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Common bacterial responses in six ecosystems exposed to ten years of elevated atmospheric carbon dioxide

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Running title: soil bacterial response in six ecosystems

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ABSTRACT

Six terrestrial ecosystems in the U.S. were exposed to elevated CO_2 in single or multi-factorial experiments for more than a decade to assess potential impacts. We retrospectively assessed soil bacterial community responses in all six-field experiments and found ecosystem-specific and common patterns of soil bacterial community response to elevated CO_2. For example, six ecosystems showed a significant change ($p < 0.1$) either in bacterial biomass, richness, or community composition in surface soils exposed to elevated CO_2. However, these response indicators were highly variable. Taxa with significant ($p < 0.1$) changes in relative abundance were detected at all sites, but were largely site-specific. The most striking common trend across sites ($p < 0.05$) was a decrease up to 3.5-fold in the relative abundance of *Acidobacteria* Group 1 bacteria in soils exposed to elevated CO_2 or other climate factors. The *Acidobacteria* Group 1 response observed in exploratory clone libraries was validated at the Wisconsin aspen FACE site by 100-fold deeper sequencing and semi-quantitative PCR assays. Collectively, the results show that climate change treatments influenced dominant soil bacteria in all of the ecosystems. Detection of weak but significant impacts among dominant taxa may be a harbinger of more substantive changes among less abundant, more sensitive populations.

INTRODUCTION

Understanding how soil microbial responses to climate change differ among terrestrial ecosystems is central to predicting regional and global trends. Changes in soil microbial communities can occur as a direct response to variables associated with climate change (e.g., temperature, water availability, nitrogen deposition, concentrations of trace gases like ozone) or as responses to altered plant primary productivity in elevated CO_2. Primary producers in diverse ecosystems have shown common responses to elevated CO_2. For example, higher short term productivity, lower transpiration, increased carbon inputs to soil through litter fall and root activity [1-4], and altered nutritional quality of plant litter or root exudates are common responses of C3 plants [5-14]. However, a corresponding picture of common patterns in soil microbial community responses to climate change variables has not yet emerged. Microbial responses to elevated CO_2 have thus far been examined in single ecosystem studies [15-18]. Concerted studies of multiple ecosystems can conceivably reveal subtle trends that might otherwise be obscured by the high variability often observed in single ecosystem studies.

We initiated a concerted analysis of soil bacterial communities in six climate change field experiments in the United States. The field experiments represent different ecosystems: three tree plantations and three natural ecosystems (Table 1). The dominant species at the three plantation sites are trembling aspen (*Populus tremuloides*; Rhinelander, Wisconsin), loblolly pine (*Pinus taeda*; Durham, North Carolina), and sweetgum (*Liquidambar styraciflua*; Oak Ridge, Tennessee). The natural desert site (Mercury, Nevada) has a patchy plant cover of creosote bush (*Larrea tridentate)*, and perennial bunchgrasses (*Lycium* spp. and *Ambrosia dumosa)*, with cyanobacterial

biological soil crusts that colonize the interspaces between the sparse plant cover. These four sites used a Free-Air Carbon Dioxide Enrichment (FACE) design. The scrub oak/palmetto (*Quercus myrtifolia/Serenoa repens*; Cape Canaveral, Florida) is a brackish coastal site. The brackish tidal marsh (Chesapeake Bay, Maryland) has a dominant cover of *Spartina* and other marsh grasses. These two sites used an Open Top Chamber (OTC) design. Each field site included replicate ambient and elevated CO_2 treatments (340 to 395 ppm ambient versus 534 to 700 ppm elevated CO_2; Table 1). Every ecosystem exhibited significant increases in net primary production under elevated CO_2 [2, 6, 8, 10, 13, 14, 19-23].

Greater primary productivity, plant litter deposition [4, 24, 25], root growth and turnover [3, 9, 26-30], root exudates [11, 31-33], and changes in root depth distributions [34] under elevated CO_2 increase carbon inputs into soil. We sought to determine whether the accumulated changes in primary production induced common soil bacterial responses among ecosystems.

We performed exploratory analyses of 76 soil samples to assess changes in the size and composition of the soil bacterial communities. Given the limited size of our surveys (168 to 338 sequences per sample), detecting responses for individual taxa was constrained to a handful of the most abundant taxa at each site. Furthermore, the spatial scale for sampling inevitably restricted our analyses to the most geographically stable taxa, which may be the least sensitive to small changes in biotic and abiotic variables. Our exploratory study was therefore conservative. Nonetheless, in every ecosystem we found responses to decade-long ecosystem exposure to elevated CO_2. This paper does not attempt to detail the responses in each ecosystem; instead we focus on general patterns

across the ecosystems.

MATERIALS & METHODS

Sample collection. Soil cores were collected from four Free Air CO_2 Enrichment (FACE) field research sites and two Open Top Chamber (OTC) sites. Site characteristics and site nomenclature used in this study are provided in Tables 1 and SOM Table 1. Samples were collected between 2007 and 2008 (SOM Table 1). Three to ten soil cores from semi-random locations in a FACE ring or OTC chamber were obtained from the upper 5 to 10 centimeters (depending on the site), homogenized, and stored at -70ºC for subsequent molecular analysis. At two sites, 30-cm-deep soil cores were obtained for analysis of depth profiles and sectioned into multiple strata prior to homogenization and DNA extraction. For physical and chemical characterization, two portions of the soil samples were sieved with a 2 mm sieve, dried for one week, and sent to the New Mexico State Soil, Water, and Agricultural Testing Lab (http://swatlab.nmsu.edu/) for chemical analysis.

DNA extraction. DNA was extracted from duplicate 0.25g (TN sweetgum) or 0.5g (all other sites) soil samples using the MP Biomedical FastDNA® Spin Kit and duplicates were pooled. These FastDNA extracts were used to assess changes in soil biomass (DNA concentration) or bacterial biomass (Q-PCR assay) across all six sites. Additionally, soil samples from four sites (WI aspen, NC pine, NV desert and TN sweetgum sites) were extracted using MoBio PowerSoil™ DNA Isolation Kit. For 16S rDNA clone library construction, the FastDNA extracts were used for all sites except the WI aspen, NC pine and TN sweetgum sites. At these three sites, the MoBio extracts were

used for clone library construction, in accordance with prior practices at these sites. For each kit, the manufacturer's protocol was followed. DNA extracts were examined on 1.2% agarose gels in 0.5x Tris-borate-EDTA (TBE) with ethidium bromide and quantified using the Quant-It PicoGreen dsDNA Assay Kit (Invitrogen).

Clone libraries. For each soil sample, a 384-member library of 16S rDNA clones was created. 16S rRNA gene fragments of 740 to 760 bp were amplified from soil DNA using primers 27F (5'-AGAGTTTGATCMTGGCTCAG) [35] and 787Rb (5'-GGACTACNRGGGTATCTAAT) [36] PCR was performed in triplicate for each sample. Each 50 µl reaction consisted of 1x PCR buffer containing 1.5 mM $MgCl_2$ (Applied Biosystems), 0.2 mM of each dNTP (Applied Biosystems), 1.5U of Taq LD DNA Polymerase (Applied Biosystems), 1 µM of each primer, and 2 µl of DNA template. Each DNA template was diluted ten- or one hundred-fold in water from the original DNA extract to avoid PCR inhibition. Thermal cycling consisted of 94°C for 2 minutes; 25 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute; 72°C for 7 minutes; and 4°C storage. Product size was confirmed by gel electrophoresis. Triplicate reactions were pooled, purified (Qiagen QIAquick PCR Purification Kit), and cloned (Invitrogen TOPO TA Cloning Kit). Clones were bi-directionally sequenced with M13 primers using Sanger technology.

Sequence processing. Forward and reverse sequences were assembled with Sequencher v4.7 (Ann Arbor, MI). Potential chimeras were identified and discarded by Bellerophon [37]. Remaining sequences were aligned using the Arb-Silva automated alignment tool [38]. Aligned sequences were compiled in a single database in Arb [39].

OTU binning and taxonomic identification. All 28,546 sequences from 97 clone

libraries were placed into Operational Taxonomic Units (OTUs) using the complete-linkage clustering tool from the Ribosomal Database Project (RDP) [40]. The output was parsed with a custom-written C+ program into a matrix of OTU counts per sample. A representative sequence for each OTU at the 0.01 distance level was classified via the RDP classifier [41]. The taxonomic assignments were then applied to appropriate OTUs defined at distance thresholds of 0.03, 0.05, 0.1, 0.15, 0.2, 0.25, and 0.3.

Data analysis. Rarefaction, calculation of sample dissimilarity matrices, and nonmetric multidimensional scaling were performed using functions in the Vegan package 1.15-4 [42] in the R statistical computing platform for Mac v2.9.2 [43]. Other analyses were performed in Microsoft Excel. For trend analyses, the probability of obtaining *n* outcomes in *m* independent trials was computed from a binomial probability distribution. Unifrac tests for homogeneity of clone libraries were performed using the web-based FastUnifrac tool [44].

Bacterial 16S rDNA quantitative PCR: Primers EUB 338 and EUB 518 [35] were used for qPCR of bacterial 16S rDNA. Soil DNA samples were adjusted to 3 to 25 ng/ml in 1X Tris-EDTA (TE). PCR was performed as described in Castro *et al.* [45]. Each 30 µl reaction contained 15 µl of iQ SYBR Green Supermix (BioRad Laboratories), 1.25 mg/ml BSA (Roche Diagnostics GmbH), 1 ml of soil DNA and 133 nM of each primer. Thermal cycling conditions were as follows: one cycle of 95 ºC for 3.25 minutes; 40 cycles of 95 ºC for 15 seconds, 55 ºC for 30 seconds, and 72 ºC for 30 seconds; one cycle of 95 ºC for 1 minute; 80 cycles of 55 ºC for 10 seconds; 4 ºC storage. Reactions were performed with a MyiQ Real-Time PCR machine (BioRad Laboratories).

Quantitative DNA standards for qPCR were produced from an *E. coli* 16S rRNA

gene. A gene fragment was PCR amplified from *E. coli* DNA using primers 27F and 787Rb. Triplicate reactions were performed, pooled, purified and cloned as described for clone libraries (above). *E. coli* clones with inserts of correct size were confirmed by PCR with M13 primers. Suitable *E. coli* clones were grown overnight at 37 ^{0}C in LB broth containing 50 mg/ml of carbenicillin. Plasmid DNA was extracted using the QIAprep Spin Miniprep Kit (Qiagen) and confirmed by gel electrophoresis. Plasmid DNA was linearized by digestion with ScaI (New England Biolabs) at 37^{0}C for 2 hours followed by enzyme inactivation at 80^{0}C for 20 minutes. The 4716 bp DNA was gel-purified, quantified using the quant-it PicoGreen dsDNA Assay kit (Invitrogen), and a dilution series was prepared for use as standards.

16S rDNA Pyrotag Sequencing. 16S rDNA pyrotag data was obtained for twelve soil DNA samples from the WI aspen site. Pyrotag libraries targeting the hypervariable regions V6-V8 amplified with universal primers 926F and 1392R [46] and were sequenced using the 454-titanium platform and standard protocols [47]. PCR reactions and product purification were performed as described above. The sequences were binned into OTUs at the 97% sequence similarity level and a representative sequence of each OTU was taxonomically classified via the RDP.

Quantitative PCR for *Acidobacteria* Group 1. Quantitative PCR was performed using the Biorad iQ SyBr Green Supermix and two primer sets designed for specific detection of *Acidobacteria* Group 1. The two primer sets were 1) acidoG1_8.2 (5'-GGTGCGTGGAATTCCCGG, 5'-GCGGATTGCTTATCGCGTTAG), and 2) acidoG1_8.17 (5'-CCCTTGGGACGTAAACTCCTT, TTCCACGCACCTCTCCCA). Each assay was performed in triplicate with primers at 0.2 μM and 1 ng of soil DNA per reaction.

Cycling conditions were as follows: one cycle of 94º C for 5.0 minutes; 40 cycles of 94ºC for 15 seconds, 65ºC for 30 seconds; 91 cycles of 50.0ºC for 30 seconds; 4.0ºC storage. Melt curves were generated for every run to detect potential false positives. Standard curves were generated with purified, genomic DNA from *Acidobacterium capsulatum.*

Genbank accession numbers. The bacterial 16S rDNA clone sequences were deposited in Genbank with accession numbers XXXXXXXXXX. (Sequences available to reviewers upon request through the Editor).

RESULTS

The results are organized in four sections. The first section provides baseline results on DNA extraction bias, soil characteristics, and general similarity of the bacterial communities in the six ecosystems. The second section describes evidence of community responses to climate treatments in terms of general indicators—biomass, bacterial community richness, and composition. The third section describes the identification of responsive taxa in each ecosystem and the fourth section provides results from follow-up studies in one ecosystem—the first ecosystem from which follow up data were available.

1. Baseline results

DNA extraction bias. Two different DNA extraction methods, MP Biomedical FastDNA® Spin Kit (hereafter referred to as FastDNA) and MoBio PowerSoil™ DNA Isolation Kit (hereafter referred to as MoBio), were used in this study in order to conform with prior practices at each site. To determine the impact of these methodological differences on cross-site comparisons, we applied both methods in parallel on twelve soil samples from four sites (NV desert, NC pine, TN sweetgum, and WI aspen; SOM Table

2) and examined systematic bias in total DNA recovery and DNA composition.

The FastDNA method yielded about 7-fold (range 1 to 19) higher recovery than the MoBio method (SOM Table 3). This was consistent with earlier work in which the FastDNA method provided ten-fold higher recovery on average than the MoBio method for 25 diverse soils collected across the U.S. [48]. This bias did not influence our ecosystem analyses, because we did not attempt to compare DNA quantities between ecosystems.

A systematic bias due to extraction method, in DNA composition was not apparent in the standardized richness or Bray-Curtis dissimilarity of the 16S rDNA libraries (187 to 295 clones per library). There were no significant differences in mean richness for MoBio-derived libraries versus FastDNA-derived libraries at any of the seven taxonomic levels examined (OTU thresholds of 70, 75, 80, 85, 90, 95, and 97% sequence similarity; SOM Table 4). Similarly, no systematic bias was detected in community composition, based on the mean Bray-Curtis dissimilarity of replicate samples extracted using the same or different methods (SOM Figure 1). In other words, bacterial community heterogeneity was similar with both methods. In fact, the average dissimilarity (beta-diversity) arising from use of different methods (n = 12 comparisons) was comparable to the average dissimilarity from duplicate extractions (n = 7 pairs) or from replicate soil samples from the same plot (n = 9 pairs) (Figure 1).

Soil physical and biochemical properties. Soils at each site were characterized independently in previous studies specific for each ecosystem, but inconsistent methods impeded cross-site comparisons. Therefore, we obtained new characterization data. Owing to limited availability of soil from some sites, only two samples per treatment per

ecosystem were analyzed. Soils from the six ecosystems differed considerably in physical and chemical properties (SOM Table 5). Soil pH ranged from 3.3 to 8.0, organic matter content (0 to ≤10cm depth) ranged from 0.8 to 58%, and the concentrations of NO_3, P, Ca^{+2}, Mg^{+2}, and other major inorganic nutrients varied 20-fold or more among ecosystems. For the TN sweetgum and the FL scrub oak/palmetto sites, soil samples were collected over a depth profile; organic matter content declined 2.2 to 4.8-fold with soil depth (SOM Table 5). Based on the physical and chemical characteristics, the forest soils were most similar to one another, and the desert and estuarine marsh soils were distinctive. The decade-long elevated CO_2 treatments in each ecosystem did not cause substantial changes in soil chemistry.

Community similarity across sites. The similarity of bacterial communities generally corresponded with the similarity of soil characteristics. The 97 clone libraries created in this study for comparison of community composition contained 168 to 338 clones (average = 271). The libraries collectively represented 34 to 6980 taxa, depending on the taxonomic level examined (i.e., OTU_{70} to OTU_{97}). Bacterial communities in soils from the WI aspen, NC pine, and TN sweetgum sites were similar (based on species-level profiles), despite large differences in soil type, tree species, mean annual temperature, and spatial separation (about 69 to 1200 km). The natural NV desert, MD marsh, and FL scrub oak/palmetto ecosystems were distinct (Figure 2). There were no species-level (OTU_{97}) taxa common to all six ecosystems, but twelve of the 6980 OTUs were detected in five ecosystems. At coarser taxonomic levels, community similarity among all ecosystems increased, as expected. In ambient surface soils, 17 of 769 total taxa (from all samples) at the family/genus level (OTU_{85}) were detected in all six ecosystems

representing the following: two *Acidobacteria* families from subdivisions 1 and 3, three *Actinobacteria* families (*Mycobacteriaceae*, *Thermomonosporaceae*, *Conexibacteraceae*), a *Bacteriodetes* family (*Chitinophagaceae*), eight *Proteobacteria* families and a *Verrucomicrobia* group (SOM Table 6).

2. Effect of elevated CO_2 on general bacterial community indicators

Soil biomass. Elevated CO_2 treatments had a small and inconsistent effect on total biomass (Figure 3A, SOM Table 7). Extracted soil DNA was used as a proxy for soil biomass. Values ranged from 1.1 μg/gram of soil (FL scrub oak/palmetto, elevated CO_2, 10-30 cm depth) to 70.8 μg/gram of soil (NC pine, nitrogen fertilized, 0-5cm depth; SOM Table 7). Soil DNA increased 0.1 to 30% in four elevated CO_2 treatment comparisons, but decreased 8 to 51% in the other four cases (Figure 3A and SOM Table 7). Only the two largest changes—the 30% increase in the TN sweetgum soils and the 51% decrease in FL scrub oak/palmetto soils—were statistically significant ($p < 0.1$ and $p < 0.06$, respectively). Thus, no consistent trend was detected for the impact of elevated CO_2 on total soil biomass in surface soils. Slightly larger changes ranging from 50 to 270% occurred in soil biomass in response to soil depth (examined at two sites) or nitrogen fertilization (examined at one site) (Figure 3A and SOM Table 7).

Bacterial biomass. The influence of elevated CO_2 exposure on bacterial biomass, estimated by qPCR of 16S rRNA genes, was generally similar to the effects on total soil biomass (Figure 3B, SOM Table 7). The estimated copy number of bacterial 16S rDNA in soil ranged from 1.8×10^8 (FL scrub oak/palmetto, elevated CO_2, 10-30 cm depth) to 3.4×10^{10} (TN sweetgum, elevated CO_2). Elevated CO_2 treatments did not substantially alter bacterial biomass in surface soils. The 16S rRNA gene copy number increased in

four cases by 4 to 10%, and decreased in the other four cases by 26 to 49%. Only the 49% decrease in surface soil with elevated CO_2 at the FL scrub oak/palmetto site was significant (t-test, $p < 0.1$). In contrast, larger declines of 2.4 to 9.7 fold occurred with soil depth or with nitrogen fertilization (Figure 3A and SOM Table 7).

Community richness. Bacterial taxon richness increased in elevated CO_2 in two of six ecosystems (Table 2, SOM Table 9). To avoid potential spurious effects from imperfect binning of sequences into OTUs, we looked only for robust differences in richness—that is, significant differences at two or more successive taxonomic levels. Significant changes in community richness in response to elevated CO_2 were detected in the NV desert and WI aspen sites. Taxon richness in the NV desert creosote bush root zone soil increased 14% at two taxonomic levels, OTU_{85} and OTU_{80} ($P < 0.1$). At the WI aspen site, taxon richness increased 11 to 21% at two to five taxonomic levels (OTU_{70} to OTU_{90}, varying by treatment; $p < 0.1$) in plots exposed to elevated CO_2, elevated ozone, or both.

Community composition. The impact of elevated CO_2 exposure on community similarity was apparent in three of six ecosystems. Community similarity was initially compared using Unifrac tests for homogeneity. However, in all ecosystems except the TN sweetgum site, significant differences occurred among replicates within treatments (data not shown), demonstrating that soil heterogeneity and treatment effects could not be distinguished. As an alternative, we evaluated treatment impacts based on t-tests of the mean beta diversity among samples within and between treatments. Only robust differences occurring at two or more taxonomic levels were considered an indicator of treatment impacts. The MD marsh soil bacterial communities showed the clearest

evidence of elevated CO_2 impacts on beta diversity. Soil communities from elevated CO_2 plots were more similar to one another than communities from ambient plots (Figure 4), and significant differences occurred at five taxonomic levels (OTU_{70} to OTU_{90}; SOM Table 11). In the NV desert ecosystem, a weak signal at two taxonomic levels (OTU_{70} and OTU_{75}; SOM Table 11) occurred in creosote bush root zone soil. Similarly, a weak signal of elevated CO_2 impact at two taxonomic levels (OTU_{80} and OTU_{85}) occurred in the NC pine soil.

The collective results show that responses of bacterial biomass, community richness, and community composition under elevated CO_2 varied greatly across the ecosystems. While all six ecosystems exhibited a significant change in at least one of these general indicators, no consistent trends were apparent.

3. Responsive taxa

Changes in relative abundance. Individual taxa responsive to elevated CO_2 treatments were detected in every ecosystem. Putative responsive taxa were defined as taxa with a significant, two-fold or greater change in average relative abundance in soils under ambient versus elevated CO_2. We restricted the analysis to *adequately sampled* taxa—i.e, taxa detected in at least three replicates from ambient and three replicates from elevated CO_2 treatments (or two replicates per treatment at the sweetgum site). Although this criterion substantially reduced the number of useable taxa, it eliminated the use of missing data (zero counts). Collectively, the sites had an average of 21 OTUs (range = 2 to 75) suitable for analysis per taxonomic level per treatment comparison. We used t-tests as a *screen* for responsive taxa. Therefore, we tolerated the risk of false positives and did not apply a Bonferroni (or any other) correction for the number of tests performed. At $p <$

0.05, 45 taxa with significant responses to elevated CO_2 treatments were collectively identified among the six ecosystems (SOM Table 12). Some of these taxa showed significant responses in multiple comparisons or at multiple sites. On average, 2.7% of the adequately sampled taxa per ecosystem showed significant responses to elevated CO_2. The capacity of this analytical approach to capture higher magnitude responses was demonstrated in three ecosystems with 11 to 12% of taxa responsive to soil depth (two sites) or nitrogen deposition (one site) (SOM Table 12).

Trends across sites. Additional taxa responsive to climate change treatments were identified by a trend analysis across sites. Taxa may exhibit significant trends across sites yet have non-significant responses within individual sites owing to local, mitigating ecological factors or to sampling artifacts. The trend analysis for each taxon involved up to twelve comparisons of ambient versus elevated CO_2. For this analysis, we included only taxa that were detected in two or more replicates from ambient and from elevated CO_2 conditions and present in four or more of the twelve test cases among ecosystems. Thus, this analysis was slightly less stringent in terms of consistent detection within sites, but was considerably more stringent in terms of detection across sites. Only 214 taxa (of 14,716 total) from OTU_{75} to OTU_{97} fulfilled these criteria. Surprisingly, 21% (46 of 214) of these taxa showed significant response trends ($p < 0.1$) (SOM Table 13). The taxa belonged to eight phyla—*Acidobacteria*, *Actinobacteria*, *Proteobacteria*, *Verrucomicrobia, Nitrospira, Firmicutes, OD1, and TM7*—and represent possible targets for further ecological analysis.

Acidobacteria Group 1 and *Acidobacteria* Group 2 taxa exhibited the most consistent and robust trends (Table 4). The *Acidobacteria* Group 1 taxa that showed

significant trends included groups from the phylum/class level (OTU_{75}) to the genus/species level (OTU_{95}) and were present in four to ten of the twelve test cases of ambient soils versus soils under elevated CO_2. Group 1 showed a significant trend ($p < 0.08$), by an average of 20 to 60% across sites (Figure 5). Similarly, *Acidobacteria* Group 2 taxa showed significant trends ($p < 0.07$), generally declining in treated soils by an average of 50 to 210% across sites. Although the magnitude of these responses is small, taxa that demonstrate significant patterns across sites may be indicators of broadly occurring but difficult to measure mechanistic phenomena.

4. Validation—deeper sequencing and group-specific qPCR assays

Pyrotag sequencing at the WI aspen site. One hundred-fold deeper sequencing at the WI aspen site supported observations from the exploratory clone library surveys. Pyrotag sequencing data were not yet available at other sites, and thus were not included in this analysis. A total of 31,737 to 75,663 16S rDNA sequences (c.a. 400 nt each) were obtained from each of three replicate soil samples from ambient plots and three replicate samples from plots exposed to elevated CO_2 (SOM Table 14). The sequences were clustered into OTUs using a 99% sequence similarity. Based on the pyrotag data, the communities exposed to elevated CO_2 showed a 16% increase in richness, but the increase was not significant, consistent with results from the exploratory clone libraries (Table 2). Similarly, no significant difference was detected in the composition (beta diversity) of the libraries from ambient and elevated CO_2 treatments (data not shown). However, responsive taxa were identified using the same approach described above for the clone libraries. Of the 1313 taxa that were consistently detected in all replicates, 75 taxa showed significant ($P < 0.05$), two-fold or greater changes in average relative

abundance (SOM Table 15). The 75 taxa represent about 6% of the 1313 tested, whereas an average of 2% of the adequately sampled taxa in the corresponding clone libraries appeared responsive. Of the 75 taxa, the relative abundance of 34 taxa increased two- to five-fold, while 41 taxa decreased 2 to 11 fold under elevated CO_2. As in the exploratory libraries, the *Acidobacteria* Groups 1 and 2 declined two- to three-fold under elevated CO_2 (Figure 6), consistent with the two- to three-fold decrease observed in the clone libraries.

Quantification of specific Acidobacteria groups by qPCR. The change in abundance of *Acidobacteria* Group 1 at the WI aspen site was validated further by qPCR assays. We designed and tested two assays (data not shown) that collectively had a predicted coverage of 36% of the 8430 *Acidobacteria* Group 1 sequences obtained from the RDP. The assays showed significant ($p<0.006$), five-fold declines in *Acidobacteria* Group 1 taxa in WI aspen soil samples exposed to elevated CO_2. The declines detected by qPCR were nearly twice as large as the declines measured in 16S rDNA clone or pyrotag surveys, possibly indicating that sequence surveys underestimate the magnitude of changes in bacterial communities.

DISCUSSION

Predicting climate change impacts requires knowledge of general ecosystem response patterns and their variability. Among six ecosystems, we found evidence of patterns in bacterial population responses to a decade of elevated CO_2 treatment. About 21% of the dominant taxa detected in multiple ecosystems displayed significant ($p < 0.1$) trends, wherein the direction of taxon response (i.e. an increase or decrease in relative

abundance) under elevated CO_2 was more consistent among habitats than expected by chance. Discovering common bacterial responses among disparate ecosystems is important because it suggests the occurrence of common mechanistic phenomona under elevated CO_2 and it provides a first step toward identifying the functional details.

Ongoing efforts to predict whether ecosystems will act as carbon sources or sinks under future climate scenarios are hampered by technical difficulties in characterizing key responses (e.g. changes in soil carbon pools) and by eccentric ecosystem behavior. Eccentric behavior yields conflicting observations that cannot be usefully incorporated in climate models. For example, the above and below-ground biomass of plant communities typically increases under elevated CO_2 [2], whereas the size of soil bacterial communities does not show a similar, consistent pattern. Instead, general indicators (biomass, taxon richness, and community composition) of microbial community response have been highly variable and conflicting among experiments [30, 32, 49-59]. We found similar variability in our concerted study, demonstrating that these general indicators show eccentric, ecosystem-specific responses to elevated CO_2 treatments, and therefore do not have broad predictive value.

Specific microbial taxa that respond to climate factors have been identified in numerous site-specific studies [18, 45, 49, 51, 57, 60-63]. The taxonomic units range from individual species to groups as broad as kingdom. Our results add to growing lists of site-specific responsive taxa. Our use of replicated surveys to examine treatment impacts, which has typically been cost-prohibitive in the past, enabled identification of taxa with statistically significant changes in relative abundance under elevated CO_2. Despite the small size of the surveys (271 sequences, average) and the very limited

number of adequately sampled taxa among replicates, we identified bacterial taxa with significant ($p<0.05$), two-fold or greater changes in relative abundance under elevated CO_2 in every ecosystem. A few of the taxa showed significant responses in more than one treatment comparison, suggesting a robust response pattern. While synthesis of all responsive taxa reported here and elsewhere to identify commonalities might be fruitful, it was beyond the scope of this work.

The observation that about 2% (0 to 8% range) of the adequately sampled taxa in each ecosystem showed significant changes in relative abundance under elevated CO_2 is noteworthy. From one perspective, the fraction of the community significantly altered by elevated CO_2 treatment is quite small. The small fraction may show that soil bacterial communities are resilient in response to the array of soil physical and chemical changes associated with plant responses to elevated CO_2, such as increased litter deposition [4, 24, 25], increased litter C:N ratios [5, 20, 64], root growth and turnover [3, 9, 26-30], root exudates [11, 31-33], changes in root depth distributions [34], and possible changes in nitrogen demand [7, 17, 65-68]. Alternatively, the array of plant-associated changes may in fact present relatively small perturbations to soil bacteria. Water, nitrogen deposition, and spatial variability have been observed to influence microbial communities more than ecosystem exposure to elevated CO_2 [69-71]. Although we cannot generalize across all six sites, soil depth (examined in two ecosystems) and nitrogen deposition (examined in one ecosystem) had larger impacts than elevated CO_2, significantly impacting 11-12% (range 2 to 21%) of the bacterial taxa. A caveat is that the size of the "responsive" fraction of the bacterial community may depend on where in the abundance distribution most responses occur. We examined only the most dominant taxa, which may be less

responsive to elevated CO_2 but more responsive to other perturbations like depth and N deposition. A key factor influencing the interpretation of observed changes is the functional contribution of the individual species within an ecosystem. Does a 2% community change in the dominant members significantly alter soil function? The unknown functional relevance of most bacterial taxa in soil currently impedes ecological assessment.

The most striking finding in our study was the identification of climate-responsive taxa that showed significant trends in the direction of response ("+" or "- ") under elevated CO_2 across ecosystems. We looked for consistent directional changes based on the notion that changes in relative abundance within individual ecosystems may be too small or confounded by sampling variability to be detected by standard statistical comparison of population means. Given the small size of our surveys, the trend analysis was restricted to a small number of taxa that were highly abundant *at multiple sites*. From an intuitive perspective, taxa that are geographically widespread and "dominant" in diverse ecosystems seem inherently insensitive to subtle ecological gradients. Nonetheless, 21% of the taxa we analyzed showed significant directional trends under elevated CO_2 (SOM Table 13). *Acidobacteria* Group 1 displayed the most robust trend across the sites. Members of *Acidobacteria* Group 1 were detected in two or more replicates in five of the six ecosystems and generally declined 2 to 71% in abundance under elevated CO_2. The decline in *Acidobacteria* Group 1 at the WI aspen site was validated by using pyrotag surveys (100-fold larger than the clone libraries) and qPCR assays.

The ecological significance of the observed *Acidobacteria* Group 1 response trend

is unknown. The environmental abundance of *Acidobacterium* Group 1 is generally expected to decline as pH [72-77] and carbon supply [78] increase. Both effects are conceivable under elevated CO_2. Soil pH can increase via decomposition of organic acids [79], decomposition of organic matter low in nitrogen [80, 81], plant/microbial release of conjugate bases [82], or production of NH_3 from organic matter decomposition [83]. Increased carbon supply seems a logical consequence of increased plant productivity, and has been supported by observed increases in microbial growth rates in three long-term CO_2 enrichment experiments [84]. Fierer *et al.* [78] predicted that increased carbon supplies should provoke contrasting responses of oligotrophs (*Acidobacteria*) and bacterial phyla rich in copiotrophs. We did not find ample evidence for this response model. A robust trend of increases in copiotrophic taxa was not observed (SOM Table 13). In fact, the most robust trend contrasting with the *Acidobacteria* Group 1 declines was an *increase* in *Caulobacteriales*, an Alphaproteobacteria order containing many oligotrophs. Further study and broader validation of taxa showing significant responses within sites and common responses among sites is needed to ascertain the functional significance and mechanisms associated with these elevated CO_2 response patterns.

In conclusion, our screen for dominant taxa responsive to elevated CO_2 treatments in six ecosystems may be only the tip of the iceberg. Less abundant taxa may be more sensitive to physico-chemical gradients and therefore more dynamic in response to climate factors. If this hypothesis is true, deeper sequencing beyond the 100-fold increase achieved with 454 pyrotag sequencing could reveal far more dynamic responses of microbial communities to climate factors.

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FIGURE LEGENDS

Figure 1. Impact of DNA extraction method on the beta diversity of 16S rDNA clone libraries. MoBio Power Soil and the QBiogene FastDNA extraction methods were compared.

Figure 2. Similarity of soil microbial communities in six ecosystems. The plot shows nonmetric multidimensional scaling of Bray-Curtis values computed from OTU_{97} presence/absence profiles of 97 16S rDNA clone libraries. Stress value = 13.9. Similar results were obtained using relative abundance instead of presence/absence data. The black-filled symbols and grey-filled symbols represent surveys from MoBio DNA extracts and matching FastDNA extracts, respectively, used to evaluate DNA extraction bias.

Figure 3. Estimated soil and bacterial biomass in soil samples from six ecosystems. Panel A – Total soil (microbial) biomass as represented by extracted DNA. Panel B – Bacterial biomass estimated by qPCR of 16S rRNA genes. Error bars are 95% confidence intervals.

Figure 4. Impact of elevated CO_2 treatment on beta diversity of Maryland marsh soil bacterial communities. Bars indicate the mean beta-diversity among samples within or between treatments. Error bars are 95% confidence intervals.

Figure 5. Relative abundance of *Acidobacteria* Groups 1 and 2 (OTU_{75}) in soil from five ecosystems. Panel A - *Acidobacteria* Group 1. Panel B - *Acidobacteria* Group 2. Acidobacteria groups 1 and 2 were not detected at the Nevada desert site. For the NC pine site with N fertilization, nitrogen was added to ambient as well as elevated CO_2 plots. For the WI aspen site with added ozone, the ozone was mixed with elevated CO_2. Error bars are 95% confidence intervals.

Figure 6. Relative abundance of *Acidobacteria* subdivisions in 454 pyrotag 16S rDNA libraries for soil under ambient and elevated CO_2 at the WI aspen site. Data represent pyrotag sequences classified as "*Acidobacteria*" by the RDP with ≥ 60% confidence score. The responses of Groups 1, 22, and 25 under elevated CO_2 were significant ($p < 0.08$). Error bars are 95% confidence intervals.

Table 1. Description of the six experimental ecosystems[a].

Ecosystem	Treatments and other Factors	Field Reps	Number of 16S rRNA sequences per survey
Wisconsin (WI) aspen plantation[b]	1) Ambient 2) Elev. CO_2 (534 ppm) 3) Elev. CO_2 + O_3 (0.05 ppm) 4) O_3 (0.05 ppm)	3	1) 254, 270, 333, 295, 297 2) 291, 282, 271, 265 3) 220, 248, 226 4) 261, 254, 248
North Carolina (NC) loblolly pine plantation[b]	1) Ambient 2) Elev. CO_2 (570 ppm) 3) Ambient + N[d] 4) Elev. CO_2 + N	3	1) 274, 206, 242, 335, 238, 239, 251 2) 282, 201, 280, 323, 247, 263, 241, 230 3) 359, 368, 360 4) 359, 338, 370
Tennessee (TN) sweetgum plantation[b]	1) Ambient 2) Elev. CO_2 (545 ppm) Factor: Depth (0-5, 5-15, 15-30cm)	2	1) (282, 262), (229, 196), (239, 287) 2) (240, 259), (292, 239), (284, 196)
Florida (FL) Scrub oak / palmetto[c]	1) Ambient 2) Elev. CO_2 (700 ppm) Factor: Depth (0-10, 10-30cm)	3	1) (315, 327, 340), (332, 304, 340) 2) (314, 237, 324), (308, 235, 339)
Maryland (MD) tidal marsh[c]	1) Ambient 2) Elev. CO_2 (680-705 ppm)	5	1) 307, 235, 185, 295, 294 2) 212, 235, 278, 300, 270
Nevada (NV) Biological soil crust / creosote bush root zone[b]	1) Ambient 2) Elev. CO_2 (550 ppm) Factor: Ground cover	3	1) B[e]-(274, 275, 275), C-(203, 257, 184) 2) B-(243, 312, 187, 248, 288, 280), C-(243, 227, 168)

[a] Additional information is provided in SOM Table 1.
[b] Free Air Carbon Dioxide Enrichment design
[c] Open Top Chamber design
[d] N fertilization, $(NH_4)_2NO_3$,was supplied in a single annual dose (11.2g /m^2/yr).
[e] "B" – creosote bush root zone; "C" – biological soil crust. Soil samples were collected below the drip line of randomly selected creosote bushes and in the biological soil crusts.

Table 2. Influence of experimental factors on soil bacterial community richness[a]

		Percentage change in richness, S						
Site	**Treatment Comparison**	**OTU_{70}**	**OTU_{75}**	**OTU_{80}**	**OTU_{85}**	**OTU_{90}**	**OTU_{95}**	**OTU_{97}**
WI aspen	↑ CO_2 vs Ambient	21[a]	21	15	15	11	10[c]	7
	↑ O_3 vs Ambient	13[c]	8	13	11	6	7	4
	↑ CO_2, O_3 vs Ambient	19	22	18	10	9	8	6
NC pine	↑ CO_2 vs Ambient	2	4	5	5	6	7	7[c]
	↑ N, Ambient vs Ambient	1	9	15	15	13	16	14
	↑ CO_2, N vs Ambient	10	11	11	12	12	13	12
	↑ CO_2, N vs ↑ CO_2	-3	-10	-1	2	-1	-5	-2
	↑ CO_2, N vs ↑ N, Ambient	5	7	11	5	2	3	2
TN sweetgum	↑ CO_2 vs Ambient, 0-5 cm	-1	5	11	13	14	5	5
	↑ CO_2 vs Ambient, 5-15 cm	5	3	0	2	6	7	3
	↑ CO_2 vs Ambient, 15-30 cm	6	5	5	-3	-4	-3	-1
	Ambient 0-5 vs 15-30 cm	-4	6	13	17	17	10	7
	↑ CO_2 0-5 vs 15-30 cm	5	7	11	5	2	3	2
FL scrub oak	↑ CO_2 vs Ambient, 0-10 cm	-17	-15	-5	-6	-5	-10	-8
	↑ CO_2 vs Ambient, 10-30 cm	-3	6	16	18	17	10	7
	Ambient 0-10 vs 10-30 cm	-3	-10	-1	2	-1	-5	-2
	↑ CO_2 0-10 vs 10-30 cm	17	5	-8	-11	-16	-11	-5
MD marsh	↑ CO_2 vs Ambient	-11	-9	-8	-6	-5	-4	-2
NV desert	↑ CO_2 vs Ambient, bush	-2	15[c]	14	14	10	4	4
	↑ CO_2 vs Ambient, crust	-6	-8	-8	-4	-5	-2	0

[a]The clone libraries used for richness comparisons are shown in SOM Table 8.

[b]Significant changes (t-test, $0.10 > p > 0.001$) are highlighted. T-test = 2-tailed, equal variance; p-values decrease if variance is unequal. P values for each test are listed in SOM Table 9.

[c]P values bordering on significance ($0.15 > p > 0.10$).

Table 3. Percentage of bacterial taxa with significant ($p < 0.05$), two-fold or greater changes in relative abundance

		Percentage of testable a taxa[a]						
Site	**Treatment Comparison**	**OTU_{70}**	**OTU_{75}**	**OTU_{80}**	**OTU_{85}**	**OTU_{90}**	**OTU_{95}**	**OTU_{97}**
NC pine	↑ CO_2, N vs Ambient	16.7 (2)[b]	18.2 (4)	10.3 (3)	10.5 (4)	5.6 (2)	15.8 (3)	9.1 (1)
	↑ N, Ambient vs Ambient	16.7 (2)	17.4 (4)	15.6 (5)	8.1 (3)	10.3 (4)	4.5 (1)	
	↑ CO_2, N vs ↑ CO_2	8.3 (1)	13 (3)	10 (3)	12.2 (5)	13.5 (5)	4.2 (1)	7.7 (1)
TN sweetgum	Ambient 0-5 vs 15-30cm			4.2 (1)	3.6 (1)		5.3 (1)	
	↑ CO_2 0-5 vs 15-30cm	27.3 (3)	15.8 (3)	20 (4)	17.6 (3)	11.8 (2)	30.8 (4)	20 (1)
FL scrub oak	Ambient 0-10 vs 10-30cm	12.5 (1)	7.1 (1)	5.6 (1)	5.6 (1)	5.9 (1)	12.5 (2)	
	↑ CO_2 0-10 vs 10-30cm	14.3 (1)	27.3 (3)	23.1 (3)	23.1 (3)	18.8 (3)	10 (1)	12.5 (1)
WI aspen	↑ CO_2 vs Ambient			4.7 (2)	2.1 (1)	2.3 (1)	7.7 (1)	
	↑ O_3 vs Ambient	7.7 (1)	4.5 (1)	3.4 (1)	3.6 (1)			
	↑ CO_2, O_3 vs Ambient		4.3 (1)	7.4 (2)	3.8 (1)			
NC pine	↑ CO_2 vs Ambient		3.3 (1)			1.3 (1)	4 (3)	8.5 (4)
	↑ CO_2, N vs ↑ N, Ambient					3.4 (1)	5.6 (1)	
TN sweetgum 0-5cm	↑ CO_2 vs Ambient			4.3 (1)	4 (1)			
5-15cm	↑ CO_2 vs Ambient		5 (1)					
15-30cm	↑ CO_2 vs Ambient	20 (2)		9.5 (2)	9.5 (2)	4.8 (1)	13.3 (2)	
FL scrub oak 0-10cm	↑ CO_2 vs Ambient						13.3 (2)	9.1 (1)
10-30cm	↑ CO_2 vs Ambient		7.7 (1)	6.3 (1)			10 (1)	
MD marsh	↑ CO_2 vs Ambient				2.5 (1)	2.9 (1)	3.7 (1)	5.3 (1)
NV bush	↑ CO_2 vs Ambient				3.8 (1)	3.4 (1)	5.6 (1)	
crust	↑ CO_2 vs Ambient							

[a]Two criteria were used to define adequately sampled taxa as follows: 1) a taxon had to be detected in at least three replicate samples (or only two in the case of the TN sweetgum site) from each condition, and 2) the taxon had to exhibit a variance > 0 in relative abundance in at least one of the comparison conditions.

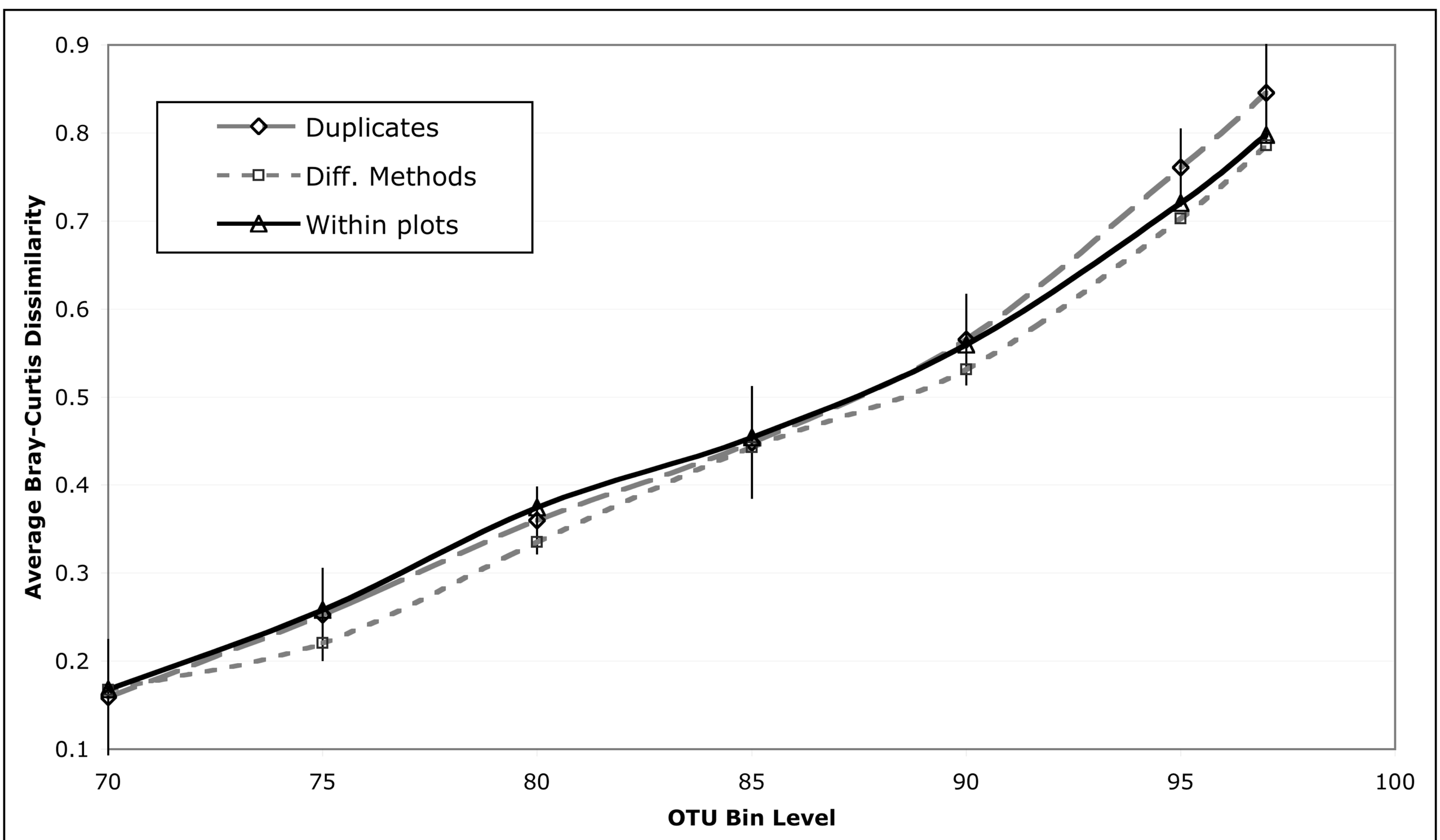
[b]Values in parentheses are the number of taxa with significant, two-fold or greater changes in relative abundance.

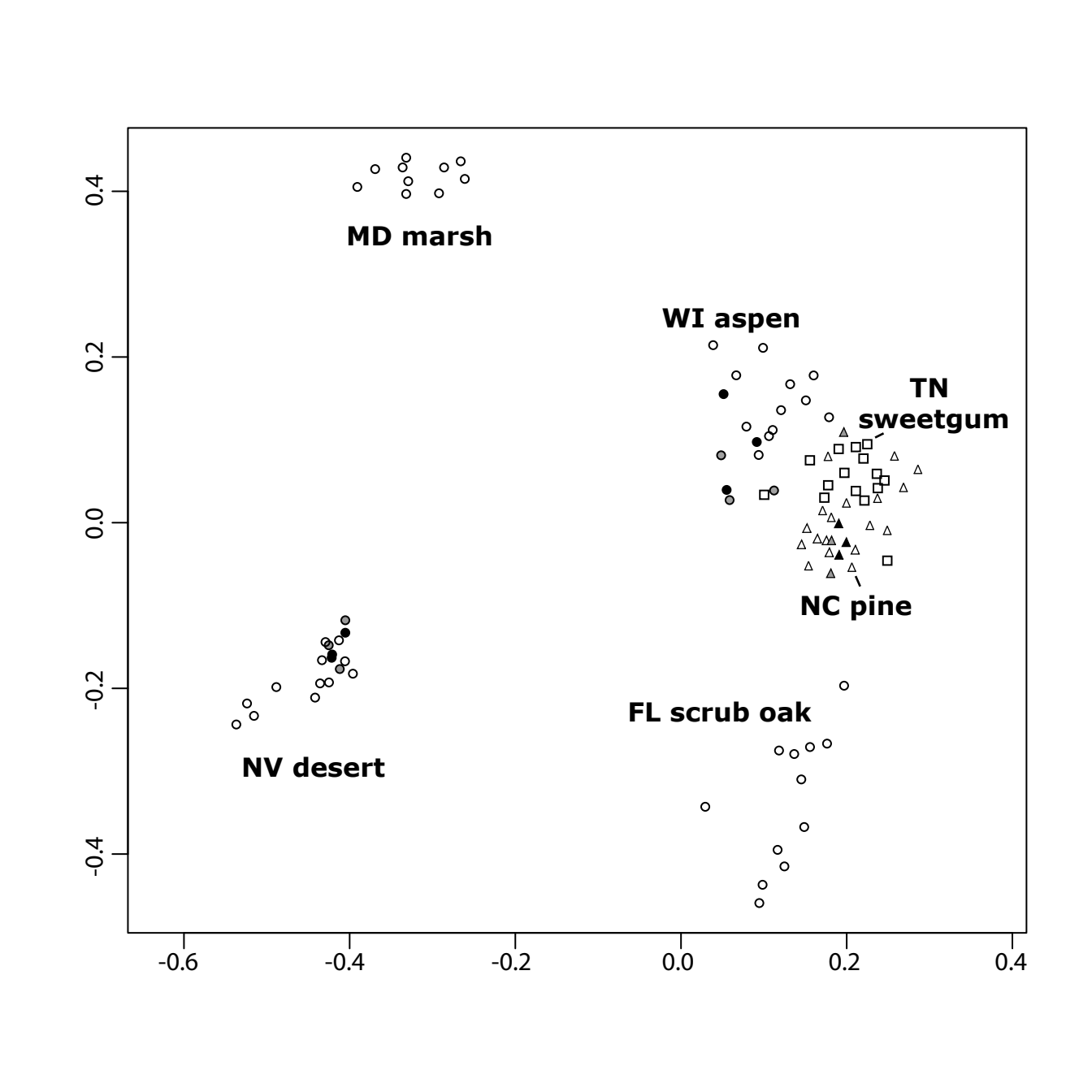
☐ - percentage ≥ 10
☐ - percentage ≥ 5
Empty cells indicate no taxa occurred with significant, two-fold or greater changes in relative abundance.

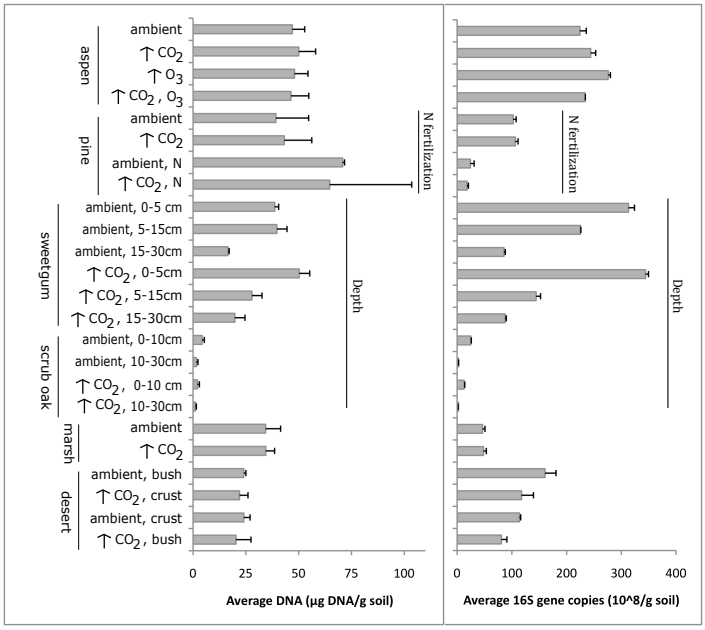
Table 4. *Acidobacteria* OTUs with significant trends across sites under elevated CO_2

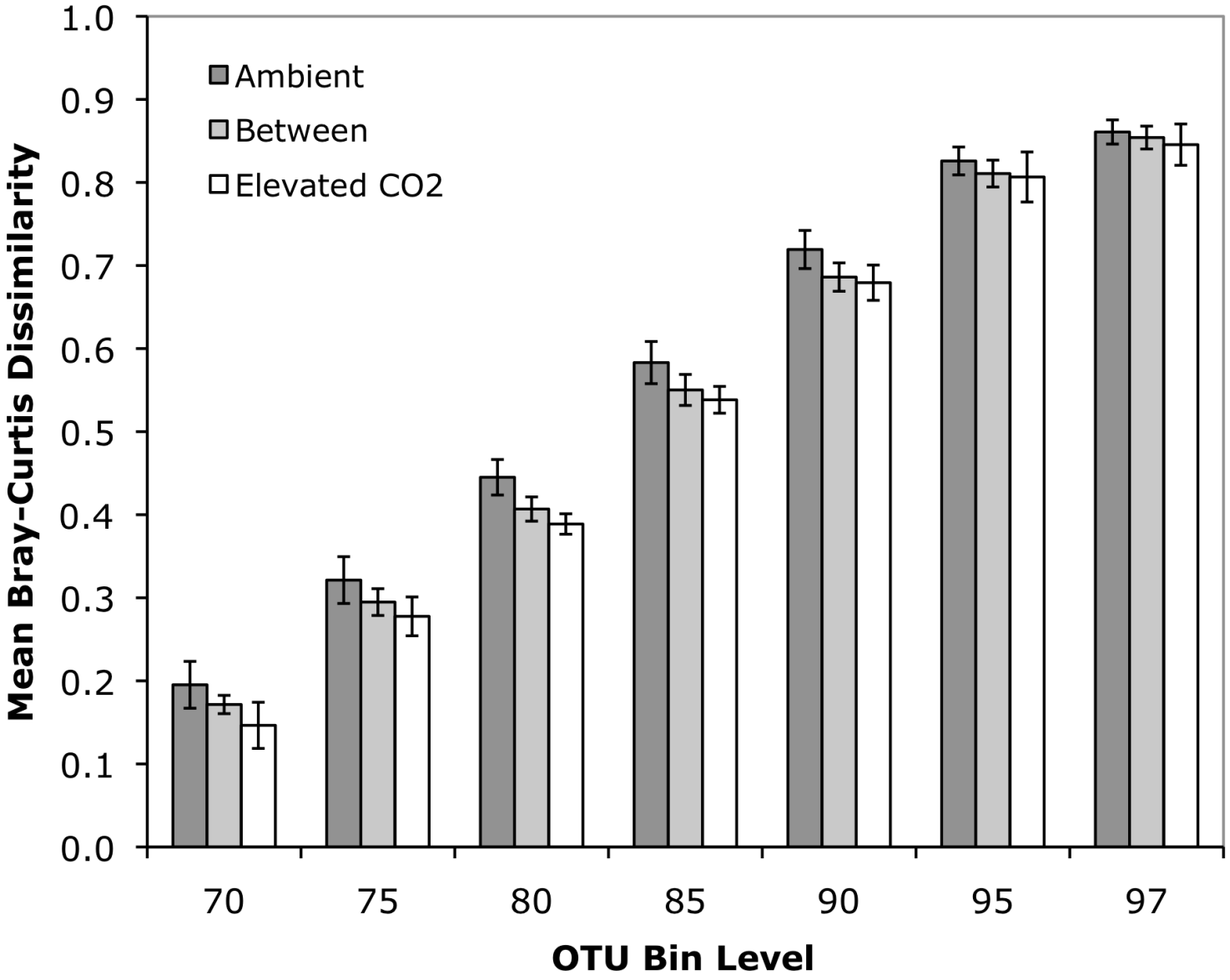
OTU level	RDP classification	p-value	NTS bush	NTS crust	FLO 0-10cm	FLO 10-30cm	MDE	NCD	NCD + N	TNO 0-5cm	TNO 5-15cm	TNO 15-30cm	WIR	WIR + O_3
95	*Acidobacteria* Gp1	0.070			+	-		-	-	-	-	-	-	+
90	*Acidobacteria* Gp1	0.070			-	-		-	-	-	-	-	+	+
90	*Acidobacteria* Gp1	0.055			-	+		-	-	-	-	-	-	-
85	*Acidobacteria* Gp1	0.070			-	-	-	-	-	-	-	+	-	+
85	*Acidobacteria* Gp1	0.031			-	-		-	+	-	-	-	-	-
80	*Acidobacteria* Gp1	0.070			-	-	-	-	-	-	-	+	-	+
80	*Acidobacteria* Gp1	0.070			-	-	-	-	+	-	-	-	-	+
80	*Acidobacteria* Gp1	0.031			-	-		-	+	-	-	-	-	-
75	*Acidobacteria* Gp1	0.044			-	-	-	-	-	-	-	+	-	+
95	*Acidobacteria* Gp2	0.063						-	-		-	-		
85	*Acidobacteria* Gp2	0.070			-	-		+	-	-	-	+	-	-
80	*Acidobacteria* Gp2	0.070			-	-		+	-	-	-	+	-	-
75	*Acidobacteria* Gp2	0.070			-	-		+	-	-	-	+	-	-

"+" and "-" indicate an increase and a decrease, respectively, in mean relative abundance of the taxon in clone libraries from elevated CO_2 compared to ambient conditions. Other taxa with a significant response trend across sites are listed in SOM Table 13. P values were calculated from a binomial distribution, treating each test case as an independent trial with a 0.5 probability of a population increase or a decrease.

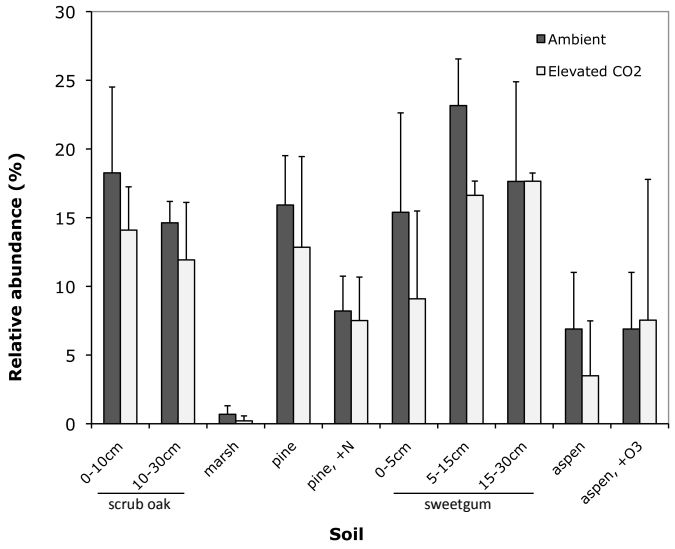

Duplicates
Diff. Methods
Within plots
Average Bray-Curtis Dissimilarity
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
70
75
80
85
90
95
100
OTU Bin Level


MD marsh
WI aspen
TN sweetgum
NC pine
FL scrub oak
NV desert
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.4
-0.2
0.0
0.2
0.4

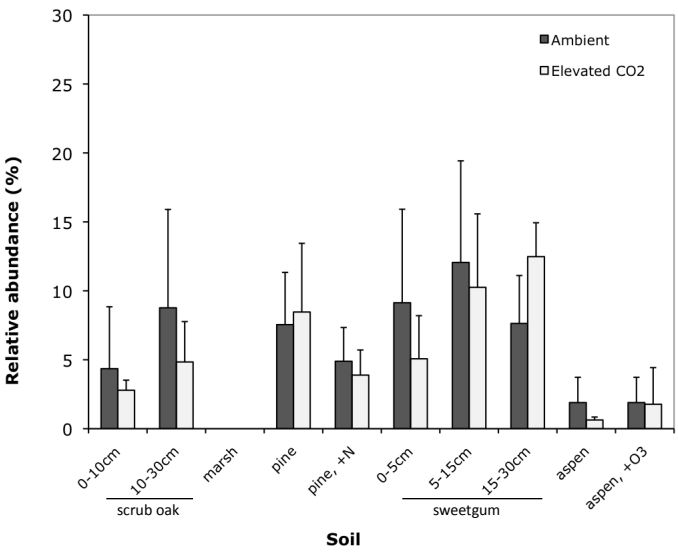

aspen
ambient
↑ CO2
↑ O3
↑ CO2 , O3
pine
ambient
↑ CO2
ambient, N
↑ CO2 , N
N fertilization
sweetgum
ambient, 0-5 cm
ambient, 5-15cm
ambient, 15-30cm
↑ CO2 , 0-5cm
↑ CO2 , 5-15cm
↑ CO2 , 15-30cm
scrub oak
ambient, 0-10cm
ambient, 10-30cm
↑ CO2 , 0-10 cm
↑ CO2 , 10-30cm
Depth
marsh
ambient
↑ CO2
desert
ambient, bush
↑ CO2 , crust
ambient, crust
↑ CO2 , bush
0
25
50
75
100
Average DNA (µg DNA/g soil)
N fertilization
Depth
0
100
200
300
400
Average 16S gene copies (10^8/g soil)

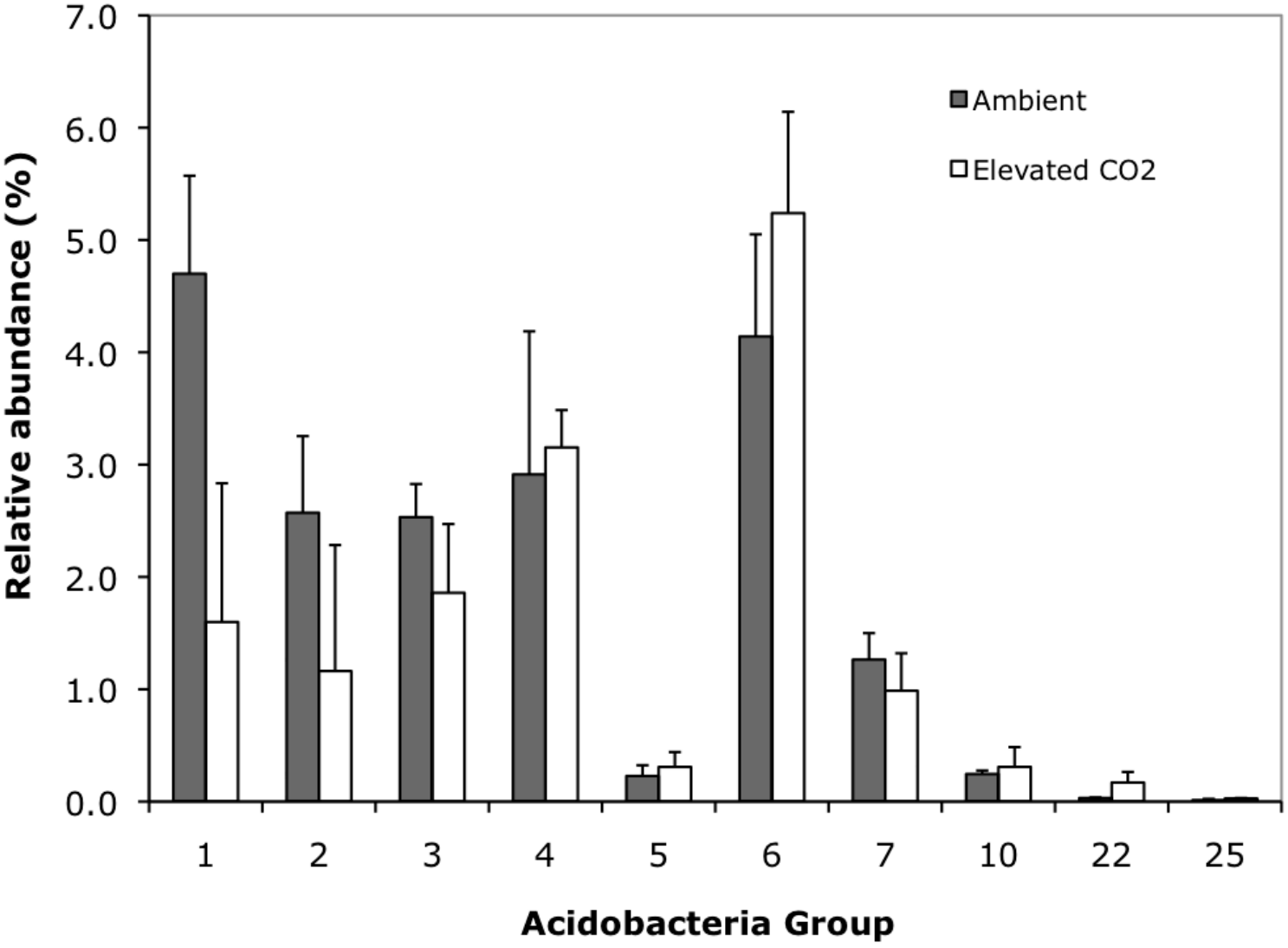

Ambient
Between
Elevated CO2
Mean Bray-Curtis Dissimilarity
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
70
75
80
85
90
95
97
OTU Bin Level

A


Ambient
Elevated CO2
Relative abundance (%)
30
25
20
15
10
5
0
0-10cm
10-30cm
marsh
pine
pine, +N
0-5cm
5-15cm
15-30cm
aspen
aspen, +O3
scrub oak
sweetgum
Soil

B


Ambient
Elevated CO2
Relative abundance (%)
30
25
20
15
10
5
0
0-10cm
10-30cm
marsh
pine
pine, +N
0-5cm
5-15cm
15-30cm
aspen
aspen, +O3
scrub oak
sweetgum
Soil


7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Relative abundance (%)
Ambient
Elevated CO2
1
2
3
4
5
6
7
10
22
25
Acidobacteria Group