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Elevated atmospheric CO_2 affects soil microbial diversity associated with trembling aspen

Celine Lesaulnier,[1,2] Dimitris Papamichail,[1,3,4] Sean McCorkle,[1] Bernard Ollivier,[2] Steven Skiena,[3] Safiyh Taghavi,[1] Donald Zak[5] and Daniel van der Lelie[1]*

[1]*Brookhaven National Laboratory, Biology Department, Building 463, Upton, New York 11973-5000, USA.*
[2]*IRD, UMR 180, IFR-BAIM, Université de Provence et de la Méditerranée, ESIL, F-13288, Marseille Cedex 09, France.*
[3]*Computer Science Department, SUNY at Stony Brook, Stony Brook, New York 11794, USA.*
[4]*Department of Computer Science, University of Miami, PO Box 248154, Coral Gables, FL 33124-4245, USA.*
[5]*School of Natural Resources and Environment, and Department of Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, MI 48109-1048, USA.*

Summary

The effects of elevated atmospheric CO_2 (560 p.p.m.) and subsequent plant responses on the soil microbial community composition associated with trembling aspen was assessed through the classification of 6996 complete ribosomal DNA sequences amplified from the Rhinelander WI free-air CO_2 and O_3 enrichment (FACE) experiments microbial community metagenome. This in-depth comparative analysis provides an unprecedented, detailed and deep branching profile of population changes incurred as a response to this environmental perturbation. Total bacterial and eukaryotic abundance does not change; however, an increase in heterotrophic decomposers and ectomycorrhizal fungi is observed. Nitrate reducers of the domain bacteria and archaea, of the phylum *Crenarchaea*, potentially implicated in ammonium oxidation, significantly decreased with elevated CO_2. These changes in soil biota are evidence for altered interactions between trembling aspen and the microorganisms in its surrounding soil, and support the theory that greater plant detritus production under elevated CO_2 significantly alters soil microbial community composition.

Received 2 February, 2007; accepted 12 October, 2007. *For correspondence. E-mail vdlelied@bnl.gov; Tel. (+1) 631 344 5349; Fax (+1) 631 344 5751.

Introduction

One of the major contributing factors associated with climate change and global warming is the ever-increasing concentration of atmospheric CO_2. With forests accounting for a large proportion of global net primary productivity (NPP) (King *et al*., 2005), much research has focused on these ecosystems as a component of the terrestrial carbon sink and their potential to mitigate the effects of this greenhouse gas. Although no widely accepted model exists accounting for subsequent plant responses, elevated atmospheric CO_2 has been documented to increase the carboxylation efficiency of Rubisco (Ceulemans and Mousseau, 1994) resulting in enhanced plant growth (Curtis and Wang, 1998), greater fine root production (Hungate *et al*., 1997) and augmentation of soil carbon allocation via secretion of root exudates from the root tips and increased turnover of fine roots (Zak *et al*., 1993; Hu *et al*., 2001). These processes result in a concomitant increase in soil microbial respiration and carbon turnover (Heath *et al*., 2005). There is no consensus on many of the secondary effects associated with these plant responses and their importance in regulating the terrestrial carbon sink remains to be determined.

The plant growth stimulation observed under elevated CO_2 has been found to be transient (Drake *et al*., 1997; DeLucia *et al*., 1999) and may be attributed to the depleting availability of mineral nitrogen (N), a concept referred to as progressive nitrogen limitation (PNL) (Field, 1999). Heterotrophic microbial communities in soil mediate organic matter transformations regulating the biogeochemical cycling of carbon (C) and N in terrestrial ecosystems. Their activities not only govern soil fertility and efficient plant growth but also contribute significantly to the cycling of essential elements (Rillig and Field, 2003). The robustness of microbial responses to changing patterns of nutrient cycling under elevated CO_2 is one of the major factors affecting the functioning of our global ecosystem (Zak *et al*., 2000).

Previous studies on our FACE experiment soil samples showed that total fungal biomass and microbial community composition did not significantly change between each of the triplicate FACE plots for either ambient or elevated CO_2 (Chung *et al*., 2006). However, significant changes in enzymatic activities were noted between ambient and elevated CO_2 treatments, indicating changes

Table 1. DNA concentrations for each domain, number of OTUs studied and predictive richness and evenness statistics.

	Mean femtomoles DNA per gram of soil (SD)		No. of sequences		Observed No. of phylotypes		Chao1 (SD)		Evenness	
Phylogeny	Ambient	Elevated	Ambient	Elevated	Ambient	Elevated	Ambient	Elevated	Ambient	Elevated
Archaea	1.66 (2.3E-03)	1.01 (9.6E-04)	1375	1399	208	100	1664 (492)	342 (93)	0.03	0.02
Bacteria	3.91 (5.7E-03)	3.85 (4.3E-03)	1155	1132	811	803	3042 (293)	4854 (605)	0.33	0.21
Eukaryotes	0.53 (6.4E-03)	0.48 (3.2E-03)	994	942	302	275	576 (58)	685 (97)	0.21	0.13
Fungi	n.d.	n.d.	205	193	60	39	187 (67)	100 (35)	0.25	0.11
Total	n.d.	n.d.	3524	3476	1381	1217	5282 (843)	5881 (795)	n.d.	n.d.

Quantitative PCR was used to determine the relative detectable DNA concentrations (in femtomoles per gram of soil) for each taxonomic domain. 16S and 18S rRNA gene clone libraries were prepared with domain specific primers. Because of differing primer specificity these values can only be used to make quantitative assessments within a single taxonomic group under ambient and elevated CO_2 and cannot be used to construe overall population makeup. The numbers of curated sequences from the ambient and elevated soil rRNA gene libraries are indicated. Based on the number of phylotypes detected, the relative richness and evenness of the respective populations were calculated using the Chao1 richness estimator using single linkage clustering and a 97% sequence similarity cut-off (Chao, 1984), and the Simpson index (Simpson, 1949) for population evenness. Standard deviations (SD) are indicated between brackets. n.d., not determined.

in either microbial community composition at lower previously unexamined phylogenetic levels, or increased metabolic activity under conditions of elevated CO_2. In order to obtain the most complete profile of the soil microbial community and how it is affected by trembling aspen's (*Populus tremuloides*) responses to elevated CO_2, we conducted an in-depth community analysis on composites of these previously characterized soils.

Results and discussion

Previous soil population studies on bacteria and eukaryotes at the domain and phylum levels showed that total microbial abundance does not significantly change under elevated CO_2 at the Rhinelander FACE site (Zak *et al.*, 2000; Chung *et al.*, 2006), which we confirmed with quantitative polymerase chain reaction (q-PCR) (Table 1). In order to address changes in microbial diversity (detection and frequency of operational taxonomic units (OTUs) and microbial richness (total number of different OTUs), a total of 5061 16S (prokaryotic and archaeal) and 1935 18S (eukaryotic) ribosomal rRNA gene clones (Table 1) were generated from total soil DNA extractions obtained from trembling aspen under ambient and elevated (560 p.p.m.) CO_2 concentrations.

An overview of bacterial, archaeal and eukaryotic community compositions for each condition is outlined in Figs 1–3. Richness estimates are presented in Table 1 and their complete analysis, using three alternative clustering methods, is presented in Table 5. Comparing the Chao1 indexes (Chao, 1984) primarily revealed an undersampling of the biodiversity present in both samples for all three domains of life as these values provide estimations of the total number of phylotypes (all OTUs) present in both communities. These primary results however, also show a significant decrease in the archaeal community diversity and an increase for the bacterial community, providing us with the first indication that we had significant population changes occurring under conditions of elevated CO_2. In addition, a general trend of decreasing evenness estimates (Simpson, 1949; Begon *et al.*, 1996; Hughes *et al.*, 2001) was observed, pointing towards a more even distribution among the different phylotypes present in the communities under conditions of elevated CO_2. The most significant changes in OTU abundance, their taxonomic assignment and statistical analysis are detailed in Table 2. The size and composition of the large taxonomic group δ-*Proteobacteria*, as well as several smaller groups (supplementary material, Table S1) showed no significant change and provide an additional internal standard further validating the comparison of these composite samples to determine microbial community composition. Complete comparisons of the community composition for all domains can be found at supplementary material, Table S1 (bacteria), Table S2 (eukaryotes) and Table S3 (archaea). Significant differences in community composition were furthermore confirmed with q-PCR (see Table 3).

Bacterial community composition

Although the total abundance of the bacterial populations in the soil under ambient and elevated CO_2 remained unchanged (Table 1), significant population rearrangements became evident upon closer examination of lower taxonomic levels. *Proteobacteria* attributed OTUs comprised approximately 40% of the bacterial soil population and no statistically significant changes in its composition were evident up to the class level, independent of CO_2 concentration (Fig. 1). Conclusions on the metabolic capabilities within this phylum are hindered by the extent of its physiological diversity, however, their abundance is consistent with previous soil bacterial community studies (Janssen, 2006). Previously undetected and significant

Table 2. Significant soil population changes due to elevated atmospheric CO_2.

Domain	Phylum	Class or order	Genus[a]	Number of OTUs[b]		*P*-value[c]
				Ambient	Elevated	
Bacteria	*Acidobacteria*	*Acidobacteria*	*Gp1*	130	38	< 0.00001
			Gp2	33	9	0.00023
			Gp3	91	48	0.00051
			Gp4	20	61	< 0.00001
			Gp6	39	61	0.01304
			Gp7	24	10	0.01640
	Actinobacteria	*Actinobacteria*	*Arthrobacter*	6	79	< 0.00001
			Lechevalieria	0	13	0.00010
	Bacteroidetes	*Sphingobacteria*	*Chitinophaga*	5	0	0.03425
			Niastella	18	32	0.02601
	Dehalococcoides	*Dehalococcoides*	*Genera_incertae_sedis*	3	12	0.01523
	Firmicutes	*Bacilli*	*Alicyclobacillus*	n.d.	5	0.02885
		Clostridia	*Thermacetogenium*	44	n.d.	< 0.00001
	Planctomycetes	*Planctomycetacia*	*Gemmata*	16	3	0.00289
	Proteobacteria	*α-Proteobacteria*	*Bradyrhizobium*	42	10	< 0.00001
			Pedomicrobium	3	10	0.04102
			Rhodoplanes	56	107	0.00003
			Rhodobium	1	8	0.01746
		β-Proteobacteria	*Thiomonas*	0	6	0.01422
			Polaromonas	0	6	0.01422
			Duganella	5	0	0.03425
		γ-Proteobacteria	*Pseudomonas*	4	19	0.00105
	TM7	*TM7*	*Genera_incertae_sedis*	2	23	< 0.00001
	Verrucomicrobia	*Verrucomicrobiae*	*Xiphinematobacteriaceae Genera_incertae_sedis*	58	27	0.00107
Eukaryota	*Fungi*	*Ascomycota*	*Cazia*	32	n.d.	< 0.00001
			Pachyphloeus	1	26	< 0.00001
		Basidiomycota	*Inocybe*	10	171	< 0.00001
			Grifola	11	n.d	0.00446
			Laccaria	36	12	0.00099
			Boletus	19	n.d.	0.00001
			Thanatephorus	11	n.d.	0.00116
			Thelephora	19	3	0.00098
			Tremellodendron	11	n.d.	0.00059
			Cryptococcus	26	n.d.	< 0.00001
			Rhodotorula	8	n.d.	0.00455
		Zygomycota	*Mortierella*	63	16	< 0.00001
Archaea	*Crenarchaea*	*Unclassified*	*OTU-14*	562	811	< 0.00001
		Crenarchaeales	*Unclassified OTU-22*	186	73	< 0.00001
			NRP-P OTU-23	147	58	< 0.00001
			Unclassified OTU-15	112	75	0.00308
		NRP-J	*OTU-25*	141	77	< 0.00001
		SCA1170	*Unclassified OTU-29*	79	227	< 0.00001
	Thermoplasmata	*Terrestrial group*	*Unclassified OTU-122*	77	31	< 0.00001

a. As defined by Garrity and colleagues (2004) and Wang and colleagues (2007).
b. Abundance of OTUs represented in individual counts.
c. *P*-value of statistical significance calculations are outlined in materials and methods and are based on the Fisher exact test. It should be noted that the threshold to determine significance (alpha value) at the genus level should be divided by the number of phylogenetic groups identified at this level according to the Bonferroni correction.
n.d. not detected.

population rearrangements begin to unveil themselves starting from the order level (Fig. 1). The most significant observations within the α-*Proteobacteria* involve a decrease in *Bradyrhizobium* ($P < 0.0001$) and *Rhodobium* ($P = 0.017$) affiliated OTUs coupled to an increase in *Rhodoplanes* (Table 2). Although far less important, the few statistically significant population changes in β-*Proteobacteria* include an increase in *Thiomonas* and *Polaromonas* ($P = 0.014$) and a decrease in *Duganella* ($P = 0.034$). All values for these observed changes in OTU abundance are further detailed in Table 2. Furthermore, no significant changes were observed within the δ- and ε-*Proteobacteria* (Table S1) whereas the γ-*Proteobacteria* showed one statistically significant variation as the abundance of OTUs affiliated with *Pseudomonas* increased fivefold ($P = 0.001$) (Table 2).

The number of OTUs affiliated with *Actinobacteria* doubled under elevated CO_2. The most significant

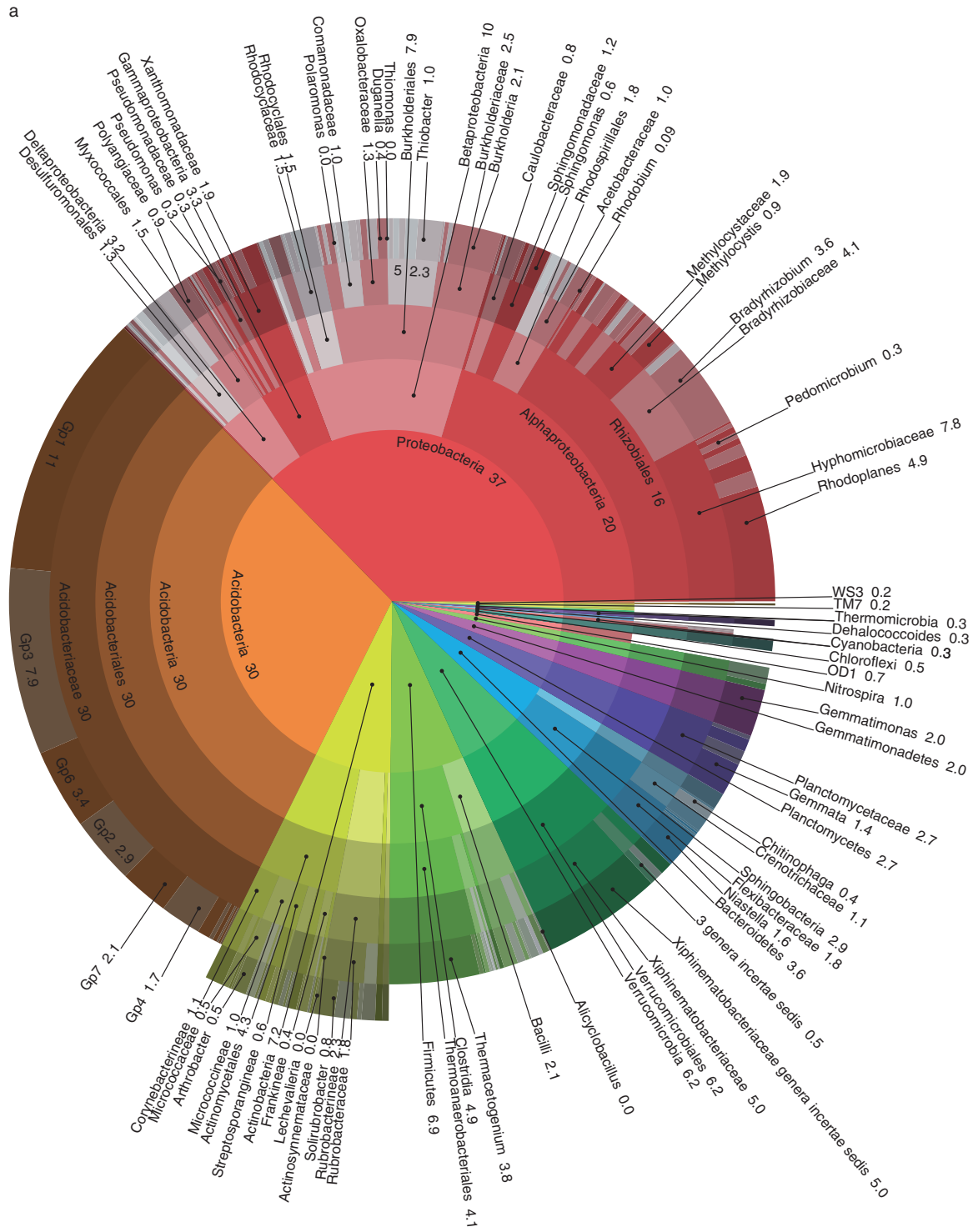

Fig. 1. Taxonomic breakdown of classified 16S rRNA gene sequences in bacterial populations from soil beneath trembling aspen under ambient (a) and elevated (b) atmospheric CO_2. Taxonomic classifications were determined according to Wang and colleagues (2007) and included 1155 ambient (a) and 1132 (b) sequences respectively. Central pie shows percentages by phyla; each outer annulus progressively breaks these down by finer taxonomic levels: class, order, family and genus in the outermost annulus. Figure numbers indicate the relative abundance, expressed as a percentage, of the different taxonomic groups. The assignments shown in the figures are based on the most statistically significant changes (all taxa in Table 2 are included).

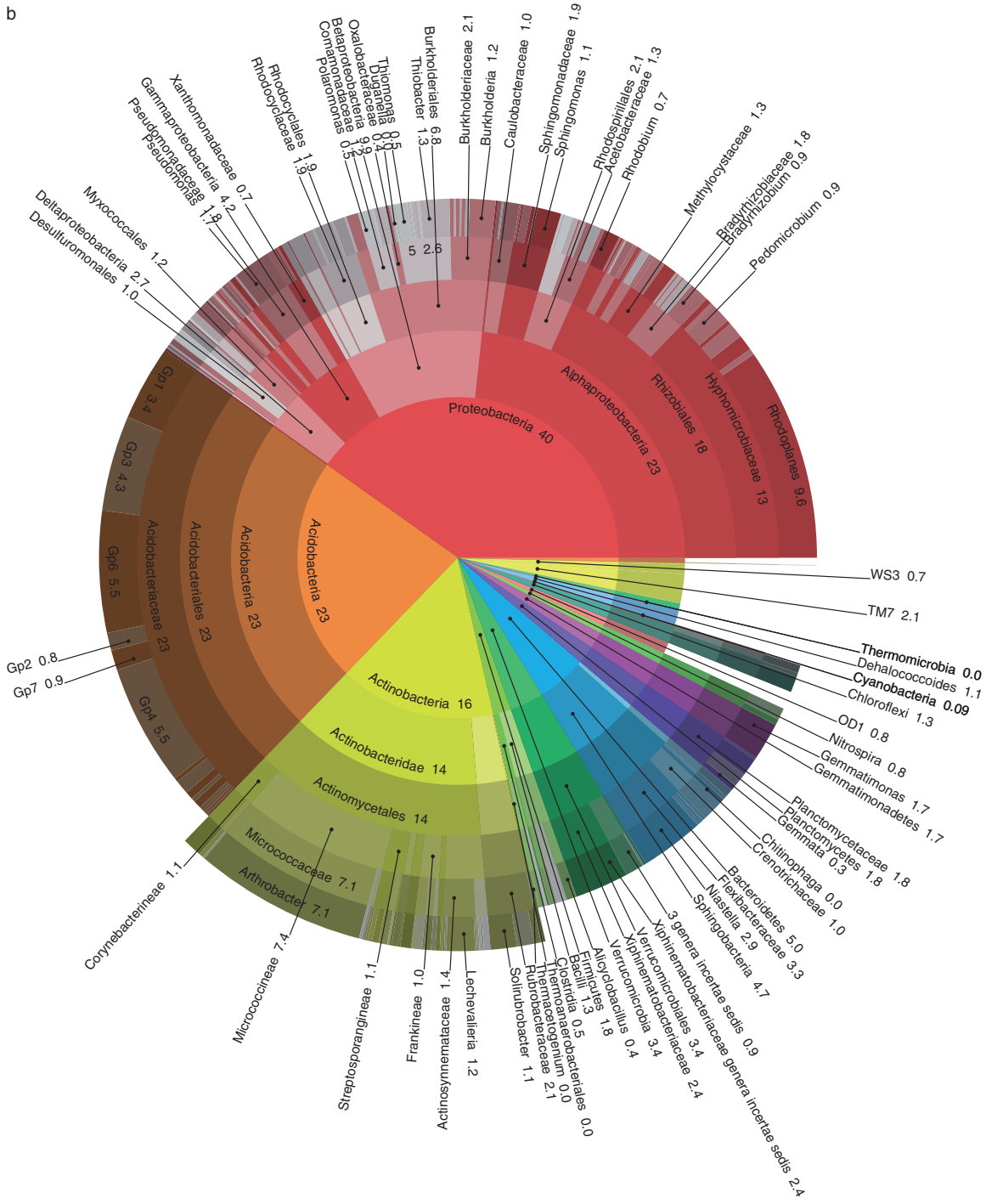

Fig. 1. *cont.*

increases occurred in the genera *Arthrobacter* ($P < 0.0001$), and *Lechevalieria* ($P = 0.0001$) whose representatives both increased in abundance by 13-fold (Table 2). These heterotrophs have been demonstrated to play an important role in the degradation of many recalcitrant forms of soil carbon, including plant cell wall components such as cellulose, hemicellulose, chitin, and are implicated in mediating important transformations regulating the biogeochemical cycling of C and N in terrestrial ecosystems (Goodfellow and Williams, 1983).

An increase in OTUs affiliated with species of *Niastella* ($P = 0.026$) and a decrease *Chitinophaga* ($P = 0.034$) were observed. These *Bacteroidetes*, characterized as *Sphingobacteria*, have known cellulolytic and chitinolytic

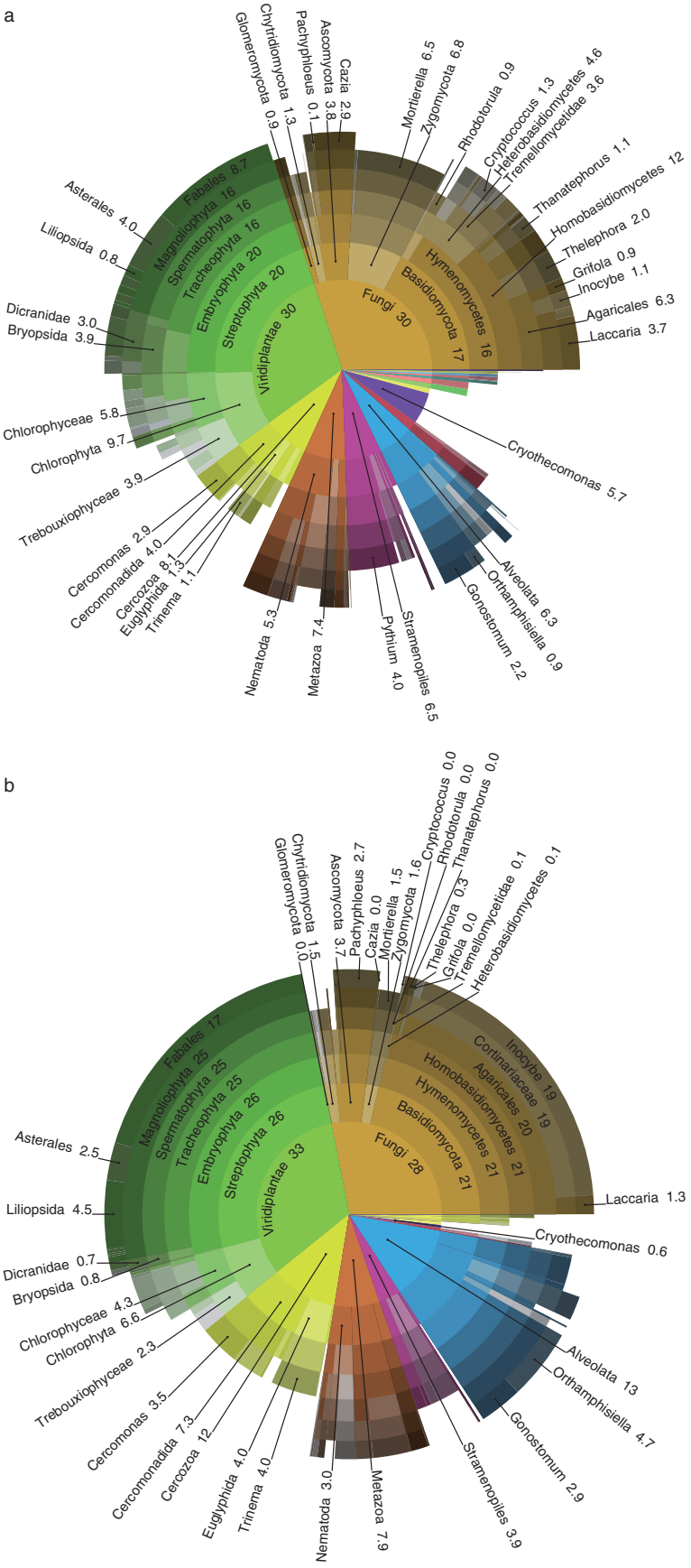

Fig. 2. Breakdown of eukaryotic populations, via NCBI taxonomic assignments of 18S rRNA gene sequences, for ambient (a) and elevated (b) atmospheric CO_2. A total of 994 ambient (a) and 942 (b) sequences were analysed respectively. Central pie shows percentages by phyla; each outer annulus progressively breaks these down by finer taxonomic levels: class, order, family and genus in the outermost annulus. Figure numbers indicate the relative abundance, expressed as percentage, of the different taxonomic groups. The assignments shown in the figures are based on the most statistically significant changes (all taxa in Table 2 are included).

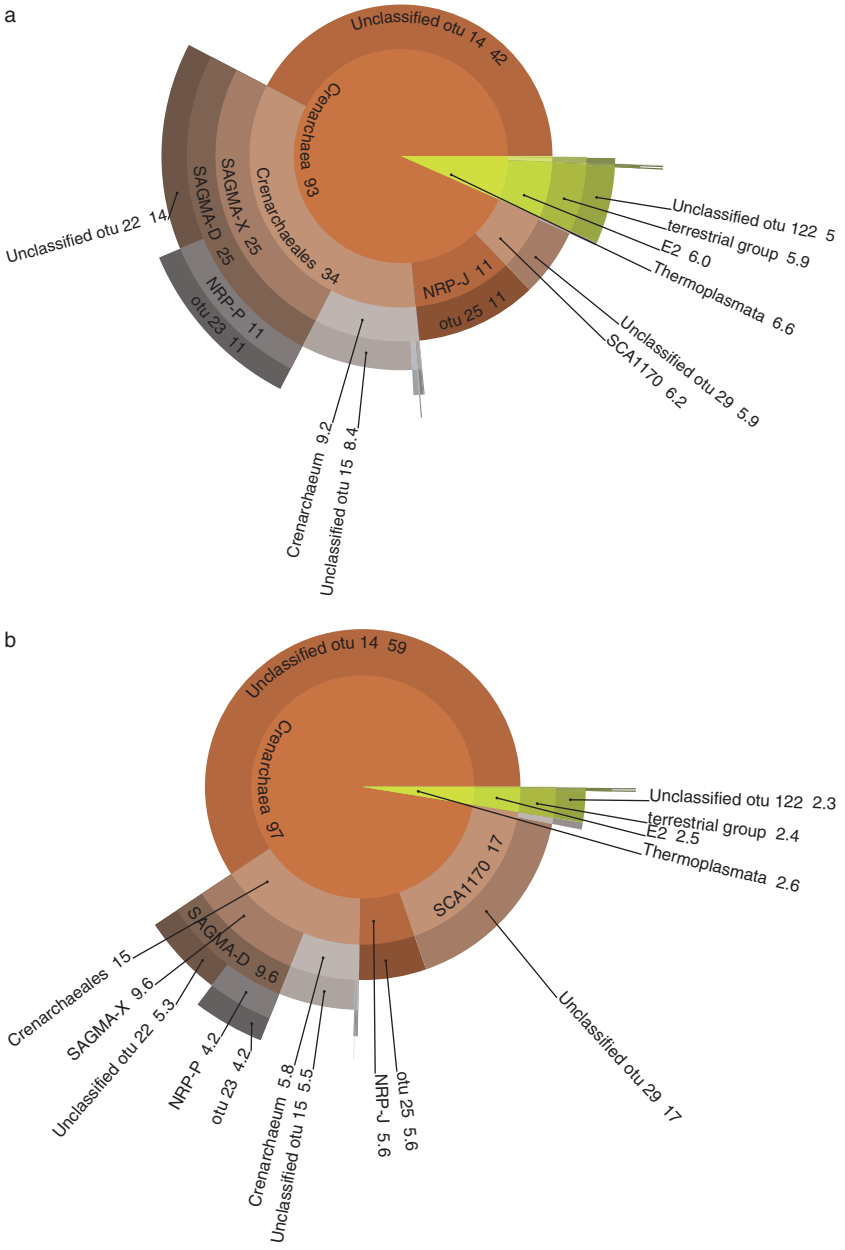

Fig. 3. Representation of archaeal populations, according to the Hugenholtz's taxonomic assignments of 16S rRNA gene sequences for ambient (a) and elevated (b) atmospheric CO_2. A total of 1375 ambient (a) and 1399 (b) sequences were analysed respectively. Figure numbers indicate the relative abundance, expressed as percentage, of the different taxonomic groups. The assignments shown in the figures are based on the most statistically significant changes (all taxa in Table 2 are included).

properties (Sly *et al.*, 1999) that enable such bacteria to metabolize both plant and fungal cells.

Increases in soil carbon, coupled to an increase in cellulolytic and chitinolytic activities have previously been noted to alter below-ground substrate availability for microbial metabolism (Larson *et al.*, 2002; Phillips *et al.*, 2002). Observed increases in the abundance of *Actinobacteria* and *Bacteriodetes* are therefore expected to result in enhanced cycling of essential elements, thereby partially governing soil fertility and plant growth efficiency (Rillig and Field, 2003).

The total number of OTUs taxonomically assigned to representatives of the phylum *Acidobacteria* decreased significantly ($P < 0.001$). Interestingly, significant increases and decreases in the abundance of sequences within this taxa show a large amount of variation. Operational taxonomic units assigned to Gp4 ($P < 0.00001$) and Gp6 ($P = 0.01$) increase significantly, whereas those grouped with the Gp1 ($P < 0.00001$), Gp2 ($P = 0.0002$), Gp3 ($P = 0.0005$) and Gp7 ($P = 0.01$) decrease (Table 2, supplementary online material). The physiological properties of this large group of soil bacteria are unclear. These observations are in part consistent with those reported by Sait and colleagues (2006) who noted an increase in *Acidobacteria* under conditions of elevated CO_2. Our q-PCR data (Table 3) also point to a similar increase in

Table 3. Results of quantitative PCR.

		Mean femtomoles DNA per gram of soil (SD)	
	Phylogeny	Ambient	Elevated
Archaea		1.66 (2.3E-03)	1.01 (9.6E-04)
	Crenarchaeota	8.00E-01 (3.47E-03)	6.29E-01 (3.99E-03)
Bacteria		3.91 (5.7E-03)	3.85 (4.3E-03)
	Bacilli	2.47E-10 (3.51E-11)	8.61E-10 (5.67E-12)
	α-*Proteobacteria*	4.78E-08 (4.73E-09)	1.66E-07 (2.02E-07)
	β-*Proteobacteria*	8.07E-10 (2.17E-10)	1.30E-09 (1.34E10)
	Firmicutes	1.16E-11 (2.34E-12)	2.71E-12 (3.79E-13)
	Acidobacterium	7.45E-12 (9.06E-13)	2.86E-11 (3.15E-12)
	Actinobacteria	5.19E-10 (6.74E-10)	2.86E-09 (3.97E-10)
	Bacteroidetes	1.28E-13 (1.07E-13)	2.68E-12 (3.12E-13)
Eukaryota		0.53 (6.4E-03)	0.48 (3.2E-03)

Quantitative PCR values were only used to confirm population trends of the major groups of microorganisms observed to change in the rRNA gene libraries. All data but those for *Acidobacteria* corresponded with our findings. The results for *Acidobacteria* were related to distant sequences being clustered within this group (i.e. sequences with high edit distance), and changes in primer annealing *Acidobacteria* population members under elevated CO_2 created biases in the quantitative PCR.

Acidobacteria; however, this overall inconsistency could be related to primer specificity. Furthermore, the growth of *Acidobacteria* has been noted to be negatively impacted by increased concentrations of organic matter (Stevenson *et al*., 2004) which has previously been observed in the soil of trembling aspen grown under elevated CO_2 (Zak *et al*., 1993; Hu *et al*., 2001). A significant decrease in the number of OTUs attributed to *Verrucomicrobia*, particularly to uncharacterized members of the *Xiphinematobacteriaceae* family ($P = 0.001$), was observed (Fig. 1; Table 2). Members of these heterotrophs are reported to be unaffected by elevated CO_2 (Sangwan *et al*., 2005); however, they have been described to be negatively impacted by soil moisture (Buckley and Schmidt, 2001), which has been noted to increase under elevated CO_2 (Zavaleta *et al*., 2003).

Finally, a significant increase in the abundance of sequences assigned to the uncharacterized phyla *TM7* ($P < 0.0001$) and *Dehalococcoides* ($P = 0.015$) was observed (Table 2).

Eukaryal community composition

Total eukaryotic DNA concentration in the soil remained unchanged under elevated CO_2 (Table 1). This is also apparent when comparing the abundance and composition of eukaryotic clones, and is in accordance with previous studies (Chung *et al*., 2006). Plant DNA comprises approximately 30% of the eukaryotic clones and remains constant under both conditions with internal modifications (Fig. 2), the most notable of which is a significant increase in *Fabales*, the order to which trembling aspen is assigned.

Fungi comprise approximately 30% of recovered OTUs in each eukaryotic library (Fig. 2). The increase in bacteria with chitinolytic activity may be implicated in why no statistically significant change in fungal abundance has been observed under elevated CO_2 at this site (Zak *et al*., 2000; King *et al*., 2005; Chung *et al*., 2006). However, fungal community composition beneath trembling aspen dramatically changed under elevated CO_2 (Fig. 2).

The results presented show an increased abundance of *Basidiomycota* though a decrease in their diversity is observed (Fig. 2, Table 2). An increased abundance of *Homobasidiomycetes* is correlated to the dominance of the ectomycorrhiza *Inocybe* ($P < 0.00001$) (Fig. 2, Table 2). This is further supported by the findings of Chung and colleagues (2006) who reported a six-fold increase in the detection of OTUs taxonomically assigned to this genus at elevated CO_2. Ectomychorrizal fungi have previously been documented to increase in abundance under elevated CO_2 (Jones *et al*., 1998) and receive a significant portion of the plant's net photosynthate (Hogberg *et al*., 2001). *Heterobasidiomycetes* abundance decreased, with only one OTU detected at elevated CO_2 (Fig. 2). The decreased abundance of the *Urediniomycetes* is also observed. These fungi are predominantly represented by plant pathogens. It has previously been reported that stimulated plant growth decreases their susceptibility to fungal plant pathogens (Chakraborty and Datta, 2003).

Zygomycota assigned OTUs decreased in abundance under elevated CO_2 with the most significant change occurring among the *Mortierella* ($P < 0.000001$) (Table 2). Although abundant in soils, the ecological role of these saprophytes is deemed to be more significant in temperate forests (Carreiro and Koske, 1992).

The number of OTUs assigned to *Ascomycota* remained constant, although under conditions of elevated CO_2 we noticed the disappearance of the dominant genus *Cazia* ($P < 0.000001$) and the incurring dominance of re-

presentatives belonging to *Pachyphloeus* ($P < 0.000001$) (Table 2). *Chytridiomycota*, however, appear to have been unaffected by the increase in atmospheric CO_2. These results suggest that elevated atmospheric CO_2 favour the symbiotic relationship between poplar and ectomychorrizal fungi belonging to the genus *Inocybe*, out-competing other fungi.

Previous studies on protists have shown that although total eukaryotic soil biomass remained unchanged, a threefold to sixfold increase has been noted in the number of protists under elevated CO_2 atmospheric (Lussenhop *et al*., 1998; Hungate *et al*., 2000; Ronn *et al*., 2003). An increase in protist population abundance was also observed in this study. Previous reports have suggested that a modification in microbial biodiversity under elevated CO_2 could be related to increased protozoan feeding. Therefore, a potential exists for future prokaryotic population bias to those organisms that replicate quickly (Ronn *et al*., 2003).

Archaeal community composition

Total archaeal soil DNA, as determined by q-PCR, decreases by 50% under elevated CO_2 (Table 1). Examination of the archaeal OTUs belonging to each library revealed that both populations were predominantly comprised of non-thermophilic *Crenarchaea* (Fig. 3). Sequences phylogenetically classified as *unclassified OTU-14 Crenarchaea* comprised the largest subdivision within this domain and their abundance increased by 40% under elevated CO_2 ($P < 0.0001$) (Table 2). A significant increase ($P < 0.0001$) was also observed for SCA1170 unclassified OTU-29 assigned OTUs (Table 2). The opposite is observed for all sequences classified as *Crenarchaeales*. The most significant changes occurred within the SAGMA-D, which showed a 60% decrease in the abundance of both *unclassified OTU-22* ($P < 0.0001$) and *NRP-P OTU-23* ($P < 0.0001$) and a 30% decrease in *Unclassified OTU-15* ($P = 0.003$) assigned sequences (Table 2). Furthermore, a 45% decrease in OTUs assigned to NRP-J OTU-25, and a 60% decrease in OTUs assigned to unclassified OTU-22 of the *Thermoplasmata* is reported ($P < 0.0001$).

Crenarchaea Chao 1 richness estimates indicate an 80% decrease in species diversity (Table 1). These changes in relative abundances, and associated varying response of different representatives, suggest that *Crenarchaea* are sensitive to the biotic effects of elevated atmospheric CO_2. A low abundance of non-thermophilic *Crenarchaea* has previously been documented in soils (Buckley and Schmidt, 2001; Simon *et al*., 2005). In contradiction to these studies, our quantitative analyses show that these organisms constitute a major group with considerable species richness (Table 1).

Community changes related to nitrogen cycling

No significant changes were noted in the population abundance of bacteria involved in nitrification, predominantly among the *Nitrosospira, Nitrobacter*, *Nitrococcus* and *Nitrospira* (*P*-values between 0.1 and 0.5, supplementary material, Table S3). An increased abundance of heterotrophic decomposers in the soil community under conditions of elevated CO_2 suggests increased rates of biomass turnover and NH_4^+ release. Their increased abundance, along with that of *Inocybe*, coupled to an increased N requirement by these organisms, may have led to reduced $NH4^+$ availability to nitrifiers. Furthermore, the increased mycorrhization of the trembling aspen root system by *Inocybe* has the potential to improve N acquisition to trembling aspen. These results further support the lack of evidence for a PNL response at the Rhinelander FACE experiment (Zak *et al*., 2000; Holmes *et al*., 2003).

The potential importance of *Crenarchaeota* governed ammonia oxidation, its upregulation in soil enriched with ammonium, and their sensitivity to increases in organic matter has recently been brought to light (Quaiser *et al*., 2002; Konneke *et al*., 2005; Treusch *et al*., 2005; Hallam *et al*., 2006; Wuchter *et al*., 2006). Their abundance and apparent adverse reaction to the biotic responses associated with elevated atmospheric CO_2 clearly demonstrates that more work is needed on the interactions of archaea in soil and plants.

Conclusions

The long-term sustainability of ecosystem productivity requires detailed knowledge of its biodiversity coupled to profound understanding of its functioning. In order to better understand the implications that elevated atmospheric CO_2 has on microbial communities, we provide the first detailed analysis profiling changes in specific groups of microbes to specific soil processes.

Initial studies on the soil core samples for both ambient or elevated atmospheric CO_2 showed that the variations between treatments are significantly greater than the intra-treatment variabilities (Chung *et al*., 2006). In order to obtain a global profile of the microbial community in each treatment and to minimize intra-treatment variability, equal amounts of these 21 samples were combined to provide one composite sample for DNA extraction and community analyses. Although having the advantage of minimizing intra-treatment variability, this methodology has, however, the drawback of removing the characterization of population variation within a treatment to subsequently compare the inter-experimental population variations. The possibility remains that intra-treatment variability could exceed the inter-treatment variability,

however, the consistency of representatives in both treatments (particularly the abundance of δ-*Proteobacteria* OTUs) suggests otherwise.

This detailed analysis on how the soil microbial community beneath trembling aspen is affected by elevated CO_2 showed a marked increase in heterotrophs, sustained total microbial abundance, and significant increases in bacterial decomposers. Furthermore, the dominance of the ectomycorrhiza *Inocybe* results in a strong decrease in fungal species diversity under elevated CO_2.

These changes in the trembling aspen microbial community composition further support previously reported increases in fine root biomass turnover rates (King *et al.*, 2005), sustaining the availability and translocation of essential nutrients required for increased plant growth under elevated CO_2. This is further supported by Hu and colleagues (2001) who noted that elevated CO_2 alters the interaction between plants and microbes in favour of plant N utilization, thus prolonging the observed increase in plant biomass production under elevated CO_2.

This is the first report of the effects of elevated atmospheric CO_2 on archaea and our data show that the abundance of *Crenarchaeota*, implicated in ammonia oxidization, decreases under conditions of elevated CO_2. The 2774 OTUs studied provide the most comprehensive profile to date of the diversity of this phylum in soil. This further enabled richness and evenness estimations for better determining the importance of the soil archaeal community of trembling aspen. Our study suggests that for these archaea, conditions of elevated atmospheric CO_2 and the observed changes in microbial community composition create a potentially limiting environment, resulting in a 50% decrease in their abundance and significant decline in species diversity.

Our results show that microbial communities appear to be altered by elevated atmospheric CO_2 and that these changes may have implications for ecosystem function, especially via effects on the cycling of essential elements. Future investigations should shed more light on how elevated atmospheric CO_2 affects the diversity of life, the complexity and functioning of microbial communities in soil, the cycling of essential elements and may further facilitate the prediction of such environmental impacts providing the key for their future correction.

Experimental procedures

Experimental design and sampling procedures

Our study was conducted at the FACE experiment in Rhinelander, WI, USA. In this experiment, factorial CO_2 and O_3 treatments are applied in a randomized complete block ($n = 3$) design. There are a total of 12 30-m-diameter-FACE rings, and within each ring, trembling aspen (*P. tremuloides*), paper birch (*B. papyrifera*), and sugar maple (*Acer saccharum*) are planted at a density of one stem per square metre. Each ring was split into three sections; half of the ring was planted with aspen; one quarter of the ring was planted with aspen and birch, and aspen and maple were planted in the remaining quarter. The trees were exposed to CO_2 and O_3 treatments beginning in May 1998. The level of elevated CO_2 was 560 p.p.m., which is 200 p.p.m. above ambient CO_2 concentration.

Seven soil cores 2 cm in diameter and 15 cm in depth were randomly collected on 1 June 2002 from each ring section. Cores were composited by ring section and immediately frozen. Soil samples were kept at –80°C prior to physical, chemical, enzymatic and molecular analysis as described previously (Chung *et al.*, 2006). For our study microbial communities present in the composite samples (each composited of three times seven soil cores) representing the ring sections planted with aspen that received either ambient CO_2 or elevated CO_2, were compared.

DNA isolation and purification

Per sample extractions were carried out in triplicate on 5 g of soil. Total soil genomic DNA was extracted using the MoBio UltraClean Soil DNA isolation kit (MoBio Laboratories, Solana Beach, CA, USA) as per manufacturer's instructions. DNA (> 23 kb) was further purified on a 0.8% agarose gel and recovered using the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Piscataway, NJ) as per manufacturer's instructions, and eluted in 50 μl ddH_2O, after which samples from the same condition were pooled.

PCR, rRNA gene clone library construction and sequencing

Primers specific for the domains archaea [21F (DeLong, 1992) – 915R (Stahl and Amann, 1991)], bacteria [8F – 1392R (Lane, 1991)] and eukarya [1F – 1520R (Lopez-Garcia *et al.*, 2001)] were used to amplify both 16S and 18S rRNA gene fragments. Polymerase chain reaction was performed on total soil metagenome DNA using 0.4 μM final concentration of primers in 1× Promega buffer (cat No. M190G, Promega, Madison, WI) containing 2 mM Mg sulfate, 0.3 mM of each dNTP and one unit of high fidelity platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) in a total volume of 50 μl. The reaction was carried out with an initial denaturing step for 5 min at 95°C, followed by 35 cycles of 60 s at 95°C, 1 min at annealing temperatures as specified in the above manuscripts, and 3 min at 72°C, with a final extension step for 8 min at 72°C. These products were then cloned into the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) as per manufacturer's instructions. All six clone libraries were sequenced at the Joint Genome Institute (http://www.jgi.doe.gov/) using PE BigDye terminator chemistry (Perkin Elmer, Boston, MA) and both vector specific M13 Forward and Reverse primers, along with internal primers for prokaryotes [341F – 907R (Muyzer *et al.*, 1995)] and eukaryotes [SR7R – SR5 (Lydolph *et al.*, 2005)] on ABI PRISM 3730 capillary DNA sequencers (Applied Biosystems, Foster City, CA).

Quantitative PCR

Per composite soil sample, q-PCR analysis of community composition was performed in triplicate on three independently isolated DNA samples. Polymerase chain reaction primers specific for the ribosomal gene of several major groups of microorganisms were gathered from the literature (Table 4). Quantitative PCR amplifications were performed on a Bio-Rad iCycler (Bio-Rad, Hercules, CA) in 96-well plate microtubes. Samples were prepared in a final volume of 25 μl using the iQ Supermix as per manufacturer's instructions. Conditions for real-time PCR amplifications were as defined in van der Lelie and colleagues (2006) with primer annealing temperatures adjusted accordingly and consisted of an initial hot-start activation step at 80°C for 30 s plus a denaturation step at 95°C for 30 s, followed by 35 cycles at 95°C for 15 s, annealing at the optimal primer hybridization temperature for 30 s, and 72°C for 1.5 min, and a final extension for 4 min at 72°C. Data analysis was carried out with iCycler software (version 3.0a; Bio-Rad, Hercules, CA) as described by Stubner (2002).

Processing of DNA data

Sequence contigs were constructed using PHRED: PHRAP software (http://www.phrap.org/) and multiple sequences were aligned using ARB (Ludwig *et al*., 2004). Putative chimeric sequences were screened using CHIMERA_CHECK (Cole *et al*., 2003).

Classification and phylogenetic tree analysis

All sequences were classified using the local alignment algorithm based on the basic local alignment search tool (BLAST) (Altschul *et al*., 1997). Bacterial sequences were related to the taxonomy outlined by Wang and colleagues (2007). Eukaryotic sequences were classified according to NCBI taxonomy (Benson *et al*., 2002; Wheeler *et al*., 2006), and archaeal sequences according to the Hugenholtz's taxonomy using the Greengenes tools (DeSantis *et al*., 2006). The ARB software package (Ludwig *et al*., 2004) was used for alignments and phylogenetic analysis of all full-length 16S and 18S rRNA gene sequences. Trees topology was evaluated using the neighbour joining and the distance matrix method with different alignment filters (gap filter, positional variability filter, maximum frequency filter). In addition, ARB was used to generate combined trees for the all three domains of life. After all reference sequences were removed, these trees were used for more robust comparisons of microbial communities and structure. *P*-test values for each of the combined trees were calculated as suggested by Martin (2002) and were < 0.0001, meaning that the observed differences in populations are significant. We also used the UniFrac test (Lozupone and Knight, 2005) with 100 simulations to verify the differences in community composition. This test gave *P*-values < 0.01. All phylogenetic trees can be viewed at http://genome.bnl.gov/FACE/.

Accession numbers

The sequences used for phylogenetic analysis are available in the GenBank database (http://www.ncbi.nlm.nih.gov)

Table 4. Primers used for rRNA gene amplification and real time quantitative PCR.

Phylogenetic group	Name	Forward 5′→3′	Reference	Name	Reverse 5′→3′	Reference
Archaea	21F	TTCCGGTTGATCCYGCCGGA	DeLong (1992)	915R	GTGCTGCCCCGCCAATTCCT	Stahl and Amann (1991)
Crenarchaeota	Cren7F	TTCCGGTTGATCCYGCCGGACC	Perevalova *et al.* (2003)	Cren518R	GCTGGTWTTACCGCGGCGGCTGA	Perevalova *et al.* (2003)
Bacteria	8F	AGAGTTTGATCMTGGCTCAG	Reysenbach and Pace (1995)	1392R	ACGGGCGGTGTGTRCA	Lane (1991)
	341F	CCTACGGGAGGCAGCAG	Lopez-Garcia *et al.* (2001)	907R	CCGTCAATTCCTTTRAGTTT	Lopez-Garcia *et al.* (2001)
Acidobacterium	Acid31	GATCCTGGCTCAGAATC	Barns *et al.* (1999)			
Actinobacteria	Actino235	CGCGGCCTATCAGCTTGTTG	Stach *et al.* (2003)	Act1159R	TCCGAGTTRACCCCGGC	Blackwood *et al.* (2005)
Bacilli	BLS342F	CAGCAGTAGGGAATCTTC	Blackwood *et al.* (2005)			
Bacteroidetes	Cfb319	GTACTGAGACACGGACCA	Manz *et al.* (1996)			
α-Proteobacteria	ADF681F	AGTGTAGAGGTGAAATT	Blackwood *et al.* (2005)	Alf685	TCTACGRATTTCACCYCTAC	Lane (1991)
β-Proteobacteria	Bet680	CRCGTGTAGCAGTGA	Overmann *et al.* (1999)	Bet680	TCACTGCTACACGYG	Overmann *et al.* (1999)
Sequencing	M13F	GTAAAACGACGGCCAGTGAA		M13R	CACACAGGAAACAGCTATGA	

Table 5. Richness and evenness estimations calculated for archaea, bacteria, eukaryotes and fungi.

Data set		Ambient									Elevated								
Clustering method		Single			Average			Complete			Single			Average			Complete		
Similarity		99%	97%	95%	99%	97%	95%	99%	97%	95%	99%	97%	95%	99%	97%	95%	99%	97%	95%
Archaea	Observed phylotypes	448	208	119	518	274	175	565	330	220	257	100	53	334	135	81	391	176	109
	Chao1 index	4069	1664	2099	3221	1430	845	2930	1146	758	1847	342	284	1635	350	211	1365	364	201
	Chao1 index SD	873	491	1205	527	305	221	427	180	155	480	94	136	309	70	56	200	55	35
	Simpson index	0.05	0.03	0.02	0.08	0.06	0.04	0.13	0.09	0.09	0.03	0.02	0.03	0.05	0.05	0.05	0.10	0.08	0.08
Bacteria	Observed phylotypes	882	811	696	884	819	730	887	819	745	839	803	738	841	805	752	845	807	762
	Chao1 index	3989	3042	2281	3966	3001	2197	3985	2927	2163	5888	4854	3858	5917	4867	3745	5975	4893	3585
	Chao1 index SD	400.7	292.6	217.9	395.9	282.1	193.6	397.8	268.8	184.0	771.9	605.3	468.8	775.8	606.9	436.9	783.6	610.2	402.1
	Simpson index	0.39	0.33	0.25	0.40	0.39	0.31	0.43	0.40	0.38	0.24	0.21	0.20	0.24	0.24	0.21	0.24	0.24	0.24
Eukaryotes	Observed phylotypes	379	302	206	387	322	243	392	330	267	358	275	216	365	299	235	373	307	259
	Chao1 index	774	576	390	771	579	389	786	583	414	914	685	445	911	686	480	902	684	496
	Chao1 index SD	73.7	58.4	49.0	71.0	53.2	35.8	72.6	51.8	34.9	111.1	96.9	60.0	107.0	86.8	61.6	101.9	83.4	57.7
	Simpson index	0.31	0.21	0.14	0.34	0.31	0.20	0.35	0.32	0.32	0.19	0.13	0.12	0.20	0.18	0.13	0.24	0.20	0.20
Fungi	Observed phylotypes	88	60	35	89	68	47	91	70	54	54	39	31	57	46	33	59	47	41
	Chao1 index	345	187	323	346	148	112	366	139	110	142	100	97	138	106	86	140	111	86
	Chao1 index SD	118.8	66.7	311.5	118.8	36.9	37.2	126.1	31.5	30.9	45.3	35.0	43.8	40.4	32.8	33.2	40.4	34.7	25.5
	Simpson index	0.41	0.25	0.14	0.41	0.36	0.19	0.41	0.39	0.36	0.18	0.11	0.10	0.18	0.16	0.12	0.26	0.16	0.14

The Chao1 non-parametric estimator was used to determine phylotype richness (Chao, 1984, 1987) and calculated on groups clustered according to specific Levenshtein edit distance values (Levenshtein, 1965). In addition to single linkage clustering commonly used for determining phylotypes (Seguritan and Rohwer, 2001), we also used average and complete linkage methods which are more appropriate to larger groupings (Kaufman and Rousseeuw, 1990). Edit distances for 99%, 97% and 95% similarity were set based on the length of the ribosomal RNA gene as 10, 30 and 50 for archaeal 16S, 14, 42 and 70 for the bacterial 16S, and 18, 54 and 90 for eukaryotic 18S rRNA genes respectively. Population equitability was calculated using the Simpson evenness index E_D (Simpson, 1949; Begon *et al.*, 1996; Hughes *et al.*, 2001).

under accession numbers EF018064–EF019217 for bacterial 16S rRNA genes representing the ambient soil community, EF019218–EF020332 bacterial 16S rRNA genes representing the elevated soil community, EF020333–EF021707 for archaeal 16S rRNA genes representing the ambient soil community, EF021708–EF023106 for archaeal 16S rRNA genes representing the elevated soil community, EF023107–EF024100 for eukaryotic 18S rRNA genes representing the ambient soil community, and EF024101–EF025042 for eukaryotic 18S rRNA genes representing the elevated soil community.

Significance

Probability values determining the significance of the observed changes in phylogenetic composition were calculated using the one-side Fisher exact test (Fisher, 1922). We test against the null hypothesis that there is no difference between subpopulation proportions. Differences in proportions follow the multinomial distribution and the Fisher test combinatorially calculates the probability of a difference with no approximations.

To account for the number of simultaneous statistical tests being performed to calculate *P*-values of the differences in the subpopulations, we adjusted the alpha value (significance threshold) by applying the Bonferroni correction. This was carried out by dividing the alpha value by the number of tests at each phylogenetic level, which equals the number of groups compared. The divisors for the bacterial 16S rRNA gene populations are: 20 for the phylum level, 27 for the class level, 52 for the order level, 120 for the family level and 273 for the genus level. The seven taxonomic levels for the eukaryotic 18S rRNA gene sequences have the following divisors: 11, 25, 39, 40, 45, 47 and 42.

Richness

Rather than using the phylogenetic grouping determined from the RDP vetted sequences, the Chao1 non-parametric estimator was used to determine phylotype richness (Chao, 1984; 1987) and calculated on groups clustered according to specific Levenshtein edit distance values (Levenshtein, 1965). In addition to single linkage clustering, commonly used for determining phylotypes (Seguritan and Rohwer, 2001), we also used average and complete linkage methods which are more appropriate to larger groupings (Table 5).

Population equitability was calculated using the Simpson evenness index E_D (Simpson, 1949; Begon *et al*., 1996; Hughes *et al*., 2001), defined as the reciprocal Simpson index D over the maximum number of phylotypes observed D_{max}:

$$E_D = \frac{D}{D_{\max}}, \text{ where } D = \frac{1}{\sum_{i=1}^{S} p_i^2}$$

Here, p_i is the proportion of the population constructed from the ith phylotype.

Kemp and Aller (2004) argued that the amount of sampling required to detect all phylotypes and reach asymptotic values of the Chao1 index (therefore significantly reducing the probability that further sampling will discover novel phylotypes) correlates well with evenness. Low evenness values (< 0.4) are indicative of under-sampling (by a factor of 8 or more), relative to the Chao1 index.

The EstimateS program (Colwell, 2005) was used to estimate the richness for the communities found under conditions of ambient and elevated CO_2 using the ACE index, this in addition to the Chao shared richness estimate (Chao *et al*., 2005). These results can be found in Table 1.

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Supplementary material

Table S1: Multilevel phylogenetic breakdown of differences between bacterial communities found beneath trembling aspen under conditions of ambient and elevated CO_2.
Each line contains a phylogenetic identifier, followed by OTU counts (in parenthesis) for ambient and elevated populations, and a p-value indicating the statistical significance of the difference in composition. Phylogentic levels are indicated by indentation: no indentation for phyla, and progressively larger indentation indicating great phylogentic depth.

Table S2: Multilevel phylogenetic breakdown of differences between eukaryotic communities found beneath trembling aspen under conditions of ambient and elevated CO_2.
Each line contains a phylogenetic identifier, followed by OTU counts (in parenthesis) for ambient and elevated populations, and a p-value indicating the statistical significance of the difference in composition. Phylogentic levels are indicated by indentation: no indentation for phyla, and progressively larger indentation indicating great phylogentic depth.

Table S3: Multilevel phylogenetic breakdown of differences between archaeal communities found beneath trembling aspen under conditions of ambient and elevated CO_2.
Each line contains a phylogenetic identifier, followed by OTU counts (in parenthesis) for ambient and elevated populations, and a p-value indicating the statistical significance of the difference in composition. Phylogentic levels are indicated by indentation: no indentation for phyla, and progressively larger indentation indicating great phylogentic depth.

ARB (http://www.arb-home.de/) tree files representing the individual and combined bacteria, eukaryotic and archaeal communities found associated with trembling aspen under conditions of ambient and elevated CO_2.

Bacteria
ARB file 1: Ambient bacteria
ARB file 2: Elevated bacteria
ARB file 3: Combined bacterial communities

Eukaryotes
ARB file 4: Ambient eukaryotes
ARB file 5: Elevated eukaryotes
ARB file 6: Combined eukaryotic communities

Archaea
ARB file 7: Ambient archaea
ARB file 8: Elevated Archaea
ARB file 9: Combined archaeal communities

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