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Divergent Responses of Forest Soil Microbial Communities under Elevated CO_2 in Different Depths of Upper Soil Layers

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ABSTRACT Numerous studies have shown that the continuous increase of atmosphere CO_2 concentrations may have profound effects on the forest ecosystem and its functions. However, little is known about the response of belowground soil microbial communities under elevated atmospheric CO_2 (eCO_2) at different soil depth profiles in forest ecosystems. Here, we examined soil microbial communities at two soil depths (0 to 5 cm and 5 to 15 cm) after a 10-year eCO_2 exposure using a high-throughput functional gene microarray (GeoChip). The results showed that eCO_2 significantly shifted the compositions, including phylogenetic and functional gene structures, of soil microbial communities at both soil depths. Key functional genes, including those involved in carbon degradation and fixation, methane metabolism, denitrification, ammonification, and nitrogen fixation, were stimulated under eCO_2 at both soil depths, although the stimulation effect of eCO_2 on these functional markers was greater at the soil depth of 0 to 5 cm than of 5 to 15 cm. Moreover, a canonical correspondence analysis suggested that NO_3-N, total nitrogen (TN), total carbon (TC), and leaf litter were significantly correlated with the composition of the whole microbial community. This study revealed a positive feedback of eCO_2 in forest soil microbial communities, which may provide new insight for a further understanding of forest ecosystem responses to global CO_2 increases.

IMPORTANCE The concentration of atmospheric carbon dioxide (CO_2) has continuously been increasing since the industrial revolution. Understanding the response of soil microbial communities to elevated atmospheric CO_2 (eCO_2) is important for predicting the contribution of the forest ecosystem to global atmospheric change. This study analyzed the effect of eCO_2 on microbial communities at two soil depths (0 to 5 cm and 5 to 15 cm) in a forest ecosystem. Our findings suggest that the compositional and functional structures of microbial communities shifted under eCO_2 at both soil depths. More functional genes involved in carbon, nitrogen, and phosphorus cycling were stimulated under eCO_2 at the soil depth of 0 to 5 cm than at the depth of 5 to 15 cm.

KEYWORDS microbial responses, elevated carbon dioxide, soil microbial community, free-air CO_2 enrichment, functional genes, forest ecosystem

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The atmospheric carbon dioxide (CO_2) concentration is closely linked to global carbon (C) cycling and has significant effects on terrestrial ecosystems (1). The concentration of global atmosphere CO_2 has increased substantially since the preindustrial period, from 280 ppm to currently over 400 ppm (2). Elevated atmospheric CO_2 (eCO_2) could strongly influence ecosystem functioning though various processes, including the soil microbial communities that mediate C sequestration and nutrient cycling (3–6). Thus, understanding the response of microbial communities to eCO_2 is crucial for understanding multiple ecosystem functions and for predicting the changes in terrestrial ecosystems in response to future climates (7, 8).

Forests currently tie up more than 85% of all terrestrial biomass carbon (C) and provide the largest terrestrial C sinks for global atmospheric CO_2 (9, 10). Numerous studies have shown that eCO_2 dramatically influenced the productivity, transpiration, nitrogen (N) use efficiency, leaf litter productivity, fine root exudation, and carbon (C) assimilation rates of trees (11–16). Such responses of forest ecosystems may further influence the belowground microbial composition and function by changing soil geochemical properties and nutrient inputs (17, 18). However, to date, accurate observations of the effect of eCO_2 on microbial communities have been largely complicated by the extremely high diversity and the complexity of measuring belowground bioprocesses (3). Some studies have shown significant changes in the composition, structure, activity, and functional capacity of soil microbial communities (3, 6, 18–22) under eCO_2, while other studies have shown no significant differences of microbial community compositions between eCO_2 and ambient CO_2 (aCO_2) conditions (21–26). Therefore, further studies at additional sites and using new analysis approaches are necessary to understand whether the structures of soil microbial communities shift under eCO_2 and how soil microbial communities respond to complex interactions of biotic and abiotic factors in forests.

Soil microbial communities are expected to play important roles in mediating many belowground processes (e.g., organic matter decomposition and nutrient cycling) in forest ecosystems (27, 28). Since nearly all soil physiochemical parameters change with soil depth (e.g., C and N availability, oxygen content, soil moisture, and temperature) (17, 29), distinct responses of soil microbial communities to eCO_2 are expected to occur at different soil depths. Previous reports showed that numerous genes for nutrient (e.g., C and N) cycling significantly increased at soil depths of 0 to 5 cm with no significant changes at depths of 5 to 15 cm in a farmland ecosystem in response to eCO_2 (17, 30). However, most studies of forest ecosystems have evaluated the effect of eCO_2 on soil microbial communities at only one depth, which could mask finer-scale differences (e.g., at different depths) (23). Plant litter and root exudation inputs to soil vary with soil depth (29, 31), which may affect soil microbial communities associated with ecological processes at different depths. Thus, it is important to further understand whether eCO_2 shifts the structure and function of microbial communities at depth-resolved scales.

Free-air CO_2 enrichment (FACE) experiments provide an opportunity to understand many aspects of eCO_2 effects on ecosystems under natural field conditions (32). It was demonstrated in several FACE experiments that the photosynthesis, net primary production (NPP), fine-root production, and turnover of trees were enhanced by eCO_2 (16, 33–36). Consequently, an increase in soil C stock and a high C/N ratio were observed under eCO_2 at a soil depth of 0 to 5 cm in a forest ecosystem, where inputs from roots and aboveground litter were greatest (37, 38). These changes may directly or indirectly affect the structure of the soil microbial community and the functional capacity associated with nutrient cycling (e.g., C and N cycling). However, how microbial communities respond to eCO_2 at different depths in forest FACE experiments remains unknown. In this study, we evaluated the soil microbial community response to eCO_2 in a deciduous forest FACE experiment site (http://face.ornl.gov/index.html), which had been exposed to eCO_2 for more than 10 years, using a functional gene array (GeoChip). As a powerful high-throughput metagenomics technology, GeoChip 3.0 assesses 292 genes of key enzymes involved in C, N, S, and P cycling, key energy metabolism, metal resistance, organic contaminant degradation, antibiotic resistance, and a phylogenetic

TABLE 1 Soil properties at different depths under eCO_2 and aCO_2[a]

Treatment	NH_4-N (mg/kg)	NO_3-N (mg/kg)	TC (%)[b]	TN (%)[c]	C/N
0- to 5-cm depth					
eCO_2	34.2 ± 2.30	3.67 ± 0.254	2.75 ± 0.145	0.203 ± 0.007	13.78 ± 0.44
aCO_2	39.4 ± 6.19	2.62 ± 0.623	2.14 ± 0.43	0.178 ± 0.018	12.47 ± 0.95
P value	0.189	**0.023**	**0.037**	0.070	0.071
5- to 15-cm depth					
eCO_2	31.26 ± 3.42	1.34 ± 0.069	2.39 ± 0.105	0.178 ± 0.011	13.67 ± 0.31
aCO_2	30.25 ± 3.39	1.00 ± 0.106	2.02 ± 0.068	0.138 ± 0.005	14.64 ± 0.32
P value	0.851	**0.035**	**0.023**	**0.012**	0.075

[a]The variables of soil properties at each depth were analyzed separately, and significances between different treatments (aCO_2 and eCO_2) were calculated by *t* tests. Significant differences ($P < 0.05$) are indicated by bold type.
[b]TC, total carbon content.
[c]TN, total nitrogen content.

marker (*gyrB*) (39). Recently, many studies demonstrated that GeoChip is an ideal tool for analyzing microbial communities from complex environments, such as soil (17, 19, 30), contaminated water (40, 41), oil fields (19, 42), bioreactor systems (43, 44), and other habitats (45). We hypothesized that the eCO_2 treatment would have significant effects on the functional diversity, composition, structure, and metabolic potential of soil microbial communities at different depths (0 to 5 cm and 5 to 15 cm) and that the plant and soil properties would have different impacts on soil microbial communities at different depths.

RESULTS

Effects of eCO_2 on plant and soil properties at two depths. The ORNL FACE experiment was constructed in 1998 in the Oak Ridge National Environmental Research Park within a sweetgum (*Liquidambar styraciflua* L.) plantation. After 10 years of elevated CO_2 (eCO_2) treatment, the plant net primary productivity (NPP) increased 9% compared with that at ambient CO_2 (aCO_2), although the enhancement of NPP was less significant (46). For soil properties, five important soil variables were measured or calculated, including soil nitrate (NO_3^--N), ammonium (NH_4^+-N), total nitrogen (TN), total carbon (TC), and C:N ratio. Elevated CO_2 greatly increased the amount of soil NO_3^--N ($P < 0.05$) and TC ($P < 0.05$) in 6 replicate soil samples at both soil depths (0 to 5 cm and 5 to 15 cm) (Table 1). However, the effects of eCO_2 on soil C:N ratios were different in these two soil layers, with an increase in the 0- to 5-cm-depth layer but a decrease at a depth 5 to 15 cm, which were marginally significant ($P < 0.1$). There was no significant difference in the NH_4^+-N contents between eCO_2 and aCO_2 at either soil depth.

Overall differences of soil microbial functional genes and phylogenetic markers. A total of 7,090 microbial function genes were detected by GeoChip 3.0 across 24 samples. The gene numbers detected under eCO_2 (3,502 ± 381) were significantly greater ($P = 0.026$) than that under aCO_2 (2,386 ± 85) at the soil depth of 0 to 5 cm. However, this difference was not significant ($P > 0.1$) at the depth of 5 to 15 cm. Similar patterns were also observed in alpha-diversity indexes. The Shannon index (H′) and the Simpson's reciprocal index (1/D) were significantly higher ($P < 0.05$) in eCO_2 at the depth of 0 to 5 cm, but no significant differences were found at the depth of 5 to 15 cm. The overall taxonomic compositions of the microbial communities were analyzed at phylum level based on GeoChip data (see Fig. S1 in the supplemental material). All detected genes were taxonomically derived from 2 archaeal phyla, 21 bacterial phyla, and 5 eukaryotic phyla. Among these phyla, functional genes derived from *Proteobacteria*, *Actinobacteria*, and *Firmicutes* had relative higher abundances than those from other phyla. eCO_2 highly increased the abundance of key genes derived from *Crenarchaeota*, *Euryarchaeota*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Firmicutes*, and *Proteobacteria* at the soil depth of 0 to cm and from *Actinobacteria* and *Proteobacteria* at the soil depth of 5 to 15 cm (see Fig. S2).

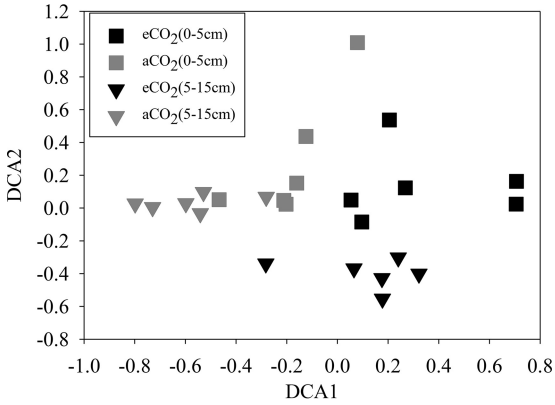


FIG 1 Detrended correspondence analysis (DCA) of all detected functional genes at soil depths of 0 to 5 cm and 5 to 15 cm under ambient CO_2 (aCO_2) and elevated CO_2 (eCO_2) conditions.

A detrended correspondence analysis (DCA) of all functional genes illustrated that eCO_2 and aCO_2 were clearly separated (Fig. 1), and similar separation by using the phylogenetic marker gene (*gyrB*) only (see Fig. S3) was observed at both soil active layers (0 to 5 cm and 5 to 15 cm), indicating that microbial functional gene structures and phylogenetic structures were both changed under eCO_2. In addition, the permutational multivariate analysis of variance (Adonis) test on all detected functional genes and *gyrB* phylogenetic markers showed that not only CO_2 treatment but also depth and their interaction had significant effects on both functional and phylogenetic structures of soil microbial communities ($P < 0.05$) (Table 2). The main factor contributing to the total variation of microbial functional gene structures was eCO_2 (12.9%), followed by depth (7.7%) and their interaction (7.7%), while the phylogenetic structures were mainly shaped by eCO_2 (12.1%), eCO_2 plus depth (9.5%), and depth (8.3%). Other statistical tests, namely, analysis of similarities (ANONISM) and a multiresponse permutation procedure (MRPP), also detected significant changes according to both CO_2 treatment and depth (see Table S1). Thus, the compositions and phylogenetic and functional gene structures of soil microbial communities had been dramatically altered by eCO_2 at both soil depths.

Effect of eCO_2 on microbial functional genes involved in C, N, and P cycling. A total of 589 ± 74 and 524 ± 41 genes involved in C cycling were detected at the depths of 0 to 5 cm and 5 to 15 cm, respectively, under eCO_2, which were higher than those under aCO_2 (399 ± 13 and 428 ± 21, respectively) (Table 3). A significant difference was observed at the depth of 0 to 5 cm ($P = 0.044$), while no significant difference was observed at the depth of 5 to 15 cm ($P = 0.087$) between aCO_2 and eCO_2. These results indicated that microbial C cycling might be altered by eCO_2.

Several important C fixation genes were further investigated, including the propionyl-CoA carboxylase (PCC) gene and the gene encoding ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO). The total signal intensities for PCC and RubisCO genes were substantially higher ($P < 0.05$) under eCO_2 than under aCO_2 at the soil depth of 0 to

TABLE 2 Adonis analysis of the effect of eCO_2, soil depth, and their interaction on the functional and phylogenetic structures of microbial communities based on all detected genes and *gyrB*

	CO_2		Depth		CO_2-depth interaction	
Gene(s)	R^2	*P* value	R^2	*P* value	R^2	*P* value
All functional genes	0.129	0.001[a]	0.077	0.007[a]	0.077	0.012[a]
Phylogenetic marker (*gyrB*)	0.121	0.001[a]	0.083	0.013[a]	0.095	0.005[a]

[a]$P < 0.05$.

TABLE 3 Distribution of key gene categories involved in carbon, nitrogen, phosphorus, and sulfur cycling

Gene category	No. ± SEM of genes at 0- to 5-cm depth			No. ± SEM of genes at 5- to 15-cm depth		
	eCO_2	aCO_2	*P* value[a]	eCO_2	aCO_2	*P* value[a]
C cycling	589 ± 74	399 ± 13	**0.044**	524 ± 41	428 ± 21	**0.087**
Acetogenesis	7 ± 1	3 ± 0	**0.043**	5 ± 0	7 ± 1	0.178
C degradation	436 ± 56	296 ± 9	**0.048**	380 ± 30	317 ± 15	0.119
C fixation	132 ± 16	90 ± 5	**0.045**	121 ± 10	94 ± 5	**0.06**
Methane	15 ± 1	11 ± 1	**0.011**	18 ± 1	11 ± 1	**<0.001**
N cycling	390 ± 43	255 ± 11	**0.019**	327 ± 29	294 ± 22	0.42
Ammonification	39 ± 5	23 ± 1	**0.021**	29 ± 3	23 ± 2	0.132
Anammox	4 ± 0	3 ± 0	0.183	4 ± 0	3 ± 0	**0.065**
Assimilatory N reduction	27 ± 2	19 ± 1	**0.021**	25 ± 2	19 ± 1	**0.038**
Denitrification	135 ± 19	87 ± 6	**0.049**	113 ± 10	97 ± 7	0.25
Dissimilatory N reduction	25 ± 3	16 ± 1	**0.033**	20 ± 3	18 ± 2	0.557
Nitrification	2 ± 0	1 ± 0	**0.038**	2 ± 0	2 ± 0	0.734
N fixation	158 ± 13	107 ± 7	**0.008**	134 ± 12	132 ± 11	0.901
P utilization	68 ± 9	49 ± 2	**0.091**	54 ± 5	47 ± 4	0.4
S cycling	177 ± 23	119 ± 5	**0.047**	135 ± 12	126 ± 7	0.601
Total	1,224 ± 148	822 ± 29	**0.036**	1,039 ± 86	895 ± 54	0.223

[a]The effects of eCO_2 on distribution of key gene categories were calculated by *t* tests. Significant differences ($P < 0.05$) indicated by bold type.

5 cm, while the significant difference was only detected for the PCC gene at the soil depth of 5 to 15 cm (see Fig. S4).

Three functional genes involved in methane production and oxidation showed distinguishable responses to eCO_2 at different depths, including *mcrA* for CH_4 production and *mmoX* and *pmoA* for CH_4 oxidation. Among these genes, a total of 4 ± 0.7 genes derived from *Archaea* for methane production and 11 ± 0.5 genes derived from *Bacteria* for methane oxidation were detected in eCO_2 samples at a soil depth of 0 to 5 cm, while 3 ± 0.4 and 8 ± 0.7 genes, respectively, were detected in aCO_2 samples. An obviously higher number of genes ($P = 0.004$) derived from *Bacteria* was observed in eCO_2 samples than that in aCO_2 samples. The difference between eCO_2 and aCO_2 was even larger at a soil depth of 5 to 15 cm. Totals of 6 ± 0.6 (*Archaea*) and 12 ± 0.7 (*Bacteria*) genes were detected in eCO_2 samples, and 3 ± 0.3 (*Archaea*) and 8 ± 0.7 (*Bacteria*) genes were detected in aCO_2 samples. eCO_2 greatly increased the numbers of detected genes derived from *Archaea* ($P = 0.002$) and *Bacteria* ($P = 0.005$) at a soil depth of 5 to 15 cm. At a soil depth of 0 to 5 cm, 12 and 3 unique genes involved in methane metabolism, including those derived from uncultured bacterium, uncultured archaeon, uncultured methanogenic archaeon, uncultured *Methanobacteriales* archaeon, uncultured euryarchaeota, and *Methanobrevibacter smithii*, were detected in eCO_2 and aCO_2, respectively, while 22 and 4 unique genes derived from uncultured bacterium, uncultured methanotrophic bacterium, *Frankia* sp., *Azoarcus* sp., *Methylosinus trichosporium*, uncultured methanogenic archaeon, uncultured *Methanobacteriales* archaeon, uncultured archaeon, *Methanofollis liminatans*, *Methanomethylovorans thermophile*, *Methanosarcina lacustris*, and uncultured *Methanosarcinales* archaeon were detected under eCO_2 and aCO_2, respectively, at a soil depth of 5 to 15 cm (see Fig. S5). The total signal intensities of these genes were increased under eCO_2 at both soil depths. Significantly higher signal intensities were observed for *mcrA* ($P = 0.001$), *mmoX* ($P = 0.033$), and *pmoA* ($P = 0.049$) under eCO_2 at the soil depth of 5 to 15 cm (see Fig. S6). However, only *pmoA* showed a dramatic difference ($P = 0.005$) between eCO_2 and aCO_2 at the soil depth of 0 to 5 cm.

In C degradation processes, eCO_2 treatment also showed positive effects in both soil layers (Fig. 2), and most of the genes had significantly higher signal intensities

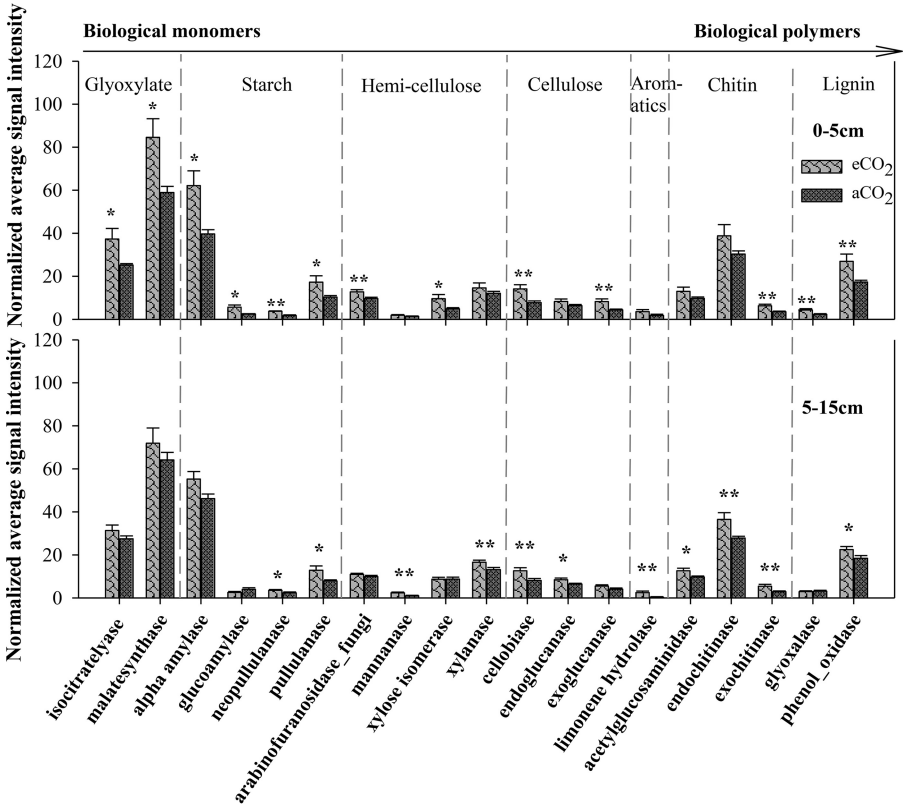


FIG 2 Normalized signal intensities of the detected genes involved in carbon degradation at the soil depths of 0 to 5 cm (top) and 5 to 15 cm (bottom). All data are presented as means ± standard errors (SEs). **, $P < 0.01$; *, $P < 0.05$ based on Student's *t* tests.

($P < 0.05$) under eCO_2 than under aCO_2. In the 0- to 5-cm-depth layer, those included genes for isocitrate lyase (*aceA*) and malate synthase (*aceB*) for the tricarboxylic acid (TCA) cycle, α-amylase (*amyA*), glucoamylase, pullulanase, and neopullulanase (*nplT*) for starch decomposition, arabinofuranosidase for hemicellulose decomposition, cellobiase and exoglucanase for cellulose decomposition, exochitinase for chitin decomposition, and glyoxalase (*glx*) and phenol oxidase for lignin decomposition (Fig. 2). At the soil depth of 5 to 15 cm, seven of the above genes were not significantly altered, including both TCA cycle genes (*aceA* and *aceB*). However, six other functional genes were dramatically changed, including two other hemicellulose degradation genes (mannanase and xylanse), one aromatics degradation gene (limonene hydrolase), one cellulose gene (endoglucanase), and two chitin degradation genes (endochitinase and acetylglucosaminidase). These results indicated that, although eCO_2 enhanced carbon degradation genes at both soil depths, the genes enriched at these two depths differed.

For N cycling processes at the soil depths of 0 to 5 cm and 5 to 15 cm, 390 ± 43 and 327 ± 29 genes, respectively were detected under eCO_2 and 255 ± 11 and 294 ± 22 genes, respectively, were detected under aCO_2. The difference is significant in the upper 0- to 5-cm-depth layer ($P = 0.019$) but not significant in the 5- to 15-cm-depth layer ($P = 0.42$) (Table 3). Under eCO_2, the total signal intensities of nine genes were substantially higher ($P < 0.05$) than those under aCO_2 in the 0- to 5-cm-depth soil layer, including *gdh* and *ureC* that are involved in ammonification, *nasA* and *nirB* involved in assimilatory N reduction, *narG* and *nirS* involved in denitrification, *nrfA* involved in dissimilatory N reduction, *amoA* involved in nitrification, and *nifH* involved in N_2 fixation (Fig. 3). However, at the soil depth of 5 to 15 cm, only the total signal intensities of three genes involved in ammonification (*gdh*), assimilatory N reduction (*nasA*), and denitrification (*narG*) were significantly ($P < 0.05$) increased under eCO_2. These results

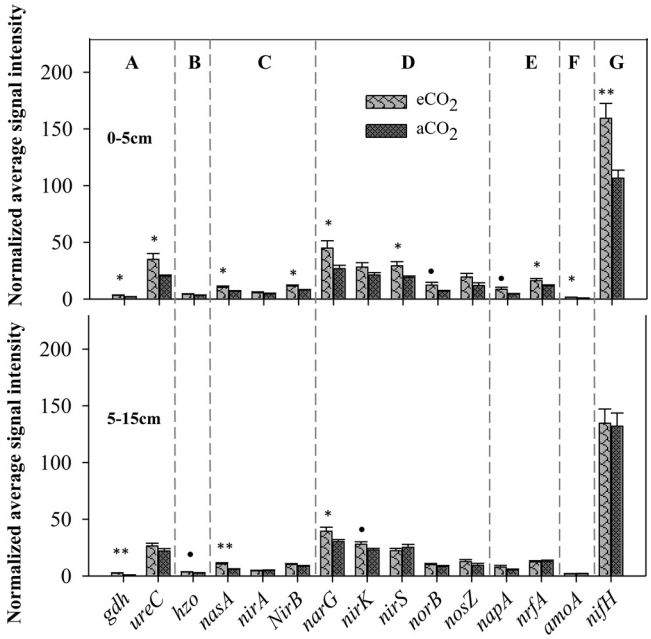


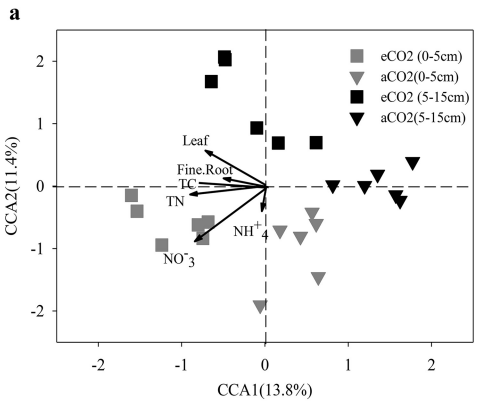
FIG 3 Normalized signal intensities of detected genes involved in the N cycle. (A) ammonification, (B) anammox, (C) assimilatory N reduction, (D) denitrification, (E) dissimilatory N reduction, (F) nitrification, and (G) N_2 fixation. All data are presented as means ± SEs. **, $P < 0.01$; *, $P < 0.05$; ●, $P < 0.1$ based on *t* tests.

suggested that soil N cycling was more active at the depth of 0 to 5 cm than at 5 to 15 cm under eCO_2.

The functional gene encoding phytase for P utilization was substantially higher ($P < 0.05$) under eCO_2 at the soil depth of 0 to 5 cm, and it was detected only in eCO_2 not in aCO_2 at the soil depth of 5 to 15 cm. For other genes, the differences were not significant between eCO_2 and aCO_2 at either depth, including for *Ppx* for inorganic polyphosphate degradation and for *Ppk* for polyphosphate biosynthesis (see Fig. S7).

Associations between the microbial functional gene structures and environmental variables. To determine how major environmental variables influence the microbial functional structure, a canonical correspondence analysis (CCA) was performed. Seven environmental variables were selected: NO_3-N, NH_4-N, TN, and TC for soil properties, and leaf litter and fine root production for plant properties (47). Totals of 13.8% and 11.4% of the constrained variations in microbial communities were explained by the first and second canonical axes, respectively (Fig. 4a). The communities under eCO_2 and aCO_2 separated clearly along the first canonical axis, while communities at the two soil depths separated along the second canonical axis (Fig. 4a). The results of the CCA showed that the microbial functional structures had significant correlations with selected soil properties ($P = 0.023$), plant properties ($P = 0.004$), and all environmental variables ($P = 0.003$) (Fig. 4b). Among these variables, NO_3-N, TN, TC, and leaf litter were significantly correlated with all detected functional genes, suggesting that they were important environmental variables in shaping the soil microbial community structure at this FACE site. Relationships between functional genes involved in C and N cycling and environmental variables at two soil depths were further analyzed by a Mantel test (see Table S2). The results showed that 9 and 8 genes involved in C and N cycling significantly correlated ($P < 0.05$) with soil or plant properties at the soil depths of 0 to 5 cm and 5 to 15 cm, respectively.

To further assess the contributions of soil and plant properties to the microbial functional structure at each soil depth, a CCA-based variation partitioning analysis (VPA) was conducted (Fig. 4c). At the soil depth of 0 to 5 cm, soil properties accounted for 35.1% of microbial community variation and plant properties accounted for 20.8%, while their



b

	F	P
Soil	1.309	**0.023***
NO_3-N	2.250	**0.001****
NH_4-N	0.859	0.797
TN	1.828	**0.003****
TC	1.45	**0.046***
Plant	1.56	**0.004****
Leaf litter	1.661	**0.015***
Fine root	0.987	0.526
Whole	1.374	**0.003****

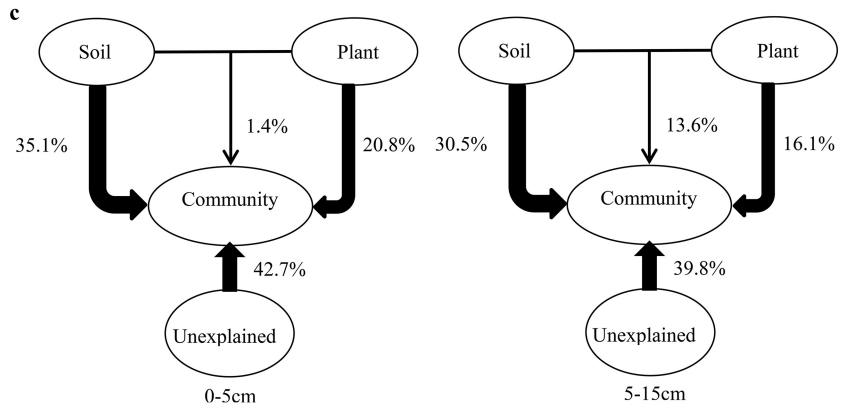


FIG 4 Canonical correspondence analysis (CCA) of GeoChip data under eCO_2 and aCO_2 with selected environmental variables (a). (b) Model significances. (c) Variation partitioning analysis (VPA) based on partial CCA in the soil depths 0 to 5 cm and 5 to 15 cm. Environmental variables, including NO_3-N, NH_4-N, TN, and TC for soil and leaf litter and fine roots for plants, were selected based on variance inflation factor (VIF) analysis.

interaction only accounted for 1.4%, with 42.7% unexplained. In comparison to those at a soil depth of 0 to 5 cm, lower amounts of variation were accounted for by soil properties (30.5%) and plant properties (16.1%) at the soil depth of 5 to 15 cm. However, the interaction of soil and plant properties accounted for a substantially higher percentage of microbial community variations (13.6%) than the 1.4% at the soil depth of 0 to 5 cm.

DISCUSSION

As soil microorganisms mediate several important aspects of nutrient (e.g., C, N, and P) cycling, understanding the responses of soil microbial communities in forest ecosystems to eCO_2 is critical to fully predict the effects of future global change. In this study, we demonstrated the long-term (>10 years) effect of eCO_2 on soil properties, functional diversity, structure and metabolic potential of microbial communities in this sweetgum forest ecosystem.

Since the response of soil microbial communities to eCO_2 remains inconclusive for FACE sites (18, 20, 23), more studies are needed to understand whether the compositions and structures of soil microbial communities are altered at eCO_2, especially across multiple ecosystems. The effects of eCO_2 treatment on soil microbial communities were observed in forest, grassland, and agriculture ecosystems (3, 8, 48), but some research also has shown a lack of significant changes in soil microbial community structures and compositions in FACE sites after 2 or 8 years of eCO_2 exposure by assaying enzymes, substrate utilization, and 16S rRNA gene clone libraries (23, 49). However, these conclusions may not be the same if eCO_2 treatment time was extended or a more comprehensive microbial technique was used for soil microbial communities and they were examined at finer scales (23, 49). It is not surprising that no significant responses

of microbial communities were detected, as the availability of soil nutrient pools were often not altered in the short-term treatment (49) and changes in the community structures would be diluted by combining different depths of soil together (23). In addition, the methods previously used for examining microbial community structure were at the phylum level, which can be imprecise for detecting the specific differences within a phylum (23, 50). As a high-resolution and high-throughput metagenomics technology (39, 50), GeoChip was employed in this study to examine the effect of eCO_2 on soil microbial communities. Our results show that soil microbial communities have significant responses ($P < 0.05$) to eCO_2 at both soil depths, which are associated with the change of soil properties, on the basis of dissimilarity tests (ANONISM and MRPP) (see Table S1 in the supplemental material). It had been substantiated by previous studies of the ORNL FACE site that eCO_2 largely increased the production of plant litter (e.g., leaves and roots) (13, 23, 37, 38), which could alter C and N storage and cycling in the soil (38). In accordance with the previous study, our research shows that total carbon content (TC) and total nitrogen content (TN) were significantly or marginally increased under eCO_2 treatment at two depths (Table 1) (38). Consequentially, these changes may result in shifts of microbial community functional diversity, composition, and structure.

As we expected, the microbial functional diversity, composition, and phylogenetic and functional structures of soil microbial communities shifted under eCO_2 at two soil depths (0 to 5 cm and 5 to 15 cm) (Fig. 1; see also Fig. S3). The results agreed well with a recent report of soil microbial communities at a FACE experimental site in a corn-soy agroecosystem (SoyFACE), which showed that after an 8-year exposure to eCO_2, the functional and phylogenetic structures of soil microbial communities had shifted at soil depths of 0 to 5 cm and 5 to 15 cm (17). Moreover, the relative abundance of functional genes derived from some key phyla was significantly enhanced, potentially indicating that the abundances of bacteria and fungi increased under eCO_2 at the two soil depths. The effects of eCO_2 on soil fungal and bacterial communities possibly occur via increased soil C input in the form of plant litter and root exudates (51, 52). This stimulatory effect of eCO_2 was also observed in other FACE experiments. The microbial and fungal abundances increased under eCO_2 treatment at a wheat field and in a biodiversity, CO_2, and N (BioCON) experiment (53, 54).

Whether the functional processes (e.g., C and N cycling) of soil microbial communities were stimulated by long-term eCO_2 is another critical issue for FACE site research. In this study, we found that a large number of the key functional genes responsible for C, N, and P cycling had significantly higher signal intensities under eCO_2 than under aCO_2, although some patterns for individual genes were different between the two depths. First, different stimulation effects of eCO_2 were observed for the detected key genes involved in C cycling between two depths. Elevated CO_2 significantly enhanced the signal intensities of 57.9% (11 of 19) of detected functional genes for C degradation at the soil depth of 0 to 5 cm and of only 31.6% (6 of 19) at the soil depth of 5 to 15 cm (Fig. 2). Moreover, eCO_2 significantly increased all functional genes for glyoxylate/TCA cycling and lignin decomposition but only one for starch decomposition at the soil depth of 0 to 5 cm, while we did not find significant differences of these genes at the soil depth of 5 to 15 cm. These significantly enriched genes are linked closely with decomposing plant litter and soil organic matter. The physiological activity of soil microorganisms is greatly driven by the input of organic substrates (27), and it has been shown that the effects of eCO_2 on plant litter production could change the substrate input to soil (36). Increases in plant litter productivity and soil C content were observed at the soil depth of 0 to 5 cm at the ORNL FACE site (Table 1) (13, 37, 38), which could stimulate plant-induced microbially mediated C decomposition in soil (17). Such effects of eCO_2 coincided with those found by previous research on soil microbial communities (3, 17, 30). However, this effect was weakened at the soil depth of 5 to 15 cm, and the total signal intensities of almost all of these genes increased only slightly under eCO_2 (Fig. 2). For C fixation, the total signal intensities of RubisCO and PCC genes increased significantly at the soil depth of 0 to 5 cm, and that for only the PCC gene increased significantly at the soil depth of 5 to 15 cm (Fig. S4), suggesting that C fixation processes

for microorganisms may be more abundant at the soil depth of 0 to 5 cm than at the depth of 5 to 15 cm. The total signal intensities of *mcrA* for methane production were enhanced under eCO_2 at two depths, and the difference was significant in the soil depth of 5 to 15 cm. In addition, the numbers of these genes derived from *Archaea* and *Bacteria* increased under eCO_2 at both depths, especially at the lower soil depth of 5 to 15 cm, where the difference was significant ($P < 0.05$). Previous studies showed that the abundance of methanogenic archaea significantly increased in rice-cultivated soil under eCO_2 (55, 56) because of the increase in the soil carbon input (e.g., root exudates) with elevated CO_2. Our results revealed that soil methane emission was potentially stimulated in response to eCO_2 in a forest ecosystem, which is in agreement with a previous report as well (30).

Second, microbially mediated soil N cycling provided a positive feedback to eCO_2. The soil C and N cycles have strong linkages in all terrestrial ecosystems, and N supply is well documented as an important constraint to limit the productivity of terrestrial ecosystems under eCO_2 at these FACE sites (36, 46, 57). Due to the high concentrations of N and carbohydrates, decaying plant materials are important for the flux of biological monomers C and N to the soil (58, 59). The production of plant materials was reported to be increased at an ORNL site under eCO_2, leading to an increasing input of C and N to the soil profile (13, 38). It remains unclear how soil microbial communities modify their functional processes (N cycling) in response to eCO_2. GeoChip data showed that the total signal intensities of *nifH* involved in N_2 fixation and *ureC* involved in ammonification significantly increased at eCO_2 at the soil depth of 0 to 5 cm and remained unchanged at the soil depth of 5 to 15 cm (Fig. 3). Such responses of these genes may increase N fixation and ammonification processes at the soil depth of 0 to 5 cm under eCO_2, which could relieve progressive N limitation at FACE sites (4, 57, 60). The total signal intensities of key genes involved in denitrification were generally increased under eCO_2, and significantly higher signal intensities were observed for *narG* and *nirS*. The results suggest that the denitrification processes could also be enhanced under eCO_2, which may result in an increase of N_2O emission as previously observed (61, 62). In addition, the key genes involved in assimilatory N reduction (*nasA* and *nirB*), dissimilatory N reduction (*nrfA*), and nitrification (*amoA*) were also significantly increased. It appears that N cycling could be entirely stimulated under eCO_2, especially at the soil depth of 0 to 5 cm, where microbial communities were more sensitive to environmental change. This could be well explained by the change of plant litter productivity. Plants may influence microbial communities directly by the changes in plant litter inputs and root exudates or indirectly by their capacity to influence soil properties (e.g., organic matter and nutrient availability) (63, 64). At this site, the C and N input to soil may be altered by the changes in leaf or fine root quantity at two depths (0 to 5 cm and 5 to 15 cm) (13, 23), which may have considerable effect on microbial communities. Previous research also reported that the increase of C and N input to the soil from fine roots under eCO_2 was mainly in deep soil (below 30 cm), which were not assessed in our study (13).

As soil microbial communities may shift with the changing of environmental variables (e.g., soil C and N concentrations) (65), it is imperative to establish links between environmental variables and microbial communities for FACE sites. Plant and soil properties were generally considered main factors that largely determine the soil microbial community structure (63, 66). The whole microbial functional composition significantly correlated with selected soil, plant, and environmental variables ($P < 0.05$) (Fig. 4b). Variations in microbial communities were explained by soil and plant properties at two depths (0 to 5 cm and 5 to 15 cm), indicating that at the soil depth of 0 to 5 cm, the microbial community is more sensitive to plant and soil properties than at the soil depth of 5 to 15 cm.

In summary, this study highlights that functional microbial communities may be altered under eCO_2 treatments in forest ecosystems. We found that eCO_2 significantly altered the microbial functional diversity, composition, and structure and increased the abundance of a large number of key functional genes involved in C and N cycling at the soil depths of 0 to 5 cm and 5 to 15 cm, which may ultimately feed back to the

ecosystem level responses to elevated CO_2 at the ORNL FACE site. In addition, the effect of eCO_2 was more pronounced at the soil depth of 0 to 5 cm, with increases of leaf litter productivity and C input to the soil. Since fine roots are critical to soil C input, further studies are needed to understand how microbial community structure and function shift in response to eCO_2 at deeper soil depths (below 15 cm) in forest ecosystems.

MATERIALS AND METHODS

Site description and experimental design. The ORNL FACE experiment was established on a sweetgum (*Liquidambar styraciflua* L.) plantation in the Oak Ridge National Environmental Research Park, TN, USA (35°54′N, 84°20′W). Five 25-m-diameter plots (46) were constructed at the FACE site, with two plots for elevated CO_2 (~560 ppm in 2008) treatment and three plots for ambient CO_2 (~405 ppm in 2008) treatment. The mean annual temperature and precipitation are 13.9°C and 1,322 mm, respectively. A more complete site description has been documented previously (33, 67). The soil at the FACE site is slightly acidic (pH 5.5 to 6.0) and is an alluvial aquic hapludult with a silty clay loam texture (68). Soil samples were collected from four plots, including two of three aCO_2 plots (plot 1 and plot 2) and two eCO_2 plots (plot 4 and plot 5). Triplicate soil samples were collected from each plot at the soil depths of 0 to 5 cm and 5 to 15 cm in July 2008 after more than 10 years of fumigation treatment. After the removal of plant roots and large stones, 24 soil samples were immediately stored at −20 or 4°C for DNA extraction or soil property analysis, respectively. Plant property data were collected from the FACE data management system (https://facedata.ornl.gov/) (69).

Soil property analysis. The total C and total N of soil samples were measured by a Leco Truspec dry combustion carbon analyzer (70), and NO_3-N and NH_4-N of were extracted from soil samples by the use of a 1.0 M KCl solution and were quantified by a flow injection autoanalyzer.

DNA extraction, amplification, labeling, and hybridization. Microbial community DNA was extracted from 5-g soil samples with the freeze-grinding method as described previously (71). DNA quality was assessed by an ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, NC) using the ratios of 260/280 nm and 260/230 nm, and DNA concentration was quantified with Quant-It PicoGreen (Invitrogen, Carlsbad, CA). Approximately 100 ng of DNA from each sample was amplified with a TempliPhi amplification kit (Amersham Biosciences, Piscataway, NJ) and labeled with Cy5 (72). The fluorescently labeled DNA was hybridized with the GeoChip 3.0 on a MAUI hybridization system (Biomicro Systems, Salt Lake City, UT) at 42°C for 12 h (39).

GeoChip data processing and statistical analysis. Gene- and group-specific probes for GeoChip 3.0 were designed by CommOligo 2.0, and the oligonucleotide probes were synthesized by Invitrogen (Carlsbad, CA). These probes were arrayed onto Corning UltraGAPS (Corning, NY) slides by a Microgrid II arrayer (Genomic Solutions, Ann Arbor, MI) as described previously (39). The microarray slides were scanned by a Pro Scan array microarray scanner (PerkinElmer, Boston, MA) with 95% laser power and 75% photomultiplier tube (PMT) gain, and the images were analyzed by ImaGene 6.0 (Biodiscovery, El Segundo, CA). Raw data were upload to the IEG microarray processing pipeline (http://ieg.ou.edu/microarray/) after the removal of poor spots with a signal-to-noise ratio (SNR) (SNR = [signal mean − background mean]/background standard deviation) of >2.0 as previously reported (73). Positive genes were those that were detected with at least two probes from 6 replicates samples. These positive genes were left for further statistical analyses.

The differences between soil properties and the total signal intensities of individual genes at eCO_2 and aCO_2 at different depths (0 to 5 cm and 5 to 15 cm) were calculated by unpaired *t* tests. The differences of microbial functional gene structures and phylogenetic structures between eCO_2 and aCO_2 at each depth were analyzed by detrended correspondence analysis (DCA), permutational multivariate analysis of variance (Adonis), analysis of similarities (ANOSIM), and a multiresponse permutation procedure (MRPP). The correlation between the microbial functional structures and environmental variables was evaluated by canonical correspondence analysis (CCA) and variation partitioning analysis (VPA). All statistical analyses were performed with R project v.3.2.1 (www.R-project.org).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01694-17.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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