) increased (Supplementary Table S1), probably because of an increase in plant biomass and a decrease in the aboveground N concentration. Similarly, eCO_2 significantly ($P<0.05$) increased soil pH and soil moisture (at depths of 0–17, 42–59 and 83–100 cm). However, no significant ($P>0.05$) changes in soil carbon, nitrogen, C/N ratio, or rates of ammonification, nitrification or net N mineralization, were observed (Supplementary Table S2). The significant differences in plant characteristics and soil properties suggest that the diversity, composition and structure of soil bacterial communities may be shifted in response to eCO_2.

Richness of soil microbial communities in response to eCO_2

The richness of soil microbial communities was examined by PhyloChip. A total of 2269 OTUs were detected at least in three samples, accounting for 26% OTUs on the PhyloChip. An average of 1916 OTUs were detected at aCO_2, which was significantly ($P=0.0281$) higher than an average of 1864 OTUs detected at eCO_2 (Table 1). All detected OTUs were taxonomically derived from two archaeal phyla and 43 bacterial phyla, 55 classes, 99 orders, 164 families and 190 subfamilies; most phylotypes were detected at both aCO_2 and eCO_2, with few detected only at aCO_2 or eCO_2 (Table 2). At the phylum level, among a total of 2269 OTUs detected, 1002 OTUs were derived from Proteobacteria, a phylum with the highest number of detectable OTUs, followed by Firmicutes with 384, Actinobacteria with 289, Bacteroidetes with 162 and Acidobacteria with 76 OTUs (Table 1). Also, based on the number of OTUs detected in each phylum, two phyla had significantly ($P<0.05$) lower numbers of OTUs detected at eCO_2 than at aCO_2, including Chloroflexi ($P=0.003$) and OP10 ($P=0.007$) (Table 1). The results indicate that the richness of soil microbial communities was decreased at eCO_2.

Overall taxonomic composition and structure of soil microbial communities in response to eCO_2

To examine if eCO_2 affects the taxonomic composition and structure of soil microbial communities, detrended correspondence analysis was performed

Table 1 Numbers of OTUs detected by PhyloChip in major phyla under aCO_2 and eCO_2 conditions

Phylum	*OTUs on PhyloChip*	*No. of OTUs detected by PhyloChip*			
		Total (%)	*aCO_2*	*eCO_2*	P *(t-test)*
Crenarchaeota	79	12 (15.1)	12.00 ± 0.00	12.00 ± 0.00	1.000
Euryarchaeota	224	2 (0.9)	1.00 ± 0.00	1.00 ± 0.00	1.000
Acidobacteria	98	76 (77.6)	67.67 ± 6.56	64.25 ± 5.96	0.195
Actinobacteria	810	289 (35.6)	233.17 ± 20.32	225.92 ± 17.24	0.356
Bacteroidetes	880	162 (18.4)	123.42 ± 14.39	119.67 ± 20.00	0.603
Chlorobi	21	11 (52.4)	9.42 ± 1.78	8.83 ± 1.59	0.406
Chloroflexi	117	44 (37.6)	37.58 ± 4.58	31.33 ± 4.75	**0.003**
Cyanobacteria	202	51 (25.2)	45.42 ± 3.37	43.00 ± 3.19	0.085
Firmicutes	2012	384 (19.1)	312.25 ± 30.54	300.75 ± 25.39	0.327
Gemmatimonadetes	15	9 (60.0)	8.67 ± 0.49	8.58 ± 0.51	0.689
Natronoanaerobium	7	5 (71.4)	4.00 ± 0.74	3.42 ± 0.79	0.076
Nitrospira	29	8 (27.6)	7.3 ± 0.98	6.5 ± 1.73	0.161
OP10	12	7 (58.3)	5.67 ± 1.07	4.58 ± 0.67	**0.007**
OP9/JS1	12	5 (41.6)	4.25 ± 1.14	3.5 ± 1.17	0.125
Planctomycetes	182	26 (14.3)	20.00 ± 4.09	17.25 ± 2.22	0.053
Proteobacteria	3170	1002 (31.6)	849.75 ± 70.62	837.58 ± 85.72	0.708
Spirochaetes	150	36 (24.0)	33.75 ± 2.93	32.67 ± 2.99	0.380
Synergistes	19	5 (26.3)	5.00 ± 0.00	5.00 ± 0.00	1.000
TM7	45	9 (20.0)	8.83 ± 0.58	8.25 ± 1.29	0.166
Verrucomicrobia	78	36 (46.1)	28.33 ± 3.92	25.75 ± 3.05	0.085
Others (<5 OTUs)	250	53 (21.2)	46.35 ± 9.37	37.67 ± 6.35	0.173
Unclassified	329	37 (11.2)	32.92 ± 1.83	31.25 ± 3.14	0.126
Total	8741	2269 (26.0)	1916.6 ± 52.03	1864.1 ± 57.25	**0.028**

Abbreviations: aCO_2, ambient CO_2; eCO_2, elevated CO_2; OTU, operational taxonomic unit.
Boldface indicates significantly changed phylotypes or all detected OTUs.

Table 2 Phylotypes detected by PhyloChip at different taxonomic levels

	Domain	*Phylum*	*Class*	*Order*	*Family*	*Subfamily*
Total no. detected phylotypes	2	45	55	99	164	190
Shared at aCO_2 and eCO_2	2	44	52	97	163	188
Only detected at aCO_2	0	1	2	1	1	2
Only detected at eCO_2	0	0	1	1	1	0

for PhyloChip signal intensity data. Overall, the majority of the samples from eCO_2 and aCO_2 were distributed in different parts of the data space, although there was some overlap. Eight of 12 aCO_2 samples were separated well from the eCO_2 samples, but four other aCO_2 samples seemed to be clustered closer to the eCO_2 than the aCO_2 samples (Figure 1). Based on the Binomial index (Anderson and Millar, 2004), three non-parametric, multivariate statistical tests, analysis of similarities, ADONIS and multi-response permutation procedure, showed significant ($P = 0.007$, 0.046 and 0.018, respectively) differences between microbial communities at aCO_2 and eCO_2. The results indicated that the overall taxonomic composition and structure of soil microbial communities was altered at eCO_2.

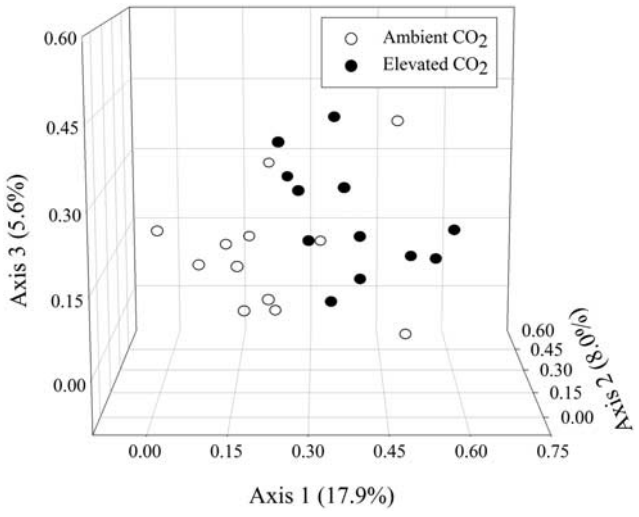


Figure 1 Detrended correspondence analysis of PhyloChip data for both aCO_2 and eCO_2 samples. Only OTUs (a total of 2269) detected in three or more samples out of 12 at aCO_2 or eCO_2 were analyzed.

Relationships between microbial communities and soil and plant properties

To link the taxonomic structure of microbial communities with soil and plant properties, Mantel tests

Table 3 Relationships of microbial phylotypes (OTUs) detected at the class level by PhyloChip to soil and plant variables revealed by partial Mantel test

In association with: Controlling			*Soil*[a]		*Plant*[b]	
			Plant[b]		*Soil*[a]	
Phylum	*Class*	*OTU no.*	$_r$M	P	$_r$M	P
All detected		2269	0.166	0.091	0.082	0.227
Acidobacteria	Acidobacteria-4	10	0.075	0.272	0.283	**0.046**
Bacteroidetes	KSA1	1	0.083	0.262	0.230	0.071
	Unclassified	8	0.039	0.306	0.144	0.053
Caldithrix	Unclassified	2	0.122	0.187	0.271	**0.040**
Chlamydiae	Chlamydiae	2	0.069	0.058	−0.003	0.478
Chlorobi	Chlorobia	11	0.143	0.088	0.096	0.195
Chloroflexi	Chloroflexi-3	2	0.168	0.106	0.192	0.069
	Chloroflexi-4	2	0.138	0.127	0.213	0.063
	Dehalococcoidetes	7	0.062	0.289	0.243	0.065
	Unclassified	6	−0.022	0.495	0.309	0.059
Coprothermobacteria	Unclassified	1	0.112	0.196	0.194	0.07
Crenarchaeota	C1	12	0.077	0.258	0.455	**0.012**
	Thermoprotei	2	0.085	0.261	0.455	**0.009**
Cyanobacteria	Cyanobacteria	48	0.147	0.145	0.315	**0.027**
Deferribacteres	Deferribacer	1	0.053	0.098	−0.057	0.919
Dictyoglomi	Dictyoglomi	1	0.158	0.174	0.423	0.029
DSS1	Unclassified	1	0.174	0.095	0.213	0.067
Firmicutes	Catabacter	7	0.131	0.162	0.249	0.060
	Symbiobacteria	2	0.154	0.144	0.354	**0.023**
	Unclassified	17	0.124	0.220	0.245	0.063
Lentisphaerae	Unclassified	3	0.178	0.084	0.212	0.065
Marinegroup-A	mgA-1	2	0.299	0.054	0.555	**0.014**
OD1	OP11-5	1	0.172	**0.038**	0.111	0.152
OP10	Unclassified	4	0.316	**0.015**	0.049	0.298
OP3	Unclassified	3	0.132	0.076	0.040	0.282
OP8	Unclassified	1	−0.177	0.920	0.329	**0.039**
OP9JS1	OP9	2	0.121	0.138	0.195	0.064
Proteobacteria	Gammaproteobacteria	330	0.326	**0.002**	0.031	0.357
	Unclassified	9	0.027	0.392	0.181	0.060
Spirochaetes	Spirochaetes	36	0.092	0.212	0.257	**0.045**
SR1	Unclassified	1	0.072	0.265	0.452	**0.020**
Synergistes	Unclassified	5	0.085	0.218	0.260	**0.030**
Thermodesulfobacteria	Thermodesulfobacteria	1	0.049	0.349	0.249	0.056
Thermotogae	Thermotogae	1	0.182	0.078	0.103	0.184
TM6	Unclassified	1	0.090	0.264	0.416	**0.035**
TM7	Unclassified	4	0.161	0.101	0.227	0.068
Unclassified	Unclassified	37	0.160	0.150	0.218	0.068
WS3	Unclassified	2	0.055	0.303	0.166	0.089

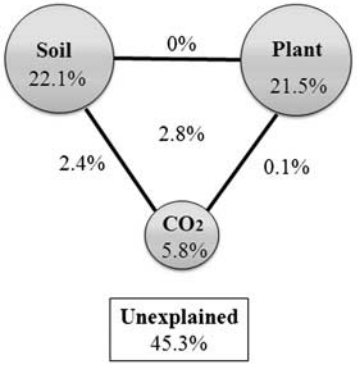
Abbreviation: OTU, operational taxonomic unit.
[a]Selected soil variables include soil %N at a depth of 10–20 cm (N10–20), soil %C and N at a depth of 10–20 cm (SCN10–20), soil pH, nitrification ($mg\,kg^{-1}\,day^{-1}$) and net N mineralization ($mg\,kg^{-1}\,day^{-1}$).
[b]Selected plant variables include total root biomass (TRB), species count from % cover (SCFPC), aboveground percentage carbon (APC), fine roots at a depth of 0–20 cm (FR0–20) and total biomass (TB).
Only classes with *P*-values <0.10 to soil or plant variables have been listed.
Boldface indicates significantly changed phylotypes or all detected OTUs.

and canonical correspondence analysis were performed. By using the BioENV procedure (Clarke and Ainsworth, 1993), five plant variables, including total root biomass (TRB), species count from % cover (SCFPC), aboveground percentage carbon (APC), fine roots at a depth of 0–20 cm (FR0–20) and total biomass (TB), were selected from 24 plant parameters (Supplementary Table S1). Similarly, five soil variables, including soil %N at a depth of 10–20 cm (N10–20), soil C/N ratio at a depth of 10–20 cm (SCN10–20), soil pH, nitrification rate ($mg\,kg^{-1}\,day^{-1}$) and net N mineralization rate ($mg\,kg^{-1}\,day^{-1}$) were selected from 20 soil parameters (Supplementary Table S2).

Based on the above selected sets of plant and soil variables, partial Mantel tests were initially performed to correlate the microbial community measured by the signal intensity of all detected 2269 OTUs with those environmental factors, and such an analysis showed that the microbial community on the whole was not correlated significantly with the soil variables ($P=0.091$) or plant variables ($P=0.227$) (Table 3). Then, we examined the correlations of the plant or soil properties with

specific microbial populations at different taxonomic levels (phylum, class, order, family and subfamily). At the phylum level, 14 phylotypes significantly ($P<0.05$) correlated with the soil or/and plant properties. For example, there was a significant ($P=0.011$) correlation between Crenarchaeota and the selected plant variables, and a significant ($P=0.037$) correlation between Proteobacteria and the selected soil variables (Supplementary Table S3). At the class level, 16 classes were significantly ($P<0.05$) correlated with the soil or/and plant characteristics. For example, γ-Proteobacteria and OP10 were significantly ($P=0.002$ and 0.015, respectively) correlated with the selected soil variables, whereas Cyanobacteria and Spirochaetes were significantly ($P=0.027$ and 0.045, respectively) correlated with the selected plant variables (Table 3). Also, there were significant ($P=0.012$ and 0.009) correlations between the microbial community and the selected plant properties for both archaeal classes, C1 and Thermoprotei, respectively (Table 3). Similarly, 48 families were detected to be correlated with the plant or soil properties (Supplementary Table S4). For example, the signal intensities of Anaplasmataceae from α-Proteobacteria and Spirochaetaceae from Spirochaetes had significant ($P=0.019$ and 0.046, respectively) correlations with the selected plant variables, and those of Enterobacteriaceae and Vibrionaceae from γ-Proteobacteria had significant ($P=0.001$ and 0.004, respectively) correlations with the selected soil variables, whereas Erysipelotrichaceae from Mollicutes was significantly correlated with both soil ($P=0.038$) and plant ($P=0.025$) properties (Supplementary Table S4). In addition, five unclassified classes and 29 unclassified families were significantly ($P<0.05$) correlated with the selected soil or plant variables, respectively, suggesting that soil and plant factors may also largely shape taxonomically uncharacterized microorganisms (Table 3 and Supplementary Table S4).

Variation partition analysis (Ramette and Tiedje, 2007) was then used to assess the contribution of CO_2, soil and plant properties to the taxonomic structure of microbial communities with the same selected variables (Figure 2). When the plant and soil variables were held constant, there was a significant ($P=0.037$) correlation between community structure and CO_2; when plant variables and CO_2 were held constant, there was a significant ($P=0.048$) correlation between community structure and soil variables; and when soil variables and CO_2 were held constant, the plant variables did not show a significant ($P=0.082$) correlation with microbial community. The single variable CO_2 was able to independently explain 5.8% of the variation observed, which was the second largest contributor based on all 11 individual variables. Five soil variables could independently explain 22.1% of the variation, and five plant variables could explain 21.5% of the variation (Figure 2). Also, the interactions between CO_2 and soil variables, CO_2 and plant variables, and soil and plant variables, and among CO_2, soil and plant variables, were 0.1%, 2.4%, 0.0% and 2.8%, respectively (Figure 2). In addition, 45.3% of the variation remained unexplained (Figure 2). The above statistical analyses suggest that CO_2 has a direct effect on the microbial community, and that both soil and plant properties are almost equally important for shaping microbial communities through indirect CO_2 effects in this grassland ecosystem.



CO_2	5.8%
pH	5.3%
N10-20	4.9%
SCN10-20	4.3%
Net N mineralization	3.2%
Nitrification	3.1%
TRB	6.5%
FR0-20	5.8%
SCFPC	4.7%
APC	3.9%
TB	3.6%

Figure 2 Variation partition analysis of the effects of CO_2, soil and plant variables on the phylogenetic structure of soil microbial communities. The BioENV procedure was used to identify common sets of soil and plant variables important to the microbial community. The same sets of soil or plant variables were used for variation partition analysis and partial Mantel tests (Table 3; Supplementary Table S3 and Supplementary Table S4). The concentrations of CO_2 are 368 p.p.m. for ambient and 560 p.p.m. for elevated environments; soil variables included soil %N at a depth of 10–20 cm (N10–20), soil C and N ratio at a depth of 10–20 cm (SCN10–20), soil pH, nitrification rate ($mg\,kg^{-1}\,day^{-1}$) and net N mineralization rate ($mg\,kg^{-1}\,day^{-1}$); plant variables included total root biomass (TRB), species count from % cover (SCFPC), aboveground percentage carbon (APC), fine roots at a depth of 0–20 cm (FR0–20) and total biomass (TB).

Significantly changed and unique OTUs

To examine effects of eCO_2 on microbial community composition, both significantly changed and unique OTUs were identified. Among 2269 OTUs detected, 2075 were shared by aCO_2 and eCO_2 samples, and 194 unique OTUs were only detected at aCO_2 (123) or eCO_2 (71), respectively. For those shared OTUs, a response ratio was calculated for each OTU based on its signal intensity. A total of 194 OTUs were significantly ($P<0.05$) decreased and only 13 were significantly ($P<0.05$) increased at eCO_2 (Table 4). Most phyla, including relatively abundant ones (for example, Crenarchaeota, Acidobacteria, Chloroflexi, Planctomycetes, Verrucomicrobia), did not have OTUs detected with increased signal intensities at eCO_2, and only a few phyla (for example, Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria) had OTUs with both increased and decreased signal intensities at eCO_2 (Table 4), which is consistent with the general trend that the richness decreased at

Table 4 Numbers of shared OTUs detected at both aCO_2 and eCO_2 based on the hybridization signal intensity and unique OTUs detected only at aCO_2 or eCO_2

Phylum	*Shared OTUs*		*Unique OTUs*	
	Decrese–eCO_2	*Increase–eCO_2*	*aCO_2*	*eCO_2*
Crenarchaeota	12	0	0	0
Euryarchaeota	1	1	1	1
Acidobacteria	15	0	3	2
Actinobacteria	10	1	26	17
Bacteroidetes	4	2	8	9
Chlorobi	1	0	0	0
Chloroflexi	11	0	5	0
Cyanobacteria	4	1	4	0
Firmicutes	29	2	25	11
Gemmatimonadetes	2	0	0	0
Natronoanaerobium	0	0	1	0
Nitrospira	4	0	0	0
OP10	2	0	2	0
OP9/JS1	3	0	0	0
Planctomycetes	9	0	6	1
Proteobacteria	55	6	33	29
Spirochaetes	2	0	0	0
Synergistes	1	0	0	0
TM7	0	0	0	0
Verrucomicrobia	16	0	4	1
Others (<5 OTUs)	6	0	3	0
Unclassified	7	0	2	0
Total	194	13	123	71

Abbreviations: aCO_2, ambient CO_2; eCO_2, elevated CO_2; OTU, operational taxonomic unit.
Increase–CO_2 or Decrease–eCO_2 indicates the signal intensity of an OTU was significantly higher or lower at eCO_2, respectively.

eCO_2. The signal intensities of 6 and 56 OTUs were significantly ($P<0.05$) increased and decreased, respectively, in Proteobacteria. Specifically, two OTUs were increased and 12 decreased in α-Proteobacteria, 2 and 2 in β-Proteobacteria, 1 and 2 in ε-Proteobacteria, and 1 and 7 in γ-Proteobacteria, respectively, but all 31 OTUs derived from δ-Proteobacteria were decreased at eCO_2 (Figure 3). Similarly, 29 and 2 (OTU3497 and OTU3254) OTUs were significantly ($P<0.05$) decreased and increased, respectively, in Firmicutes, which were mostly derived from two classes, Clostridia and Bacilli (Supplementary Figure S1). Among 194 unique OTUs, 123 and 71 were from aCO_2 and eCO_2, respectively, and those OTUs were largely derived from the most abundant phyla, such as Proteobacteria, Firmicutes and Actinobacteria (Table 4 and Supplementary Table S5). The analysis of significantly changed and unique OTUs further confirms that the phylogenetic composition of soil microbial communities changed in response to eCO_2.

Significantly changed microbial populations at eCO_2

To understand what specific microbial populations may be affected by eCO_2, we mapped OTUs detected to microbial populations at the phylum or lower levels, and significantly changed populations were identified by response ratio based on PhyloChip hybridization signal intensity. At the phylum level, five phyla, including one archaeal phylum (Crenarchaeota) and four bacterial phyla (Chloroflexi, OP10, OP9/JS1, Verrucomicrobia), showed significantly ($P<0.05$) decreased signal intensities, but most abundant phyla (for example, Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Acidobacteria) remained unchanged at eCO_2 (Figure 4). A further examination of those significantly changed phyla showed that those changes occurred in some specific microbial groups at the class or lower levels. In the phylum of Chloroflexi, a significant decrease of signal intensities at eCO_2 was observed in three orders (Chloroflexi-1a, 1b, 1f) of the class Anaerolineae, and the class of Dehalococcoidetes, whereas the signal intensities of other classes (for example, Chloroflexi-3, Chloroflexi-4, Thermomicrobia) did not change significantly (Supplementary Figure S2A). In the phylum of Verrucomicrobia, all significant changes appeared to occur in the order of Verrucomicrobiales from the class of Verrucomicrobiae, in which three families (Verrucomicrobiaceae, Verrucomicrobia subdivision-3, Verrucomicrobia subdivision-7) and an unclassified phylotype had significantly ($P<0.01$) decreased signal intensities, although the other two families (Verrucomicrobia subdivision-5 and Xiphinematobacteraceae) detected did not show significant changes in signal intensity at eCO_2 (Supplementary Figure S2B). Crenarchaeota is an archaeal phylum showing significantly ($P<0.01$) decreased signal intensities at eCO_2, and such decreases were observed in three orders (C1a, C1b, Cenarchaeales) from two classes (C1 and Thermoprotei) (Supplementary Figure S3A). In addition, significant decreases of signal intensity were seen in two less characterized phyla (OP10 and OP9/JS1), with one from an unclassified class in OP10 (Supplementary Figure S3B) and the other from an unclassified order of JS1class in OP9/JS1 (Supplementary Figure S3C).

Although significant changes were not observed at eCO_2 for the most abundant phyla at the phylum level based on summed intensities, such significances were detected at the class or lower taxonomic levels for some phyla. In the phylum Proteobacteria, the signal intensity of the AMD clone order of δ-Proteobacteria was significantly ($P<0.05$) decreased although no significant changes were detected at the class level (Supplementary Figure S4). In the phylum Firmicutes, the signal intensities of the family Syntrophomonadaceae in the order of Clostridiales and an unclassified order in the class of Clostridia significantly ($P<0.05$) decreased at eCO_2, as did an unclassified phylotype ($P<0.01$), although no significant changes were observed in other phytolypes (for example, Bacilli, Mollicutes) (Supplementary Figure S5). Also, three groups of Actinobacteria showed significantly decreased signal intensities at eCO_2, which included the order Acidimicrobiaceae and an unclassified phylotype in the class Acidimicrobiales; the order Bifidobacteriaceae in the class Bifidobacteriales and

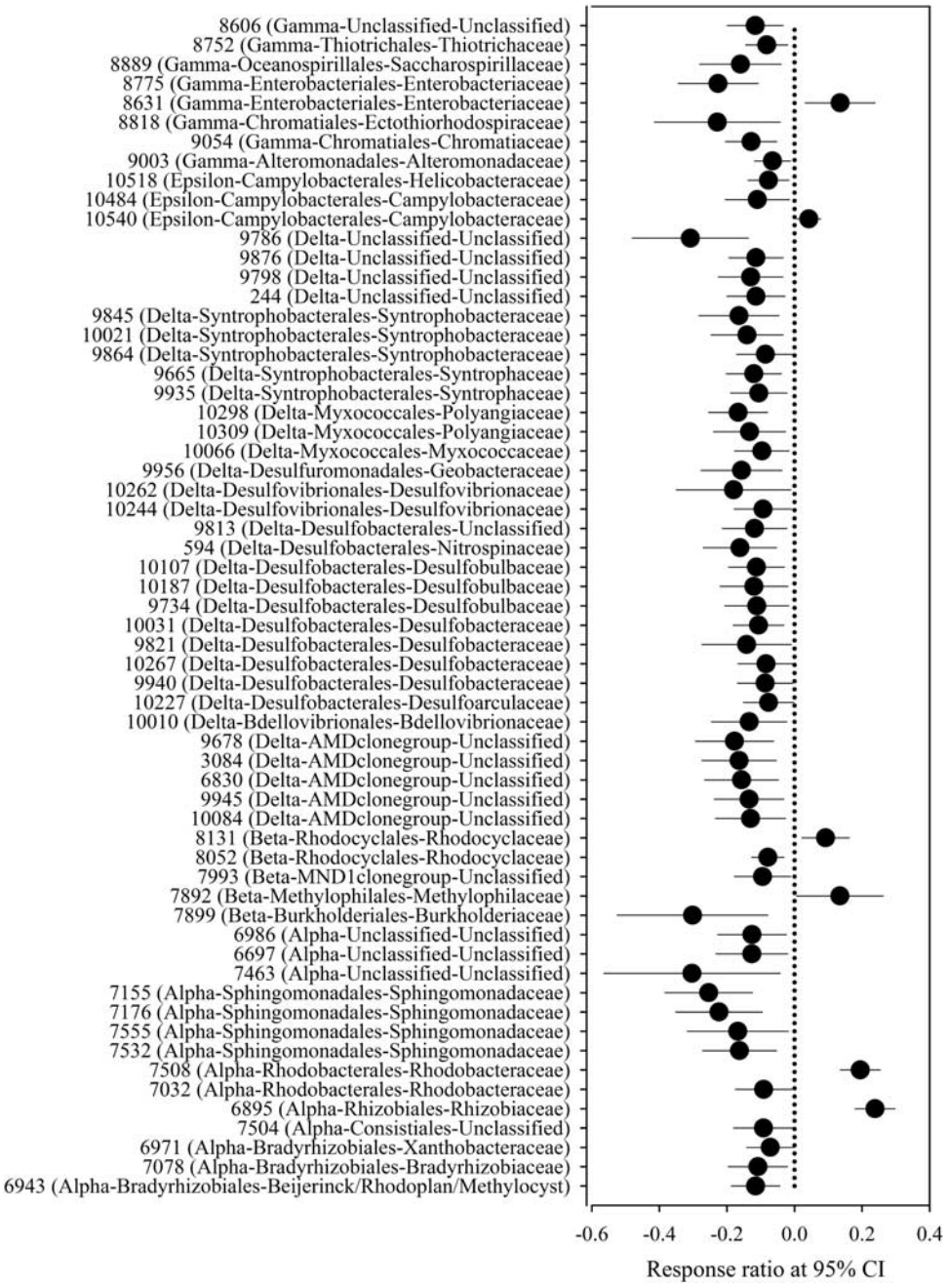


Figure 3 Significantly changed OTUs in the phylum of Proteobacteria at eCO_2 by using the response ratio method (Luo *et al.*, 2006) at 95% confidence interval.

an unclassified phylotype at the phylum level, and interestingly, no significant changes in signal intensity were observed in the largest class Actinomycetales (Supplementary Figure S6). In addition, no significant changes were observed in the phylum of Bacteroidetes (Supplementary Figure S7) or Acidobacteria even at the class, order or family level (Supplementary Figure S8). Those results indicate that eCO_2 significantly affected some specific microbial populations at different taxonomic levels, such as phylum, class, order and family, and those phylotypes generally appeared to have decreased signal intensities at eCO_2.

Discussion

The long-term sustainability of ecosystem productivity requires detailed knowledge of its biodiversity coupled to profound understanding of its functioning. To better understand the implications of eCO_2 on microbial communities, we used PhyloChip to comprehensively survey the richness, composition and structure of soil microbial communities in the BioCON grassland. Our results showed that eCO_2 significantly altered the microbial community diversity, composition and structure, especially for particular microbial populations at the OTU level.

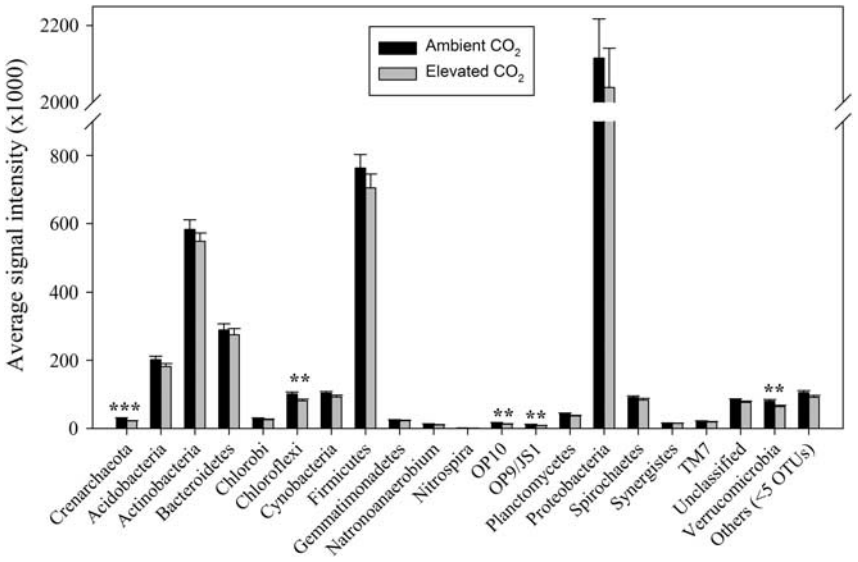


Figure 4 Average PhyloChip hybridization signal intensities for aCO_2 and eCO_2 samples at the phylum level. Significance was tested by response ratios (Luo *et al.*, 2006). $***P<0.01$; $**P<0.05$.

Such microbial population changes were significantly correlated with soil and plant properties.

This study provides a comprehensive survey of the microbial richness and composition of grassland soil microbial communities. Previous studies with 16S rRNA-based analyses using clone libraries (Janssen, 2006; Lesaulnier *et al.*, 2008), microarrays (for example, PhyloChip) (Cruz-Martinez *et al.*, 2009; DeAngelis *et al.*, 2009; Yergeau *et al.*, 2009), pyrosequencing (Roesch *et al.*, 2007; Fulthorpe *et al.*, 2008; Campbell *et al.*, 2010; Eilers *et al.*, 2010; Uroz *et al.*, 2010) and other approaches (Drigo *et al.*, 2007, 2008, 2009, 2010; Feng *et al.*, 2009) show that soil microbial communities are highly diverse and complex. In this study, 2269 OTUs affiliated 45 phyla, 55 classes, 99 orders, 164 families and 190 subfamilies were detected. Proteobacteria was the most well-represented phylum, with α-, β-, δ-, γ- and ε-Proteobacteria, as well as unclassified classes, detected. This group of bacteria has considerable morphological, physiological and metabolic diversity, which are of great importance to global carbon, nitrogen and sulfur cycling (Kersters *et al.*, 2006). Firmicutes were detected as the second most prevalent phylum in terms of the number of OTUs, and Bacilli, Clostridia and Mollicutes were found to be major classes in this grassland ecosystem. Some previous cloning analyses may underestimate this group as cells or spores are known to be difficult to lyse during DNA extraction (Janssen, 2006). The members of the Actinobacteria phylum are a group of Gram-positive bacteria that have an important role in organic matter turnover and carbon cycling, such as decomposition of cellulose and chitin, and Actinomycetales and Acidimicrobiales were found to be major classes in the BioCON site. A previous study showed that long-term organic and inorganic amendments significantly altered the Actinobacterial community structure but not its diversity (Piao *et al.*, 2008). Bacteroidetes are the fourth most prevalent group of bacteria detected in this study, with three major classes (Bacteroidetes, Flavobacteria, Sphingobacteria). Acidobacteria are among the most dominant phyla in soil-borne microbial communities, and generally are classified into eight classes (Handelsman, 2004). The diversity of Acidobacteria in soil was recently examined using different approaches, and a higher proportion of Acidobacteria was observed in bulk soil than in rhizosphere soil (Kielak *et al.*, 2008). Therefore, this study provides a comprehensive survey of the richness and composition of soil microbial communities at this grassland ecosystem.

Elevated atmospheric CO_2 may affect soil microbial communities in both direct and indirect ways. In a previous study, soil CO_2 flux increased $0.57\,mmol\,m^{-2}\,s^{-1}$ or 16% on average at eCO_2 conditions as compared with aCO_2 conditions in the BioCON site (Craine *et al.*, 2001). Also, a recent study in the same site showed that the abundance of key genes involved in microbial C and N fixation, and labile C degradation, was significantly increased at eCO_2 (He *et al.*, 2010b). Those results suggest that eCO_2 may directly impact soil microbial community structure and function. However, as CO_2 concentrations in the pore space of soil generally are between 2000 and 38 000 p.p.m., much higher than those in the atmosphere even under aCO_2 condition, the direct effects of eCO_2 on soil microbial communities may be negligible compared with potential indirect effects, such as increased plant carbon inputs to soil and changes in soil properties (Drigo *et al.*, 2008). The data presented here reflect this idea: CO_2 alone explained 5.8% of the total variation of microbial community structure, compared with soil variables at 22.1% and plant variables at 21.5%.

Also, eCO_2 significantly increased plant productivity, whole-plot total N, soil pH and soil moisture, and decreased whole-plot plant N, aboveground and belowground N concentrations. The results suggest eCO_2 may directly and indirectly affect soil microbial communities, and the indirect effects appear to make more of a contribution to shaping the soil microbial communities.

Effects of eCO_2 on plant and soil properties are expected to modify taxonomic microbial community composition and structure, and regulate ecosystem functioning. First, it is indicated by differential responses of soil microbial populations to eCO_2. Increases in soil carbon, coupled to an increase in cellulolytic and chitinolytic activities, were noted to alter the availability of soil substrates for microbial metabolism (Larson *et al.*, 2002; Phillips *et al.*, 2002). Previous studies showed increases in the abundance of Actinobacteria and Bacteroidetes at eCO_2 (Sait *et al.*, 2006; Lesaulnier *et al.*, 2008). However, we did not see significant changes in the total signal intensities for Actinobacteria, Bacteroidetes or other most abundant phyla at the phylum level in response to eCO_2. In α-Proteobacteria, a significant increase in the abundance of OTUs related to Rhodobium and a significant decrease in the abundance of OTUs related to Bradyrhizobium were detected in the trembling aspen FACE study (Lesaulnier *et al.*, 2008), and a recent study showed a stimulation of purple phototrophic α- and β-Proteobacteria in a flooded paddy soil by eCO_2 (Feng *et al.*, 2009), which are generally consistent with this study. Also, the preference of Acidobacteria in bulk soil has been suggested to be a result of the oligotrophic lifestyle for many members of this phylum (Fierer *et al.*, 2007). A higher input of organic matter into soil at eCO_2 may be favorable for carbon polymer-degrading or fast-growing microorganisms, which presumably outcompete Acidobacteria. If true, the signal intensity of Acidobacteria may remain unchanged or decrease at eCO_2, which was observed in this study. In addition, a decrease in the abundance of Crenarchaea and Verrucomicrobia at eCO_2 was observed previously (Lesaulnier *et al.*, 2008), which is consistent with our observation in this study. Members of Verrucomicrobia are reported to be negatively impacted by soil moisture (Buckley and Schmidt, 2001), which has been shown to increase at eCO_2 in the BioCON site (Reich *et al.*, 2001; He *et al.*, 2010b) and other sites (Zavaleta *et al.*, 2003), which is largely due to reduced stomatal conductance of plants (Kandeler *et al.*, 2008). Second, significant correlations are observed between environmental factors and microbial communities. A negative impact of increased concentrations of organic matter on the growth of Acidobacteria was observed previously (Stevenson *et al.*, 2004), and consistently, Acidobacteria-4 populations were found to be correlated significantly with plant variables in this study. Also, more carbon input into soil may affect autotrophic populations, such as Chloroflexis and Cyanobacteria. Indeed, a decrease in the signal intensity of those phyla was observed in this study. pH has been considered an important factor affecting the diversity and structure of soil microbial communities (Fierer and Jackson, 2006), and our Mantel analysis showed a significant correlation between soil properties, including pH and γ-Proteobacteria or OP10. In this study, soil pH was ~6.2 in aCO_2 plots and it significantly increased to ~6.5 in eCO_2 plots, suggesting a possible shift of microbial community composition and structure. Therefore, our results indicate that both soil and plant properties, such as soil pH, moisture and plant biomass, significantly affect the microbial richness, composition and structure, which may determine or modify ecosystem functioning.

The central hypothesis of this study was that, at eCO_2, an increase in plant biomass (Reich *et al.*, 2006) and soil carbon inputs (Adair *et al.*, 2009), and associated microenvironmental changes (Reich, 2009; He *et al.*, 2010b), would stimulate microbial, especially bacterial growth, which would lead to significant changes in the richness, composition, structure and function of soil microbial communities. Previous studies of effects of eCO_2 on soil microbial communities showed variable responses. For example, a study conducted at a trembling aspen FACE experiment site in Wisconsin, USA, showed an increase in heterotrophic decomposers and a decrease in nitrate reducers of the domain bacteria and archaea, although the total bacterial abundance did not change (Lesaulnier *et al.*, 2008). By contrast, no detectable effects on microbial community structure, microbial activity, potential soil N mineralization or nitrification rates were observed at a sweetgum FACE experiment in Tennessee, USA (Austin *et al.*, 2009). For testing our core hypothesis, this study had several strengths: (i) It was conducted at a well-designed BioCON experimental site, with 12 replicates for each CO_2 condition, so that the effects of eCO_2 on soil microbial communities could be robustly examined; (ii) PhyloChip is considered a powerful tool for a comprehensive survey of microbial richness and composition (DeAngelis *et al.*, 2009; Rastogi *et al.*, 2010), which may overcome the limitations of cloning-based approaches and (iii) this study was conducted in a grassland ecosystem with defined plant species, which minimizes the effects of plant diversity and composition on soil microbial communities. Indeed, consistently with some previous studies in grasslands (Schortemeyer *et al.*, 1996; Drissner *et al.*, 2007), our results indicated that eCO_2 had significant effects on the richness, composition and structure of soil microbial communities. First, such changes are reflected in a decrease in richness, which generally agrees with previous studies (Begon *et al.*, 1996; Hughes *et al.*, 2001). Similarly, a significant decrease in richness was observed in some phyla (for example, Chloroflexi, OP10). Second, more OTUs were found

to have decreased signal intensities and fewer OTUs with increased signals at eCO_2 among shared OTUs, and more unique OTUs were detected at aCO_2 than eCO_2. Third, based on PhyloChip signal intensities, although some specific microbial populations, especially from the most abundant phyla, remained unchanged at the phylum level, significant changes were apparent at the class or lower levels, suggesting the microbial composition was altered at eCO_2 at finer taxonomic scales. Finally, the taxonomic structure is different between aCO_2 and eCO_2 samples as shown by statistical analyses (for example, detrended correspondence analysis, analysis of similarities, ADONIS, multi-response permutation procedure). Recently, a study using a comprehensive functional gene array, GeoChip 3.0 (He *et al.*, 2010a), also demonstrated that the functional composition and structure of soil microbial communities were significantly altered at eCO_2 (He *et al.*, 2010b), which may be due to eCO_2-induced shifts in microbial populations. The results suggest that the richness, composition and structure of soil microbial communities shift in response to eCO_2.

PhyloChip has been considered a powerful tool to comprehensively and rapidly analyze microbial communities. Specifically, as such a microarray-based technology has a defined probe set and targets known populations, it minimizes or eliminates sampling artifacts, including under-sampling, unequal sampling and random sampling (Zhou *et al.*, 2008), making it a preferable approach for community-scale comparison of microbial communities, as has been demonstrated in this study. Like other high-throughput technologies, however, PhyloChip has its limitations. For example, PhyloChip only detects known sequences already present in a database at the time of probe design, so the G2 PhyloChip used in this study may not fully cover the species richness of soil microbial communities, and a follow-up study using the G3 PhyloChip could prove beneficial. To discover unknown 16S rRNA genes, future investigations may use high-quality, full-length sequencing as a complementary approach to further understand the taxonomic and phylogenetic diversity, composition, structure and function of the soil microbial communities in this grassland ecosystem.

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