

# Common bacterial responses in six ecosystems exposed to 10 years of elevated atmospheric carbon dioxide

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## Summary

Six terrestrial ecosystems in the USA were exposed to elevated atmospheric CO<sub>2</sub> in single or multifactorial 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<sub>2</sub>. Soil bacterial composition differed greatly across the six ecosystems. No common effect of elevated atmospheric CO<sub>2</sub> on bacterial biomass,

richness and community composition across all of the ecosystems was identified, although significant responses were detected in individual ecosystems. The most striking common trend across the sites was a decrease of up to 3.5-fold in the relative abundance of *Acidobacteria* Group 1 bacteria in soils exposed to elevated CO<sub>2</sub> or other climate factors. The *Acidobacteria* Group 1 response observed in exploratory 16S rRNA gene clone library surveys was validated in one ecosystem by 100-fold deeper sequencing and semi-quantitative PCR assays. Collectively, the 16S rRNA gene sequencing approach revealed influences of elevated CO<sub>2</sub> on multiple ecosystems. Although few common trends across the ecosystems were detected in the small surveys, the trends may be harbingers of more substantive changes in less abundant, more sensitive taxa that can only be detected by deeper surveys.

## 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 under elevated atmospheric CO<sub>2</sub>. Primary producers in diverse ecosystems have shown common responses to elevated CO<sub>2</sub>. Higher short-term productivity, lower transpiration, increased carbon inputs to soil through litter fall and root activity (Karnosky, 2003; Ainsworth and Long, 2005; Ainsworth and Rogers, 2007; Lukac *et al.*, 2009), and altered nutritional quality of plant litter or root exudates are common responses of C3 plants (Zak *et al.*, 1993; Cotrufo *et al.*, 1994; Couteaux *et al.*, 1995; Randlett *et al.*, 1996; King *et al.*, 2001; Weatherly *et al.*, 2003; Hall *et al.*, 2005; Haase *et al.*, 2007; Parsons *et al.*, 2008; Phillips *et al.*, 2009). However, a corresponding picture of common patterns in soil microbial community responses to climate change variables has not yet emerged. Microbial responses to elevated atmospheric CO<sub>2</sub> have thus far been examined in single ecosystem

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**Table 1.** Description of the six experimental ecosystems.<sup>a</sup>

Ecosystem	Treatments and other factors	Total time (years)	Field reps	Number of 16S rRNA sequences per survey
Wisconsin (WI) aspen plantation <sup>b</sup>	1) Ambient 2) Elev. CO <sub>2</sub> (534 p.p.m.) 3) Elev. CO <sub>2</sub> + O <sub>3</sub> (0.05 p.p.m.) 4) O <sub>3</sub> (0.05 p.p.m.)	9	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 <sup>b</sup>	1) Ambient 2) Elev. CO <sub>2</sub> (570 p.p.m.) 3) Ambient + N <sup>c</sup> 4) Elev. CO <sub>2</sub> + N	11	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 <sup>b</sup>	1) Ambient 2) Elev. CO <sub>2</sub> (545 p.p.m.) Factor: Depth (0–5, 5–15, 15–30 cm)	9	2	1) (282, 262) (229, 196) (239, 287) 2) (240, 259) (292, 239) (284, 196)
Florida (FL) Scrub oak/palmetto <sup>d</sup>	1) Ambient 2) Elev. CO <sub>2</sub> (700 p.p.m.) Factor: Depth (0–10, 10–30 cm)	11	3	1) (315, 327, 340) (332, 304, 340) 2) (314, 237, 324) (308, 235, 339)
Maryland (MD) tidal marsh <sup>d</sup>	1) Ambient 2) Elev. CO <sub>2</sub> (680–705 p.p.m.)	21	5	1) 307, 235, 185, 295, 294 2) 212, 235, 278, 300, 270
Nevada (NV) Biological soil crust/creosote bush root zone <sup>b</sup>	1) Ambient 2) Elev. CO <sub>2</sub> (550 p.p.m.) Factor: Ground cover	10	3	1) B <sup>e</sup> -(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 Table S1.

b. Free Air Carbon Dioxide Enrichment design.

c. N fertilization (NH<sub>4</sub>)<sub>2</sub>NO<sub>3</sub>, was supplied in a single annual dose (11.2 g m<sup>-2</sup> per year).

d. Open Top Chamber design.

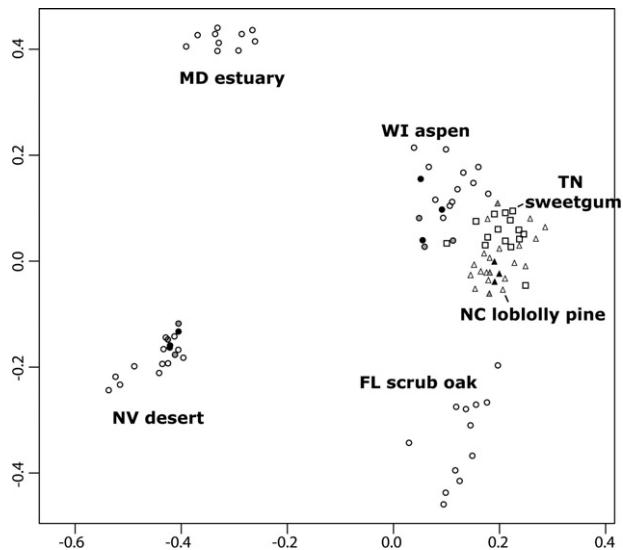
e. 'B' – creosote bush root zone; 'C' – biological soil crust. Soil samples were collected below the drip line of randomly selected creosote bushes and from the biological soil crusts.

studies (Zak *et al.*, 2000; Rustad *et al.*, 2001; Treseder, 2004; Kelly *et al.*, 2010). 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 elevated atmospheric CO<sub>2</sub> field experiments in the USA. The field experiments represent different ecosystems: three tree plantations and three natural ecosystems (Tables 1 and S1). The dominant species at the three plantation sites were trembling aspen (*Populus tremuloides*; Rhinelander, Wisconsin), loblolly pine (*Pinus taeda*; Durham, North Carolina), and sweetgum (*Liquidambar styraciflora*; Oak Ridge, Tennessee). The natural desert site (Mercury, Nevada) had a patchy plant cover of creosote bush (*Larrea tridentata*), 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<sub>2</sub> treatments (340–395 p.p.m. ambient versus 534–700 p.p.m. elevated CO<sub>2</sub>; Table 1). Every ecosystem exhibited significant increases

in net primary production under elevated CO<sub>2</sub> (Zak *et al.*, 1993; Couteaux *et al.*, 1995; Smith *et al.*, 2000; Weatherly *et al.*, 2003; Ainsworth and Long, 2005; Nowak *et al.*, 2004; Hall *et al.*, 2005; Heath *et al.*, 2005; Housman *et al.*, 2006; Iversen *et al.*, 2008; Parsons *et al.*, 2008). Increased carbon inputs into soil under elevated CO<sub>2</sub> occur through greater primary productivity, via plant litter deposition (Karnosky, 2003; Lichter *et al.*, 2008; Hoosbeek and Scarascia-Mugnozza, 2009), root growth and turnover (King *et al.*, 2001; Larson *et al.*, 2002; Matamala *et al.*, 2003; Norby *et al.*, 2004; Godbold *et al.*, 2006; Lukac *et al.*, 2009), root exudates (Cheng, 1999; Patterson *et al.*, 2008; Pendall *et al.*, 2008; Phillips *et al.*, 2009), and changes in root depth distributions (Iversen, 2010). We sought to determine whether the aggregate changes in primary production induced common soil bacterial responses among ecosystems.

We performed exploratory analyses with 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 taxa present in multiple sites, 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



**Fig. 1.** Similarity of soil microbial communities in six ecosystems. The plot shows nonmetric multidimensional scaling of Bray–Curtis values computed from OTU<sub>97</sub> presence/absence profiles of 97 16S rRNA gene 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.

elevated CO<sub>2</sub>. This paper does not attempt to detail the responses in each ecosystem; instead we focus on general patterns across the ecosystems.

## Results

The results are organized in four sections. The first section describes the general similarity of the bacterial communities in the six ecosystems as measured by 16S rRNA clone library sequences. The second section describes evidence of community responses to climate factors using composite metrics – biomass, bacterial community richness and composition. The third section describes the responses of individual taxa. The fourth section describes a trend analysis to discover common taxon responses among the ecosystems and an associated validation effort at one of the six sites.

### *Soil bacterial communities across the ecosystems were very different, but some common taxa occurred*

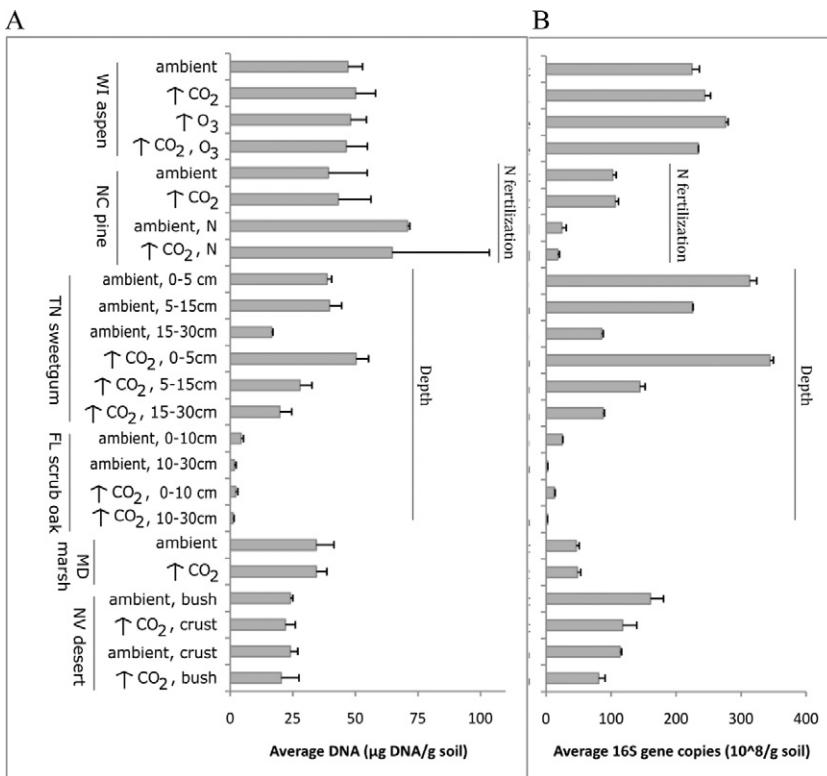
The level of similarity of bacterial communities generally corresponded with degree of similarity in measured soil characteristics (Fig. 1, Table S2). Each of the 97 16S rRNA gene clone library surveys created in this study for comparison of community composition contained 168–338 clones (average = 271). The libraries collectively represented 34 to 6980 taxa, depending on the taxonomic level examined (i.e. OTU<sub>70</sub> to OTU<sub>97</sub>). Bacterial communi-

ties in soils from the WI aspen, NC pine and TN sweetgum sites were the most similar (based on species-level profiles), although the sites have large differences in soil type, tree species, mean annual temperature and spatial separation (about 69–1200 km) (Fig. 1). The natural NV desert, MD marsh and FL scrub oak/palmetto ecosystems were distinct. There were no species-level (OTU<sub>97</sub>) taxa common to all six ecosystems, but 12 of the 6980 species-level OTUs were detected in five ecosystems. At coarser taxonomic levels, community similarity among all ecosystems increased, as expected. In ambient CO<sub>2</sub> surface soils, 17 of 769 total taxa (from all samples) at the family/genus level (OTU<sub>85</sub>) 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 (Table S3).

### *Significant changes in general community metrics (biomass, richness and beta diversity) were sparse and ecosystem-specific*

**Soil biomass.** Elevated CO<sub>2</sub> treatments showed a small and inconsistent effect on total biomass (Fig. 2A, Table S5). Extracted soil DNA was used as a proxy for biomass. In surface soils, DNA increased 0.1–30% in four elevated CO<sub>2</sub> treatment comparisons, but decreased 8–51% in the other four cases (Fig. 2A and Table S4). Only the two largest changes – a 30% increase in the TN sweetgum soils and a 51% decrease in FL scrub oak/palmetto soils – were statistically significant ( $P = 0.092$  and  $P = 0.051$  respectively). Thus, no consistent trend was detected for the impact of elevated CO<sub>2</sub> 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) (Fig. 2A and Table S4).

**Bacterial biomass.** The influence of elevated CO<sub>2</sub> exposure on bacterial biomass, estimated by qPCR of 16S rRNA genes, was generally similar to the effects on total soil biomass (Fig. 2B, Table S4). The estimated copy number of bacterial 16S rRNA genes in soil ranged from  $1.8 \times 10^8$  to  $3.4 \times 10^{10}$ . Elevated CO<sub>2</sub> treatments did not substantially alter bacterial biomass in surface soils (0 to 5 or 10 cm). The 16S rRNA gene copy number increased in half the cases by 4–10%, and decreased in the other cases by 26–49%. Only the 49% decrease in surface soil with elevated CO<sub>2</sub> at the FL scrub oak/palmetto site was significant ( $P = 0.084$ ). Larger declines of 2.4- to 9.7-fold occurred with soil depth or with nitrogen fertilization (Fig. 2B and Table S4).

**Fig. 2.** Estimated soil and bacterial biomass in soil samples from six ecosystems.

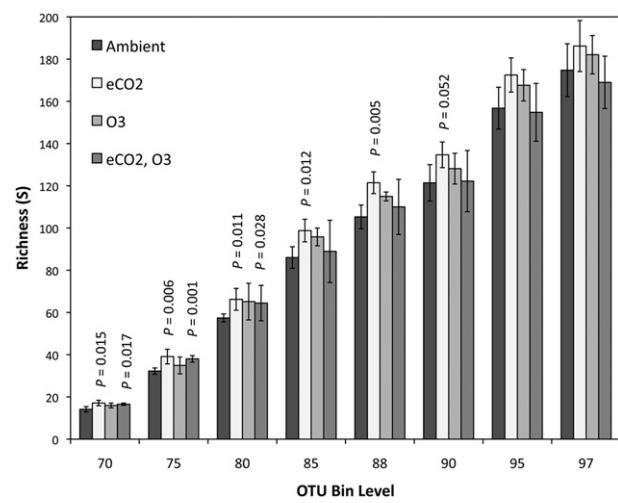
A. Total soil (microbial) biomass as represented by extracted DNA.

B. Bacterial biomass estimated by qPCR of 16S rRNA genes. Error bars are 95% confidence intervals.

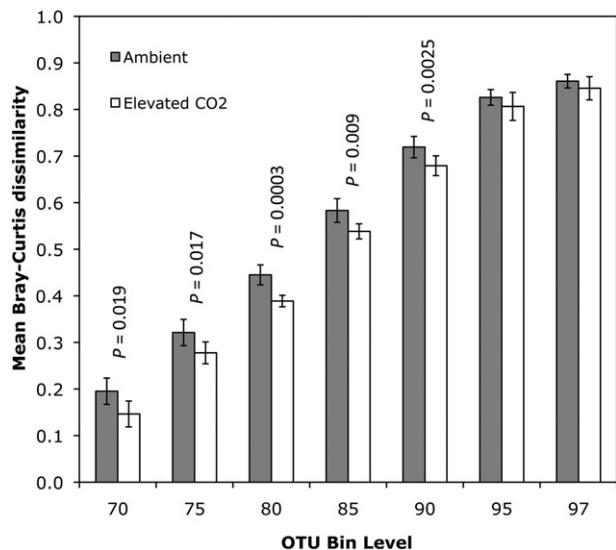
**Community richness.** In one of six ecosystems, bacterial taxon richness increased under elevated CO<sub>2</sub> (Table S5). At the WI aspen site, taxon richness increased 11–22% at various taxonomic levels (OTU<sub>70</sub> to OTU<sub>90</sub>,  $P < 0.1$ ) in plots exposed to elevated CO<sub>2</sub> or a combination of elevated CO<sub>2</sub> and ozone (Fig. 3). The WI aspen site had the highest magnitude of changes in richness among the six ecosystems.

**Community composition.** Three of five ecosystems (the TN sweetgum site was not included in this analysis because of insufficient replicates) showed evidence of treatment effects on community similarity. The similarity of communities from treated plots versus ambient plots was initially compared using UniFrac tests for homogeneity. However, in all ecosystems, significant differences occurred among replicates (data not shown), demonstrating that soil heterogeneity and treatment effects could not be distinguished by UniFrac permutation tests. As an alternative, we evaluated treatment effects based on *t*-tests of the mean beta diversity among samples within treatments. The MD marsh soil bacterial communities showed the clearest evidence of elevated atmospheric CO<sub>2</sub> impacts on beta diversity. At five taxonomic levels (OTU<sub>70</sub> to OTU<sub>90</sub>; Table S6), soil communities from elevated CO<sub>2</sub> plots were significantly more similar to one another than communities from ambient plots (Fig. 4), suggesting a homogenizing effect of selection at this site.

The results were obtained using the quantitative version of the Bray-Curtis index, which gives abundant taxa the most weight. In the qualitative (presence–absence) Bray-Curtis index, taxa with lower abundance exert a larger effect. With the qualitative index, three ecosystems



**Fig. 3.** Average richness of soil bacterial community surveys from treatments at the WI aspen site. Bars indicate the mean richness (number of OTUs) among samples within each treatment. Error bars are 95% confidence intervals. *P*-values were obtained from *t*-tests of each treatment compared with the ambient condition. Only the *P*-values that remained significant after correcting for the false discovery rate at  $P < 0.1$  are shown.



**Fig. 4.** Impact of elevated CO<sub>2</sub> treatment on beta diversity of MD marsh soil bacterial communities. Bars indicate the mean beta diversity among samples within each treatment. Error bars are 95% confidence intervals.

showed significant elevated CO<sub>2</sub> effects on community similarity. These were the WI aspen site, the NC pine site and the MD marsh site (Table S6). We did not observe a consistent pattern of communities becoming more similar under elevated CO<sub>2</sub> compared with communities from ambient plots.

Collectively, the results show that significant changes in bacterial biomass, community richness, and community composition under elevated CO<sub>2</sub> were sparse and ecosystem-specific. Although significant treatment effects were measured at individual sites, a decade of exposure to elevated CO<sub>2</sub> had no significant common effect on bacterial biomass, community richness or community composition across the sites.

#### Individual taxa were screened for potentially significant, ecosystem-specific responses to treatment factors

Although community size and structure were generally resilient under elevated CO<sub>2</sub> (results above), significant responses may have occurred among individual taxa. Individual taxa with twofold or greater changes in abundance under elevated CO<sub>2</sub> treatments were detected in every ecosystem (Table 2). We restricted the analysis to adequately sampled taxa – i.e. taxa detected in at least

**Table 2.** Percentage of bacterial taxa with two-fold or greater changes in relative abundance and *P*-values less than 0.05.

Site	Treatment comparison	Percentage of testable taxa showing significant change <sup>a</sup>						
		OTU <sub>70</sub>	OTU <sub>75</sub>	OTU <sub>80</sub>	OTU <sub>85</sub>	OTU <sub>90</sub>	OTU <sub>95</sub>	OTU <sub>97</sub>
Elevated CO <sub>2</sub>	↑ CO <sub>2</sub> vs Ambient				4.7 (2)	2.1 (1)	2.3 (1)	7.7 (1)
	↑ O <sub>3</sub> vs Ambient		7.7 (1)	4.5 (1)	3.4 (1)	3.6 (1)		
	↑ CO <sub>2</sub> , O <sub>3</sub> vs Ambient		4.3 (1)	7.4 (2)	3.8 (1)			
NC pine	↑ CO <sub>2</sub> vs Ambient			3.3 (1)		1.3 (1)	4 (3)	8.5 (4)
	↑ CO <sub>2</sub> , N vs ↑ N, Ambient					3.4 (1)	5.6 (1)	
TN sweetgum	↑ CO <sub>2</sub> vs Ambient				4.3 (1)	4 (1)		
	↑ CO <sub>2</sub> vs Ambient, 0–5 cm		5 (1)					
	↑ CO <sub>2</sub> vs Ambient, 5–15 cm							
FL scrub oak	↑ CO <sub>2</sub> vs Ambient, 15–30 cm	20 (2)		9.5 (2)	9.5 (2)	4.8 (1)	13.3 (2)	
	↑ CO <sub>2</sub> vs Ambient, 0–10 cm		7.7 (1)	6.3 (1)			13.3 (2)	9.1 (1)
	↑ CO <sub>2</sub> vs Ambient, 10–30 cm					2.5 (1)	3.7 (1)	5.3 (1)
MD marsh	↑ CO <sub>2</sub> vs Ambient					3.8 (1)	3.4 (1)	5.6 (1)
NV bush	↑ CO <sub>2</sub> vs Ambient							
NV crust	↑ CO <sub>2</sub> vs Ambient							
Nitrogen fertilization	↑ CO <sub>2</sub> , N vs Ambient	16.7 (2) <sup>b</sup>	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 <sub>2</sub> , N vs ↑ CO <sub>2</sub>	8.3 (1)	13 (3)	10 (3)	12.2 (5)	13.5 (5)	4.2 (1)	7.7 (1)
Depth	Ambient 0–5 vs 15–30 cm			4.2 (1)	3.6 (1)		5.3 (1)	
	↑ CO <sub>2</sub> 0–5 vs 15–30 cm	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 10–30 cm	12.5 (1)	7.1 (1)	5.6 (1)	5.6 (1)	5.9 (1)	12.5 (2)	
	↑ CO <sub>2</sub> 0–10 vs 10–30 cm	14.3 (1)	27.3 (3)	23.1 (3)	23.1 (3)	18.8 (3)	10 (1)	12.5 (1)

**a.** Two criteria were used to define adequately sampled taxa as follows: (i) 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 treatment and (ii) the taxon had to exhibit a variance  $> 0$  among replicate samples in relative abundance.

**b.** Values in parentheses are the number of taxa with twofold or greater changes in relative abundance and *P*-values  $< 0.05$ .

■ – table values  $\geq 10$ .

□ – table values  $\geq 5$ .

Empty cells indicate no taxa occurred with significant, twofold or greater changes in relative abundance.

three replicates from ambient and three replicates from elevated CO<sub>2</sub> treatments (or two replicates per treatment at the TN sweetgum site) at each site. Although this criterion substantially reduced the number of useable taxa, it avoided the problematic use of missing data (zero counts). On average, each site had 21 OTUs (range = 2–75) suitable for analysis per taxonomic level per treatment comparison. At  $P < 0.05$ , 45 taxa (various taxonomic levels) with twofold or greater changes in abundance under elevated CO<sub>2</sub> were identified among the six ecosystems from the 133 taxa tested (Table S7). On average, 2.7% of the adequately sampled taxa in each ecosystem showed significant responses to elevated CO<sub>2</sub>. In comparison, 11–12% of taxa were responsive to soil depth (examined at two sites) or nitrogen deposition (examined at one site).

It is possible that most of the taxa that appeared responsive under elevated CO<sub>2</sub> were false positives that had low  $P$ -values by chance. After correcting for the false discovery rate with a  $P$ -value of 0.1, only one group of *Beta-proteobacteria* from the 15–30 cm soil depth at the TN sweetgum site was significant (Table S7). However, application of the false discovery rate, which reduces the risk of false positives but increases the risk of false negatives, may be excessively stringent with these exploratory data. The combination of small sample sizes, substantial spatial heterogeneity and low replication weakens the resolving power and can obscure true positives. Thus, it is worthwhile to consider the taxa in Table S8 as *candidate* responsive taxa (i.e. possible indicator species) that may be corroborated by evidence in future field studies. We illustrate the excessive stringency of the false discovery rate below with 100-fold deeper pyrotag surveys.

Pyrotag surveys at the WI aspen site were conducted to validate observations from the exploratory clone library surveys. The pyrotag surveys used a different PCR primer set and sequencing technology, providing a measurement that was both higher quality and orthologous to the clone library surveys. A total of 31 737–75 663 16S rRNA gene sequences (about 400 nt each) were obtained from each of three replicate soil samples from ambient plots and three replicate samples from plots under elevated CO<sub>2</sub> (Table S9). The sequences were clustered into OTUs using a 97% sequence similarity threshold. Of the 1313 taxa that were consistently detected in all replicates, 93 taxa had  $P$ -values less than 0.05. Seventy-five of these (about 6% of total) showed twofold or greater changes in average relative abundance (Table S10). Of the 75 taxa, the relative abundance of 34 taxa increased 2- to 5-fold, while 41 taxa decreased 2- to 11-fold under elevated CO<sub>2</sub>. After applying the false discovery rate (FDR), none of the taxa were significant. However, if all of the taxa were false positives occurring by chance, as implied by the FDR results, the distribution of  $P$ -values from the 1313 total

taxa would be uniformly distributed. The observed distribution was significantly different from a uniform distribution (chi-squared test and Kolmogorov–Smirnov test,  $P < 10^{-10}$ ) with far more low  $P$ -values (i.e.  $P < 0.1$ ) than expected by chance. Thus, application of the FDR is too stringent for this dataset. The set of 75 taxa may contain some false positives, but clearly there are many true, responsive taxa. Identifying exactly which taxa are true positives will require additional corroborating data.

*Some taxa do not have significant changes in abundance within a single site, but have a common, significant response pattern across sites*

Taxa with common response patterns to climate change treatments across sites were identified by a trend analysis. Taxa may exhibit significant trends across sites yet have non-significant responses within individual sites owing to local, mitigating ecological factors or to sampling artefacts. The trend analysis for each taxon involved up to 12 comparisons of ambient versus elevated CO<sub>2</sub>. For this analysis, we included only taxa that were detected in two or more replicates from ambient and from elevated CO<sub>2</sub> conditions and present in four or more of the twelve test cases among ecosystems. Thus, the trend 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<sub>75</sub> to OTU<sub>97</sub> fulfilled these criteria. Eighteen percent (39 of 214) of the taxa showed significant response trends ( $P < 0.1$ ) (Table S12). The taxa belonged to eight phyla – *Acidobacteria*, *Actinobacteria*, *Proteobacteria*, *Verrucomicrobia*, *Nitrospira*, *Firmicutes*, *OD1* and *TM7* – and represent possible targets for further ecological analysis.

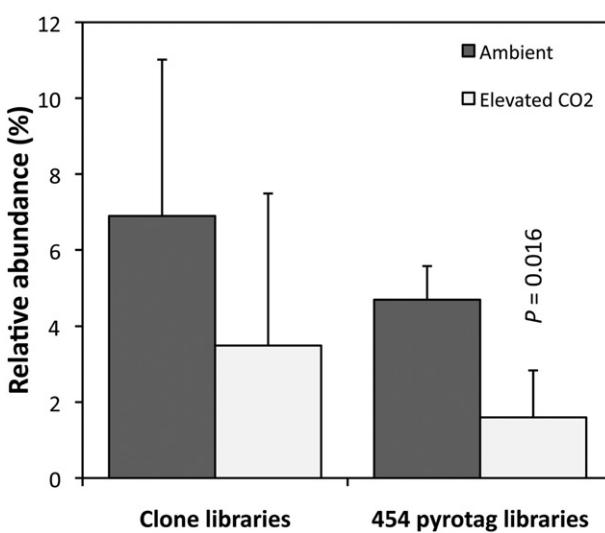
*Acidobacteria* Group 1 and *Acidobacteria* Group 2 taxa exhibited the most consistent and robust trends (Table 3). The *Acidobacteria* Group 1 taxa that showed significant trends included groups from the genus/species level (OTU<sub>95</sub>) to the subdivision level (OTU<sub>75</sub>) and were present in 4–10 of the 12 test cases of ambient soils versus soils under elevated CO<sub>2</sub> (Table 3). Relative abundance of *Acidobacteria* Group 1 (at the subdivision level) under elevated CO<sub>2</sub> conditions was reduced by an average of 25% across all the sites. Individual site responses ranged from a 9% increase to a 69% reduction (Table S8). Similarly, the average decline of *Acidobacteria* Group 2 was 18% across sites. Although the magnitude of these responses is small, taxa that demonstrate significant patterns across sites are noteworthy as possible indicators of broadly occurring but difficult to measure mechanistic phenomena.

The decline in *Acidobacteria* Group 1 was validated by pyrotag surveys and qPCR assays at the WI aspen site. In

**Table 3.** Acidobacteria OTUs with significant trends across sites under elevated CO<sub>2</sub>.

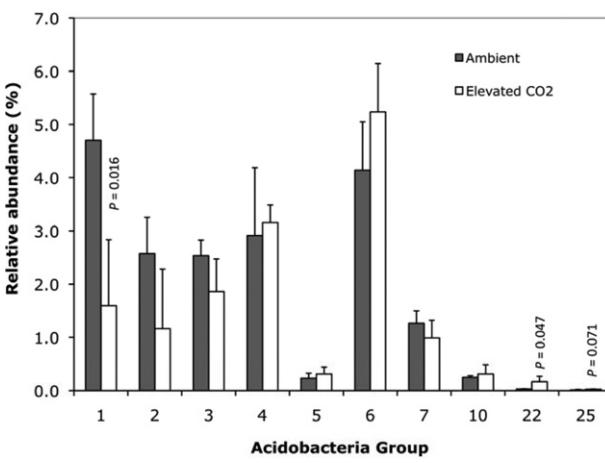
OTU level	RDP classification	P-value	WI aspen	WI aspen + O <sub>3</sub>	NC pine	NC pine + N	TN sweetgum 0–5 cm	TN sweetgum 5–15 cm	TN sweetgum 15–30 cm	FL scrub oak 0–10 cm	FL scrub oak 10–30 cm	MD marsh
95	Acidobacteria Gp1	0.090	+	+	+	+	+	+	+	+	+	+
90	Acidobacteria Gp1	0.090	+	+	+	+	+	+	+	+	+	+
90	Acidobacteria Gp1	0.063	+	+	+	+	+	+	+	+	+	+
85	Acidobacteria Gp1	0.090	+	+	+	+	+	+	+	+	+	+
85	Acidobacteria Gp1	0.035	+	+	+	+	+	+	+	+	+	+
80	Acidobacteria Gp1	0.090	+	+	+	+	+	+	+	+	+	+
80	Acidobacteria Gp1	0.090	+	+	+	+	+	+	+	+	+	+
80	Acidobacteria Gp1	0.035	+	+	+	+	+	+	+	+	+	+
75	Acidobacteria Gp1	0.055	+	+	+	+	+	+	+	+	+	+
95	Acidobacteria Gp2	0.063	+	+	+	+	+	+	+	+	+	+
85	Acidobacteria Gp2	0.090	+	+	+	+	+	+	+	+	+	+
80	Acidobacteria Gp2	0.090	+	+	+	+	+	+	+	+	+	+
75	Acidobacteria Gp2	0.090	+	+	+	+	+	+	+	+	+	+

'+' and '-' indicate an increase and a decrease, respectively, in mean relative abundance of the taxon in clone libraries from elevated CO<sub>2</sub> compared with ambient conditions. Other taxa with a significant response trend across sites are listed in Table S8. 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. The listed taxa were not adequately detected at the NV desert site.



**Fig. 5.** Decline in abundance of *Acidobacteria* Group 1 in soil at the WI aspen site measured by clone library and pyrotag surveys. Error bars are 95% confidence intervals.

a pattern similar to the clone library trend analysis, the relative abundance of *Acidobacteria* Group 1 (at the subdivision level) in the pyrotag surveys declined between 2- and 3-fold under elevated CO<sub>2</sub> (Fig. 5). Because the variance in population estimates in the pyrotag surveys was smaller than in the clone libraries, the *Acidobacteria* Group 1 decline was significant ( $P = 0.016$ ). The pyrotag surveys showed declines or increases in other *Acidobacteria* groups, and some of the changes were significant (Fig. 6). 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



**Fig. 6.** Relative abundance of *Acidobacteria* groups detected in pyrotag surveys of soil under ambient and elevated CO<sub>2</sub> at the WI aspen site. Data represent pyrotag sequences classified as '*Acidobacteria*' by the RDP with a confidence score  $\geq 60\%$ . The abundance of each group was computed as the sum of all species-level (OTU97) clusters within each group. Error bars are 95% confidence intervals.

collectively had a predicted coverage of 36% of the 8430 *Acidobacteria* Group 1 sequences obtained from the RDP. The assays showed significant ( $P < 0.006$ ) declines between 3- and 4-fold in the abundance of *Acidobacteria* Group 1 taxa in soil samples under elevated CO<sub>2</sub>. Based on these results, further analysis of the function and response of *Acidobacteria* Group 1 under ecosystem exposure to elevated CO<sub>2</sub> is warranted.

## Discussion

Predicting climate change impacts requires knowledge of general ecosystem response patterns and their variability. Among six ecosystems, we found patterns in bacterial population responses to a decade of elevated CO<sub>2</sub> treatment. About 18% of the dominant taxa detected in multiple ecosystems displayed significant ( $P < 0.1$ ) cross-site response patterns, wherein the direction of taxon response (i.e. an increase or decrease in relative abundance) under elevated CO<sub>2</sub> was more consistent among habitats than expected by chance. Discovering common bacterial response patterns among disparate ecosystems is important because it suggests the occurrence of common mechanistic phenomena under elevated CO<sub>2</sub>, and it provides a first step towards identifying 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 site-specific ecosystem behaviour. Site-specific behaviour yields conflicting observations that cannot be usefully incorporated in climate models. General indicators (biomass, taxon richness and community composition) of microbial community response to climate factors have been highly variable and conflicting among experiments (Lipson *et al.*, 2002; Montealegre *et al.*, 2002; Phillips *et al.*, 2002; Niklaus *et al.*, 2003; Ebersberger *et al.*, 2004; Kandeler *et al.*, 2006; Lipson *et al.*, 2006; Carney *et al.*, 2007; Chung *et al.*, 2007; Denef *et al.*, 2007; Drissner *et al.*, 2007; Lesaulnier *et al.*, 2008; Paterson *et al.*, 2008). We found similar variability in our concerted study. Our results demonstrate that these general community metrics showed ecosystem-specific responses to elevated CO<sub>2</sub> treatments, and therefore do not have broad predictive value across a wide range of ecosystems.

The soil bacterial communities were generally resilient despite the array of physical and chemical changes in soil associated with plant responses to elevated CO<sub>2</sub>. Changes in soil that may provoke microbial responses under elevated CO<sub>2</sub> include increased litter deposition (Karnosky, 2003; Lichter *et al.*, 2008; Hoosbeek and Scarascia-Mugnozza, 2009), increased litter C : N ratios (Cotrufo *et al.*, 1994; Gill *et al.*, 2002; Heath *et al.*, 2005), increased root growth and turnover (King *et al.*, 2001; Larson *et al.*,

2002; Matamala *et al.*, 2003; Norby *et al.*, 2004; Godbold *et al.*, 2006; Lukac *et al.*, 2009), altered root exudates (Cheng, 1999; Paterson *et al.*, 2008; Pendall *et al.*, 2008; Phillips *et al.*, 2009), changes in root depth distributions (Iversen, 2010), and possible changes in nitrogen demand (Hungate *et al.*, 1994; 1996; Treseder, 2004; Finzi *et al.*, 2007; Haase *et al.*, 2007; Niboyet *et al.*, 2010).

Soil microbial taxa that are potentially responsive to elevated CO<sub>2</sub> and other climate change factors have been identified in numerous site-specific studies (Zak *et al.*, 2000; Montealegre *et al.*, 2002; Chung *et al.*, 2005; Moscatelli *et al.*, 2005; Carney *et al.*, 2007; Denef *et al.*, 2007; Drigo *et al.*, 2009; Talhelm *et al.*, 2009; Castro *et al.*, 2010). The taxonomic groups range from species to phyla. Our results add to the growing lists of potentially responsive taxa. We identified bacterial taxa with potentially significant ( $P < 0.05$ ), twofold or greater changes in relative abundance under elevated CO<sub>2</sub> in every ecosystem. The observation that only a small fraction (about 2%) of the taxa are potentially responsive demonstrates the resilience of the communities. Water, nitrogen deposition and spatial variability appear to influence microbial communities more than ecosystem exposure to elevated CO<sub>2</sub> (Bloor *et al.*, 2009; Garten *et al.*, 2009; Ge *et al.*, 2010). Although we cannot generalize across all six sites, soil depth (examined in two ecosystems) and nitrogen deposition (examined in one ecosystem) had larger effects than elevated CO<sub>2</sub>, significantly affecting 11–12% (range from 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. Dominant taxa may be less responsive to elevated CO<sub>2</sub> effects compared with less abundant taxa. Much deeper surveys or targeted approaches will be needed to address this ambiguity.

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<sub>2</sub> 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 spatial heterogeneity to be detected by standard statistical comparison of population means. Given the size of our surveys, the trend analysis was restricted to a small number of taxa that were highly abundant at multiple sites. About 18% of the taxa analysed showed significant directional trends under elevated CO<sub>2</sub> (Table S8). *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 declined up to 3.5-fold in relative abundance under elevated CO<sub>2</sub>. The decline in *Acidobacteria* Group 1 at the WI aspen site was validated by pyrotag surveys (100-fold larger than the clone librar-

ies) and qPCR assays. Moreover, these higher-quality measurements revealed statistically significant declines that were not apparent in the clone library surveys, illustrating the value of deeper coverage and more targeted detection strategies in quantifying responses. The value of identifying indicator taxa for community response to climate change factors cannot be overstated. Given a set of indicator genes or species, targeted studies that clarify spatial and temporal responses are feasible at lower cost and higher resolution.

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 (Sait *et al.*, 2006; Eichorst *et al.*, 2007; Hartman *et al.*, 2008; Upchurch *et al.*, 2008; Jones *et al.*, 2009; Lauber *et al.*, 2009) and carbon supply (Fierer *et al.*, 2007) increase. Both effects are conceivable under elevated CO<sub>2</sub>. Soil pH can increase via decomposition of organic acids (Rukshana *et al.*, 2011), decomposition of organic matter low in nitrogen (Kretzschmar *et al.*, 1991; Barekzai and Mengel, 1992), plant/microbial release of conjugate bases (Li *et al.*, 2008), or production of NH<sub>3</sub> from organic matter decomposition (Ingelog and Nohrstedt, 1993). 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<sub>2</sub> enrichment experiments (Blagodatskaya *et al.*, 2010). Increased carbon supply is predicted to provoke contrasting responses of bacterial phyla rich in oligotrophs (e.g. *Acidobacteria*) versus copiotrophs (Fierer *et al.*, 2007). We did not find ample evidence for this response model. A robust trend of increases in copiotrophic taxa was not observed (Table S8). In fact, the most robust trend contrasting with the decline of *Acidobacteria* Group 1 was an increase in *Caulobacterales*, an *Alpha-proteobacteria* order known to contain oligotrophs. Thus, the functional significance of these responses under elevated CO<sub>2</sub> requires further investigation.

In conclusion, our screen for dominant taxa responsive to elevated CO<sub>2</sub> 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 more dynamic responses of microbial communities to climate factors.

## Experimental procedures

### Sample collection

Soil cores were collected from four Free Air CO<sub>2</sub> 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 S1. Samples were collected between 2007 and 2008 (Table S1). Three to ten soil cores from random locations in a FACE ring or OTC chamber were obtained from the upper 5–10 cm (depending on the site), homogenized, and stored at -70°C for subsequent molecular analysis. At the NV desert site, separate sets of samples were collected from shrub rhizospheres and inter-space biocrusts. At the FL scrub oak and TN sweetgum sites, 30 cm deep soil cores were obtained for analysis of depth profiles and sectioned into multiple strata before homogenization and DNA extraction. For physical and chemical characterization, two portions of the soil samples were sieved with a 2 mm sieve, dried for 1 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 0.25 g (TN sweetgum) or 0.5 g (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 rRNA gene 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 0.5× Tris-borate-EDTA (TBE) with ethidium bromide and quantified using the Quant-It PicoGreen dsDNA Assay Kit (Invitrogen).

Because two different DNA extraction methods were used in this study, we determined the impact these methodological differences might have on cross-site comparisons. We applied both methods in parallel on 12 soil samples from four sites (NV desert, NC pine, TN sweetgum, and WI aspen; Table S11) and examined systematic bias in total DNA recovery and DNA composition. We did not observe a systematic bias of extraction method on bacterial community richness, diversity or composition (Figs S1 and S2). Although the FastDNA method yielded an average of sevenfold higher recovery than the MoBio method (Table S11), this did not influence our ecosystem analyses, because only FastDNA extracts were used to evaluate treatment effects on community biomass.

### Clone libraries

For each soil sample, a 384-member library of 16S rRNA gene clones was created. 16S rRNA gene fragments of 740–760 bp were amplified from soil DNA using primers 27F (5'-AGAGTTTGATCMTGGCTCAG) (Lane, 1991) and 787Rb (5'-GGACTACNRGGGTATCTAAT) (Kuske *et al.*, 2006). PCR was performed in triplicate for each sample. Each 50 µl reaction consisted of 1× PCR buffer containing 1.5 mM MgCl<sub>2</sub>

(Applied Biosystems), 0.2 mM of each dNTP (Applied Biosystems), 1.5 U of Taq LD DNA Polymerase (Applied Biosystems), 1  $\mu$ M of each primer, and 2  $\mu$ l of DNA template. Each DNA template was diluted 10- or 100-fold in water from the original DNA extract to avoid PCR inhibition. Thermal cycling consisted of 94°C for 2 min; 25 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; 72°C for 7 min; 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 bidirectionally sequenced with M13 primers using Sanger technology.

#### Sequence processing

Forward and reverse sequences were assembled with Sequencher v4.7 (Ann Arbor, MI, USA). Potential chimeras were identified and discarded by Bellerophon (Huber *et al.*, 2004). Remaining sequences were aligned using the Arb-Silva automated alignment tool (Pruesse *et al.*, 2007). Aligned sequences were compiled in a single database in ARB (Ludwig *et al.*, 2004).

#### 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) (Cole *et al.*, 2009). The output was parsed with a custom-written C+ program into a matrix of OTU counts per sample (Table S12). A representative sequence for each OTU at the 0.01 distance level was classified via the RDP classifier (Wang *et al.*, 2007). 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 (Oksanen *et al.*, 2009) in the R statistical computing platform for Mac v2.9.2 (Team, 2009). Other analyses were performed in Microsoft Excel. For trend analyses, the probability of obtaining  $\geq n$  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 (Hamady *et al.*, 2010). The false discovery rate correction for multiple testing (Yoav and Yekutieli, 2001) was performed using the R software package (<http://www.r-project.org/>).

#### Bacterial 16S rRNA gene quantitative PCR

Primers EUB 338 and EUB 518 (Lane, 1991) were used for qPCR of the bacterial 16S rRNA gene. Soil DNA samples were adjusted to 3–25 ng ml<sup>-1</sup> in 1× Tris-EDTA (TE). Each PCR was performed in triplicate as described in Castro and colleagues (Castro *et al.* 2010). Each 30  $\mu$ l reaction con-

tained 15  $\mu$ l of iQ SYBR Green Supermix (Bio-Rad Laboratories), 1.25 mg ml<sup>-1</sup> 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°C for 3.25 min; 40 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s; one cycle of 95°C for 1 min; 80 cycles of 55°C for 10 s; 4°C storage. Reactions were performed with a MyiQ Real-Time PCR machine (Bio-Rad Laboratories).

Quantitative DNA standards for qPCR were produced from an *Escherichia 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°C in Luria-Bertani (LB) broth containing 50 mg ml<sup>-1</sup> 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 *Scal* (New England Biolabs) at 37°C for 2 h followed by enzyme inactivation at 80°C for 20 min. 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 rRNA gene pyrotag sequencing

16S rRNA gene pyrotag data were obtained for 12 soil DNA samples from the WI aspen site. Pyrotag libraries targeting the hypervariable regions V6–V8 were constructed using universal primers 926F and 1392R (Kunin *et al.*, 2010) and were sequenced using the 454-titanium platform and standard protocols (Engelbrektson *et al.*, 2010). 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: (i) acidoG1\_8.2 (5'-GGTGCCTGGAATTCCCGG, 5'-GCGGATTGCTATCGCGTTAG), and (ii) acidoG1\_8.17 (5'-CCCTTGGGACGTAAACTCCTT, TTCCACGCACCTCTC CCA). Each assay was performed in triplicate with primers at 0.2 mM and 1 ng of soil DNA per reaction. Cycling conditions were as follows: one cycle of 94°C for 5 min; 40 cycles of 94°C for 15 s, 65°C for 30 s; 91 cycles of 50°C for 30 s; 4°C 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

Representative bacterial 16S rRNA gene clone sequences were deposited in GenBank with Accession No. JQ366086–JQ387568.

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## Supporting information

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

**Fig. 1.** Impact of DNA extraction method on 16S rRNA gene library similarity. For each method, beta diversity values were

computed for the three samples from each of four sites, and the global average and 95% confidence interval (shown in figure) were computed from the 12 beta diversity values. The mean beta diversity for samples from FastDNA extractions was not significantly different from the mean for samples from MoBio extracts at any classification level, demonstrating a lack of systematic bias.

**Fig. 2.** Impact of DNA extraction method on the beta diversity of 16S rRNA gene clone libraries. MoBio Power Soil and the QBiogene FastDNA extraction methods were compared. 'Duplicates' represents seven pairs of libraries that were derived from DNA extracted in two independent trials from the same seven soil samples. 'Within plots' represents libraries that were extracted from independent soil samples from the same field plot.

**Table S1.** Soil sample collection from six experimental ecosystems.

**Table S2.** Chemical and physical characterization of soil samples from six ecosystems.

**Table S3.** Most common taxa at the family/genus level (OTU85) in ambient surface soils across sites.

**Table S4.** Soil biomass (DNA) and bacterial biomass (qPCR) estimates for soil samples from six ecosystems.

**Table S5.** Percentage change in community richness and associated *t*-test results.

**Table S6.** *t*-tests for differences in average community similarity (beta diversity).

**Table S7.** Responsive taxa with a twofold or greater change in relative abundance in clone libraries and *P*-values < 0.05.

**Table S8.** Taxa with significant trends across sites.

**Table S9.** Richness of Wisconsin aspen pyrotag libraries.

**Table S10.** Putative responsive taxa from pyrotag surveys of WI aspen site with twofold or greater changes in abundance and *P*-values < 0.05.

**Table S11.** Impact of DNA extraction method on DNA recovery.

**Table S12.** Code for standardization of 16S rRNA gene surveys for composition analysis in R.

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