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Effects of Elevated Atmospheric CO_2 on Rhizosphere Soil Microbial Communities in a Mojave Desert Ecosystem

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Abstract

The effects of elevated atmospheric carbon dioxide [CO_2] on microbial communities in arid rhizosphere soils beneath *Larrea tridentata* were examined. Roots of *Larrea* were harvested from plots fumigated with elevated or ambient levels of [CO_2] using Free-Air CO_2 Enrichment (FACE) technology. Twelve bacterial and fungal rRNA gene libraries were constructed, sequenced and categorized into operational taxonomical units (OTUs). There was a significant decrease in OTUs within the Firmicutes (bacteria) in elevated [CO_2], and increase in Basiomycota (fungi) in rhizosphere soils of plots exposed to ambient [CO_2]. Phylogenetic analyses indicated that OTUs belonged to a wide range of bacterial and fungal taxa. To further study changes in bacterial communities, Quantitative Polymerase Chain Reaction (QPCR) was used to quantify populations of bacteria in rhizosphere soil. The concentration of total bacteria 16S rDNA was similar in conditions of enriched and ambient [CO_2]. However, QPCR of Gram-positive microorganisms showed a 43% decrease in the population in elevated [CO_2]. The decrease in representation of Gram positives and the similar values for total bacterial DNA suggest that the representation of other bacterial taxa was promoted by elevated [CO_2]. These results indicate that elevated [CO_2] changes structure and representation of microorganisms associated with roots of desert plants.

Keywords

microbial ecology; bacterial communities; fungal communities; arid rhizosphere soil; FACE

1. INTRODUCTION

Current estimates suggest that the atmospheric concentration of CO_2 ([CO_2)] will increase from ca. 380 ppm (~ μmol mol^{-1}) at present to between 550 and 600 ppm by the year 2050 (Houghton et al., 2001). The increase in atmospheric [CO_2] is projected to have an impact on many terrestrial ecosystems, including arid and semi-arid lands (Norby et al., 2001; Nowak et al., 2004). Since arid/semiarid ecosystems make up a considerable fraction of our biosphere (~40% of the earth's terrestrial surface area), their response to elevated [CO_2] needs to be understood. Many studies characterizing the effects of elevated [CO_2] on terrestrial ecosystems have focused on the response of plants and shown that plant

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physiology and production changes significantly. From a soil microbiology perspective, interesting plant changes occurring in elevated [CO_2] are those of increased root exudation and root growth, and changes in the quality and quantity of plant litter. How these altered plant processes affect soil microbial communities, which in turn modulate nutrient availability and many other soil processes, is only beginning to be addressed.

Evidence supporting the concept that exposure to elevated [CO_2] alters the composition of soil bacteria associated with roots is provided in part by extensive research in plant physiology. For example, photosynthetic rate has been shown to increase in elevated [CO_2] (Ellsworth et al., 2004). This increase is associated with many changes in plant function, such as increased growth, higher carbon (C) root exudation, and higher litter quantity but low litter quality (Billings et al., 2003; Cotrufo et al., 1994; Cotrufo and Ineson, 1995; King et al., 1997). Importantly, the increase in plant photosynthate is concomitant with a higher net C assimilation and associated with many changes belowground, such as increased root biomass (Rogers et al., 1994) and increased total rhizodeposition and changes in chemical composition of root exudates (Rogers et al., 1994; Sadowsky and Schortenmeyer, 1997). Since roots are the primary additional C input source into the soil, heterotrophic microbial populations colonizing the soil attached to roots (rhizosphere) or the bulk soil in close proximity to roots may be affected (Marilley et al., 1999; Montealegre et al., 2000; Sadowsky and Schortenmeyer, 1997). Observations in non-desert ecosystems have shown higher rates of microbial respiration in conditions of CO_2 enrichment as compared to control conditions (Dhillion et al., 1995; Rogers et al., 1992; Runion et al., 1994; Williams et al., 2000). In desert soil, which is often low in organic matter, the additional C substrate from plant roots to soil is expected to have a more pronounced impact than in soils with a higher content of organic matter (Buyanovsky et al., 1982). Soil respiration of ecosystems exposed to elevated [CO_2] increases with little or no contribution of root respiration, which suggests microbial respiration as a major contributor in the altered C cycle (Phillips et al., 2006). Further, N cycling and other nutrient transformations in soil, also modulated by soil microbial activities, are affected in conditions of elevated [CO_2] (Apple et al., 2005; Billings et al., 2002[1], 2004;).

In spite of the significant effects of elevated [CO_2] on plant physiology and soil processes in arid and semiarid ecosystems, there are only a few experimental attempts to understand how microbial diversity varies with varying concentrations of atmospheric CO_2. In this study, fungal and bacterial diversity associated with roots of the dominant evergreen shrub *Larrea tridentata* were surveyed using rDNA assays and quantified by PCR (QPCR) methods. We hypothesized that root-associated bacterial communities would change in structure, but also in taxa representation, with shifts in species composition and functional group structure representing more carbon-rich (higher C:N ratio) soil conditions.

2. MATERIALS AND METHODS

2.1 Experimental site

The field experiments took place at the Nevada Desert FACE (Free-Air CO_2 Enrichment) Facility (NDFF; Jordan et al., 1999) located within the Department of Energy's Nevada Test Site (NTS), about 100 km from the city of Las Vegas, NV, USA (36°49′N, 115°55′W, 965–970 m elevation). The NDFF has been in operation since April 1997, consisted of three control circular plots fumigated at ambient [CO_2] (~380 ppm), and three plots at elevated [CO_2] (550 ppm). Within the FACE rings, an intact Mojave Desert ecosystem is exposed continuously to the target atmospheric CO_2 concentrations (Jordan et al., 1999), although conditional shut-downs occurred during high winds (> 7 m s^{-1}) and freezing temperatures. During daylight hours, when plants are photosynthetically active, CO_2 fumigation occurred over 95% of the time on an annual basis.

Each 28 m diameter experimental plot was equipped with a pivoting walkway from which all plant and soil sampling was performed (Jordan et al., 1999). Therefore, all soil surfaces in the plots were left undisturbed throughout the multi-year experiment, preserving the biological soil crust community which covered ca. 20% of the surface and is active in fixing atmospheric nitrogen. Precipitation inputs from Jan-May 2007 were 56 mm, versus a mean of 70 mm from 1997 to 2007.

2.2 Soil and rhizosphere sampling procedures

Five to seven *Larrea* plants located inside each FACE ring were randomly chosen for fine root harvest in May 2007 (an approximately average rainfall year in the region). Visible fine roots were collected by a hand trowel to maximum depth of 50 cm. Roots and soil from each *Larrea* plant were placed in a separate plastic bag, placed immediately in an ice chest, and stored at −20°C within one hour after collection. Within 24 hours after collection, samples were transferred to and kept at −80°C until further processing. Fine roots were processed for DNA within a period of one to three months after collection.

2.3 DNA isolation and purification

Roots were visually inspected, sieved and fine roots <1 mm in diameter were collected with sterile instruments. Three hundred mg of fine roots from each FACE ring, collected from three to five *Larrea* plants, were used to form a composite sample per plot. Roots were placed in a 50 mL Falcon centrifuge tube (BD Biosciences, San Jose, CA), containing 8 mL of sterile phosphate buffer saline (pH 7.4) and vortexed vigorously for 20 s. Roots were removed and the remaining rhizhosphere suspension was centrifugated at 13,000 rpm for 10 min. After centrifugation the pellet was used for DNA extraction with the Fast DNA Spin Kit for Soil and FastPrep FP120 bead beater instrument (QBioGene, Carlsbad, CA, USA).

2.4 PCR

Polymerase chain reaction (PCR) is an enzymatic process that rapidly amplifies specific DNA sequences (Saiki *et al.*, 1985). This technique can be used to detect specific microorganisms by amplifying DNA or RNA sequences unique to the organism of interest regardless of the physiological state of the organism. PCR amplification is especially useful in the detection of slow-growing organisms, non-culturable organisms, or those that are difficult to grow in the laboratory (Buttner *et al.*, 2002).

The bacterial 16S rRNA gene was amplified by PCR from rhizosphere soil DNA using universal bacterial primers f8-27 and r1510 (Appendix A) (Integrated DNA Technologies, San Diego, CA, USA). PCR was performed in a total volume of 50 μL using the following reagents: One μL of template DNA (approximately 0.06 μg DNA), 125 μg of bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA), 1.25 mM $MgCl_2$ (Omnipur), 200 μM deoxynucleoside triphosphates (Promega, Madison, WI, USA), 0.63 μM and 0.8 μM, respectively, of primers f8-27 and r1510 (Appendix A), and 1.25 U Go*Taq* polymerase (Promega). The following PCR reaction conditions were performed in a Biorad iCycler: 95°C for 2 min, 32 cycles (95°C for 30 s, 56°C for 35 s, 72°C for 1:30 min), and 72°C for 10 min. The PCR product was purified in affinity columns (Qiagen, Valencia, CA, USA). The fungal 18S rRNA gene was amplified by PCR from rhizosphere soil DNA using universal fungal primers nu-SSU-0817-5′ and nu-SSU-1536-3′ (Appendix A) (Integrated DNA Technologies). Fungal PCR reaction was identical to the protocol used to amplify the bacterial 16S rRNA gene above, with the following changes: 400 nM of fungal primers (as suggested by Borneman and Hartin, 2000), and 5 min of final PCR elongation.

2.5 Construction and sequencing of S rRNA gene clone libraries

Construction and sequencing of the 18S rRNA (fungal) and 16S rRNA (bacterial) genes required several steps: (1) generating PCR products;(2) cloning of PCR products into plasmid vectors;(3) transforming *Escherichia coli* cells with recombinant plasmids containing cloned rRNA genes (this step is required to propagate recombinant plasmids for subsequent analyses); (4) preparing the recombinant plasmids for sequencing; and (5) sequencing individual clones. Aliquots from the PCR products obtained from each of the plots were combined to generate a master sample from either ambient or elevated [CO_2]. This pooling of soil samples has been previously used at other FACE sites on microbial communities (Lesaulnier et al., 2008; Lipson et al., 2005). This sampling strategy provided well-mixed, diverse samples from each [CO_2] treatment that were compared using phylogenetic tools. The PCR products from each of the tested conditions were cloned into pGEMT-easy vector by ligation (Promega) and the ligation reactions were transformed into competent XL1-Blue *E.coli* cells (Stratagene, La Jolla, CA, USA) using the manufacturer's protocol. Transformants containing individual clones were selected on Luria-Bertani (Sigma-Aldrich) agar plates containing 50 mg mL^{-1} carbenicillin (Calbiochem, San Diego, CA. USA), 4 μL mL^{-1} Isopropyl β-D-1-thiogalactopyranoside (IPTG) and 0.8 μL mL^{-1} 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal) (Promega), and incubated at 37°C overnight. White colonies, which indicated the presence of cloned 16S or 18S rRNA fragments, were transferred to Costar round-bottom 96-well plates (Corning Life Sciences, Corning, NY, USA) containing LB broth and carbenicillin and incubated at 37°C overnight. Each of the clones were individually sequenced using the DyDeoxy terminator technique at the Nevada Genomics Center, Reno. This effort resulted in 407 and 371 good quality 16S and 18S rRNA sequences, respectively. Of note, close to 10% of the clones were discarded due to poor quality sequencing.

2.6 Classification of sequences and phylogenetic tree construction

Bacterial phylogenetic affiliations using an 80% confidence threshold were assigned with the "Classifier" function through the Ribosomal Database Project Release 9 (RDP, East Lansing, MI, USA). Bacterial 16S rRNA and fungal 18S rRNA gene sequences were assembled, aligned and edited using MEGA 4. MEGA (Molecular Evolutionary Genetics Analysis) 4 is a software tool to conduct automatic and manual sequence alignment, and inferring phylogenetic trees (Tamura et al., 2007). The ARB (latin, "arbor"= tree, this software is a graphically oriented package comprising various tools for sequence database handling and data analysis (Ludwig et al., 2004) software package was used to generate phylogenetic trees using rDNA sequences. Chimeric sequences were identified with Bellerophon (Huber et al., 2004).

2.7 Significance

For analysis on the observed changes in phylogenetic composition, probability values determining significance were calculated using a one-sided Fisher exact test (Fisher, 1922), which calculates the probability of a difference with no approximations. The null hypothesis is that there are no differences between the representation of taxa found in elevated and ambient [CO_2] treatments. The differences in proportions of taxa found under the different [CO_2] treatments follow a multinomial distribution. To account for the number of simultaneous statistical tests being performed to calculate *p*-values on the differences in subpopulations, the α value (significance threshold) was adjusted by applying the Bonferroni correction. That is, the α value was divided by the number of groups compared at the phylum, class, and genus phylogenetic level. The denominator values (Bonferroni correction factors) for the bacterial 16S rRNA gene populations were 11 for phylum, 16 for class, and 294 for the genus level. The three taxonomic levels for the fungal 18S rRNA gene sequences had 3 divisors for phylum, 11 for class, and 64 for the genus level.

2.8 Richness, Genetic and Phylogenetic Analyses

Chao (1984) introduced a non-parametric estimator for species richness that uses the form $S = D + (a^2/2b)$, where S is the total number of species in a community, D is the number of species discovered/observed in the sample, a is the number of species observed just once, and b is the number of species observed just twice. The Chao 1 estimator (Colwell & Coddington, 1994), used when Chao is applied to a single collection of specimen, was applied to estimate species richness in our OTU-based analyses. The standard deviation was computed using $b [(a/(4b))^4 + (a/b)^3 + (a/(2b))^2]$ (Colwell and Codington, 1994. Chao1 (Chao, 1984) and rarefaction analyses, which describe richness, relied on distance matrices generated by the neighbor-joining method, and the Jukes-Cantor correction. These distance matrices were generated using MEGA 4. The Chao1 and rarefaction analyses were implemented using DOTUR. DOTUR (Distance based OTU and Richness) is a computer program that uses a distance matrix as the input file and assigns sequences to OTUs using nearest neighbor clustering algorithm. After sequences were assigned to OTUs, the frequency data for each distance level is used to construct rarefaction for the number of species observed, and Chao1 richness estimator as a function of sampling effort (Schloss and Handelsman, 2005).

The Fst test uses the formula $\mathbf{F_{ST} = (\theta_T - \theta_W)/\theta_T}$ where θ_T is the total genetic diversity for all communities combined, and θ_W is the average within-sample diversity for all the communities being compared. This test determines whether the diversity observed in each of the [CO_2] treatments is similar to the total diversity observed in both treatments combined. The null hypothesis for the F_{ST} test was the level of genetic diversity within each [CO_2] treatment community was equal to the level of diversity of the two [CO_2] treatments combined, or $F_{ST} \approx 0$ (on a scale of 0 “no difference” to 1 “unique”). Statistical significance was calculated by randomly assigning sequences to populations and 1,000 permutations using Arlequin 3.11 (Excoffier et al., 2005). The P-test (Martin, 2002) was used to calculate probability values for the bacterial and fungal populations, and was performed by UniFrac (Lozupone et al., 2006). One thousand randomly permuted trees were generated and the tree lengths needed to evolve the different communities obtained from the two [CO_2] treatments were calculated. Statistical significance is inferred from determining the expected number of changes between the two [CO_2] communities. The hypothesis was that the sequences from each community were not randomly clustered.

2.9 Accession numbers

GU200736 to GU200934 and GU200935 to GU201121 were assigned to bacterial clones obtained from Elevated [CO_2] and ambient conditions, respectively. Accession numbers GU201122 to GU201303 and GU201304 to GU201492 were assigned to fungal clones from elevated [CO2] and ambient conditions (http://www.ncbi.nlm.nih.gov).

2.10 Test microorganisms for QPCR

Cell cultures and DNA from referenced microorganisms were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). One representative from all bacterial phyla, several additional species isolated from the Nevada Test Site (NTS), and fungal and human DNA for cross-reactivity testing were obtained (Appendix B).

2.11 Development of primers and probes for Gram-positives for QPCR

A Gram-positive group-specific oligonucleotide probe was developed from probe RW03 (Greisen et al., 1994) and renamed Gram+P (Appendix A). Basic Local Alignment Search Tool algorithm (BLAST, National Institutes of Health, Bethesda, MD, USA) search results showed that the probe region is specific for Gram-positive bacteria (data not shown).

Critical PCR primer design parameters, such as melting temperature, and guanine and cytosine (G+C) nucleotide base percentage values were verified for candidate primer sets using Primer Express software (Applied Biosystems, Foster City, CA, USA). The primers and probe (Appendix A) were purchased from Operon Biotechnologies (Huntsville, AL) and Applied Biosystems, respectively. The amplification of the greatest number of target bacterial phyla and the strength of the signal resulting from amplification of 5 to 10 ng of template DNA were the criteria to select the final primer and probe. These primers and probe set were further evaluated on the ability to amplify the DNA of representative bacteria from every bacterial phylum, genera previously characterized from the subsurface, as well as cross-reactivity with non-bacterial organisms (Appendix B).

2.12 Construction of QPCR standards

Quantitative PCR (QPCR) provides a means of determining the initial concentration of target DNA template in the sample. The theory of quantification is based on the exponential accumulation of the amplification product during PCR, which is dependent on the concentration of the initial template and the efficiency of the reaction (Crotty *et al*., 1994). Two sets of standards were constructed, one for the test cultures and one for the rhizosphere samples. The DNA from *Bacillus atrophaeus* or 16S rDNA PCR amplification products were used to construct quantitation standards. For the test microorganisms, the QPCR quantification standard was prepared from a purified suspension, ranging from 10^0 to 10^5 spores, of *Bacillus atrophaeus* (U.S. Army Dugway Proving Ground, Dugway, UT, USA). These spores were enumerated electronically with a Coulter Multisizer II (Beckman Coulter, Miami, FL, USA), and used for DNA extraction and purification as previously detailed (Buttner et al., 2001). The amount of DNA extracted from test microorganisms and the quantity of PCR products used to construct quantitation standards were measured spectrofluorometrically using the Quant-iT PicoGreen assay (Invitrogen, Carlsbad, CA, USA) in a 96-well flat-bottom black polystyrene assay plate (Costar; Corning), with a Flx800 Microplate Fluorescence Reader (BioTek, Winooski, VT, USA). Standards containing known DNA concentrations were prepared according to the PicoGreen assay instructions, and concentrations of samples were determined using a standard curve. Data analysis was done with the KCjunior software (BioTek). The *Ct* values from the standards provide absolute quantification of *B. atrophaeus* templates and were used to estimate the concentration of DNA templates in samples. Ct refers to the PCR cycle at which fluorescence (i.e., amplification product) is first detected; and is inversely proportional to the log of the initial DNA template concentration. Concentration values for the unknown samples were extrapolated from the standard curve by the software and reported as the mean of two replicates.

To quantitate rhizosphere DNA, we constructed standards from a composite of six PCR reactions (one reaction for each of the six FACE ring rhizosphere soil samples). The PCR reaction and purification was performed as detailed in the “PCR” section of Materials and Methods. Equal volumes of the resulting DNA products from all six FACE rings were combined to form a composite DNA mixture to make the standards. The DNA concentration of this composite was measured spectrofluorometrically using the Quant-iT PicoGreen assay as described above. Ten-fold serial dilutions of PCR products were used to construct a six-point standard curve, beginning with the concentration of 29.7 ng and 2.97 ng for universal and Gram-positive, respectively, per QPCR reaction. Quantitation was achieved by amplification of standards and samples using the developed primers GF3 and GR1, and probe Gram+P (Appendix A). Standards were amplified in triplicate under the same conditions as the unknown samples.

2.13 Quantification of DNA

The ABI Prism 7900HT Fast PCR System (Applied Biosystems) was the instrument used for QPCR analysis. Soil DNA was amplified by QPCR using the universal bacterial probes and primers (Appendix A) as specified by Nadkarni et al. (2002), and the following QPCR conditions. For Universal QPCR, final amplification concentrations and conditions for a 25 μl reaction volume were as follows: 5 μl DNA template (approximately 0.3 μg DNA), 12.5 ul 2X Universal Master Mix (Applied Biosystems), 0.1 % BSA (Sigma, St Louis, MO, USA), 0.2 μM forward primer NadF, 0.5 μM reverse primer NadR, 0.15 μM probe UnivP (Appendix A), and sterile nuclease-free water. Cycling conditions were the Applied Biosystems default Standard Mode: 50°C × 2 min, 95°C × 10 min, followed by 40 cycles of 95°C × 15 sec and 60°C × 1 min.

For Gram-positive QPCR, amplification concentrations and conditions for a 25 μl reaction volume were as follows: 5 μl DNA template, 15 ul DNase-treated FAST Universal Master Mix (Applied Biosystems), 0.1 % BSA (Sigma), 0.9 μM each of forward GF3 and reverse primers GR1, 0.2 μM probe Gram+P (Appendix A), and sterile nuclease-free water. Cycling conditions were the Applied Biosystems default Fast Mode (95°C × 20 sec, followed by 40 cycles of 95°C × 1 sec and 60°C × 20 sec). Due to positive PCR results obtained in no-template control samples with the Gram-positive primers and probe, DNase treatment of the master mix was performed to enzymatically digest residual contaminant DNA present in the FAST Universal Master Mix. A 10-fold dilution of Turbo DNase (Ambion, Foster City, CA, USA) and accompanying 10X Turbo DNase Buffer was added to the 2X FAST Universal Master Mix (Applied Biosystems) in a ratio of 1 μl: 1.5 μl: 12.5 μl, respectively. The mixture was incubated in a 37°C water bath for 3 hrs with shaking at 50 rpm, and by treatment for 30 min. at 75°C with vortexing and pulse centrifugation every 10 min.

Results from the experiments that tested the QPCR primers and probes showed that 11 of the 12 Gram-positive microorganisms belonging to the phyla Firmicutes and Actinobacteria were amplified with the GF3/GR1 primers and the Gram+P probe (Appendix B). Of the 26 non-target Gram-negative organisms tested, only *Borrelia burgdorferi* was amplified with this protocol. The use of universal primers showed that No *Borrelia*, or other Spirochaetes, were detected in any of the environmental clone libraries of the same studied environment, thus their effect on the quantification of the target populations was not expected to be significant. No cross-reactivity was observed with fungal or human DNA.

After amplification, all QPCR data were analyzed using the ABI Prism 7900 HT Fast PCR System. Using the concentrations assigned to each standard, the software constructed a standard curve of *Ct* value versus concentration. Concentration values for the experimental samples were extrapolated from the standard curve (not shown) and reported as the mean of six replicates. To assess the concentration of DNA of bacterial microorganisms in rhizosphere soil from the quantitative standard curves, the following formula was used: $b = \log^{-1}((y-7.775)/-4.470)$, where b = log of DNA concentration of total bacterial 16S rDNA, y = QPCR Ct value, 7.775 = y-intercept, −4.470 = slope. The correlation coefficient of the straight line was 0.993. Similarly, the following formula was used to determine the concentration of Gram-positives: $G = \log^{-1}((y-16.657)/-3.538)$, where G = log of DNA concentration of Gram-positive microorganisms, y = QPCR Ct value, 16.657 = y-intercept, −3.538 = slope. The correlation coefficient of the straight line was 0.967.

3. RESULTS

3.1 Richness estimates

Richness estimates are shown for bacterial and fungal communities under conditions of ambient and elevated [CO_2] in Table 1. The Chao1 predicted values for richness of bacterial

communities were similar for both [CO_2] treatments, whereas the Chao1 values for fungal communities indicated that elevated [CO_2] enhanced the development of fungal communities, although they were much smaller than those observed for bacteria.

3.2 Microbial community composition

Among *Bacteria*, Proteobacteria OTUs (all subdivisions) comprised approximately 50% of the Mojave Desert soil populations and were similar in the two [CO_2] environments (Table 2). However, at the class level among Proteobacteria, the γ-proteobacteria showed a significantly higher representation in ambient compared to elevated [CO_2].

The total number of OTUs taxomically assigned to the phylum Actinobacteria (high G+C Gram-positive) doubled under elevated [CO_2] (Table 2). However, the abundance of Firmicutes, which are low G+C Gram-positive organisms, was significantly lower under elevated [CO_2] than in ambient controls. This decrease in Firmicutes is mainly attributed to members of the “Bacilli” class, whose representatives decreased in abundance by 4-fold in elevated [CO_2]. Other non- Proteobacteria and a small portion of rRNA gene sequences that could not be assigned a taxonomic group made up the rest of the sequences recovered from the rhizosphere soil, and there were no significant differences in their OTU representation in the two [CO_2] treatments (Table 2).

3.3 Fungal community composition

Examination of the fungal OTUs revealed the presence of three major phyla, with Ascomycota comprising 82% of sequences within the fungal libraries (Table 3). That the majority of fungal clones fell within this phylum is interesting and may indicate biases due to PCR or DNA extraction. However, this is unlikely because our DNA extraction and PCR protocols have been previously used in studies of many types of soils and have shown no noticeable phylum biases (Blackwood et al., 2005; Eder et al., 1999; Lesaulnier et al., 2008). In addition, the observed increase of abundance in the ascomycota was mostly due to increases in the number of clones obtained in the elevated [CO_2] treatment.

Within the Ascomycota, representatives of two classes showed significant increases in abundance at elevated [CO_2] (Table 3). Within Eurotiomycetes, there was a significant increase of OTUs assigned to the order Onygenales, specifically among a cluster that include *Arachnomyces*, *Kraurogymnocarpa*, and others that grouped closely with these species (Table 3). The other significant increase in the Ascomycota involves the class currently classified as “Dothideomycetes et Chaetothyriomycetes insertae sedis”, of which members are virtually absent from ambient [CO_2] rhizosphere soils.

The OTUs classified to a large group within Mitosporic Ascomycota were not detected as often in conditions of elevated [CO_2]. Basidiomycota comprised approximately 16% of recovered OTUs in the fungal library. The OTUs classified to this phylum were found mostly in the class Agaricomycetes, which showed a significant increase in elevated [CO_2] (Table 3). Among this class, a highly significant change was noted among *Lepista* (Table 3). Other non-significant changes in phyla representation contributed very little to the fungal libraries.

3.4 Bacterial and fungal Fst and P-tests

Population pairwise F_{st} values suggest bacteria in elevated [CO_2] were different from ambient controls (0.018 on a scale of 0 “no difference” to 1 “unique”, $p \leq 0.00001$). Similarly, fungi populations obtained from elevated [CO_2] were different from populations found under ambient controls (0.022, $p \leq 0.00001$). These results imply that there was significantly less genetic diversity within each [CO_2] treatment community than for both

communities combined. The P-test results showed that the bacterial and fungal communities in the two [CO_2] treatments were phylogenetically different (both $p < 0.001$).

3.5 Quantification of DNA

To further examine the differences in bacterial communities described above, QPCR was used to measure bacterial and Gram-positive microorganisms. The concentrations of bacterial 16S rDNA in rhizosphere soil samples, standardized by gram of fine root weight, were determined to be similar in conditions of elevated and ambient [CO_2] (Table 1). In contrast, the values for Gram-positives in ambient [CO_2] were 43% higher than those seen in elevated [CO_2], although Table 1 suggests a higher value for Gram positives than for total bacteria on a gram per fine root basis. This discrepancy is observed because the amplification efficiency of the universal primers used to generate the fragments for QPCR was lower than that of the Gram positive primers used in this study. Because these biases are maintained through our analysis, the Ambient-to-Elevated ratio is a better measure of changes in abundance than the absolute value (Table 1).

4. DISCUSSION

We studied the effects of elevated [CO_2] on microbial communities associated with roots of the desert shrub *Larrea tridentata* after 10 years of elevated [CO_2] exposure at the Nevada Desert FACE Facility (NDFF). Changes in microbial communities were evident at the qualitative and quantitative levels. We used 16S rDNA analysis to examine microbial communities, which are still informative and used to examine environments as complex as the human colon (Lupp et al., 2007). Even though the methods used here yield less information than more powerful sequencing techniques in the study of more complex environments, these new methods are costly and were not readily available when the NDFF study ended in 2007. Moreover, rarefaction curves and Chao1 indicated that the sampling efforts presented here allowed adequate analysis of community structure for bacteria at the phylum and fungi at the class taxonomic levels (data not shown).

Although the difference in species richness between [CO_2] treatments was slight among bacteria, fungi showed pronounced differences (Table 1). The fungal communities obtained from the elevated [CO_2] plots were richer in phylotypes than from ambient [CO_2]. Overall, small numbers of fungal phylotypes were observed in these Mojave Desert soils, which suggests that fungal populations are not diverse in arid rhizosphere soils, in general. This is congruent with data from fungal studies obtained by other research at the Nevada Test Site (NTS) (Apple et al., 2002; Titus et al., 2002), which showed that mycorrhizal colonization is not high at any time of the year.

Examination of the taxonomic groups obtained from rhizosphere soil of *Larrea* revealed that the phylogenetic diversity of bacteria and fungi in enriched [CO_2] is distinct from those observed in ambient [CO_2]. Further examination of proportional representation of bacterial taxa indicated that there were some population rearrangements at the phylum level.

Based on OTUs, there was no [CO_2] effect on the distribution of phyla. Among *Bacteria*, Proteobacteria OTUs (all subdivisions) comprised approximately 50% of the Mojave Desert soil bacterial population in the two treatments (Table 2), and their abundance was consistent with previous soil bacterial community studies (Janssen, 2006; Lesaulnier et al., 2008; Lipson et al., 2005). Proteobacteria showed no significant changes in composition at the phylum and class levels. Although γ-Proteobacteria were decreased in ambient [CO_2] compared to other classes of Proteobacteria (Table 2), due to this group's diverse physiological and metabolic capabilities, it is difficult to ascribe this group's role in nutrient transformation in the rhizosphere.

The Gram-positive communities showed mixed responses to elevated [CO_2]. The representation of Actinobacteria displayed a significant increase in elevated [CO_2], while the most significant decreases occurred in the phylum Firmicutes. This is mainly attributed to members of the “Bacilli” class, whose representatives decreased four-fold in abundance in elevated when compared to ambient [CO_2] (Table 2). Although the decrease in Gram-positive microorganisms was also observed with our QPCR assays, the decrease of the Bacilli in elevated [CO_2] seen in this study is not in agreement with studies conducted recently by other researchers from other habitat types (Lesaulnier et al., 2008; Lipson et al., 2005). The discrepancy between this and other studies may be due to differences in the response of plant species to [CO_2] and the ecosystems examined, as this is the only study to date in an arid ecosystem. None of the other phyla showed a significant change. This is in contrast to the reported results that under conditions of elevated [CO_2], there was an increase among Bacteroidetes (Lesaulnier et al., 2008) in forest soil.

Fungal community composition in rhizosphere soil of *Larrea* dramatically changed under elevated [CO_2]. Results are consistent with fungal data in semi-arid FACE soil (Lipson et al., 2005). The total fungal DNA concentration in the soil was not determined in this study. However, Lesaulnier *et al.* (2008) reported changes in fungal community composition and observed no change in eukaryotic DNA concentration in elevated [CO_2], while others reported no statistically significant change in fungal abundance at the same site (Chung et al., 2006; King et al., 2005; Zak et al., 2000). In these studies, changes observed in fungal composition were not associated with increases in fungal biomass (as measured by DNA content), which suggests that changes in composition resulted from population displacement.

Fungal OTU examination revealed that Ascomycota comprised 82% of sequences belonging to fungal libraries (Table 3); however, ascomycotes often accounted for 75% of all described fungi (Taylor et al., 2006). The results presented show a significant increase in abundance at the class level within Ascomycota (Table 3). This increase of diversity within Ascomycota in elevated [CO_2] soil is correlated with the increase in OTUs categorized to the temporarily-classified class “Dothideomycetes et Chaetothyriomycetes insertae sedis”. Members of this group can often be found as endophytes or epiphytes of living plants, and also as saprobes degrading cellulose, keratin and other complex carbohydrates in dead or partially digested plant matter in leaf litter. Thus, elevated [CO_2] may trigger a nexus in the production of plant or root matter which is processed preferentially by this fungal class. Other researchers also noted that saprotrophic fungi associated with *Adenostoma fasciculatum* and other chaparral shrubs are stimulated (Lipson et al., 2005; Rillig and Allen, 1998; Treseder et al., 2003).

In contrast, the OTUs classified to a large group within Mitosporic Ascomycota (ascomycotic fungi which have no sexual state) were significantly decreased in conditions of elevated [CO_2]. This may be the first report in which an effect of elevated [CO_2] is observed in this group of ascomycotes.

The significant decrease of OTUs classified to Agaricomycetes in elevated [CO_2] is in sharp contrast to previous findings that ectomychorrhizal fungi, a majority of which are basidiomycetes, increase in abundance under elevated [CO_2] (Jones et al., 1998). In a recent study at the NDFF, Clark et al. (2009) found that elevated [CO_2] did not increase any measures arbuscular mycorrhizal fungi, including root colonization, extra-radical hyphal length, or glomalin-related soil proteins. Therefore, the significant decreases among OTUs classified to Basidiomycota in elevated [CO_2] suggest that basiomycotes and fungal-plant associations are not promoted in response to an increase in [CO_2] in this arid ecosystem.

Members of Glomeromycota form arbuscular mycorrhizae (AM) with plants. Thus, the low amount of OTUs assigned to Glomeromycota observed are in agreement with previous findings by Apple et al. (2008), who noted AM colonization is low at the NTS during all times of the year. However, this is in contrast to data presented by others who worked in semi-arid FACE sites, who found a stimulation of AM in soil under chaparral plants (Lipson et al., 2005) and changes in AM species composition (Rillig and Allen, 1998) in elevated [CO_2]. The difference in findings suggests that *Larrea* and its rhizosphere respond differently to elevated [CO_2] than in other semi-arid plant ecosystems.

Put in the context of biogeochemistry, our results suggest that changes in below-ground (root) processes, effected by elevated [CO_2], mediate shifts in composition and abundance of microbial communities. Although early results at the Nevada FACE site showed no increases in fine root production and turnover at elevated CO_2 (Phillips et al., 2006), more recent results (prior to the sampling in this study) showed an increase in fine root turnover and probably carbon exudation by roots (R. Nowak, unpublished data). This was accompanied by higher soil respiration rates (Jasoni et al., 2005) and increased microbial activity, as measured by extracellular enzyme activities and community-level physiological profiles (Jin and Evans, 2007). Thus, we speculate that the additional C source allocated toward *Larrea* roots is preferentially used by ascomycotes (Table 3), while decreases were also observed in the abundance of firmicutes (Table 2). These changes in structure and abundance are likely to alter transformations and availability of nutrients other than C. For example, Schaeffer et al. (2003) and Gallardo and Schlesinger (2005) showed that in the Mojave and Chihuahuan Deserts, respectively, N may become more limiting to plants if elevated [CO_2] increases soil C, which results in increased soil organic matter and reduced N availability

In conclusion, the experiments reported here indicate that elevated [CO_2] alters the composition and abundance of microbial communities associated with roots of *Larrea*, a dominant shrub in the Mojave Desert. The observed phylogenetic changes in microbial communities support recent reports that examine soil microbes by other methods (Jin and Evans, 2010) and observations in which ecosystem functions and productivity, as measured by nutrient transformations and nutrient availability, were also affected.

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Appendices

Appendix A. Bacterial and fungal primer and probe sequences used in this study

Primer or Probe	Sequence	Reference
GF3	aamycggaggaaggtggggatg	This study
GR1	ggccattgtagcacgtgtgt	This study
Gram+P	6-FAM-acgtcaaatcatcatgccccttatgtc-TAMRA	Modified from Greisen et al. (1994)
f8–27	agagtttgatcctggctcag	Eder et al. (1999)
r1510	ggttaccttgttacgactt	Eder et al. (1999)
nu-SSU-0817-5′	ttagcatggaataatrraatagga	Borneman and Hartin (2000)
nu-SSU-1536-3′	attgcaatgcyctatcccca	Borneman and Hartin (2000)

Primer or Probe	Sequence	Reference
NadF	tcctacgggaggcagcagt	Nadkarni et al. (2002)
NadR	ggactaccagggtatctaatcctgtt	Nadkarni et al. (2002)
UnivP	6-FAM-cgtattaccgcggctgctggcac-TAMRA	Nadkarni et al. (2002)

Appendix B. Microorganisms used in the development and testing of Gram-positive PCR primers and probes. The QPCR resulted in identification of 27 Phyla using Gram-positive bacterial primers and probes

Phylum	Class	Organism	ATCC #	Gram	Mean Ct, n=2 (SD)
Crenarchaeota	Thermoprotei	*Aeropyrum pernix*)	700893D	−	40.00
Euryarchaeota	Methanococci	*Methanococcus maripaludis*	43000D	−	40.00
Aquificae	Aquificae	*Hydrogenothermus marinus*	BAA-483	−	40.00
Thermotogae	Thermotogae	*Thermotoga maritima*	43589D	−	40.00
Thermodesulfobacteria	Thermodesulfobacteria	*Thermodesulfobacterium commune*	33708	−	40.00
Deinococcus-Thermus	Deinococci	*Deinococcus radiodurans*	13939D	+	40.00
Chrysiogenetes	Chrysiogenetes	*Chrysiogenes arsenatis*	700172	−	40.00
Chloroflexi	Chloroflexi	*Chloroflexus aurantiacus*	29366	−	40.00
Thermomicrobia	Thermomicrobia	*Thermomicrobium roseum*	27502	−	40.00
Nitrospirae	Nitrospira	*Thermodesulfovibrio yellowstonii*	51303	−	40.00
Deferribacteres	Deferribacteres	*Geovibrio thiophilus*	BAA-311	−	40.00
Cyanobacteria	Cyanobacteria	*Nostoc* sp.	29133D	−	40.00
Chlorobi	Chlorobi	*Chlorobium tepidum*	49652D	−	40.00
Proteobacteria	Alphaproteobacteria	*Methylobacterium extorquens*	14718D	−	40.00
	Betaproteobacteria	*Hydrogenophaga pseudoflava*	700892	−	40.00
	Gammaproteobacteria	*Shewanella oneidensis*	700550D	−	40.00
	Deltaproteobacteria	*Desulfovibrio vulgaris* subsp. *vulgaris*	29579D	−	40.00
		Geobacter sulfurreducens	51573D	−	40.00
Firmicutes	Bacilli	*Bacillus cereus*	14579D	+	17.04 (0.09)
		Bacillus halodurans Nielsen	BAA-125D	+	15.43 (0.06)
		Bacillus subtilis	82D	+	18.35 (0.50)
	Clostridia	*Clostridium difficile*	9689D	+	15.43 (0.03)
		Clostridium perfringens	13124D	+	18.63 (0.00)
		Desulfosporosinus meridiei	BAA-275	+	18.97 (2.18)
Actinobacteria	Actinobacteria	*Arthrobacter crystallopoietes*	15481	+	26.96 (0.19)
		Cellulomonas fimi	484	+	26.60 (0.06)
		Gordonia rubripertincta	14352	+	26.63 (0.18)
		Microbacterium flavescens	13348	+	28.27 (0.07)
		Micrococcus luteus	53598D	+	40.00
		Rhodococcus erythropolis	27854	+	24.72 (0.10)
Planctomycetes	Planctomycetacia	*Planctomyces maris*	29201	−	40.00

Phylum	Class	Organism	ATCC #	Gram	Mean Ct, n=2 (SD)
Chlamydiae	Chlamydiae	*Parachlamydia acanthamoebae*	VR-1476	−	40.00
Spirochaetes	Spirochaetes	*Borrelia burgdorferi*	35210D	−	20.47 (0.18)
Fibrobacteres	Fibrobacteres	*Fibrobacter intestinalis*	43854	−	40.00
Acidobacteria	Acidobacteria	*Geothrix fermentans*	700665	−	40.00
Bacteroidetes	Bacteroides	*Bacteroides fragilis*	25285D	−	40.00
Fusobacteria	Fusobacteria	*Ilyobacter insuetus*	BAA-291	−	40.00
Verrucomicrobia	Verrucomicrobia	*Verrucomicrobium spinosum*	43997	−	40.00
Dictyoglomi	Dictyoglomi	*Dictyoglomus thermophilum*	35947	−	40.00
Ascomycota	Ascomycetes	*Stachybotrys chartarum*	MYA-3310	N/A	40.00
Ascomycota	Ascomycetes	*Aspergillus fumigatus*	36607	N/A	40.00
Chordata	Mammalia	Human DNA	N/A	N/A	40.00

N/A - not applicable

Table 1

Predicted species richness for *Bacteria* and *Fungi* and quantitative PCR of total bacterial or Gram-positive 16S rDNA in *Larrea* rhizosphere soil exposed to elevated or ambient [CO_2].

Phylogeny	[CO_2]	No. of sequences[a]	Observed No. of phylotypes[b]	Chao1 (SD)[c]	QPCR mean nanogram DNA per gram of fine roots SD (Ratio Ambient to Elelvated)	
					Total	Gram-pos
Bacteria	Ambient	199	130	522 (343, 853)	2.53 ±0.37 (1.10)	34.02 ±6.69 (1.77)
	Elevated	208	164	535 (385, 788)	2.29 (±0.18)	19.20 (±3.65)
Fungi	Ambient	189	15	16 (15, 22)	N.D.	N.D.
	Elevated	182	49	64 (54, 92)	N.D.	N.D.

[a] The number of sequences used to calculate the species richness.

[b] The number of identified phylotypes (or OTUs) detected at 97% sequence similarity cut-off.

[c] Relative richness employing the Chao1 (Chao, 1984) richness estimator, using single linkage clustering and 97% sequence similarity cutoff, for microbial communities under the different CO_2 treatments. (SD) = Standard deviations

N.D. = not done

Table 2

Abundance of bacterial OTUs represented as individual counts for elevated and ambient [CO_2] samples of rhizosphere soil beneath *Larrea tridentata*.

		Number of OTUs		
Phylum	**Class**	**Elevated**	**Ambient**	***P*-value**
Acidobacteria	Acidobacteria	13	5	0.09
Actinobacteria	Actinobacteria	36	18	8.39E-03
Bacteroidetes	Flavobacteria	2	0	0.49
	Sphingobacteria	9	6	0.60
Chloroflexi	Cloroflexi	N.D.	1	0.49
Firmicutes	“Bacilli”	10	40	3.11E-06
Planctomycetes	Planctomycetacia	4	1	0.37
Proteobacteria	Alphaproteobacteria	59	44	0.17
	Betaproteobacteria	18	24	0.32
	Deltaproteobacteria	5	9	0.28
	Gammaproteobacteria	10	25	7.28E-03
	Unclassified Proteobacteria	7	4	0.54
Gemmatimonadetes	Gemmatimonadetes	3	3	1.00
TM7	TM7 Genera Incertae sedis	8	6	0.78
Verrucomicrobia	Verrucomicrobiae	4	2	0.68
Unclassified		20	11	0.13
Total		**208**	**199**	N/A

P-values were calculated based on the Fisher exact test and are outlined in materials and methods. The threshold to determine significance (alpha value) at the phylum, class, and genus level should be adjusted by the Bonferroni correction, also detailed in materials and methods.

N.D. = not detected, N/A = not applicable.

Table 3

Abundance of fungal OTUs represented in individual counts of rhizosphere soil samples from elevated and ambient [CO_2].

			Number of OTUs		
Phylum	**Class or Group**	**Genus**	**Elevated**	**Ambient**	***P*-value**
Ascomycota	Dothideomycetes		33	26	0.25
	Dothideomycetes et Chaetothyriomycetes insertae sedis		19	1	9.77E-06
	Eurotiomycetes				0.06
		Arachnomyces, Kraurogymnocarpa, and other closely related species	27	3	1.40E-06
		Other	20	30	0.18
	Letiomycetes		2	6	0.28
	Pezizomycetes		16	18	0.86
	Sordariomycetes		25	28	0.88
	Mitosporic Ascomycota				8.23E-04
		Madurella	N.D.	10	0.02
		Other	10	21	0.06
	Other Ascomycota		5	4	0.74
Basiomycota	Agaricomycetes				1.10E-03
		Lepista	N.D.	14	9.78E-05
		Other	13	21	3.33E-03
	Other Basiomycota		8	4	0.25
Glomeromycota			3	2	0.68
Total			**181**	**188**	N/A

P-values were calculated based on the Fisher exact test and are outlined in materials and methods. The threshold to determine significance (alpha value) at the phylum, class, and genus level should be adjusted by the Bonferroni correction, also detailed in materials and methods.

N.D. = not detected, N/A = not applicable.