

Elevated Carbon Dioxide Alters the Structure of Soil Microbial Communities

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Pyrosequencing analysis of 16S rRNA genes was used to examine impacts of elevated CO₂ (eCO₂) on soil microbial communities from 12 replicates each from ambient CO₂ (aCO₂) and eCO₂ settings. The results suggest that the soil microbial community composition and structure significantly altered under conditions of eCO₂, which was closely associated with soil and plant properties.

The concentration of atmospheric CO₂ is increasing, largely due to human activities, and it is projected to reach 700 μmol/mol by the end of this century (15, 17). Although it is well established that elevated CO₂ (eCO₂) stimulates plant growth and primary productivity (2, 19, 23), the impact of eCO₂ on soil microbial communities remains poorly understood (5, 7, 11–14, 18, 22, 28). Pyrosequencing of 16S rRNA genes has been used to analyze the diversity, composition, structure, and dynamics of microbial communities from different habitats, such as soil (1, 6, 10, 24, 25, 27), water (4, 21), fermented foods (16), and human gut (26). In this study, we aimed to (i) examine effects of eCO₂ on the taxonomical diversity, composition, and structure of soil microbial communities and (ii) link soil and plant properties with the microbial community composition and structure by the use of tag-encoded pyrosequencing of 16S rRNA genes.

To address those issues, this study was conducted within the BioCON (Biodiversity, CO₂, and Nitrogen) experiment site located at the Cedar Creek Ecosystem Science Reserve in Minnesota (23). For this study, 24 soil samples (12 from ambient CO₂ [aCO₂] settings, 12 from eCO₂ settings, and all with 16 plant species and without an added nitrogen supply) were collected in July 2007. eCO₂ plots have been treated since 1997 during the plant growing season (May to October) every year, and each sample was composited from five soil cores at a depth of 0 to 15 cm. Details are provided in Materials and Methods in the supplemental material.

The V4 and V5 regions of 16S rRNA genes were amplified using a conserved primer pair with a unique 6-mer oligonucleotide sequence (barcode) at the 5' end for each sample (see Table S1 in the supplemental material). After preprocessing of all reads, 30,008 and 29,091 high-quality sequences were obtained for aCO₂ and eCO₂ samples, respectively. The numbers of sequences from different samples ranged from 1,854 to 3,087 for aCO₂ samples and from 1,698 to 3,299 for eCO₂ samples (see Table S2 in the supplemental material). All sequences were aligned using the RDP Infernal Aligner, and the complete linkage clustering method was used to define operational taxonomic units (OTUs) using 97% identity as a cutoff, resulting in 2,527 and 2,354 OTUs for aCO₂ and eCO₂, respectively (see Table S2 in the supplemental material). The average Shannon indices were 6.58 and 6.51 for aCO₂ and eCO₂ samples, respectively (see Table S2 in the supplemental material).

However, no significant ($P > 0.05$) differences were seen in the numbers of sequences, OTUs, or Shannon diversity index between aCO₂ and eCO₂ samples. In total, 3,500 OTUs were detected for at least 2 of 12 aCO₂ or eCO₂ samples, phylogenetically deriving from one known archaeal phylum and 16 known bacterial phyla as well as unclassified phylotypes. Most phylotypes were detected at both aCO₂ and eCO₂, with only a few detected at only aCO₂ or eCO₂ (see Table S3 and Fig. S1 in the supplemental material). At the phylum level, 811 (23.2%) OTUs were derived from *Proteobacteria*, a phylum with the highest number of detectable OTUs, followed by *Actinobacteria* (596; 17.0%), *Bacteroidetes* (448; 12.8%), and *Acidobacteria* (369; 10.5%) (Table 1).

To examine whether eCO₂ affects the taxonomical composition and structure of soil microbial communities, principal coordinate analysis (PCoA) was performed with the relative abundance values of 454 pyrosequencing data. Two distinct clusters were formed, and aCO₂ samples were well separated from eCO₂ samples (Fig. 1). The results of three nonparametric multivariate statistical tests, ANOSIM (8), Adonis (3), and MRPP (20), showed significant differences ($P = 0.001$ and $\delta = 0.481$, $P = 0.003$ and $R = 0.209$, and $P = 0.001$ and $R^2 = 0.082$, respectively) based on the abundance of all OTUs detected at aCO₂ and eCO₂, and such significances ($P < 0.05$) were also observed at the phylum level, including *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, DP10, *Proteobacteria*, TM7, and WS3 (see Table S4 in the supplemental material). The results indicated that the overall taxonomic composition and structure of soil microbial communities shifted in response to eCO₂.

To further examine effects of eCO₂ on microbial communities, we analyzed both significantly changed and unique OTUs. Among

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TABLE 1 Numbers of OTUs detected under aCO₂ and eCO₂ conditions

| Domain and phylum | No. of OTUs | | <i>P</i> (unpaired <i>t</i> test) | |
|-------------------------|------------------------|------------------|-----------------------------------|------|
| | Total ^a (%) | Avg ^b | | |
| | | aCO ₂ | eCO ₂ | |
| <i>Archaea</i> | | | | |
| <i>Crenarchaeota</i> | 10 (0.28) | 4.42 ± 1.73 | 3.17 ± 1.59 | 0.08 |
| Unclassified | 1 (0.03) | | | |
| <i>Bacteria</i> | | | | |
| <i>Acidobacteria</i> | 369 (10.50) | 112.33 ± 20.08 | 91.08 ± 19.11 | 0.01 |
| <i>Actinobacteria</i> | 596 (17.00) | 179.83 ± 26.18 | 179.25 ± 25.52 | 0.96 |
| <i>Bacteroidetes</i> | 448 (12.80) | 105.58 ± 24.54 | 100.17 ± 21.07 | 0.57 |
| BRC1 | 2 (0.57) | 0.17 ± 0.39 | 0.25 ± 0.45 | 0.63 |
| <i>Chlamydiae</i> | 36 (1.03) | 3.08 ± 2.39 | 3.92 ± 3.00 | 0.46 |
| <i>Chloroflexi</i> | 94 (2.69) | 17.33 ± 4.23 | 14.92 ± 6.04 | 0.27 |
| <i>Cyanobacteria</i> | 3 (0.86) | 0.75 ± 0.87 | 0.17 ± 0.39 | 0.04 |
| <i>Firmicutes</i> | 118 (3.4) | 28.58 ± 5.65 | 24.33 ± 5.99 | 0.09 |
| <i>Gemmatimonadetes</i> | 86 (2.46) | 22.83 ± 5.81 | 17.92 ± 5.52 | 0.05 |
| <i>Nitrospirae</i> | 6 (0.17) | 3.00 ± 1.48 | 2.00 ± 1.04 | 0.07 |
| OP10 | 8 (0.23) | 1.42 ± 1.51 | 0 | 0.00 |
| <i>Planctomycetes</i> | 251 (7.17) | 42.00 ± 8.82 | 39.67 ± 12.37 | 0.60 |
| <i>Proteobacteria</i> | 811 (23.2) | 207.08 ± 30.79 | 220.33 ± 30.22 | 0.30 |
| TM7 | 52 (1.49) | 7.83 ± 3.79 | 6.25 ± 4.11 | 0.34 |
| <i>Verrucomicrobia</i> | 127 (3.63) | 29.33 ± 8.32 | 22.92 ± 8.01 | 0.07 |
| WS3 | 6 (0.17) | 1.00 ± 0.74 | 0.83 ± 1.19 | 0.68 |
| Unclassified | 454 (13.00) | | | |
| Total unclassified | 22 (0.63) | | | |
| Total | 3,500 (100) | 846.75 ± 108.71 | 800.5 ± 122.03 | 0.34 |

^a Data represent total numbers of OTUs detected by pyrosequencing across all 24 samples.

^b Data represent average numbers of OTUs detected using 12 samples under aCO₂ or eCO₂ conditions.

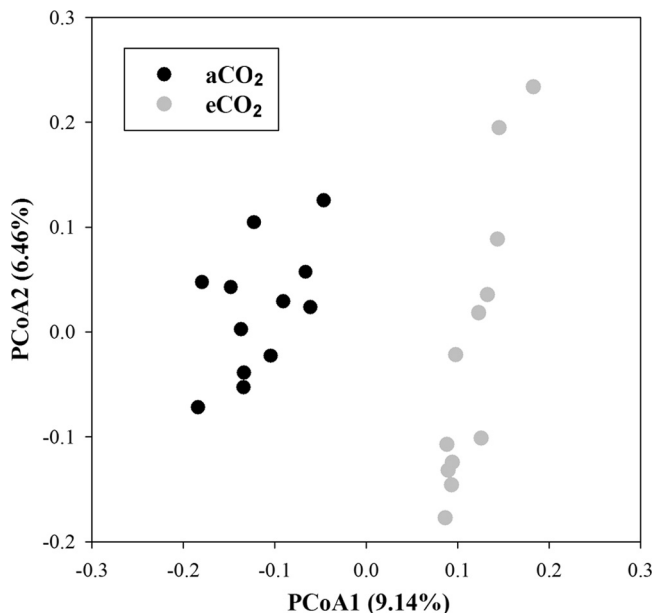


FIG 1 Principal coordination analysis (PCoA) of soil microbial community structure based on the relative abundances of OTUs detected by 454 pyrosequencing.

3,500 OTUs detected, 1,381 were shared by aCO₂ and eCO₂ samples, and 1,146 and 973 unique OTUs were detected at aCO₂ and eCO₂, respectively. For those shared OTUs, 27 were significantly ($P < 0.05$) decreased and 36 were significantly ($P < 0.05$) increased at eCO₂ (see Table S5 in the supplemental material). For example, 26 significantly changed OTUs were found in *Actinobacteria*, with 7 decreased and 19 increased at eCO₂ (see Table S5 and Fig. S2 in the supplemental material). Also, 11 OTUs were significantly ($P < 0.05$) changed at eCO₂, with 9 increased and 2 decreased in *Proteobacteria*, and 5, 3, 2, and 1 OTUs were derived from *Alpha*-, *Beta*-, *Delta*-, and *Gammaproteobacteria*, respectively (Fig. 2; see also Table S5 in the supplemental material). In addition, significantly ($P < 0.05$) changed OTUs in other phyla, such as *Acidobacteria*, *Bacteroidetes*, and *Planctomycetes*, were observed (Fig. 2; see also Table S5 in the supplemental material). A large percentage (60.5%) of unique OTUs was detected by pyrosequencing, with 1,146 (32.7%) at aCO₂ and 973 (27.8%) at eCO₂, and those OTUs were largely derived from the most abundant phyla (see Table S5 in the supplemental material). For example, 213 and 243 unique OTUs were detected at aCO₂ and eCO₂, respectively, for *Proteobacteria*, and 170 and 150 for *Actinobacteria*. The analysis of significantly changed and unique OTUs confirmed that the taxonomic composition and structure of soil microbial communities significantly changed at eCO₂.

To understand whether microbial populations differentially respond to eCO₂, detected OTUs were mapped to their associated

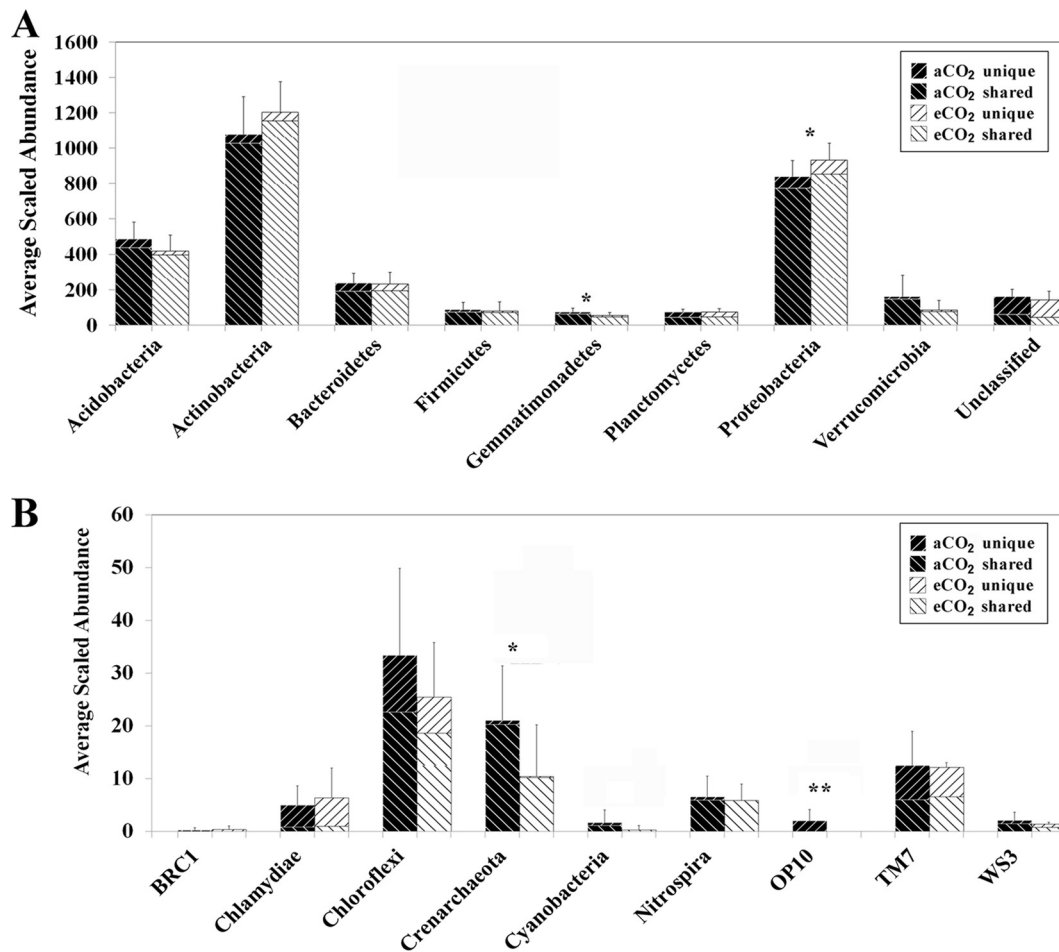


FIG 2 Averages of scaled relative abundances of detected OTUs at the phylum level for both aCO₂ and eCO₂ samples. Data are presented as means \pm standard errors, with 12 samples for each CO₂ condition. Generally, each bar has two portions: the top part is for unique OTUs detected only at aCO₂ or eCO₂ and the bottom part for shared OTUs detected under both aCO₂ and eCO₂ conditions. *, $P < 0.05$; **, $P < 0.01$.

populations at the phylum or lower levels. Based on relative abundances of all OTUs in a given phylotype with at least six OTUs detected, significantly changed populations were identified by response ratio (19). At the phylum level, four phyla, including one archaeal phylum (*Crenarchaeota*) and three bacterial phyla (*Proteobacteria*, *Gemmatimonadetes*, and DP10), showed significant ($P < 0.05$) changes in their relative abundances, but other phyla, including *Actinobacteria*, the most abundant phylum, did not show significant changes in relative abundances at eCO₂ (Fig. 3). A further examination of those significantly changed phyla showed that those changes occurred in some specific microbial populations at the class or lower levels. In *Proteobacteria*, the relative abundances of some orders, such as *Caulobacterales* of *Alphaproteobacteria*, *Myxococcales* of *Deltaproteobacteria*, and *Xanthomonadales* of *Gammaproteobacteria*, significantly ($P < 0.05$) increased at eCO₂ (see Fig. S3 in the supplemental material). Also, although significant changes were not seen at eCO₂ for the most abundant phyla at the phylum level, such significances were detected at class or lower taxonomic levels. For example, in *Acidobacteria*, significant ($P < 0.05$) changes were observed in the relative abundances in the classes of Gp1, Gp2, and Gp3 but not in other classes (see Fig. S4A in the supplemental material); in *Verrucomicrobia*, all

significant ($P < 0.05$) changes appeared to be decreased in *Spartobacteria* but not in other classes (see Fig. S4B in the supplemental material); in *Firmicutes*, the relative abundances of unclassified phylotypes were significantly ($P < 0.05$) decreased (see Fig. S4C in the supplemental material). These results indicated that eCO₂ might differentially affect some specific microbial populations at different taxonomic levels such as phylum, class, and order, with decreased or increased relative abundances at eCO₂. Recently, a study using a comprehensive functional gene array, GeoChip 3.0 (12), demonstrated that the functional composition and structure of soil microbial communities were significantly altered at eCO₂ in BioCON, which may have been due to eCO₂-induced shifts in microbial populations. However, the linkage between taxonomical populations and their functions of soil microbial communities needs further investigations.

To link the microbial community structure with soil and plant properties, Mantel tests were performed. First, using the BioENV procedure (9), four plant variables, including the belowground carbon percentage (BPC), aboveground carbon percentage (APC), aboveground total biomass (ATB) and total biomass (TB), and four soil variables, including the proportion of soil moisture at the depth of 0 to 17 cm (PSM0–17), the percentage of C at the

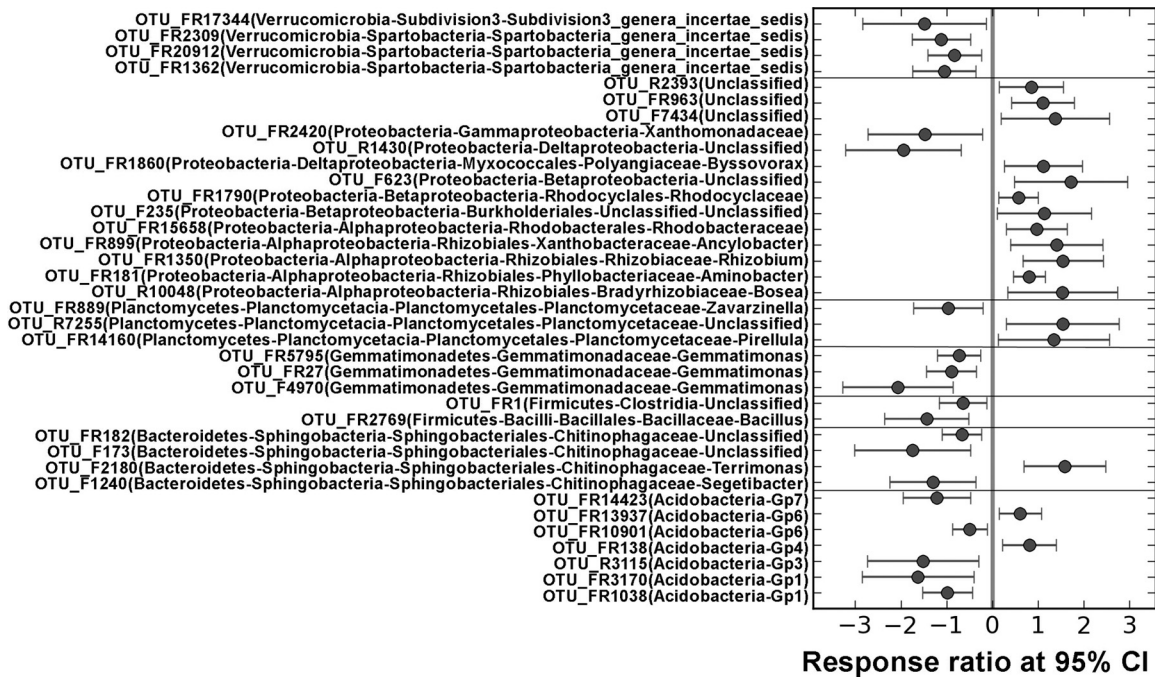


FIG 3 Significantly changed OTUs in the phyla of *Acidobacteria*, *Bacteroidetes*, *Firmicutes*, *Gemmatimonadetes*, *Planctomycetes*, *Proteobacteria*, *Verrucomicrobia*, and unclassified phylotypes at elevated CO₂ determined using the response ratio method at a 95% confidence interval.

depth of 0 to 10 cm (C0–10), the percentage of N at the depth of 0 to 10 cm (N0–10), and net N mineralization, were selected for partial Mantel tests (see Table S6 in the supplemental material). Second, partial Mantel tests were performed to correlate the microbial community measured by the relative abundances of all detected 3,500 OTUs with the selected sets of plant and soil variables described above. Although the microbial community was not significantly ($P > 0.05$) correlated with plant variables or soil variables (see Table S6 in the supplemental material) at the community level, significant correlations were observed with specific microbial populations at different taxonomic levels (phylum, class, order, family, and genus). At the phylum level, five phyla (*Firmicutes*, *Proteobacteria*, *Chlamydiae*, *Gemmatimonadetes*, and *Nitrospirae*) were significantly ($P < 0.05$) correlated with the selected plant properties (see Table S6 in the supplemental material). At the class level, nine classes were significantly ($P < 0.05$) correlated with soil or plant characteristics. *Bacilli*, *Clostridia*, *Alphaproteobacteria*, *Chlamydiae*, *Nitrospirae*, and an unclassified phylotype from the TM7 phylum were significantly ($P < 0.05$) correlated with the selected plant variables, subdivision 3 of *Verrucomicrobiae* ($P = 0.05$) with soil variables, and *Gammaproteobacteria* ($P < 0.05$) with both soil and plant variables (Table 2). Similarly, significant ($P < 0.05$) correlations between the microbial community and the selected plant and/or soil properties were detected in 12 orders (see Table S7 in the supplemental material), 23 families (see Table S8 in the supplemental material), and 48 genera (see Table S9 in the supplemental material). In addition, many unclassified phylotypes were significantly ($P < 0.05$) correlated with the selected soil or/and plant variables, suggesting that soil and plant factors may also shape taxonomically uncharacterized microbial communities (Table 2; see also Table S6, S7, S8, and S9 in the supplemental material). The results presented above suggest that the variables selected greatly influenced the taxonomic

composition and structure of microbial communities in this grassland ecosystem.

In summary, this study used pyrosequencing technologies to examine the taxonomic diversity, composition, and structure of soil microbial communities in a grassland ecosystem and their responses to eCO₂. The microbial community composition and structure significantly shifted at eCO₂, potentially due to differen-

TABLE 2 Partial Mantel analysis of the relationship between the relative abundance of class and soil or plant properties^a

| Phylum | Class | Soil, ^b partial | | Plant, ^c partial | |
|------------------------|----------------------------|----------------------------|----------|-----------------------------|----------|
| | | <i>r</i> | <i>P</i> | <i>r</i> | <i>P</i> |
| All detected OTUs | | -0.018 | 0.508 | 0.266 | 0.065 |
| <i>Firmicutes</i> | <i>Bacilli</i> | 0.184 | 0.118 | 0.379 | 0.020 |
| | <i>Clostridia</i> | 0.040 | 0.380 | 0.316 | 0.014 |
| <i>Proteobacteria</i> | <i>Alphaproteobacteria</i> | 0.137 | 0.139 | 0.300 | 0.012 |
| | <i>Betaproteobacteria</i> | 0.220 | 0.077 | 0.470 | 0.003 |
| | <i>Gammaproteobacteria</i> | 0.317 | 0.023 | 0.353 | 0.021 |
| TM7 | Unclassified | -0.065 | 0.621 | 0.415 | 0.010 |
| <i>Chlamydiae</i> | <i>Chlamydiae</i> | 0.192 | 0.157 | 0.356 | 0.026 |
| <i>Nitrospira</i> | <i>Nitrospira</i> | -0.033 | 0.520 | 0.404 | 0.013 |
| <i>Verrucomicrobia</i> | Subdivision 3 | 0.289 | 0.049 | -0.193 | 0.922 |

^a Only significantly ($P < 0.05$) changed phylotypes are shown.

^b Selected soil variables included proportion soil moisture at the depth of 0 to 17 cm (PSM0–17), percent C at the depth of 0 to 10 cm (C0–10), percent N at the depth of 0 to 10 cm (N0–10), and net N mineralization.

^c Selected plant variables included below ground carbon percent (BPC), above ground carbon percent (APC), aboveground total biomass (ATB), and total biomass (TB).

tial responses of specific microbial populations to eCO₂. Plant and soil properties, such as plant biomass, soil moisture, and soil C and N contents, may greatly shape the microbial community composition and structure and regulate ecosystem functioning in this grassland.

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