Effects of elevated carbon dioxide on soil microbial communities of the Mojave Desert

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EFFECTS OF ELEVATED CO$_2$ ON SOIL MICROBIAL COMMUNITIES
OF THE MOJAVE DESERT

by

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Bachelor of Science
University of Minnesota
1991

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University of Minnesota
1998

A dissertation submitted in partial fulfillment
of the requirements for the

Doctor of Philosophy Degree in Biological Sciences
School of Life Sciences
College of Sciences

Graduate College
University of Nevada, Las Vegas
December 2008
Dissertation Approval
The Graduate College
University of Nevada, Las Vegas

December 3, 2008

The Dissertation prepared by
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Entitled
Effects of Elevated CO₂ on soil microbial communities of the Mojave Desert

is approved in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Biological Sciences

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ABSTRACT

Effects of Elevated CO$_2$ on Soil Microbial Communities of the Mojave Desert

by

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The objective of this study is to determine soil microbial community structure in the framework of increased atmospheric carbon availability associated with global change. Since arid ecosystems make up a considerable fraction of our biosphere, their response to global change will be of significance. Further, implication for plant and ecosystems productivity in arid environments may be linked to changes in diversity among microbial communities, since the cycling of essential elements in the soil is often mediated by microbes. The following are three sets of experiments designed to examine the changes in microbial diversity associated with a global change factor, an increase in atmospheric carbon dioxide (CO$_2$).

To start the examination of the differences caused by elevated atmospheric CO$_2$, bacterial communities living in soil associated with Larrea tridentata, a dominant Mojave Desert shrub, were studied. Terminal restriction length polymorphism (T-RFLP) analysis, and preliminary phylogenetic analysis, of 16S rDNA amplified from soil indicated that there is an alteration in the soil bacterial community structure between ambient and elevated CO$_2$ conditions. Preliminary richness estimation indicated that the studied environments have been greatly undersampled. The analysis also revealed T-RFLP may not be a suitable method to study the microbial diversity in the studied environment, and more extensive 16S rDNA sampling is necessary to determine the effects of elevated atmospheric CO$_2$ on the diversity of soil microbes in an intact desert environment.
The effects of elevated CO₂ on soil microbial communities were further tested through microbial community surveys by the construction of larger environmental gene libraries to identify the dominant operational taxonomic units (phytotypes) among bacterial and fungal communities. To better understand how community composition relates to environmental changes, a multi-faceted approach consisting of qualitative and quantitative methods to assess community diversity was employed to characterize the microbial communities found in the different CO₂ treatments. Significant changes in the bacterial and fungal community structures in enriched CO₂ conditions were observed.

The final experiments used to determine the structure of microbial communities relied on the quantitation of total and specific groups of bacteria. Quantitative polymerase chain reaction (QPCR) using TaqMan® technology was employed to infer population density of total and Gram-positive bacteria in rhizosphere soil DNA exposed to enhanced and ambient CO₂. The QPCR results indicate that the density of bacteria is similar in the two CO₂ treatments, while Gram-positive microorganisms decreased by 44% in ambient relative to those observed in enriched CO₂ conditions. These experiments also suggest that a cumulative change in many fungal phyla was observed in conditions of elevated CO₂, while change among bacteria was group-specific.
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ABREVIATIONS

%  percent
°  degree
<  less than
=  equal to
>  greater than
≈  approximately
≤  equal or less than
µL  microliter
µM  micromolar
16S  16 Svedberg
18S  18 Svedberg
AM  arbuscular mycorrhizae
ARB  Latin arbor: tree
ATCC  American Type Culture Collection
BLAST  Basic Local Alignment Search Tool
BSA  bovine serum albumin
C  Celcius
CI  confidence intervals
cm  centimeter
CO₂  carbon dioxide
Ct  cycle threshold
DDH  DNA-DNA hybridization
DNA  deoxyribonucleic acid
DOTUR  Distanced-Based OTU and Richness
FACE  Free Air CO₂ Enrichment
fg  femtogram
FISH  fluorescent in situ hybridization
Fst  fixation index
g  gram
G+C  guanine and cytosine
IPTG  isopropyl-beta-D-thiogalactopyranoside
LB  Luria-Bertani
MgCl₂  magnesium chloride
min  minute
mL  milliliter
mm  millimeter
mM  millimolar
N  nitrogen
NCBI  National Center for Biotechnology Information
NDFF  Nevada Desert FACE Facility
ng  nanogram
nt  nucleotide
NTS  Nevada Test Site
OTU  operational taxonomic unit
PCR  polymerase chain reaction
pH  power of hydrogen
pM  picomolar
ppm  parts per million
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<td>$R^2$</td>
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ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Eduardo Robleto for his guidance and knowledge throughout the past five years. I am also grateful to members of my advisory committee, Drs. Dennis Bazylinski, Ronald Yasbin, Ernesto Abel-Santos, and Mark Buttner for their advice and support.

I thank members of the Robleto laboratory: Christy Pybus for critical reading of my thesis, and fellow graduate students Rowena Manalang, Katila Pipitone, and Holly Martin for their friendship and scientific discussions. I would also like to thank members of the Buttner laboratory: Patricia Cruz, Vanessa Stevens, and Joanne Henry for their friendship and technical advice.

I would like to thank two former mentors. First is Sue Marshall, my personal cheerleader, who laced my science education with large doses of love. Second is Dr. Raymond Sicard, a scientist who trained me with great conviction. The guidance I received from them provided the foundations on which my career rests.

Above all, I am most grateful to my parents, Lieu Mai, Lich and Phan Nguyen, and sister, Linh Hanley, for their incredible support, love and guidance in my academic career. To my beloved little Tran boys, Addison and Noah, I want to encourage you to chase your dreams. Lastly, much gratitude to my husband, Lich Tran, whose love and devotion make it possible for me to keep my eyes on the prize.
CHAPTER 1

PRELIMINARY STUDIES ON THE EFFECTS OF ELEVATED CO\textsubscript{2} ON MICROBIAL COMMUNITIES IN MOJAVE DESERT SOIL

1.1 Abstract

The response of bacterial communities to ambient and elevated atmospheric carbon dioxide (CO\textsubscript{2}) using Free Air CO\textsubscript{2} Enrichment (FACE) technology was investigated. The goal of this study was to determine bacterial community structure in the framework of increased carbon availability associated with global change. Bacterial communities living in soil associated with \textit{Larrea tridentata}, a dominant Mojave Desert shrub, were studied. Terminal restriction length polymorphism (T-RFLP) analysis of 16S rDNA amplified from soil suggests that there are changes in density in bacterial components of soil communities between ambient and enriched CO\textsubscript{2} conditions. Also, a preliminary phylogenetic analysis using 16S rRNA gene environmental libraries showed alterations in the soil bacterial community structure between ambient and elevated CO\textsubscript{2} conditions. Preliminary richness estimation using the Chao1 algorithm indicated that the studied environments have been greatly undersampled. The analysis shows T-RFLP may not be a suitable method to study the microbial diversity in this environment, and more extensive 16S rDNA sampling is necessary to determine the effects of elevated atmospheric CO\textsubscript{2} on the diversity of soil microbes in an intact desert environment.

1.2 Introduction

Due to anthropogenic activities since the start of the Industrial Revolution, the atmospheric concentration of CO\textsubscript{2} has increased from about 280 parts per million (ppm) to over 370 ppm. Current estimates suggest that this range will lie between 450 and 600 ppm by the year 2050 (25). The steady increase in atmospheric CO\textsubscript{2} has been demonstrated to have an
impact on many terrestrial ecosystems. Arid and semi-arid lands will likely be affected (38).

Since arid ecosystems make up a considerable fraction of our biosphere (30-40% of the earth's terrestrial surface excluding Antarctica) their response to global warming may significantly affect resource availability to plants and animals.

Elevated CO₂ affects these ecosystems by influencing plant physiological processes, which in turn have an effect on soil microbial communities associated with these plants. The study of plant-associated soil microbial communities is important because microbes modulate nutrient cycling, organic matter metabolism, soil neogenesis and improvement of soil structure (4, 33). Although short-term plant responses to CO₂ enrichment are well studied, our knowledge of the effects of elevated atmospheric CO₂ on soil microbial populations is very limited. Therefore, in order to gain a better understanding of whole ecosystem responses to global change, it is imperative to examine the effects of atmospheric CO₂ on soil microbial communities.

1.2.1 Plant physiology and microbial communities in response to CO₂ enrichment

Decades of CO₂ enrichment studies have provided significant data and an improved understanding about the response of plants from various ecosystems to elevated CO₂. Evidence supporting the concept that exposure to elevated CO₂ results in a shift towards heterotrophy is provided in part by extensive research in plant physiology. A number of plant parameters are increased in conditions of elevated CO₂, such as photosynthetic ability, litter production, and root exudation (7, 10, 11, 21).

In general, observations in numerous plant studies elucidate that elevated CO₂ confers increased photosynthetic ability. This increase in plant photosynthesis is associated with many changes in plant biochemistry, such as high carbon (C) and nitrogen (N) root exudates, litter quantity, and low litter quality (9, 15, 16, 26). Importantly, the increase in plant photosynthate is also associated with many changes underground, such as increased root biomass due to a higher net C assimilation (44), to increased total rhizodeposition and to change in chemical composition of root exudates (44, 46). Since roots are the primary additional C input source into the soil, it is reasonable to predict that there will be changes in the heterotrophic microbial populations colonizing the soil attached to roots (rhizosphere) or the bulk soil in close proximity to
roots (46). In desert soil, which is often poor 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 (12). Plant roots do create a selective environment for microbial populations according to Marilley et al. (1999), who demonstrated that the number of operational taxonomic units (OTUs) increased as the distance from the root increased. Similarly, using a Swiss pastureland FACE system, Montealegre et al. (2000) demonstrated that there is a difference between microbial communities in rhizosphere and bulk soil.

The type of microbial processes in root-associated soil affected by exposure to elevated CO\(_2\) are consistent with microbial metabolic activities seen in environments that are rich in fixed C. Soil respiration of plant ecosystems in conditions of elevated CO\(_2\) increases with little or no contribution of root respiration (40). Further, N cycling and other nutrient transformations in soil, all of which are modulated by soil microbial communities, are affected in conditions of elevated CO\(_2\) (3, 6, 7). Interestingly, studies examining degradative and catabolic enzymatic activities of soil extracts in other ecosystems, in conditions of elevated CO\(_2\), showed significant increases in these activities compared to those observed in conditions of ambient CO\(_2\) (17, 19, 30, 57). For example, Dhillion et al. (1996) studied soil associated with the Mediterranean annual grass Bromus madritensis and reported that elevated CO\(_2\) increases levels of dehydrogenase, cellulose, phosphatase, and xylanase. Further, other reports indicate increases in the number of culturable heterotrophs in rhizospheres of white clover (48) and in perennial ryegrass Lolium perenne (34). Elevated CO\(_2\) conditions also affect soil fungal populations in semi-arid ecosystems by producing community composition changes, by alterations in population size, and by increasing extracellular enzymatic activities (30, 42, 50), which ultimately indicates a shift to heterotrophy or decomposition.

1.2.2 Technology for microbial community assessment

Very little is known about how microbial diversity varies with varying concentrations of atmospheric CO\(_2\). Pure cultures of microorganisms have been traditionally classified by their morphological and physiological characteristics; however, these traits are not very useful for evolutionary classifications or the study of microbial communities. Furthermore, the reliance on
traditional culture methods, which detect only a fraction of microorganisms (2), makes this technique impractical for characterizing the diversity of microorganisms in natural environments.

The development of techniques to analyze the small-subunit rRNA gene (rDNA) obtained from the environment provides culture-independent means to study microbial communities. One example of such a method used to determine microbial community structure is terminal restriction fragment length polymorphism (T-RFLP) (14). While T-RFLP is a cost-effective method, it does not always provide the resolution needed to characterize the complex structure of highly diverse microbial communities. A more comprehensive depiction of microbial community composition can be obtained by extensive sequence analysis of ribosomal deoxyribonucleic acid (rDNA) clone libraries (11, 52). By using rDNA sequences for statistical and phylogenetic comparisons, researchers can estimate the species richness, establish sampling needs, and identify unique or numerically dominant strains/groups, as well as determine microbial community structure under different environmental conditions.

Metagenomics, the study of genetic material recovered directly from environmental samples, approach to examine microbial diversity is another way to capture the microbial populations that had been missed by cultivation-based methods. Unlike the approaches used in this study, metagenomics does not center on the amplification of rDNA samples, but focuses on cloning and sequencing. Although this approach provides the information about types of organisms (53) and the possible metabolic processes (51) in the community, large samples and sequencing (which are expensive) are often needed to fully resolve the genomes of microbial community members in an ecosystem.

In addition to these culture-independent means to profile microbial community structure, quantitative PCR methods, such as the TaqMan® fluorogenic PCR system (Applied Biosystems, Foster City, CA) permits quantification of rDNA taken from environmental samples (49). This method will be discussed in detail in Chapter 4. The results from QPCR studies can provide insights into the relative density of target populations in the environments under study, and confirm observations obtained from environmental libraries.
1.2.3 Free Air CO\textsubscript{2} Enrichment (FACE) technology

Researchers have investigated the effects of elevated atmospheric CO\textsubscript{2} on soil microbial community structure (23, 27, 54, 56); however, the results have been varied and inconclusive. It has been speculated that a major factor influencing the patterns of microbial response to CO\textsubscript{2} enrichment is the experimental system used to provide the additional C (22). Studies that examine microbial diversity in soils associated with plants grown in man-made constructs, such as greenhouse or growth chamber systems, may not be applicable to field situations. Some of the experimental difficulties involving the delivery of additional C in field settings can be addressed by employing FACE technology, because it is a system that can expose intact ecosystems to varying levels of atmospheric CO\textsubscript{2} uniformly. FACE technology has been used to study the effects of elevated CO\textsubscript{2} on specific soil microbes (43, 45), and on soil microbial community composition. For example, both Schortemeyer \textit{et al.} (1996) and Montealegre \textit{et al.} (2000), used Swiss grassland FACE technology and observed a shift in the representation of \textit{Rhizobium leguminosarum} bv. \textit{trifolii} in the rhizosphere of white clover plants subjected to elevated CO\textsubscript{2} levels. Similarly, the structures of microbial communities were modified by CO\textsubscript{2} enrichment in five Sphagnum peatlands mini-FACE sites, while the total microbial biomass remained unchanged (36). In contrast, studies conducted by Deiglmayr \textit{et al.} (2004) found that the structure of the nitrate-reducing microbial community was not affected by CO\textsubscript{2} enrichment, but rather by season and soil pH. In summary, although these researchers used FACE technology to deliver CO\textsubscript{2} to the experimental units, shifts in microbial community structure in response to CO\textsubscript{2} enrichment depends on many factors that include type of plants studied, soil type, soil nutrient conditions, the experimental system, and the specific diversity of microbes present in the system.

Further, there have been few molecular studies aimed at elucidating how changes in atmospheric CO\textsubscript{2} affect bacterial diversity in a natural arid ecosystem. An improved understanding of how soil microbial populations respond to the global change phenomena is vital. Soil microbes play major roles in ecosystem productivity due to their abilities to sequester or make nutrients available to plants. Their examination will take on a more urgent role in the
future, as expanding human development places many stressors on already fragile arid and semi-arid ecosystems in the American southwest and globally.

1.2.4 Hypothesis and objectives

The overall hypothesis in this study is that in soil associated with the desert shrub *Larrea tridentata*, elevated CO$_2$ conditions produce shifts in the heterotrophic microbial community compositions. It is reasonable to speculate, based on the plant-mediated increase of C input into the ecosystem, that increased levels of CO$_2$ will lead to an increased heterotrophic or decomposing activity. This hypothesis is supported by observations in other ecosystems which illustrate higher values of microbial respiration in conditions of CO$_2$ enrichment as compared to control conditions (19, 43, 45, 55). For example, both Rogers *et al.* (1992) and Runion *et al.* (1994), working at different cotton FACE sites using dehydrogenase assays as a measure of heterotrophy, reported that total microbial respiratory activity was significantly greater in elevated CO$_2$ conditions.

The objectives of the study were to generate and analyze T-RFLP fragments, and to construct two small 16S rDNA libraries from soil cores collected in May 2003 and October 2004 from the Nevada FACE site. These soil cores were obtained from plots which were fumigated with ambient or elevated CO$_2$.

1.3 Materials and Methods

1.3.1 Experimental site

The field experiments took place at the Nevada Desert FACE Facility located within the Department of Energy's Nevada Test Site (NTS), about 100 km (62 miles) from the city of Las Vegas, Nevada (Fig. 1.1A). FACE technology, such as the system employed at NTS, allows researchers to enrich the CO$_2$ concentration in large study plots (25 m in diameter) without influencing other ecosystem factors (39). The NTS FACE site (24) has been in operation since April 1997, and consists of six experimental circular plots (rings), which are three control rings of ambient level, three experimental rings at 550 ppm of CO$_2$, plus three non-blower control rings (Fig. 1.1B). Within the FACE rings, native Mojave Desert soil and vegetation (Fig. 1.1C) is
exposed daily to the target atmospheric CO\textsubscript{2} concentrations, except when high wind or low temperature conditions forces conditional system shut-downs.

The site of study is an intact natural environment, but the extraction of soil and roots from plants within the FACE rings required destruction of plants. Because the different types of research (including, but not limited to plant ecophysiology and soil nutrient studies) conducted in these field plots is multidisciplinary, large destructive sampling is not allowed and hampered our studies. In general, researchers whose areas of study are FACE-technology and natural environments, are restricted to obtaining three to four subreplicates (6, 8, 30) per FACE ring to minimize disturbance to the natural study environment.

1.3.2 Soil sampling procedures

The sampling took place during May 2003 and October 2004. Information regarding the average ambient temperature, humidity, and water volumes in soil for all sampling times at the NTS is at: http://www.unlv.edu/Climate_Change_Research/Data_Bases/data_index.

Within each plot, soils from two conditions were sampled: i) Soil associated with the roots of desert bush Larrea, and ii) soil associated with no plants or containing mature cryptobiotic crust (denoted as interspace). Sampling cores (dimensions: 2 cm diameter x 5 cm depth) were used to collect soil associated with Larrea plants from three rings exposed to elevated CO\textsubscript{2} and three ambient CO\textsubscript{2} rings. Within each FACE ring, three randomly Larrea plants were chosen for sampling. Two cored soil samples were taken from the base of each of the three plants. All six soil cores (two cores x three Larrea plants) for each FACE ring were pooled together, and mixed inside a gallon-size Ziploc plastic bag (SC Johnson, Racine, WI). The total number of soil cores was 72 (six FACE rings x six per ring x two types of soil). This procedure was also conducted for the interspace condition. To avoid further plot damage and disturbance, the sampling procedure was conducted from a suspended platform within the ring (Fig. 1.1D) to minimize disturbing the study site. Upon returning to the field station, the soil samples were immediately separated from plant roots and large rock particles after passing through a 1 mm sieve, and stored at -20°C while in transport, and at -80°C prior to further manipulations.
1.3.3 DNA isolation and purification

DNA was extracted from soil collected in May 2003 and October 2004 using a bead-beating FastDNA Spin Kit for Soil (Qbiogene, Carlsbad, CA), which allows a maximum amount of soil of 0.5 g per single preparation to be processed. Total DNA was collected from 0.5 g of soil samples using spin columns and the FastPrep FP120 bead beater instrument (QBioGene, Carlsbad, CA).

1.3.4 PCR for T-RFLP analysis

The 16S rRNA gene was amplified by standard PCR from soil DNA using universal 16S rDNA primers (530F and 6-FAM-labeled 1494R) (11) to produce labeled amplicons of 964 base-pairs in length. Primers were purchased from Integrated DNA Technologies (San Diego, CA). The PCR reaction was performed in a total volume of 50 µL using the following reagents at the indicated final concentrations or amounts: One µL of template DNA, 125 µg of bovine serum albumin (Sigma-Aldrich Corp., St. Louis, MO), 1.25 mM MgCl₂ (Omnipur), 200 µM deoxynucleoside triphosphates (Promega, Madison, WI), 0.63 pM and 0.8 pM, respectively, of primers 530F and 1494R (IDT, San Diego, CA), and 1.25 U GoTaq polymerase (Promega, Madison, WI). The following PCR reaction conditions in a Biorad iCycler were: 95°C for 3 min, 32 cycles (95°C for 30 s, 56°C for 35 s, 72°C for 1:10 min), and 72°C for 5 min. The PCR product
was purified in spin columns (Qiagen, Valencia, CA), and restriction digested using Rsal and TaqI (Promega, Madison, WI). Quantification of peak heights and fragment lengths was performed at the Nevada Genomics Center (Reno, Nevada).

1.3.5 Construction of 16S rRNA gene clone libraries and sequencing

The construction of 16S rRNA clone libraries was designed to represent the spatially averaged bacterial communities within each CO₂ treatment; therefore, spatial subreplicates (all 18 soil cores from each CO₂ treatment) were combined (Fig. 1.1B). This sampling strategy provided well-mixed, diverse samples from each CO₂ treatment that were compared using phylogenetic tools. The pooling of subreplicates from FACE rings to construct environmental libraries for the construction of phylogenetic tree was previously performed by Deiglmayr et al. (2004), who studied nitrate reducers structure in grass monocultures, Lipson et al. (2005), who examined microbial diversity in a natural semi-arid chaparral environment, and most recently by LeSaulnier et al. (2008) who examined microbial diversity in quaking Aspen. This strategy provided a homogeneous composite sample since replicates from each of the two treatments (three rings each) were pooled. Furthermore, this experimental design was not hampered by other research constraints such as small FACE rings, CO₂ fumigation only during the day, and no ambient CO₂ control ring.

DNA was extracted from soils using a bead-beating FastDNA Spin Kit for Soil (Qbiogene, Carlsbad, CA). Total DNA was collected from 0.5 g of soil samples using spin columns and the FastPrep FP120 bead beater instrument (QBiogene, Carlsbad, CA). Soil 16S rDNA was PCR amplified in the same manner as described above, with the exception that primers were not labeled with 6-FAM. The PCR product was purified using spin columns (Qiagen, Valencia, CA) and cloned into pGEMT-easy vector (Promega, Madison, WI). XL1-Blue (Stratagene, La Jolla, CA) competent cells were transformed with ligation reactions containing vector and PCR products using the manufacturer's protocol. Transformed cells were selected on Luria-Bertani (Sigma-Aldrich Corp., St. Louis, MO) agar plates containing 50 mg/mL carbenicillin (Calbiochem, San Diego, CA), 4 μL/mL Isopropyl β-D-1-thiogalactopyranoside (IPTG) and 0.8 μL/mL 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (XGal) (Promega, Madison, WI) which were incubated at
37°C overnight. White colonies were transferred to Costar round-bottom 96-well plates (Corning Life Sciences, Corning, NY) containing LB broth and carbenicillin and incubated at 37°C overnight. Clones were subjected to PCR to confirm the presence of the correct insert size and were sequenced at the Nevada Genomics Center, Reno. A total of 40 sequences were generated, with 20 for the soil collected in May 2003 and the remainder for soil collected on October 2004.

1.3.6 Sample size analysis

To estimate total species richness, 16S clones were processed using DOTUR v1.53 (47). Furthest neighbor assignment analysis from DOTUR (Distanced-Based OTU and Richness) ((47) described in Chapter 3) and the Chao1 (13) estimator ($S_{\text{Chao1}}$) were used to determine total species richness. This analysis used the criteria of 97% or greater sequence identity to indicate a distinct phylotype/OTU.

1.3.7 Classification of sequences and phylogenetic analysis

Prior to phylogenetic analysis, and to eliminate repeat sequences, the 16S rDNA clones obtained in this study were searched using BLASTN (1), with parameters of $E=0.0$ and sequence identities of 98% or greater. Also, sequence chimeras were identified using the CHECK_CHIMERA program from Ribosomal Database Project II Release 8.1 (RDP, East Lansing, Michigan). Two archaeal sequences were eliminated from analysis, and unique bacterial sequences were matched to the nearest neighbor and aligned using the RDP II Release 9.42. Sequences from samples and from reference organisms were manually edited using ARB (from Latin arbor: tree) (32), and sequences of poor quality were identified by visual inspection of sequence alignments. Poor quality, short sequences and sequences showing vast misalignments were eliminated prior to phylogenetic dendrogram construction. Trees were constructed using the software program ARB and by the neighbor-joining method.
1.4 Results and Discussion

1.4.1 T-RFLP analysis

The T-RFLP methodology was used to examine the effects of elevated CO$_2$ on total bacterial communities associated with roots of Larrea and with cryptobiotic crust. T-RFLP or RFLP has shown to be an effective tool for soil microbial community analysis (18, 27, 31) and is based on the generation of signature profiles that correspond to polymorphisms of 16S rDNA fragments.

![Figure 1.2. Terminal restriction fragment length polymorphism (T-RFLP) of soil microbial communities exposed to ambient and elevated CO$_2$. 16S rDNA was restriction digested with Rsal and TaqI. Soil associated with Larrea (left graph) and in interspace/cryptobiotic crust (right graph) in response to ambient (solid) and elevated (dots) CO$_2$ treatments.](image)

Figure 1.2 illustrates TRFLP profiles of soil microbial communities as affected by exposure to elevated CO$_2$ and shows differences presence and intensity in profiles between samples taken from root-associated soil and cryptobiotic crust. In summary, our results suggest that there are changes in density in bacterial components of soil communities.

Results (Fig. 1.2) showed no apparent changes in peaks among total bacterial populations in soil associated with Larrea and in cryptobiotic crust in response to CO$_2$ enrichment, although there were some changes in the intensities of the peaks. The T-RFLP fragments approaching 100 and 400 bp in length were present in both conditions and in both
types of soil samples. However, 400 bp fragments in soils associated with *Larrea* were absent or undetected in conditions of elevated CO$_2$. In contrast, the interspace soils had marked differences in intensity. These findings suggest that while there were no major differences in number of fragments among the soils sampled, there were changes in the abundance of specific microbial lineages. Furthermore, the small number of peaks in both graphs (Fig. 1.2) indicates that the diversity of microbes in the Mojave Desert soil is low. This may be explained by the possibility that the two restriction enzymes used in the study did not effectively discriminate among 16S rDNA fragments, or that T-RFLP might not be an appropriate method to examine microbial community structure in this environment. Therefore, a more comprehensive method is needed.

1.4.2 Preliminary 16S rDNA environmental libraries to estimate diversity

The T-RFLP analysis indicates some redistribution of soil microbial communities found in these two CO$_2$ habitats. To conduct a more detailed study of the effects of CO$_2$ on the structure of soil microbial communities, four (two CO$_2$ conditions x two different seasons) preliminary environmental 16S rDNA libraries were generated from plots maintained under either elevated CO$_2$ or ambient conditions. The collection of soil cores associated with *Larrea*, which was used to construct environmental libraries, is described in the materials and methods section.
Figure 1.3. Phylogenetic tree of bacterial 16S rDNA clones from desert soil associated with Larrea. Tree was inferred by neighbor-joining analysis of 964 homologous positions of sequence from each reference organism or clone. Scale bar represents 10 changes per 100-nt sequence positions. Clones isolated from ambient or elevated CO\(\text{2}\) conditions are indicated by “Ambient” or “Elevated” in the name, respectively. Phyla are listed on the right side of illustration, with Family names in parentheses.
Preliminary phylogenetic analysis (Fig. 1.3) confirmed the presence of previously reported bacterial species found in arid soil. Other researchers have noted that microbial diversity in the American southwest arid lands appears to be dominated by yet-to-be cultured Acidobacteria species (5, 20, 21, 28), and other less-abundant bacterial phyla such as Proteobacteria, Gram-positives, Bacteroides-Cytophaga-Flexibacter, Nitrospira, Verrucomicrobia, and Plantomycetes. In some deserts, Gram-positive sporeformers are dominant (41), with Actinomycetes composing 50% of the total microbial biota.

The dendrogram (Fig. 1.3) of the bacterial 16S rRNA gene sequences suggests CO₂-enrichment alters the diversity of bacterial lineages in desert soil associated with Larrea. Clones from both experimental CO₂ conditions are well represented among phyla Actinobacteria, Proteobacteria, and Acidobacteria. Much is known about members of these phyla; the abundance of these groups in many diverse environments is due to the versatility of their extensive metabolism. For example, Actinobacteria are Gram-positive, high G+C bacteria, with most members of this phylum found in the soil where they play an important role in decomposition of organic material. Also, most Proteobacteria members are also heterotrophic and include many of the bacteria responsible for N fixation. Lastly, Acidobacteria is a novel phylum that is ubiquitous in many different habitats, particularly soil.

On the other hand, the phylotypes detected in the phyla Cyanobacteria, Deinococcus-Thermus, and Bacteroides are unique to either ambient or elevated CO₂ condition (Fig. 1.3). For example, in elevated CO₂ conditions, only Cyanobacteria phylotypes are present. Cyanobacteria are bacteria capable of photosynthesis and N fixation. In contrast, clones unique to phylum Deinococcus-Thermus, which is a group of bacterium that are very resistant to environmental hazards such as radiation, heat, and desiccation (35), are exclusively present in conditions of ambient CO₂. Although their presence in the harsh desert environment is expected, it is interesting that members of this phylum are only detected in conditions of ambient CO₂. Likewise, clones unique to the Bacteroidetes phylum are only present in ambient CO₂ conditions, which is unexpected since members of this phylum are heterotrophic and are widely distributed in soil (33).
1.4.3 Chao1 species richness estimator

The cutoff of 97% or greater sequence criterion to distinguish among phylotypes is used frequently by many researchers. In a review of 16S rDNA libraries data obtained from various aquatic and non-aquatic environments, Kemp et al. (2004) observed that 81 of the 91 studies reviewed employed these cutoff values. An in-depth discussion of this criterion can be found in Chapter 3. Since this is a preliminary study involving only 30 clones, the sequences obtained from enriched and ambient CO$_2$ conditions were pooled for Chao1 analyses.

As predicted, the Chao1 species richness estimates suggest that these environments have been grossly undersampled. Here, it is predicted that a total of 218 (95% CIs, 89 to 620) distinct DNA sequences are required to adequately describe the diversity of the 16S rRNA gene in the studied environments; the 30 sequences obtained is approximately 13% of the 218 phylotypes estimated. A study involving a larger sequencing effort will increase the number of OTUs identified, and will most likely give a better resolution of the community structure in an intact desert ecosystem. Most importantly, more detailed studies will test the hypothesis that in soil associated with the desert shrub Larrea tridentata, there is a shift in the heterotrophic microbial population in elevated CO$_2$ conditions.

1.5 Conclusion

Overall, it seems that T-RFLP showed little difference in the types of microbes in ambient and elevated CO$_2$, and did not provide enough resolution of community structure. A more detailed study composed of preliminary bacterial 16S rDNA libraries supported the inadequacy of using T-RFLP methods to study microbial communities in our environment. Nevertheless, the examination of the 16S rDNA libraries suggests some differences in the bacterial community structure. Further, preliminary richness estimates using the Chao1 algorithm indicates that the study environments have been greatly undersampled, and a more robust sampling effort is necessary. Taken as a whole, the results on this chapter support the notion that elevated CO$_2$ is selecting for some bacterial lineages and warrants a closer inspection. This can be achieved by
a more comprehensive construction of clone libraries (Chapter 2) to determine the diversity of soil microbes in an intact desert environment.

1.6 References


CHAPTER 2

DIVERSITY ANALYSES OF THE EFFECTS OF ELEVATED CO$_2$ ON
MICROBIAL COMMUNITIES ASSOCIATED WITH LARREA

2.1 Abstract

In this chapter, experiments designed to study the effects of elevated CO$_2$ on microbial communities found in soils and also associated with roots of Larrea tridentata are presented. The analyses are based on rDNA technology and use a collection of tests that examine many aspects of species diversity. Different types of analyses for the measurement of diversity are reviewed and discussed in this chapter. Twelve bacterial and fungal rRNA gene libraries were constructed, and 1,056 clones (600 bacterial and 456 fungal) were sequenced, categorized into operational taxonomical units (OTUs), and analyzed. Species richness estimators indicate that bacterial populations were undersampled and that sampling among fungal populations was adequate. Phylogenetic analyses indicated that the different OTUs belonged to a wide range of bacterial and fungal taxa. Among the main differences observed at the bacterial community level, there was a significant decrease in Firmicutes in elevated CO$_2$ conditions. Among fungal populations, OTUs classified to the phylum Ascomycota, particularly ones belonging to Eurotiomycetes and "Dothideomycetes et Chaetothyriomycetes insertae sedis," increased, while mitosporic ascomycotes significantly decreased, in elevated CO$_2$ conditions. Furthermore, the rhizosphere soils of plots exposed to ambient CO$_2$ harbored a significantly higher proportion of OTUs taxonomically assigned to Basiomycota, especially among Agaricomycetes.

2.2 Introduction

The concentration of atmospheric carbon dioxide (CO$_2$) has increased steadily due to anthropogenic activities after the industrial revolution. The effects of elevated CO$_2$ on plant
responses in terrestrial systems have provided much data; however, the responses of microbial communities to rising atmospheric CO$_2$ concentrations in arid ecosystems have not been examined in detail. The hypothesis in this study is conditions of elevated atmospheric CO$_2$ cause shifts in soil microbial communities of the Mojave Desert. To test the hypothesis, the species richness, genetic and phylogenetic diversity of bacterial and fungal communities inhabiting the rhizosphere of a dominant desert bush, which was fumigated with elevated or ambient levels of CO$_2$, were determined.

2.2.1 Measurements of diversity

The unit of analysis in diversity measurements prior to the 1970's has historically been species phenotype. Since then, new criteria used to delineate species are based on relatedness of bacterial genomes. Conventionally, isolates that have $>70\%$ genome DNA-DNA hybridization (DDH) are considered the same species (19). For studies that examine the 16S rRNA genes, researchers frequently choose a $\geq 97\%$ sequence identity to differentiate between species because lineages below this similarity threshold usually have DDH values below 70\% (59). For example, in a review of 16S rDNA libraries data obtained from various aquatic and non-aquatic environments, Kemp et al. (2004) observed that 81 of the 91 studies employed these cutoff values. However, instead of using the term species, many researchers use the term operational taxonomic units (OTU) or phylotype, to group related 16S rRNA gene sequences.

Community diversity can be measured using many methods, however, the study presented here uses three main types of analyses (and select tests associated with them) to characterize the bacterial and fungal communities found in the different CO$_2$ treatments. The analyses and tests used in this study provide a multi-angle approach to assess community diversity, and are summarized in Table 2.1 and explained below.
Table 2.1. Categories of diversity measurements, adapted from Lozupone et al. (2008). The tests associated with each category of diversity measurement are shown in bold. * The theta (\( \Theta \)) calculation was not reported for a single community, but used in the calculation for significance in the Fst test for communities from both CO\(_2\) treatments. A) and C) show alpha while B) and D) show examples of beta diversity measures.

<table>
<thead>
<tr>
<th>Only presence/absence of taxa considered</th>
<th>Measurement of diversity within a single community (( \alpha )-diversity)</th>
<th>Measurement of diversity shared among two or more communities (( \beta )-diversity)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A) Qualitative ( \alpha )-diversity (Richness)</td>
<td>B) Qualitative ( \beta )-diversity (Divergence-based) Phylgenetic test (P-test)</td>
</tr>
<tr>
<td></td>
<td>Choao1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rarefaction</td>
<td></td>
</tr>
<tr>
<td>Additionally accounts for the number of times that each taxon was observed</td>
<td>C) Quantitative ( \alpha )-diversity</td>
<td>D) Quantitative ( \beta )-diversity (Divergence-based) Fst</td>
</tr>
<tr>
<td></td>
<td>Theta (( \Theta ))</td>
<td></td>
</tr>
</tbody>
</table>

2.2.2 Diversity-based analysis

Alpha (\( \alpha \))-diversity is usually characterized using the total number of species (species richness) within a given community (Table 2.1A, 2.1C). In this study, \( \alpha \)-diversity measurements were used to estimate number of species/OTUs for either elevated or for ambient CO\(_2\) conditions. Beta (\( \beta \))-diversity (Table 2.1B, 2.1D) is often characterized using the number of species shared between two communities (measurements of the partitioning of biological diversity among communities or along an environmental gradient). Extrapolation of \( \beta \)-diversity in this study allows comparison of the genetic and phylogenetic diversity between microbial communities in different CO\(_2\) treatments (discussed later in this chapter). The following two \( \alpha \)-diversity methods are used in this study to assess diversity.

a.) Choao1 richness estimator (9)

Large clone libraries have been used to detect unusual microbes and to determine microbial community structures from soil obtained from diverse environments (6, 41). A problem with this type of study is determining a representative sample size that would correlate to diversity in the test environment. The Choao1 richness estimator (Table 2.1A) has been applied frequently to microbial data to estimate the total richness from a sample (13, 25, 56, 57).
b.) Rarefaction analysis

Although the implicit assumption in diversity surveys is that the total diversity in a community has been exhaustively sampled, this practice is not always practical or warranted. Rarefaction analysis (Table 2.1A) is one technique that can be used to estimate the total phylogenetic diversity that would be obtained with exhaustive sampling, and can also be used to evaluate whether diversity has been sampled adequately. A rarefaction curve is a plot of phylotype richness (number of species recorded at a pre-determined level for species identity, such as >97% sequence similarity), against level of sampling effort (number of clones sequenced). The species richness of an environment can be predicted using curve-fitting methods to estimate the asymptote (25).

2.2.3 Quantitative measures of diversity

The presence or absence of a species may be used to measure changes in the relative abundance of lineages. A drawback of quantitative measures that use amplified 16S rDNA to define OTUs is that frequency of appearance may be altered by biases during the process of amplification. A couple of such biases are primer specificity during PCR and differences in rRNA copy number between bacteria (69). It is thus always desirable to confirm abundance estimates using other methods such as QPCR (Chapter 4). This study uses the Fst test, which includes theta (θ) in the calculation for significance (Table 2.1C-D), described below to quantitatively assess the genetic diversity and relative abundance of bacterial and fungal populations in conditions of enriched and ambient CO₂.

a.) The Fst test

The fixation index (FST) (52) measures the genetic diversity between two communities by comparing the genetic diversity within each community to the total genetic diversity of the communities combined (40). It was employed for the analysis of microbial diversity by Martin et al. (2002), and has been commonly applied in molecular evolution and population genetics. It identifies cases in which more sequence variation exists between two communities than within a single community. The Fst test is based on sequence alignments and may be altered by biases.
2.2.4 Divergence-based analysis of diversity

Recently, there has been much interest in methods for characterizing diversity within and between microbial communities (15, 38, 40, 53). Divergence-based methods account for the fact that not all species or phylotypes within the sample are equally related to each other, and they imply that gene similarity often correlates with phenotypic similarity (34, 55). Divergence-based measures are especially well suited to the evaluation of microbial communities because of the availability of databases for, and the ease to generate, 16S and 18S rRNA gene sequences. In this study, microbial communities are evaluated using PCR products that were generated with primers that amplify sequences from microorganisms from a very large taxonomic range.

a.) The P-test

The Phylogenetic test (P-test, also known as the permutation tail probability test) (Table 2.1B), was introduced by Martin in 2002 as a method to determine whether independent microbial communities are significantly different. The P-test has also been broadly applied in microbial ecology (7, 14, 35, 49, 58). The P test measures the significance of the association between environment and phylogeny, and determines whether members of the two communities are randomly distributed over a phylogenetic tree. When diversity indices (such as the \( F_{ST} \) test described above) are coupled with phylogenetic measurements of diversity like the P-test, they become more powerful for testing for differences between communities (38, 40, 68). This is because phylogenetic tests provide extra resolution to community analysis by accounting for divergence between sequences.

2.3 Materials and Methods

2.3.1 Sampling procedures

Five to seven Larrea plants located inside each FACE ring were randomly chosen for fine root harvest in May 2007. These fine roots (and the rhizosphere soils attached to them) were collected within one hour of above-ground plant harvest. Large tap roots were broken with a pick-
ax, and 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 quart-sized Ziploc bag (SC Johnson, Racine, WI). Root bags were placed immediately in ice 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 processing. Fine roots were processed for DNA within a period of one to three months after collection.

2.3.2 DNA isolation and purification

Root bags were thawed on ice and sieved through a sterile 2 mm mesh screen, which trapped small rocks and roots but allowed soil to go through. Rocks and roots trapped on mesh were visually inspected, and fine roots <1 mm in diameter were collected by 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. Roots were placed in a 50 mL Falcon centrifuge tube (BD Biosciences, San Jose, CA), and 8 mL of sterile phosphate buffered saline (pH 7.4) were added to roots. Saline and roots were vortexed vigorously for 20 s. Next, roots were gently pressed by tweezers against the side of the centrifuge tube to drain additional liquid and removed. Liquid containing suspended rhizosphere soils were concentrated by centrifugation at 13,000 rpm for 10 min. The resulting rhizosphere soil-pellet was processed by a DNA bead-beating extraction kit: Fast DNA Spin Kit for Soil (Qiagen, Carlsbad, CA). Total DNA was collected from rhizosphere soil samples using spin columns and the FastPrep FP120 bead beater instrument (QiBioGene).

2.3.3 PCR

The bacterial 16S rRNA gene was amplified by PCR from rhizosphere soil DNA using universal bacterial primers f8-27 and r1510 (16) (Table 2.2) (Integrated DNA Technologies, San Diego, CA). PCR was performed in a total volume of 50μL using the following reagents: One μL of template DNA, 125 μg of bovine serum albumin (Sigma-Aldrich, St. Louis, MO), 1.25 mM MgCl₂ (Omnipur), 200 μM deoxynucleoside triphosphates (Promega, Madison, WI), 0.63 μM and 0.8 μM, respectively, of primers f8-27 and r1510 (Table 2.2), and 1.25 U GoTaq polymerase (Promega, Madison, WI). 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 spin columns (Qiagen, Valencia, CA). 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’ (5) (Table 2.2) (Integrated DNA Technologies). The PCR reaction was identical to the protocol used to amplify the bacterial 16S rRNA gene above, with the following minor changes: An amount of 40 μM was used for fungal primers, and the final PCR elongation step was 5 min.

### Table 2.2. The sequences of primers used for PCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>f8-27</td>
<td>agagtttgatccttgctcag</td>
<td>Eder et al.</td>
</tr>
<tr>
<td>r1510</td>
<td>ggtacctgttagctgactt</td>
<td>Eder et al.</td>
</tr>
<tr>
<td>nu-SSU-0817-5’</td>
<td>ttgcatggataatcttagga</td>
<td>Borneman et al.</td>
</tr>
<tr>
<td>nu-SSU-1536-3’</td>
<td>attgcaatgcyclatccca</td>
<td>Borneman et al.</td>
</tr>
</tbody>
</table>

2.3.4 Construction and sequencing of 16S rRNA gene clone libraries

Environmental 16S rDNA libraries were constructed as previously described in the materials and methods portion of Chapter 1. In brief, the PCR products were purified using spin columns (Qiagen) and cloned into the pGEMT-easy vector (Promega). XL1-Blue competent cells (Stratagene, La Jolla, CA) were transformed with ligation reactions containing insert and vector as specified by the manufacturer’s protocol. Transformed cells were selected on Luria-Bertani (Sigma-Aldrich Corp., St. Louis, MO) agar plates containing 50 mg/mL carbenicillin (Calbiochem, San Diego, CA), 4 μL/mL IPTG and 0.8 μL/mL XGal (Promega, Madison, WI), which were incubated at 37°C overnight. White colonies were transferred to round-bottom 96-well plates containing LB broth and carbenicillin and incubated at 37°C overnight. Representative clones were screened by PCR to confirm that the size of the insert was correct. Clones were sequenced using the DyDeoxy terminator technique at the Nevada Genomics Center, Reno.

2.3.5 Processing of DNA data

Table 2.3 lists the number of clones sequenced and suitable clones that were curated from each of the six FACE rings. A total of 1,834 clones were sequenced in the combined twelve
clone libraries. Sequences of poor quality (i.e. sequencing errors, too short, incorrect insert size, vector) were discarded from analysis to yield a total of 778 analyzable “curated” sequences. Of these, 407 were bacterial sequences (208 and 199 sequences, respectively, from elevated and ambient CO$_2$ conditions). A total of 371 fungal sequences were deemed of good quality, with 182 and 189 arising from elevated and ambient CO$_2$ conditions, respectively.

Table 2.3. The number of clones sequenced and curated from each of the six FACE rings. Curated clone sequences must contain >700 bases, have the correct bacterial 16S or fungal 18S rRNA inserts, and were used in all other analysis.

<table>
<thead>
<tr>
<th>FACE Ring #</th>
<th>Bacterial Clones</th>
<th></th>
<th>Fungal Clones</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sequenced</td>
<td>Curated *</td>
<td>Sequenced</td>
<td>Curated *</td>
</tr>
<tr>
<td>1</td>
<td>88</td>
<td>68</td>
<td>76</td>
<td>63</td>
</tr>
<tr>
<td>2</td>
<td>88</td>
<td>70</td>
<td>76</td>
<td>64</td>
</tr>
<tr>
<td>3</td>
<td>112</td>
<td>70</td>
<td>76</td>
<td>55</td>
</tr>
<tr>
<td>Elevated CO$_2$ Total</td>
<td>288</td>
<td>208</td>
<td>228</td>
<td>182</td>
</tr>
<tr>
<td>4</td>
<td>88</td>
<td>72</td>
<td>76</td>
<td>59</td>
</tr>
<tr>
<td>5</td>
<td>88</td>
<td>55</td>
<td>76</td>
<td>69</td>
</tr>
<tr>
<td>6</td>
<td>136</td>
<td>72</td>
<td>76</td>
<td>61</td>
</tr>
<tr>
<td>Ambient CO$_2$ Total</td>
<td>312</td>
<td>199</td>
<td>228</td>
<td>189</td>
</tr>
</tbody>
</table>

To show that inter-exceeded intra-treatment variability, pairwise Fst comparisons were applied to replicate clone libraries of each CO$_2$ treatment condition. Bacterial sequences between ambient CO$_2$-treated FACE rings 1-3 were compared to each other, and the same procedures were repeated to compared sequences from elevated CO$_2$-treated FACE rings 4-6. The significances of the differences between sequences obtained from replicate FACE rings were determined at $\alpha \leq 0.0001$. The procedure was duplicated to compare fungal sequences found in the replicate FACE rings exposed to the different CO$_2$ treatments.

2.3.6 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, Michigan). Bacterial 16S rRNA and fungal 18S rRNA gene sequences were assembled, aligned and edited with the computer program MEGA 4 (60). The ARB (Latin arbor: tree) (39) software
package was used to generate phylogenetic trees using rDNA sequences. Tree topology was evaluated using neighbor-joining and distance matrix methods. In addition, fungal classification was accomplished by using a combination of ARB-generated tree and lineage-specific analysis using UniFrac (36). Finally, phylogenetic assignments of fungal sequences were accomplished by employing the NCBI BLAST for comparison against the GenBank 18S rRNA database (National Center for Biotechnology Information, Bethesda, MD), and UniProt Taxonomy database (66). Chimaeric bacterial and fungal sequences were identified with Bellerophone (24).

2.3.7 Significance

For analysis on the composition of microbial community structures, probability values determining significance were calculated using the one-sided Fisher exact test (18), and the 2-tail p-values (1) were reported. The null hypothesis is that there is no difference between subpopulation proportions found in elevated and ambient CO$_2$ concentrations. To account for the number of simultaneous statistical tests being performed to calculate p-values on the differences in subpopulations, the $\alpha$ value (significance threshold) was adjusted by applying the Bonferroni correction. That is, the $\alpha$ value was divided by the number of groups compared at the phylum, class, and genus phylogenetic level. The divisors (Bonferroni correction factors) for the bacterial 16S rRNA gene populations were: 11 for the phylum level, 16 for the class level, and 294 for the genus level. The three taxonomic levels for the fungal 18S rRNA gene sequences had the following divisors: 3 for the phylum level, 11 for the class level, and 64 for the genus level.

2.3.8 Richness analysis: Chao1 and rarefaction analyses

Chao1 and rarefaction analysis relied on distance matrices generated by the neighbor-joining method, and the Jukes-Cantor correction. These distance matrices were generated by MEGA 4. The Chao1 and rarefaction analyses were implemented using the computer program DOTUR (Distanced-Based OTU and Richness) (51), a statistics computer software program for defining OTUs based on the genetic distances between sequences and determining species richness.
2.3.9 Fst and P-test analyses

Theta (θ) (40), the unit of measurement for the Fst test, is the average divergence between two randomly chosen sequences or individuals in a population. The Fst test uses the formula $F_{ST} = (\theta_T - \theta_W) / \theta_T$ where $\theta_T$ is the total genetic diversity for all communities combined, and $\theta_W$ is the average within-sample diversity for all the communities being compared. Statistical significance was calculated by randomly assigning sequences to populations and 1,000 permutations using the computer software Arlequin 3.11 (17). 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 $F_{ST} = 0$ (on a scale of 0 "no difference" to 1 "unique").

The $P$-test was used to calculate probability values for the bacterial and fungal populations. One-hundred randomly permuted trees were generated and the tree lengths needed to evolve the different communities obtained from the two CO$_2$ conditions were calculated. The fraction of the permuted trees that have a lower parsimony count than the true tree (lengths frequencies distributions of random trees compared to the tree length of the original bacterial community) was calculated to produce a $p$-value. Statistical significance is inferred from determining the expected number of changes between the two CO$_2$ communities. The hypothesis is the sequences from each community are not randomly clustered. This analysis was performed by using the computer program UniFrac (36).

The $F_{ST}$ and $P$ tests were also applied to the sequence data with the exclusion of select taxa to calculate the importance of these groups to the microbial populations found in each CO$_2$ treatment. For bacteria, the $F_{ST}$ and $P$ tests were performed on phylogenetic trees that excluded either the Firmicutes, or Actinobacteria, or α-Proteobacteria. For fungi, the OTUs belonging to Basiomycota, or all members of major classes belonging to Ascomycota were systematically removed, and Fst and P tests were applied to the rest of the tree.
2.4 Results

2.4.1 Chao1

Richness estimates are shown for bacterial and fungal communities under conditions of elevated and ambient CO₂ in Table 2.4. The Chao1 predicted values for richness for bacterial communities were similar for both conditions of CO₂; however, at this level of sampling, more bacterial phylotypes are observed in elevated CO₂. Twenty-five percent and 31% of the predicted bacterial species were observed in ambient and elevated CO₂, respectively. Table 2.4 also shows that the Chao1 predicted richness for fungal communities under ambient CO₂ treatment is much lower than the predicted species richness for communities under elevated CO₂. Approximately 94% and 77% of the predicted fungal species were observed in ambient and elevated CO₂, respectively.

Table 2.4. Summary of the predicted species richness for Bacteria and Fungi. a) The number of sequences used to calculate the species richness. b) The number of identified phylotypes (or OTUs) at the existing level of sampling (sequencing). c) The Chao1 predictive richness for microbial communities under the different CO₂ treatments.

<table>
<thead>
<tr>
<th>Phylogeny</th>
<th>CO₂ Treatment</th>
<th>No. of sequences a</th>
<th>Observed No. of phylotypes b</th>
<th>Chao1 (SD) c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>Ambient</td>
<td>199</td>
<td>130</td>
<td>522 (343, 853)</td>
</tr>
<tr>
<td></td>
<td>Elevated</td>
<td>208</td>
<td>164</td>
<td>535 (385, 788)</td>
</tr>
<tr>
<td>Fungi</td>
<td>Ambient</td>
<td>189</td>
<td>15</td>
<td>16 (15, 22)</td>
</tr>
<tr>
<td></td>
<td>Elevated</td>
<td>182</td>
<td>49</td>
<td>64 (54, 92)</td>
</tr>
</tbody>
</table>

2.4.2 Rarefaction

At the experimental levels of sequencing effort presented here, a higher number of bacterial phylotypes were observed in conditions of elevated CO₂ than in ambient CO₂ (Table 2.4). Figure 2.1 illustrates that the bacterial phylogenetic diversity rarefaction curves appear linear and do not level off (reach asymptotes). The absence of plateaus from the bacterial rarefaction curves suggest that additional sequences may provide more information regarding the diversity of bacterial species found under the two CO₂ treatments. The curves also show a slight difference in species richness between ambient and elevated CO₂. At the current level of sampling, more OTUs are observed in elevated CO₂ conditions.
On the contrary, comparative fungal rarefaction curves of the phylotypes identified in both CO$_2$ treatments showed very different trajectories. The rarefaction curves for fungal isolates clearly showed a plateau and strongly suggest that sampling saturation had been reached. The curves also show a marked difference in species richness between ambient and elevated CO$_2$. Similarly to bacterial rarefaction curves, at the current level of sampling, more fungal OTUs are observed in elevated CO$_2$ conditions.

![Rarefaction Analyses of Bacteria and Fungi in Elevated and Ambient CO$_2$](image)

**Figure 2.1.** Rarefaction plot of Bacteria and Fungi in elevated and ambient CO$_2$. The grey and black lines represent the amount of bacterial and fungal clones sequenced, respectively, versus the operational taxonomic units (OTUs) identified. A phylotype (or OTU) is defined by distance level of 97% sequence identity.

2.4.3 $Fst$ and $P$ tests

a.) Bacterial and fungal $Fst$ and $P$-tests

A comparison of bacteria obtained from conditions of elevated and ambient CO$_2$ showed a population pairwise $Fst$ value of 0.0177 (on a scale of 0 "no difference" to 1 "unique"). This value suggests that bacterial populations obtained from the two CO$_2$ treatments are genetically
slightly different from each other, and that this difference is highly significant (Table 2.5). These results imply that there is significantly less genetic diversity within each CO₂ treatment community than for both communities combined. For *Fungi*, this analysis showed a similar Fst result to that found with bacteria; however, the value for the population pairwise test is 0.0224 (Table 2.5). This suggests, and is corroborated by the Chao1 and rarefaction analyses, that the fungi in elevated CO₂ treatment are genetically different than those in ambient conditions, and that this difference is highly significant.

The P-test results for both bacteria and fungi in the two CO₂ treatments also showed a significant difference (Table 2.5). The p-values < 0.05 was considered significant, and p < 0.01 was considered highly significant.

<table>
<thead>
<tr>
<th>Types of test</th>
<th>Population Pairwise Fst</th>
<th>Fst p-value</th>
<th>P-test p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial Tree</td>
<td>0.0177</td>
<td>≤ 0.00001</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Bact Tree Without Firmicutes</td>
<td>0.00696</td>
<td>≤ 0.00195</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Fungal Tree</td>
<td>0.0224</td>
<td>≤ 0.00001</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

b.) *Fst and P-tests with exclusion of select taxa*

In order to provide a more robust analysis of the differences in microbial diversity between the different CO₂ treatments, and to attribute this difference to a specific group, the F_st and P-tests were also applied on select taxa on the phylogenetic tree (i.e. solely on some taxa and/or with the exclusion of select taxa from the whole tree). The bacteria P-test results remained similar as the full tree when members of Firmicutes, Actinobacteria, or γ-Proteobacterial were removed from full tree analysis (data not shown). When Fst tests were applied to sequence data without each of these groups, the significance level without Actinobacteria and α-Proteobacteria remained the same as in the full tree analysis (data not shown). However, the p-value increased when Fst test was applied without Firmicutes (Table 2.5). Further, population pairwise comparisons between all bacterial sequences and bacterial sequences without Firmicutes showed a corresponding decrease as well (Table 2.5).
For fungi, the OTUs belonging to Basiomycota, and the all major classes belonging to Ascomycota were removed systematically, and Fst and P tests were applied to the rest of the tree. No significant changes were detected in the p-values of these tests (data not shown).

c.) Pairwise Fst test on sequences from replicate FACE rings

To show that inter- exceeded intra-treatment variability, pairwise Fst comparisons were applied to replicate clone libraries of each CO\textsubscript{2} treatment condition. The bacterial results showed that the sequences found in the elevated CO\textsubscript{2} replicate libraries are similar (Fig. 2.2). However, bacterial sequences isolated from ambient CO\textsubscript{2} conditions, especially FACE ring 4, are significantly different from the other two replicate rings.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>FACE Ring #</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fungi</th>
<th>FACE Ring #</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 2.2. Results of bacterial (top figure) and fungal (bottom figure) pairwise Fst analyses between replicate FACE rings. Rings 1-3 were treated with elevated, and rings 4-6 treated with ambient, atmospheric CO\textsubscript{2}. Negative (-) or positive (+) symbols denote non-significant or significant differences, respectively, between sequences obtained from replicate FACE rings at α ≤ 0.0001.

Similarly, Figure 2.2 revealed that fungal sequences arising from ambient CO\textsubscript{2} conditions from all replicate FACE rings are not significantly different. However, sequences obtained from elevated CO\textsubscript{2}-treated ring 2 were significantly different from the other two replicate rings. These results suggest that the sequences from the three duplicate libraries are genetically similar to each other. However, of the six FACE rings from which the libraries were obtained, the
sequences from one ring may be significantly different from ones found in the other two replicate rings.

2.4.4 Microbial community composition

Among Bacteria, Proteobacteria OTUs (all subdivisions) comprised approximately 50% of the Mojave Desert soil populations in the two environments (Fig. 2.3). At the class level among Proteobacteria, the Gamma (γ)-proteobacteria appeared the most significant (Table 2.6). However, when the threshold to determine significance, α, at the class level is divided by the number of phylogenetic groups (Bonferroni correction) Gammaproteobacteria is not significant at α=0.05.

![Composition of Bacterial 16S rRNA Clones](image)

**Figure 2.3. Bacterial soil population changes due to elevated atmospheric CO₂.** The values in the brackets denote the percentage of OTUs that were assigned to each phylum, according to the Ribosomal Database Program at 80% threshold.

The Gram-positive communities showed mixed responses in elevated CO₂. Although the total number of OTUs taxonomically assigned to the phylum Actinobacteria (high G+C Gram-positive) doubled under elevated CO₂, this increase was not significant. On the other hand, significant decreases in CO₂ enrichment occurred in the phylum Firmicutes, which are low G+C Gram-positive organisms (Table 2.6). This decrease in Firmicutes is mainly attributed to
members of the "Bacilli" class, whose representatives decreased in abundance by a 4-fold in elevated CO$_2$ when compared to ambient CO$_2$ conditions (Table 2.6).

Table 2.6. Abundance of bacterial OTUs represented in individual counts. N.D. = not detected. The p-value of statistical significance calculations are outlined in material and methods and are based on the Fisher exact test. The p-value highlighted is significant at $\alpha = 0.05$ with Bonferroni correction. The threshold to determine significance ($\alpha$ value) at the phylum and class levels should be divided by the number of phylogenetic groups identified at this level according to the Bonferroni correction.

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Class</th>
<th>Number of OTUs</th>
<th>Elevated</th>
<th>Ambient</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidobacteria</td>
<td>Acidobacteria</td>
<td>13</td>
<td>5</td>
<td></td>
<td>0.093</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>Actinobacteria</td>
<td>36</td>
<td>18</td>
<td></td>
<td>8.39E-03</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>Flavobacteria</td>
<td>2</td>
<td>0</td>
<td></td>
<td>0.499</td>
</tr>
<tr>
<td></td>
<td>Sphingobacteria</td>
<td>9</td>
<td>6</td>
<td></td>
<td>0.602</td>
</tr>
<tr>
<td>Chloroflexi</td>
<td>Cloroflexi</td>
<td>0</td>
<td>1</td>
<td></td>
<td>0.489</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>&quot;Bacilli&quot;</td>
<td>10</td>
<td>40</td>
<td></td>
<td>3.11E-06</td>
</tr>
<tr>
<td>Planctomycetes</td>
<td>Planctomycetacia</td>
<td>4</td>
<td>1</td>
<td></td>
<td>0.373</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>Alphaproteobacteria</td>
<td>59</td>
<td>44</td>
<td></td>
<td>0.171</td>
</tr>
<tr>
<td></td>
<td>Betaproteobacteria</td>
<td>18</td>
<td>24</td>
<td></td>
<td>0.328</td>
</tr>
<tr>
<td></td>
<td>Deltaproteobacteria</td>
<td>5</td>
<td>9</td>
<td></td>
<td>0.284</td>
</tr>
<tr>
<td></td>
<td>Gammaproteobacteria</td>
<td>10</td>
<td>25</td>
<td></td>
<td>7.28E-03</td>
</tr>
<tr>
<td></td>
<td>Unclassified</td>
<td>7</td>
<td>4</td>
<td></td>
<td>0.544</td>
</tr>
<tr>
<td>Gemmatimonadetes</td>
<td>Gemmatimonadetes</td>
<td>3</td>
<td>3</td>
<td></td>
<td>1.000</td>
</tr>
<tr>
<td>TM7</td>
<td>TM7 Genera Incertae sedis</td>
<td>8</td>
<td>6</td>
<td></td>
<td>0.788</td>
</tr>
<tr>
<td>Verrucomicrobia</td>
<td>Verrucomicrobia</td>
<td>4</td>
<td>2</td>
<td></td>
<td>0.686</td>
</tr>
<tr>
<td>Unclassified</td>
<td></td>
<td>20</td>
<td>11</td>
<td></td>
<td>0.137</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>208</td>
<td>199</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Other non-Proteobacteria and a small portion of rRNA gene sequences that could not be assigned a taxonomic group make up the rest of the sequences recovered from the rhizosphere soil (Fig. 2.3). These taxonomic groups include Acidobacteria, Bacteroidetes, TM7, Gemmatimonadetes, Verrucomicrobia, Planctomycetes, and Chloroflexi.

2.4.5 Fungal community composition

Examination of the fungal OTUs revealed that Ascomycota comprised 82% of sequences within the fungal libraries. The results also show a significant increase in abundance at the class level within the Ascomycota (Table 2.7). This increase of diversity in CO$_2$ soil is correlated to the dominance of the representatives classified to two classes. Within Eurotiomycetes, there was a
significant increase of OTUs assigned to the order Onygenales in elevated CO₂, specifically among a cluster that include *Arachnomyces*, *Kraurogymnocarpa*, and others that grouped closely with these species (Table 2.7). 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 CO₂ rhizosphere soils.

In contrast to the increase seen in the above classes, OTUs classified to a large group within Mitosporic Ascomycota (a heterogeneous group of ascomycotic fungi whose common characteristic is the absence of a sexual state) were not detected as often in conditions of elevated CO₂, when compared to ambient CO₂ (Table 2.7).

Basidiomycota comprised approximately 16% of recovered OTUs in the fungi library. Results show OTUs classified to this phylum were found mostly in the class Agaricomycetes, and there is a significant decrease in ambient CO₂ (Table 2.7). Looking further among the genera of this class, a highly significant change was noted among *Lepista* (Table 2.7), because no member of this genus was detected in conditions of elevated CO₂. Other non-significant changes in phyla representation such as Glomeromycota contributed very little to the fungal libraries, and members of phyla Chytridiomycota and Zygomycota were not detected in the rhizosphere soils of either CO₂ treatment.
Table 2.7. Abundance of fungal OTUs represented in individual counts. N.D. = not detected. The P-value of statistical significance calculations are outlined in material and methods and are based on the Fisher exact test. The P-values highlighted are significant at and \( p = 0.05 \) respectively, with Bonferroni correction at the class or species levels. The threshold to determine significance (a value) at the phylum, class, and genus levels should be divided by the number of phylogenetic groups identified at this level according to the Bonferroni correction.

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Class or Group</th>
<th>Genus</th>
<th>Number of OTUs</th>
<th>Elevated</th>
<th>Ambient</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascomycota</td>
<td>Dothideomycetes</td>
<td></td>
<td>33</td>
<td>26</td>
<td>0.259</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dothideomycetes et</td>
<td>Chaetothyriomycetes insertae sedis</td>
<td>19</td>
<td>1</td>
<td>9.77E-06</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Euromycetes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.0581</td>
</tr>
<tr>
<td></td>
<td>Arachnomyces, Kraurogymnocarpa, and other closely related species</td>
<td></td>
<td>27</td>
<td>3</td>
<td>1.40E-06</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td></td>
<td>20</td>
<td>30</td>
<td>0.175</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Letiomycetes</td>
<td></td>
<td>2</td>
<td>6</td>
<td>0.284</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pezizomycetes</td>
<td></td>
<td>16</td>
<td>18</td>
<td>0.858</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sordariomycetes</td>
<td></td>
<td>25</td>
<td>28</td>
<td>0.882</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mitosporic Ascomycota</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8.23E-04</td>
</tr>
<tr>
<td></td>
<td>Madurella</td>
<td></td>
<td>N.D.</td>
<td>10</td>
<td>0.00175</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td></td>
<td>10</td>
<td>21</td>
<td>0.0606</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Other Ascomycota</td>
<td></td>
<td>5</td>
<td>4</td>
<td>0.747</td>
<td></td>
</tr>
<tr>
<td>Basidiomycota</td>
<td>Agaricomycetes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.10E-03</td>
</tr>
<tr>
<td></td>
<td>Lepista</td>
<td></td>
<td>N.D.</td>
<td>14</td>
<td>9.78E-05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td></td>
<td>13</td>
<td>21</td>
<td>3.33E-03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Other Basidiomycota</td>
<td></td>
<td>3</td>
<td>2</td>
<td>0.251</td>
<td></td>
</tr>
<tr>
<td>Glomeromycota</td>
<td></td>
<td></td>
<td>3</td>
<td>2</td>
<td>0.680</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>181</td>
<td>188</td>
<td>0.680</td>
<td></td>
</tr>
</tbody>
</table>

2.5 Discussion

In order to study changes in microbial diversity, a total of 600 16S bacterial and 456 18S fungal ribosomal rRNA gene clones were sequenced from total soil DNA extractions obtained from *Larrea* rhizosphere soil under ambient and elevated CO\(_2\) concentrations (Table 2.3). The clone libraries were designed to represent the spatially averaged bacterial communities within each treatment, and therefore, spatial replicates were combined. This approach serves to provide well-mixed samples for the application of phylogenetic and statistical analysis.

Previous work measuring biotic, abiotic and other performance aspects at the Nevada FACE site showed very little variability between the elevated and ambient FACE rings (28). To
control for the possibility that intra-treatment variability could exceed the inter-treatment variability, the following methods were employed: (i) Instead of the common method of combining subsamples for each treatment condition, which minimizes intra-treatment variability, equal amounts of Larrea fine roots from each of the six experimental FACE ring were processed independently.  
(ii) A total of twelve bacterial and fungal clone libraries (six FACE rings x bacterial 16S and fungal 18S rRNA) were constructed instead of forming one composite clone library for each CO₂ treatment condition. Pairwise comparisons applied to replicate clone libraries of each CO₂ treatment condition showed that inter- exceeded intra-treatment variability.  
(iii) The quantity and distribution of OTUs in each FACE ring and in each CO₂ treatment also served as a control measure for inter-treatment variability. For example, the consistency of representatives assigned to different taxonomic groups in both treatments, particularly the Proteobacteria, which composes 50% of all bacterial clone libraries (Fig. 2.3), also suggest that intra-treatment variability did not exceed that of inter-treatment.  
(iv) Furthermore, significant differences in bacterial Gram-positive populations, a major group of bacteria which composes roughly 25% of the clone libraries, were confirmed by another quantitative method, QPCR (Chapter 3).

2.5.1 Species richness and sampling

The Chao1 total richness estimates for bacteria and fungi gave the first indications that there are differences in microbial diversity in these groups in the different CO₂ treatments. Chao1 analysis also suggests that the bacterial populations have been undersampled. Although the analysis revealed that more sampling is needed, it is often impractical to exhaustively sequence all 16S rRNA ribotypes in a soil community; therefore, many researchers chose to generate much smaller clone libraries to represent total diversity (21, 35, 58). Increasing the number of sequences is unlikely to alter the results of the bacterial phylogenetic analysis for several reasons: First, the unique sequences generated in this study represent a significant subsample of the total diversity, an average of about 34%, based on the Chao1 estimate of distinct ribotypes for this community. Second, the major phyla are represented in bacterial clone libraries and have also been detected in other soil studies involving atmospheric CO₂ enrichment (33, 35). Lastly, in order for further sequencing to alter the results of the P or Fst analysis, entirely new clades that
contain sequences from only one CO₂ condition must emerge. Thus, although the Chao1 analysis revealed that bacterial populations were undersampled, it is unlikely that the remaining unsequenced ribotypes would drastically change the results.

Table 2.4 shows that although the difference in species richness is slight among bacteria, fungi showed a pronounced difference. The analysis demonstrated that fungal populations have been adequately, or as with the sampling of the ambient CO₂ populations exhaustively, sampled. In addition, the Chao1 species richness estimations of the total number of phylotypes present in the fungal communities of the CO₂ treatments are highly dissimilar from each other (Table 2.4). This suggests that the fungal communities obtained from the elevated CO₂ treatment are richer and have about 4-fold more phylotypes than ones from ambient conditions. Interestingly, small numbers of fungal phylotypes were observed in the two CO₂ treatments (Table 2.4), 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 NTS (2, 64), which shows that mycorrhizal colonization is not high at any time of the year.

2.5.2 Community structure

The genetic diversity of the bacterial and fungal communities in the different CO₂ treatments was examined by testing for the F_{ST} and for the influence of exposure to CO₂ on phylogeny (P test). The data showed that the bacteria isolated from environments of ambient and elevated CO₂ are significantly different, both genetically and phylogenetically, from each other (Table 2.5). Fungi showed a similar result to those observed for bacteria. These results provide strong statistical support for the hypothesis and show a clear differentiation between communities in the two CO₂ treatments among bacteria and fungi.

a.) Bacterial community composition

Examination of the taxonomic groups obtained from rhizosphere soil of *Larrea* revealed that the phylogenetic diversity of bacteria and fungi in enriched CO₂ is distinct from those observed in ambient CO₂. Upon closer assessment, it became apparent that there were some population rearrangements at the species level. Likewise, analysis of fungal clone sequences obtained from both CO₂ treatments showed comparable abundance of sequences at the phyla
level; however, significant increases and decreases in the abundance of sequences within certain taxa showed a large amount of variation at the class levels.

The numbers of OTUs affiliated with all bacterial groups were found in both CO$_2$ environments and on almost all branches of the phylogenetic tree, suggesting bacteria belonging to these taxa are widely distributed in the two environments. Among *Bacteria*, Proteobacteria OTUs (all subdivisions) were dominant and comprised approximately 50% of the bacterial Mojave Desert soil population in the two environments (Fig. 2.3), and their abundance is consistent with previous soil bacterial community studies (26, 33, 35). 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 2.6), it is not possible to conclude this group's heterotrophic contributions, since γ-Proteobacteria is a large and diverse group in physiological and metabolic capability. Also, further analysis of this subphylum is not possible as there are too few sequences for order-level comparisons.

The Gram-positive communities, which composed approximately 25% of the total OTUs in the environmental clone libraries, showed mixed responses in elevated CO$_2$. The representation of Actinobacteria displayed a non-significant trend to increase in elevated CO$_2$. Many of these heterotrophs take part in the degradation of many recalcitrant forms of soil C and cellulose and lignin, and are mediators in the biogeochemical cycling of C and nitrogen (N) in terrestrial ecosystems (20). On the other hand, the most significant decreases in CO$_2$ enrichment occurred in the phylum Firmicutes, which are low G+C Gram-positive organisms. This is mainly attributed to members of the "Bacilli" class, whose representatives decreased by a four-fold in abundance in elevated when compared to ambient CO$_2$ conditions (Table 2.6). Although the decrease in Gram-positive microorganisms is confirmed by QPCR assays of the same studied environment (Chapter 3), the decrease of the Bacilli in elevated CO$_2$ seen in this study is not in agreement with studies conducted recently by other researchers (33, 35). The discrepancy between this and other studies may be due to differences in the ecosystems examined, which included forest and semi-arid lands. Future attempts to gain an understanding of functional
differentiation between microbial communities inhabiting rhizospheres found in ambient and elevated CO₂ conditions might begin by focusing on the biology of the Bacilli.

None of the other phylum showed a significant change. This is in contrast to the reported results that under conditions of elevated CO₂, there is an increase among Bacteroidetes (33) and Acidobacteria (47). However, these results are congruent with reports from other studies of the effects of elevated CO₂ on soil microbes. For example, Verrucomicrobia was shown to be unaffected by elevated CO₂ (48).

b.) Fungal community composition

Fungal community composition in rhizosphere soil of Larrea roots dramatically changed under elevated CO₂. The greatest differences were found in several classes in the phylum Ascomycota, and among Basidiomycota. Results are consistent with fungal data in semi-arid FACE soil (35). The total fungal DNA concentration in the soil was not determined in this study, but Lesaulnier et al. (2008) and Chung et al. (2006) found no change in fungal and eukaryotic DNA concentration in conditions of elevated CO₂.

Examination of the fungal OTUs revealed that sac-producing Ascomycota comprised of 82% of sequences belonging to fungal libraries (Table 2.7). This is not surprising since ascomycotes often accounted for some 75% of all described fungi (61). The most well-known members of this phylum include yeasts, powdery mildew, penicillin molds, and cup fungi.

In agreement with the rarefaction analysis, which showed an increase in fungal diversity in conditions of elevated CO₂, the results presented also show a significant increase in abundance at the class level within Ascomycota (Table 2.7). This increase of diversity in CO₂ soil is correlated to the OTUs categorized to the temporarily-classified class “Dothideomycetes et Chaetothyriomycetes insertae sedis”, because members of this class were absent from ambient CO₂ rhizosphere soils. Members of this class 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. Their significant increases provide strong evidence for the hypothesis that there is an increase in heterotrophy associated with elevated CO₂. These results agree with other researchers who also noted that saprotrophic fungi
associated with *Adenostoma fasciculatum* and other chaparral plants are stimulated (35, 46, 65). The increased root biomass associated with elevated CO$_2$ conditions probably stimulated fungi that thrive on both dead and live roots (35).

In contrast to the increase seen in the above classes, OTUs classified to a large group within Mitosporic Ascomycota (a heterogeneous group of ascomycotic fungi whose common characteristic is the absence of a sexual state) were significantly decreased in conditions of elevated, when compared to ambient, CO$_2$. To our knowledge, this is the first report in which an effect of elevated CO$_2$ condition is observed in this group of ascomycotes.

Basidiomycota are differentiated from other fungi because of their ability to produce sexual spores on club-like structures called basidia. Notable members of this phylum are mushrooms, puffballs, and smuts. Results show OTUs classified in this phylum were found mostly in the class Agaricomycetes, and there is a significant decrease at this level in elevated CO$_2$. This is in sharp contrast to previous findings that ectomycorrhizal fungi, a majority of which are basidiomycetes, have previously been documented to increase in abundance under elevated CO$_2$ (27) and receive a significant portion of the plant's net photosynthate (22). Further, a significant change was noted among *Lepista*. Members of this genus were not detected in conditions of elevated CO$_2$, and some members of this group are known for their saprotrophic abilities, growing on wood litter. Overall, the significant decreases among OTUs classified to Basidiomycota in elevated CO$_2$ suggest that basiomycotes did not thrive in response to CO$_2$ enhancement.

Other non-statistically significant fungal phyla such as Glomeromycota contributed very little to the fungal libraries. Members of this phylum form arbuscular mycorrhizae (AM) with plants; the data are in accord with previous findings by Apple *et al.* (2008), who observed that AM colonization is low at the NTS all times of the year and specifically lower in spring (when this study was conducted). 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 *A. fasciculatum* and other chaparral plants (35) and changes in AM species composition (46), in elevated CO$_2$. 
Members of the phyla Chytridiomycota and Zygomycota were not detected. This is not surprising since Chytridiomycota are mostly aquatic and not expected to be present in arid environments due to their demands for high moisture. However, members of this phylum have been reported in soils under trembling aspens, where they appear to have been unaffected by the increase in atmospheric CO$_2$ (33). Zygomycota assigned OTUs, although abundant in soils of temperate forests (8), were not detected in rhizosphere soils of the Mojave Desert.

Fungal Chao1 richness estimates indicate a four-fold increase in species diversity under elevated CO$_2$ (Table 2.4). These changes in relative abundances, and associated varying response of different representatives, suggest that fungal species belonging to the phylum Ascomycota are very responsive to the effects of elevated atmospheric CO$_2$. These results suggest that elevated atmospheric CO$_2$ favors the symbiotic relationship between fine roots of Larrea and ectomycorrhizal fungi belonging to the phylum Ascomycota. On the other hand, basidiomycotes were outcompeted by other fungal species in conditions of elevated CO$_2$.

Furthermore, in Fungi, no significant changes were detected with each deletion of OTUs belonging to a major class. This implies that a cumulative change in all the fungal phyla, rather than in a specific group, was observed in conditions of elevated CO$_2$.

2.5.3 Microbial community changes related to root growth, soil respiration, and nitrogen cycling

Nowak et al. (2004) reported that both aboveground and belowground production generally increased with CO$_2$ enrichment in bog, forest, and grassland systems. Similarly, the Mojave Desert system above-ground production was significantly enhanced by CO$_2$ enrichment (23, 43, 54). Above-ground production due to an increase in CO$_2$ usually translates to an increase in fine root production in other environments (42). Since the rhizosphere is defined as the soil environment directly under the influence of living roots (31), it is intuitive that an increase in root production due to elevated CO$_2$ would result in higher C deposition, thereby increasing the microbial biomass and extracellular enzymatic activity of inhabitants of the rhizospheres (46). This notion was supported by data from other FACE systems. For example, in Pinus taeda, it has been suggested that increased mycorrhizal growth (62) or root exudation (44) under elevated CO$_2$ may be the source of increased microbial respiration (50). Further, in FACE system studies
involving microbial diversity of chaparral plants, Lipson et al. (2005) found that the respiratory physiology of the microbial community shifted in response to elevated CO$_2$, and that this effect could be caused by stimulated root respiration.

In stark contrast to previous findings, many researchers at the NTS FACE location have shown that in the Mojave Desert, fine roots production under *Larrea* did not increase in elevated CO$_2$. For example, Phillips et al. (2006) showed that in *Larrea*, the increase in soil respiration was not due to increased fine root standing crop, production, and mortality (thus decomposition). Likewise, at the same study site, several researchers (2, 3, 4) observed that root respiration in elevated CO$_2$ may be due to increased microbial activity and respiration. Altogether, researchers at the NDFF have reported that it is very unlikely that the C source for increased microbial activity is root biomass.

Although the increased *Larrea* plant growth did not translate to increased fine root biomass (thus root turnovers rates) under elevated CO$_2$ in the Mojave Desert, current data suggests that fungi became significantly diversified and shifted toward heterotrophy to take advantage of the limited availability of essential nutrients found in the *Larrea* rhizosphere. Perhaps, with their vast network of mycelia, certain fungal species can spread beyond the rhizosphere to obtain other below-ground sources of nutrients (32). Further, the increased mycorrhization of the *Larrea* root stem by fungal species has the potential to improve soil nutrient acquisition (63). Also, it could be speculated that the increase in heterotrophy among fungi is sequestering the ammonium which would otherwise be used by bacterial nitrifiers (33). This is supported by the observations from this and other FACE studies (33, 35), which showed that there were no significant changes in the abundance of bacteria involved in nitrification in elevated CO$_2$.

2.5.4 Future work

The continuation in the quantification of significant bacterial and fungal groups in arid soil (as determined by clone libraries analysis) using QPCR is also necessary. For example, because bacterial Gram-positives showed a significant decrease in elevated CO$_2$, this study employed QPCR to target this group (Chapter 3). Also, the quantification of smaller taxonomic groups of
microorganisms inhabiting the rhizosphere may provide additional insights into the microbial community structures in CO\textsubscript{2} enrichments. These analyses will serve to confirm the differences in diversity detected in this research, and provide a foundation for comparisons to data reported by researchers at the same or different FACE sites.

This research shows the community structure of rhizosphere bacteria and fungi communities differ in response to elevated CO\textsubscript{2} conditions. However, other microbial communities inhabiting the root environments of plants have been shown to respond to changes in levels of CO\textsubscript{2}. For example, a comprehensive survey of microbial diversity by Lesaulnier et al. (2008), who investigated the effects of elevated and ambient levels of CO\textsubscript{2}, has shown that the composition of OTUs classified to Archaea associated with quaking aspens are significantly altered in elevated CO\textsubscript{2}. Thus, analysis of the archeal communities will be performed in the future to determine the diversity of this community in CO\textsubscript{2} enrichment in arid soil.

Furthermore, future studies can address changes in microbial communities in different conditions, such as wet and dry, or comparing microbial communities in different desert plants. An example of this is work is by Billings et al. (2002), who examined N cycling processes at the NTS FACE facility, which suggests that the effects of elevated CO\textsubscript{2} on soil microbial activity may not be consistent for all seasons and types of desert plants. Lastly, other approaches which examine functional genes (70) and enzymatic activities (11, 12) can be employed to determine changes in soil microbial communities of the studied environment.

2.6 Conclusion

This work reports the effects of elevated atmospheric CO\textsubscript{2} on bacterial and fungal communities inhabiting the rhizosphere of a major Mojave Desert plant. The results of this study show that bacterial and fungal communities are significantly altered by elevated atmospheric CO\textsubscript{2}. Since the cycling of essential elements in the soil is often mediated by microbes, these results also suggest that changes in diversity among microbes within these two groups may have implications for plant productivity and for functions of desert ecosystems. This research dovetails with many ongoing research efforts in the same experimental plots, specifically ones which
examine the functioning of microbial communities in soil, in specific nutrient pools, in cycling of
essential elements, and soil respiration. The combined efforts should shed more light on how a
major global change phenomenon, such as the increase in atmospheric CO$_2$, affects the diversity
of life in the Mojave Desert environments.

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CHAPTER 3

QUANTITATIVE ANALYSES OF THE EFFECTS OF ELEVATED CO₂ ON BACTERIAL COMMUNITIES ASSOCIATED WITH LARREA

3.1 Abstract

In the Mojave Desert, there is compelling statistical and phylogenetic evidence which support the notion that high carbon dioxide (CO₂) levels produce a shift in the species composition of arid soil bacterial and fungal populations (Chapter 2). It was hypothesized that changes in microbial community structures observed in enriched atmospheric CO₂ are coupled to changes in population density. To test this hypothesis, universal quantitative PCR (QPCR) employing TagMan® technology was used to quantify the total amplifiable specific small-subunit 16S rDNA in rhizosphere soil samples. These samples were obtained from the desert shrub Larrea tridentata, which was exposed to elevated or to ambient atmospheric CO₂. Also, QPCR protocol, primers and probe were developed to quantify Gram-positive microorganisms in the same rhizosphere soil samples. The composite of purified 16S rRNA PCR products amplified from all CO₂ treatment plots were used as standards for quantification. It was concluded that the concentration of total bacteria 16S rDNA, standardized by fine root weight, were similar in conditions of enriched and ambient CO₂. The QPCR protocol, primers, and probe developed for quantifying bacterial Gram-positive microorganisms from rhizosphere soil samples showed a relative 44% decrease in the population of Gram-positives in conditions of elevated CO₂ compared to ambient conditions. These results indicate that elevated CO₂ changes composition and population dynamics of microorganisms associated with roots of desert plants.
3.2 Introduction

Very little is known about microbial diversity at the Nevada Test Site (NTS), with just over 60 isolates cultured from samples in the laboratory. A variety of analysis methods were traditionally used to characterize the diversity of microorganisms in this environment (13, 14, 17). However, among these, the culture approach is often limited since only about 1% of microbes can be cultured from environmental samples (1, 5). Microscopy provides data on total cell concentrations, but discrimination between genera or species data are lost. Fluorescent in situ hybridization (FISH) is often used to identify microorganisms, however, this method may be better suited to visualize the distribution of target microorganisms in aquatic environments because there is less background fluorescence (36). Lastly, although phospholipid analysis provides information on microbial biomass and can be used to classify organisms by physiological traits, specific identification is also not possible.

The development of techniques to analyze the small-subunit rRNA gene (rDNA) obtained from environmental samples provides culture-independent means to specifically detect and enumerate target microorganisms. Culture-independent molecular methods based on polymerase chain reaction (PCR) have greatly advanced the study of microbial communities, and have been used to characterize a variety of environments (6, 20, 30, 32). The method that is currently most commonly used in microbial population research is to amplify the 16S rDNA gene sequences in a sample, followed by cloning and sequencing of the DNA, and then identifying the microorganisms by comparing the sequences with databases. However, the cloning and sequencing approach can be labor intensive, and in some diversely populated environments, only a very small percentage of the population is identified.

3.2.1 Quantitative polymerase chain reaction (QPCR)

In addition to its utility in conducting environmental surveys, PCR may also be used to directly quantify populations of yet-to-be cultured bacteria. Quantitative PCR (QPCR) or real-time PCR is a real time assay that measures product accumulation with fluorogenic probes and enables the sensitive, specific detection and enumeration of target organisms. This technique is widely applied and has been used in studies on clinical and environmental samples (15, 26, 34,
Further, QPCR studies can provide insights into the quantities of rDNA present in environmental samples, can determine the relative density of the target populations in the studied environments, and can corroborate data obtained in environmental libraries.

Currently, the most common types of QPCR assays commercially available include SYBR® Green, TaqMan®, and molecular beacon. The SYBR® Green dye has a high affinity to double-stranded DNA, and emits light when excited. Despite having the advantage of being inexpensive and sensitive, a major disadvantage is SYBR Green will bind to any double-stranded DNA in the reaction. This may include primer-dimers and other non-specific reaction products, which may result in inaccurate data by overestimating template concentration.

Alternative QPCR assays to SYBR Green include TaqMan® and molecular beacon. The TaqMan® assay requires the following events: 1) The binding of a forward primer and reverse primer to a specific location on the target DNA. 2) The binding of a fluorescent dye-containing TaqMan® probe in the internal region of the PCR product. 3) Cleavage of the probe by the 5' exonuclease activity of the polymerase (which thereby separating the quencher from the reporter located on the ends of the probe) allows the reporter to produce a fluorescent signal when DNA is being synthesized. In slight contrast to the TaqMan® assay, the probe for the molecular beacon assay forms a hairpin that can anneal to its target sequence unless it is displaced by the DNA polymerase. When the hairpin structure is disrupted (as when probe is bound to the target DNA), the molecular beacon probe also produces a fluorescent signal, which indicates DNA synthesis.

The detected fluorescent signal for all above QPCR assays is reported as the cycle threshold (Ct) value. The Ct value is the cycle in which fluorescence is first detected above a background signal, and is inversely proportional to the concentration of DNA in the sample. In all QPCR assays, the presence of PCR inhibitors can result in false-negatives and reduce sensitivity. Due to the inclusion of the probe, probe-based QPCR systems are more specific than dye-based assays. However, a drawback to probe-based QPCR assays is the time-consuming development of DNA primers and probes that can effectively amplify a large variety of organisms.
3.2.2 Advantages and limitations of QPCR

The QPCR approach has several advantages and limitations for the characterization of environmental samples. The speed, sensitivity and the potential for high throughput processing of samples are superior compared to other methods. One limitation of QPCR is the requirement for protocol validation using environmental samples. Many primers and probes in existing QPCR protocols were designed based on existing sequence information in databases and have not been subjected to rigorous empirical testing (22).

One significant obstacle to the absolute determination of bacterial numbers by real-time PCR based on 16S rDNA is the variation in the number of rDNA gene copies in a given species at the time of analysis (26), potentially leading to overestimating the population of target microorganisms within an environmental sample. Despite this drawback, for characterization of environmental microbial populations, other methods are likely far less sensitive or precise. Lastly, inhibition of the PCR assay by environmental or biological compounds can reduce assay sensitivity or result in false negatives. This issue can be minimized by the use of effective DNA extraction and purification methods, and appropriate internal positive controls to detect inhibition of the QPCR assay.

3.3 Materials and Methods

3.3.1 Experimental design

The use of QPCR is an effective method to quantify specific bacterial groups. Hence the experimental plan presented below will employ QPCR to test the hypothesis that shifts in microbial community structure observed in elevated CO$_2$ conditions are coupled with changes in population density. This study has two sets of experiments. First, QPCR assays employing TaqMan® technology and a previously published primers and probe set were used to quantify the total amplifiable bacterial small-subunit 16S rDNA in rhizosphere soil samples. These soil samples were obtained from the root of the desert shrub *Larrea tridentata*, exposed to elevated or ambient atmospheric CO$_2$ (see Chapters 1 and 2 for more details on experimental design of field plots at the Department of Energy's Nevada Test Site (NTS)). Second, a TaqMan® protocol was
developed and used to enumerate Gram-positive microbial populations in the same rhizosphere soil samples. This group of microorganisms was selected because, in addition to composing approximately 25% of the environmental bacterial clone libraries of desert rhizosphere soils, they shift in community structure in conditions of elevated CO$_2$ (Chapter 2). The Gram-positive PCR amplification conditions were optimized, and quantitation standards were prepared from a composite of PCR products of rhizosphere soil from all CO$_2$ treatment plots. This QPCR protocol was tested against bacterial and non-bacterial organisms.

### 3.3.2 Test microorganisms

Cell cultures and DNA from certified microorganisms were obtained from the American Type Culture Collection (ATCC, Manassas, VA). At least one representative from all bacterial phyla was obtained, and several additional species isolated from the NTS were also included. Thus, a total of 39 bacterial species were obtained, as well as fungal and human DNA for cross-reactivity testing (Table 3.1).
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<th>ATCC #</th>
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</tr>
<tr>
<td>Fusobacteria</td>
<td>Fusobacteria</td>
<td><em>Fusobacter insuetus</em></td>
<td>Neg</td>
<td>BAA-291</td>
</tr>
<tr>
<td>Verrucomicrobia</td>
<td>Verrucomicrobia</td>
<td><em>Verrucomicrobi um spinosum</em></td>
<td>Neg</td>
<td>43997</td>
</tr>
<tr>
<td>Dictyoglomi</td>
<td>Dictyoglomi</td>
<td><em>Dictyoglomus thermophilum</em></td>
<td>Neg</td>
<td>35947</td>
</tr>
<tr>
<td>Ascomycota</td>
<td>Ascomycetes</td>
<td><em>Stachybotrys chartarum</em></td>
<td>N/A</td>
<td>3310</td>
</tr>
<tr>
<td>Ascomycota</td>
<td>Ascomycetes</td>
<td><em>Aspergillus fumigatus</em></td>
<td>N/A</td>
<td>36607</td>
</tr>
<tr>
<td>Chordata</td>
<td>Mammalia</td>
<td><em>Human DNA</em></td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>
3.3.3 Rhizosphere soil samples from NTS FACE facility

The complete description of the collection of rhizosphere soil samples is detailed in the Materials and Methods section of Chapter 2. Briefly, the field experiments took place at the Nevada Desert Free-Air Carbon Exchange (FACE) Facility located within the NTS. The sampling took place during May 2007. Five to seven *Larrea* plants located inside each FACE ring were randomly chosen for fine root harvest.

3.4.4 DNA extraction and quantitation

For this study, DNA from test cultures (Table 3.1) and rhizosphere soil samples were used. Also, the DNA from *Bacillus atrophaeus* or 16S rDNA PCR amplification products was used to construct quantitation standards. For the test organisms, DNA was extracted using the standard protocol developed by Buttner *et al.* (2001). The DNA from FACE soil samples were extracted as detailed in the Materials and Methods section of Chapter 2. 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) in a 96-well flat-bottom black polystyrene assay plate (Costar; Corning, Corning, NY), with a Fx800 Microplate Fluorescence Reader (BioTek, Winooski, VT). 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).

3.3.5 Development of primers and probes for Gram-positives

A Gram-positive group-specific oligonucleotide probe, RW03 (11), was identified for potential use with the TaqMan® QPCR technology. The RW03 probe sequence was checked for cross-hybridization by sequence comparison using the Basic Local Alignment Search Tool algorithm (BLAST, National Institutes of Health, Bethesda, MD).

The sequence of the oligonucleotide probe RW03 was located in the 16S rRNA gene of each representative organism previously found in the NTS (i.e., *Desulfosporosinus* sp. strain A10, *Desulfosporosinus* sp. strain STP12, *Clostridium perfringens*, *C. lundense*, *Thermoterrabacterium ferrireducens*, *Bacillus subtilis*, *B. cereus*, *B. solfarensis*, *Cellulomonas fermentans*, *C.*
denverensis, Microbacterium flavescens, and M. arborescens). The sequences corresponding to the 16S rRNA gene of each organism were obtained from GenBank (National Center for Biotechnology Information, National Institutes of Health), and the twelve sequences were aligned using the Brixoft SourceEdit V4 R3 software program (www.brixoft.net). To develop a set of primers to be used with probe RW03 in the quantification of Gram positive DNA, the 16S rDNA alignments of the species above described were assessed visually and inspected for regions of homology. 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). The primers and probe (Table 3.2) were purchased from Operon Biotechnologies (Huntsville, Alabama) 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 used 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 (Table 3.1).

Table 3.2. Bacterial primer and probe sequences designed in this study or previously published. Wobble (interchangeable) bases are indicated by the letters m and y (m = adenine or cytosine; y = cytosine or thymine; * = modified in this study). P in the name denotes probe sequences.

<table>
<thead>
<tr>
<th>Primer or Probe</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GF3</td>
<td>aamycggaggaaggtggggatg</td>
<td>This study</td>
</tr>
<tr>
<td>GR1</td>
<td>ggccttggtagcctgtgt</td>
<td>This study</td>
</tr>
<tr>
<td>Gram+P</td>
<td>6-FAM-acgtcaaatcatgtgctggttc-TAMRA</td>
<td>Greisen et al. (1994)*</td>
</tr>
<tr>
<td>f8-27</td>
<td>agagtttgatccctgggtcag</td>
<td>Eder et al. (1999)</td>
</tr>
<tr>
<td>r1510</td>
<td>ggtacctgtttacgtt</td>
<td>Eder et al. (1999)</td>
</tr>
<tr>
<td>NadF</td>
<td>tcctacggaggggcaagt</td>
<td>Nadkarni et al. (2002)</td>
</tr>
<tr>
<td>NadR</td>
<td>ggactacgaggtatcctaactcgttt</td>
<td>Nadkarni et al. (2002)</td>
</tr>
<tr>
<td>UnivP</td>
<td>6-FAM-cgtattaccgcggctgctgctgac-TAMRA</td>
<td>Nadkarni et al. (2002)</td>
</tr>
</tbody>
</table>
3.3.6 Quantification of DNA

The ABI Prism 7900 HT Sequence Detection System (Applied Biosystems) was used for QPCR analysis. Soil DNA was amplified by QPCR using the universal bacterial probes and primers (Table 3.2) as specified by Nadkarni et al. (2002), and the following QPCR conditions. For Universal PCR, Standard Mode was utilized and final amplification concentrations and conditions for a 25 µl reaction volume were as follows: 5 µl DNA template, 12.5 ul 2X Universal Master Mix (Applied Biosystems), 0.1 % BSA (Sigma, St Louis, MO), 0.2 µM forward primer NadF, 0.5 µM reverse primer NadR, 0.15 µM probe UnivP (Table 3.2), and sterile nuclease-free water. Cycling conditions were the Applied Biosystems default Standard Mode: 50°C x 2 min, 95°C x 10 min, followed by 40 cycles of 95°C x 15 sec and 60°C x 1 min.

For Gram-positive PCR, FAST Mode was utilized and 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 (Table 3.2), and sterile nuclease-free water. Cycling conditions were the Applied Biosystems default Fast Mode: 95°C x 20 sec, followed by 40 cycles of 95°C x 1 sec and 60°C x 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, Inc., Foster City, CA) 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.

For the test microorganisms, the QPCR quantitation standard was a purified suspension of Bacillus atrophaeus spores (U.S. Army Dugway Proving Ground, Dugway, UT). These spores were enumerated electronically with a Coulter Multisizer II (Beckman Coulter, Inc., Miami, FL), and the DNA extraction and purification methods used to process samples were previously detailed (7). Briefly, this consists of enzymatic, heat, and cold treatments, followed by DNA
purification by Pellet Paint protocol (Novagen, Madison, WI). Quantitation was achieved by amplification of standards containing DNA extracted from suspensions of known cell concentrations (10⁵ to 10⁶ templates per reaction) of B. atrophaeus. The Ct values from the standards provide absolute quantitation of B. atrophaeus templates and were used to estimate the total concentration of DNA templates in samples. Concentration values for the unknown samples were extrapolated from the standard curve by the software and reported as the mean of two replicates.

For the rhizosphere samples, the QPCR quantitation standard was a composite of six PCR reactions (one reaction for each of the six FACE ring rhizosphere soil sample). The PCR reaction was performed in a total volume of 50 µL using the following reagents at the indicated final concentrations or amounts: One µL of template DNA, 125 µg of bovine serum albumin (Sigma-Aldrich, St. Louis, MO), 1X GoTaq Buffer, 1.25 mM MgCl₂, 200 µM deoxynucleoside triphosphates (Promega, Madison, WI), 0.63 µM and 0.8 µM, respectively, of primers f8-27 and r1510 (10) (Integrated DNA Technologies, San Diego, CA) (Table 3.2), and 1.25 U GoTaq 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 spin columns (Qiagen, Valencia, CA). 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 2.97 ng per QPCR reaction. Quantitation was achieved by amplification of standards and samples using the developed primers GF3 and GR1, and probe Gram+P (Table 3.2). Standards were amplified in triplicate under the same conditions as the unknown samples.

After amplification, all QPCR data were analyzed using the software provided with the ABI Prism 7900 HT SDS. 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 (Figs. 3.1-3.2) and reported as
the mean of six replicates. To assess the concentration of DNA of bacterial and Gram-positive microorganisms in rhizosphere soil from the quantitative standard curves, the following formulas were used:

\[ b = \text{antilog} \left( y - 7.775 \right) - 4.470 \]

Where \( b \) = log of DNA concentration of total bacterial 16S rDNA, \( y \) = QPCR Ct value, 7.775 = y-intercept, -4.470 = slope

\[ G = \text{antilog} \left( y - 16.657 \right) - 3.538 \]

Where \( G \) = log of DNA concentration of Gram-positive microorganisms, \( y \) = QPCR Ct value, 16.657 = y-intercept, -3.538 = slope

3.4 Results

3.4.1 Testing of Gram-positive primers and probes

Sequence alignments for Gram-positive organisms demonstrated that the RW03 probe (11) is located in a highly conserved region of the 16S rRNA gene (data not shown). Gram-positive primers were designed in relation to a modified probe RW03 using the Desulfovosporosinus sp. strain A10 16S rRNA gene as the template. The probe was modified by shortening the length by one nucleotide at the 5’ end to comply with the guidelines for probe design specified by Primer Express software, and was renamed Gram+P (Table 3.2). BLAST search results showed that the probe region is specific for Gram-positive bacteria (data not shown).

The primer combinations GF3/GR1 and probe Gram+P (Table 3.2) underwent preliminary testing of positive and negative control samples and DNA from selected microorganisms (Table 3.1). The addition of BSA was found to further improve amplification efficiency; therefore, the final QPCR protocol for the Gram-positive assay included the use of BSA in the reaction (0.1% final concentration).
The Gram-positive QPCR protocol was evaluated for amplification of DNA from test microorganisms from all phyla (Table 3.3). A Ct value of 40 represented a negative result. Results 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 (Table 3.3). The only organism's DNA that did not amplify was from Micrococcus; however, the Micrococcineae's contributions to the environmental clone libraries is miniscule (Chapter 2), and therefore not expected to affect QPCR results. *Deinococcus radiodurans* (Table 3.3), although classified as a Gram-positive due to its unusual outer cell wall, is of the phylum Deinococcus-Thermus. The DNA from this organism was not amplified with the primers used in this study. Since this study was aimed at detection of Firmicutes and Actinobacteria, the detection of members of Deinococcus-Thermus is outside the scope of the study. Interestingly, one of the 26 non-target Gram-negative organisms tested, *Borrelia burgdorferi*, was amplified with this protocol. No *Borrelia* or other Spirochaetes were detected in any of the environmental clone libraries of the same studied environment (Chapter 2), thus their effect on the quantification of the target populations is not expected to be significant. There was no cross-reactivity observed with fungal or human DNA.
Table 3.3. The QPCR results obtained for 27 Phyla using Gram-positive bacterial primers and probe. Ten nanograms of bacterial DNA were used per PCR assay with one exception, C. perfringens (5 ng). Human DNA consisted of 15 nanograms, and fungal DNA consisted of $2.77 \times 10^6$ S. chartarum templates and $3.97 \times 10^5$ A. fumigatus templates. Two replicates were amplified for each DNA sample with one exception D. meridiei ($n=4$). Ct value is inversely proportional to the concentration of DNA measured, and a Ct value of 40 represents a negative result (QPCR = quantitative polymerase chain reaction; Neg = Gram-negative; Pos = Gram-positive; N/A = not applicable)

<table>
<thead>
<tr>
<th>Organism</th>
<th>Gram Classification</th>
<th>Mean Ct value (n=2)</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aeropyrum pernix (Archaea)</td>
<td>Neg</td>
<td>40.00</td>
<td>---</td>
</tr>
<tr>
<td>Methanococcus maripaludis (Archaea)</td>
<td>Neg</td>
<td>40.00</td>
<td>---</td>
</tr>
<tr>
<td>Hydrogenothermus marinus</td>
<td>Neg</td>
<td>40.00</td>
<td>---</td>
</tr>
<tr>
<td>Thermotoga maritima</td>
<td>Neg</td>
<td>40.00</td>
<td>---</td>
</tr>
<tr>
<td>Thermodesulfobacterium commune</td>
<td>Neg</td>
<td>40.00</td>
<td>---</td>
</tr>
<tr>
<td><strong>Deinococcus radiodurans</strong></td>
<td>Pos</td>
<td>40.00</td>
<td>---</td>
</tr>
<tr>
<td>Chrysiogenes arsenatis</td>
<td>Neg</td>
<td>40.00</td>
<td>---</td>
</tr>
<tr>
<td>Chloroflexus aurantiacus</td>
<td>Neg</td>
<td>40.00</td>
<td>---</td>
</tr>
<tr>
<td>Thermomicrobiurn roseum</td>
<td>Neg</td>
<td>40.00</td>
<td>---</td>
</tr>
<tr>
<td>Thermodesulfovibrio yellowstonii</td>
<td>Neg</td>
<td>40.00</td>
<td>---</td>
</tr>
<tr>
<td>Geovibrio thiophilius</td>
<td>Neg</td>
<td>40.00</td>
<td>---</td>
</tr>
<tr>
<td>Nostoc sp.</td>
<td>Neg</td>
<td>40.00</td>
<td>---</td>
</tr>
<tr>
<td>Chlorobium tepidum</td>
<td>Neg</td>
<td>40.00</td>
<td>---</td>
</tr>
<tr>
<td>Methylobacterium extorquens</td>
<td>Neg</td>
<td>40.00</td>
<td>---</td>
</tr>
<tr>
<td>Hydrogenophaga pseudoflava</td>
<td>Neg</td>
<td>40.00</td>
<td>---</td>
</tr>
<tr>
<td>Shewanella oneidensis</td>
<td>Neg</td>
<td>40.00</td>
<td>---</td>
</tr>
<tr>
<td>Desulfovibrio vulgaris subsp. vulgaris</td>
<td>Neg</td>
<td>40.00</td>
<td>---</td>
</tr>
<tr>
<td>Geobacter sulfurreducens</td>
<td>Neg</td>
<td>40.00</td>
<td>---</td>
</tr>
<tr>
<td><strong>Bacillus cereus</strong></td>
<td>Pos</td>
<td>17.04</td>
<td>0.09</td>
</tr>
<tr>
<td><strong>Bacillus halodurans Nielsen et al.</strong></td>
<td>Pos</td>
<td>15.43</td>
<td>0.06</td>
</tr>
<tr>
<td><strong>Bacillus subtilis</strong></td>
<td>Pos</td>
<td>18.35</td>
<td>0.50</td>
</tr>
<tr>
<td><strong>Clostridium difficile</strong></td>
<td>Pos</td>
<td>15.43</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>Clostridium perfringens</strong></td>
<td>Pos</td>
<td>18.63</td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Desulfosporosinus meridiei</strong></td>
<td>Pos</td>
<td>18.97</td>
<td>2.18</td>
</tr>
<tr>
<td><strong>Arthrobaeter crystallopoites</strong></td>
<td>Pos</td>
<td>26.96</td>
<td>0.19</td>
</tr>
<tr>
<td><strong>Cellulomonas fimis</strong></td>
<td>Pos</td>
<td>26.60</td>
<td>0.06</td>
</tr>
<tr>
<td><strong>Gordonia rubripertincta</strong></td>
<td>Pos</td>
<td>26.63</td>
<td>0.18</td>
</tr>
<tr>
<td><strong>Microbacterium flavescens</strong></td>
<td>Pos</td>
<td>28.27</td>
<td>0.07</td>
</tr>
<tr>
<td><strong>Micrococcus luteus</strong></td>
<td>Pos</td>
<td>40.00</td>
<td>---</td>
</tr>
<tr>
<td><strong>Rhodococcus erythropolis</strong></td>
<td>Pos</td>
<td>24.72</td>
<td>0.10</td>
</tr>
<tr>
<td>Planctomyces maris</td>
<td>Neg</td>
<td>40.00</td>
<td>---</td>
</tr>
<tr>
<td>Parachlamydia acanthamoebae</td>
<td>Neg</td>
<td>40.00</td>
<td>---</td>
</tr>
<tr>
<td>Borrelia burgdorferi</td>
<td>Neg</td>
<td>20.47</td>
<td>0.18</td>
</tr>
<tr>
<td>Fibrobacter intestinalis</td>
<td>Neg</td>
<td>40.00</td>
<td>---</td>
</tr>
<tr>
<td>Geothrix fermentans</td>
<td>Neg</td>
<td>40.00</td>
<td>---</td>
</tr>
<tr>
<td>Bacteroides fragilis</td>
<td>Neg</td>
<td>40.00</td>
<td>---</td>
</tr>
<tr>
<td>Ilyobacter insuetus</td>
<td>Neg</td>
<td>40.00</td>
<td>---</td>
</tr>
<tr>
<td>Verrucomicrobiurn spinosum</td>
<td>Neg</td>
<td>40.00</td>
<td>---</td>
</tr>
<tr>
<td><strong>Dictyoglomus thermophilum</strong></td>
<td>Neg</td>
<td>40.00</td>
<td>---</td>
</tr>
<tr>
<td>Stachybotrys chartarum</td>
<td>N/A</td>
<td>40.00</td>
<td>---</td>
</tr>
<tr>
<td>Aspergillus fumigatus</td>
<td>N/A</td>
<td>40.00</td>
<td>---</td>
</tr>
<tr>
<td>Human DNA</td>
<td>N/A</td>
<td>40.00</td>
<td>---</td>
</tr>
</tbody>
</table>
3.4.2 Quantification of DNA in rhizosphere soil samples

a.) Universal QPCR

The QPCR studies employing TaqMan® technology and a published primer and probe set were used to quantify the total amplifiable bacterial specific small-subunit 16S rDNA from rhizosphere soil samples treated with elevated or ambient CO₂. The concentrations of bacterial 16S rDNA in elevated and ambient CO₂ were determined from the standard curve (Fig. 3.1), which shows the relationship between the threshold cycle and the detectable amount of bacterial 16S rDNA. The concentrations of bacterial 16S rDNA, standardized by gram of fine root weight, were determined to be similar in conditions of elevated and ambient CO₂ (Table 3.4).

![Standard Curve Plot](image)

Figure 3.1. Relation between the threshold cycle and the detectable amount of bacterial 16S rDNA. Each point represents an amount of 16S rDNA corresponding to the Ct value using the universal TaqMan® QPCR probe and primers set. The Ct is when the fluorescence is first detected crossing the threshold of 0.05 during the QPCR assay. The Ct values are in the range of 4-32 for standard dilutions (■), which are the equivalents of 29.7 ng to 2.97 fg of template DNA per reaction, plotted against the log of the concentration. The Ct values for rhizosphere soil samples (■) are plotted on the standard curve. R², the correlation coefficient of the straight line, is 0.993.

b.) Gram-positive QPCR

The Gram-positive PCR protocol, primers and probe was applied for amplification of 16S rDNA from Gram-positive microorganisms from rhizosphere soil, which were obtained from plots...
treated with elevated or ambient CO\textsubscript{2}. The relation between the threshold cycle and the detectable amount of bacterial Gram-positive 16S rDNA in the experimental samples are shown in Fig. 3.2. The DNA quantity for each of three replicates, mean and standard deviation were calculated (Table 3.4). The values for Gram-positives in ambient were 44% higher than those seen in elevated CO\textsubscript{2}.

![Standard Curve Plot](image_url)

**Figure 3.2. Relation between the threshold cycle and the detectable amount of bacterial Gram-positive 16S rDNA.** Each point represents an amount of 16S rDNA corresponding to the Ct value using the Gram-positive TagMan® QPCR probe and primers set. The Ct is when the fluorescence is first detected crossing the threshold of 0.05 during the QPCR assay. The Ct values are in the range of 17-37 for standard dilutions (■), which are the equivalents of 2.97 ng to 2.97 fg of template DNA per reaction, plotted against the log of the concentration. The Ct values for rhizosphere soil samples (■) are plotted on the standard curve. The correlation coefficient of the straight line, R\textsuperscript{2}, was 0.967.

Table 3.4. Quantitative PCR results of total bacterial or Gram-positive 16S rDNA in *Larrea* rhizosphere samples, which were exposed to elevated or ambient CO\textsubscript{2}. * Values were used to confirm population trends of the major groups of microorganisms observed to change in the rRNA gene libraries (Chapter 2).

<table>
<thead>
<tr>
<th>Assay</th>
<th>Mean concentration of DNA per gram of fine roots (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Elevated CO\textsubscript{2}</td>
</tr>
<tr>
<td>Bacteria QPCR</td>
<td>2.29 (±0.18) ng</td>
</tr>
<tr>
<td>Gram-positives QPCR</td>
<td>19.20 (±3.65) ng</td>
</tr>
</tbody>
</table>
3.5 Discussion

3.5.1 Application of Gram-positive assay to environmental samples

This research shows that rDNAs from different bacterial phyla were amplified with different efficiencies (Table 3.3). The concentrations of test organisms measured in this study are relative to the concentrations obtained by amplification of known concentrations of the standard, B. atrophaeus. Therefore, the measurements for test organisms are estimations of microbial concentrations based on similar amplification efficiencies of sample populations with B. atrophaeus. However, since environmental samples are seldom composed of one species, other standards are necessary when applying these assays to mixed populations of bacteria. The DNA used to construct the standard curve to quantitate the rhizosphere samples was a composite of conventional PCR products of bacterial 16S rDNA isolated from the same environment. This approach lessens the need to compensate for differences in 16S rDNA copy for different bacterial species. Although DNA template type may amplify differently, it was shown to be independent of amplification efficiency (33).

3.5.2 FACE site rhizosphere soil samples

Although the F$_{ST}$ and P tests measure the diversity within each sample and compare diversity between CO$_2$ treatments (Chapter 2), diversity can also be measured quantitatively. The QPCR amplification efficiency varies between microorganisms amplified with universal primers and probe; however, as the assay narrows to the Gram-positives (and the number of different target microorganisms decreases), it is expected that the problems and uncertainty in measured concentrations will also decrease (3).

The QPCR analysis showed that the amounts of bacterial 16S rRNA per gram of rhizosphere fine roots were similar among both CO$_2$ treatments (Table 3.4). The results are similar to several studies in which there was no difference in the total number of bacteria among elevated and ambient CO$_2$ treatments (19, 28, 31, 37). In contrast, several authors have seen an increase in bacterial numbers in conditions of elevated CO$_2$ (16, 23, 29). Although numerous researchers have found that microbial respiration was significantly higher in elevated CO$_2$ conditions in various environments (9, 27, 29, 38), data from semi-arid environments
demonstrated a significant increase in total microbial biomass in elevated CO₂; however, this does not necessarily translate to increased bacterial biomass, but to an increase in fungal biomass (21).

The relative proportions of SSU rDNAs of Gram-positive bacteria were decreased by 44% in conditions of elevated CO₂. This estimated decrease in Gram-positive microorganisms shows a similar trend to that observed in phylogenetic studies (Chapter 2). Those studies show that the representation of Gram-positive bacteria decreased by 20% in clone libraries from an elevated CO₂ environment when compared to ambient CO₂. The differential changes in microbial community structures in elevated CO₂ (25, 39) detected while employing molecular methods (8, 12, 18, 23, 24), have been explored previously. For example, Schortenmeyer et al. (2000) found some species increased by two-fold in a natural scrub ecosystem, and Montealegre et al. (2002) reported soil microbial community composition changes occurred in the rhizosphere soil of white clover plants grown in elevated CO₂. Billings et al. (2004) suggested that shifts in soil microbial structure in the Mojave Desert may occur with elevated CO₂.

The QPCR assays in this study were designed to quantify species belonging to the phyla Firmicutes and Actinobacteria. Results in Actinobacteria suggested an increasing growth trend in elevated CO₂; the Firmicutes significantly decreased in elevated CO₂ (Chapter 2). Thus, it is reasonable to conclude that the overall net decrease among Gram-positives, seen in the clone libraries (Chapter 2) and in these QPCR studies, are the results of the significant decrease of Firmicutes in elevated CO₂. The changes observed in these QPCR studies are not in agreement with those of Lipson et al. (2005), who did not report a significant increase in any bacterial phylum among the rRNA gene libraries of soil associated with chaparral plants obtained from conditions of elevated and ambient CO₂. It must be noted that the clone libraries used by Lipson et al. (2005) to assess bacterial community structure were small, which may limit the sensitivity in detection of specific taxonomic groups. In a similar study which uses QPCR, the overall trends in Gram-positive microorganisms seen in forest soil (19) are in contrast to those observed in this study. In the forest environment, QPCR studies show a decrease in Actinobacteria and increase in Firmicutes in elevated CO₂ conditions, the opposite of the results reported here.
The lower concentration of Gram-positives in elevated, when compared to ambient, CO₂ observed in this study may be related to the biology of the Firmicutes and other factors which are unique to arid environments. A decrease in their representation in elevated CO₂ may be due to members of the Firmicutes group's ability to sporulate in conditions of environmental and nutrient stress (4) found in arid soil. This notion is supported by work which shows that in the desert, although there is an influx of labile C in conditions of elevated CO₂ to the soil, other nutrients remain very limiting. For example, Schaeffer et al. (2003) and Gallardo et al. (1995) showed that in the Mojave and Chihuahuan Desert respectively, N may become more limiting to plants if elevated CO₂ increases soil C, which results in increased soil biomass and reduced N availability. Furthermore, other data show no significant increases in the populations of nitrifiers in conditions of elevated CO₂ in the desert (Chapter 2) and in other ecosystems (19). The increased demands of heterotrophic bacterial and fungal communities (Chapter 2) in the Mojave Desert may further reduce the low overall soil nutrient availability in conditions of elevated CO₂. Taken together, nutrient limitations and other stressors that are unique to the Mojave Desert soil may generate an environment of high nutritional stress for Firmicutes in elevated CO₂.

3.6 Conclusion

Although elevated CO₂ levels have no effects on the total quantity of bacterial DNA, the QPCR data demonstrate that they have a negative effect on Gram-positive microorganisms. These observations provide strong support for the hypothesis that in arid environments, changes in microbial community structures observed in enriched atmospheric CO₂ are coupled with changes in population density. Further, the QPCR protocol, primers and probe developed for the TaqMan® system have the potential for use in characterization of bacterial Gram-positive populations in desert rhizosphere soil, as well as in other environments. The developed QPCR assays should contribute to providing a comprehensive molecular microbiological approach to measuring microorganisms in environmental samples.
3.7 References


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Effects of elevated CO$_2$ on microbial communities of the Mojave Desert

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