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Biological Soil Crusts Microbiomes: An Exploration and Investigation of Factors Influencing Biocrust Microbial Communities in the Mojave Desert, USA

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of the requirements for the degree of

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Plant Pathology

by

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ABSTRACT OF THE DISSERTATION

Biological Soil Crusts Microbiomes: An Exploration and Investigation of Factors Influencing Biocrust Microbial Communities in the Mojave Desert, USA

by

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Dr. Jason E. Stajich, Chairperson

Biological soil crusts (biocrusts) are major components in the dryland environment. They contain diverse microbial communities with crucial functions to dryland ecosystems. However, very little is known about these microorganisms because the biocrusts are defined by their external morphology which oftentimes neglect these unseen world. To better understand biocrusts microbial communities, extensive and integrated microbial research (external morphology and microorganisms) on biocrust is needed. Therefore, in my dissertation work I aimed to 1) summarize previous and current research, and identify knowledge gaps about biocrust microbes to provide baseline understanding about biocrust microorganisms, 2) identify key factors that influenced the biocrust microbes from three domains of life (archaea, bacteria, and fungi), and 3) to further expand our understanding about temporal/seasonal effect on these microorganisms. We employed amplicon-based metabarcoding sequencing to investigate biocrusts microbial communities. In chapter 2, our results showed that biocrusts microorganism are diverse and we are only at the beginning of biocrust microbes investigation because many microorganisms cannot be identified which could be new

to science. We have modified sequencing protocols that work well with JTNP biocrusts and a baseline knowledge about biocrust microbes was established. Then, in chapter 3, we expanded our study to cover the Mojave Desert. To test our hypotheses that geography, soil depth, and crust types influenced biocrust microbial communities, 5 common biocrust types samples were collected from 4 sites across the Mojave desert. The results showed that 1) Biocrust in central Mojave were distinct from southern Mojave site, 2) biocrust surface harbored different microorganisms from subsurface soil, and 3) crust types displayed different community signatures. Moreover, microbials hubs species that connect biocrust microbes together were also identified. Lastly, chapter 4 concluded this dissertation by improving our baseline knowledge documenting how these microbial communities changes over the year and investigate how weather patterns that influenced these changes in microbial communities. We hypothesized, biocrust microbial communities were dynamic and weather including temperature, precipitation, wind speed, atmospheric pressure, humidity, and dew point influenced biocrust microbial composition. These findings not only improve our baseline understandings about biocrust microorganisms but also provide essential information for future biocrust management and conservation.

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Chapter 1

General introduction

1.1 The importance of drylands

Although their importance is rarely recognized, drylands cover about 40% of the earth land surface [1]. Drylands area is expanding and has been predicted to increase about 10% by the end of 21st century because of global warming and human activities [2]. However, climate change and global warming research largely focus on the tropical region while drylands/desert environment research has just begun to pay off. Recent research has shown that drylands impact worldwide processes such as nutrient deposition, global dust transportation, and local dust storm [3]. Carbon storage in some dryland areas with vegetation is 60% more efficient than the global average and net carbon uptake is comparable to the European pine forest which should bring more attention to dryland vegetation and how they contribute to the earth's ecosystem [3, 4]. Nevertheless, vegetation to stabilize soil surface is spare in drylands and dust can be problematic in this unstable soil area. The dust clouds also carry microorganisms over very long distances which may include

pathogens that can affect the health of all living organisms where these aerially suspended particles settling down [5]. As a result, mechanisms that can stabilize dryland soil surfaces are important for ecosystem stability. Researchers have found a key component to dryland ecosystem called "Biological Soil Crusts" (biocrusts) that can not only stabilize the soil but also contributing to many other functions in this environment.

1.2 Biological soil crusts: a living skin of drylands

Biocrusts are recognized as a living skin and a critical zone of drylands which covers the top few millimeters of the soil surface [1]. Biocrusts are important due to their diverse organismal assemblages and their essential functional roles in dryland environment. Biocrusts are the aggregation of soil particles with very diverse biota including both microscopic and macroscopic organisms such as eukaryotic algae, bacteria, cyanobacteria, fungi, bryophytes, lichens, and microarthropods [6–9]. Although water availability is very limited in dryland environment, these organisms are able to survive the harsh conditions and thrive together as an important functional units of drylands. Diverse biocrusts assemblages contribute to many essential functional roles including stabilizing soil surface, preventing soil erosion, mediating nutrient cycles, fertilizing subsurface soil, protecting vegetation, and assisting with vegetation regeneration [6, 8–10]. While biocrust has been recognized as an essential component of drylands, there are many aspects of biocrusts that still need to be explored and investigated such as exploring overall biocrust microbial community composition and structure, defining the factors that contribute to complexity and variety of

biocrust microbiome, and describing potential biocrust microbial functional roles in dryland environments from key species identified in biocrust microbiome.

1.3 Knowledge gaps in biocrust research: biocrust microbial studies are needed.

In the past two decades, biocrust research has seen a significant development and progress. More than 100 articles on biocrust studies are published per year [8]. Interestingly, research on biocrust organisms especially diverse photoautotrophs during the past several years has paved the way for future biocrust studies which raise the question about the diversity of other heterotrophs, decomposers, and consumers within biocrust organismal community. There is no doubt that research on biocrust photoautotrophic organisms have advanced the most and we learned much about the importance of eukaryotic algae, bryophytes, cyanobacteria, and lichens. However, biocrust organismal studies of heterotrophs, decomposers, and consumers are only in their infancy. Weber et al. 2016 indicated that "there are major knowledge gaps regarding the diversity and response of microbial organisms [8]." At the beginning of this project in 2015, biocrust research on heterotrophs and especially decomposers diversity was very limited. In order to overcome this limitation, we employed the next generation high throughput amplicon sequencing to identify archean, bacterial, and fungal communities in biocrusts to cover both autotrophs and heterotrophs.

1.4 Microbial studies using next generation high throughput amplicon sequencing

While traditional culture dependent and sequencing procedure has provided immense knowledge in microbial diversity research, an innovation of next generation high throughput sequencing (HTS) has changed the way we can explore the microbial world. Prior to the introduction of HTS, traditional microbial study procedures including isolating microorganisms, growing microbes in culture media, and sanger sequencing may took months or years with high cost [11]. The introduction of HTS has unfolded new possibilities in which both a lower cost and parallel sequencing to generate much more data can be achieved at the same time [11, 12]. The beginning of the HTS microbiome research era began with the human microbiome project [13]. Microbiome research is simply the study of microbial diversity that live on/in a particular study object and their activities [13, 14]. Several years after the initiation of the human microbiome project, the earth microbiome project began with a prospect to provide environmental HTS standard protocols for global microbiome studies [15–17]. To improve our understanding about the biocrust microbiome and to overcome the limitations discussed in the previous section (microbial diversity), the earth microbiome protocols which targeted archean, bacterial, and fungal communities were applied in this dissertation to investigate the biocrust microbiome and compare the findings in the context of global microbial diversity [18, 19].

1.5 Microbial diversity (microbiome) analysis

HTS generates millions of sequences from a single run; however, a microbiome project may have more than one HTS run which requires powerful software/platforms to analyze many millions of these sequences [20]. Fortunately, microbiome data analysis has been simultaneously developed and improved along with the HTS microbiome procedures. Some of the major tools that were used in this dissertation for processing sequences include QIIME [21], QIIME2 [22], AMPtk [23], USEARCH [24], VSEARCH [25], and DADA2 [26].

Together, these tools were used to process millions of raw sequences by 1) demultiplexing sequences into each sample, 2) paired-end reads were merged to produce long reads, 3) sequences were clustered into Operational Taxonomy Unit (OTU) based on similarity (e.g. conventionally, sequences will be grouped when they share 97% of the DNA sequences) or denoised into Amplicon Sequences Variant (ASV) in which the exact sequences are used (new method to group sequences based on single nucleotide changes), and 4) OTUs/ASVs were assigned to the closest taxonomy references and species tables were generated. As a result, we can identify which archaea, bacteria, and fungi are present in our biocrust samples, however, not all sequences can be identified due to limited references in the database. Then, a comparison of microbial communities between samples can be performed to investigate the differences among samples, locations, seasons etc.

1.6 Overview of the dissertation and objectives

This dissertation consists of three main chapters including Chapter 2) Exploring the microbial diversity in biological soil crusts at Joshua Tree National Park (JTNP),

Chapter 3) Insights into dryland biocrust microbiome: geography, soil depth, and crust type affect biocrust microbial communities and networks in Mojave Desert, USA, and Chapter 4) Temporal weather variation influences biocrust microbial dynamic changes. The overarching goal for this dissertation is to greatly expand our fundamental knowledge about biocrust microbial diversity and identify factors that influence microbial community which would help inform future biocrust management and restoration according to microscopic changes. Therefore, specific objectives of this dissertation are: 1) to summarize and present the current fundamental knowledge on biocrust microorganismal diversity in JTNP, investigate and develop an efficient HTS protocol for biocrust microbiome research, and provide the future direction for biocrust microbial studies, 2) to expand our understanding about biocrust microbial communities in the Mojave Desert using an efficient HTS protocol, identify factors that influence microbial diversity, and produce baseline knowledge about biocrust microbial profile from five common crust types including light algal/cyanobacteria, cyanolichen, green algal lichen, smooth moss, and rough moss crust, and 3) to evaluate whether temporal changes in JTNP including temperature, precipitation, humidity, atmospheric pressure, dew point, and wind speed have any effect on biocrust microbial communities throughout the year.

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Chapter 2

Exploring the microbial diversity in biological soil crusts at Joshua tree national park

2.1 Introduction

Up to 40% of the global land surface consists of desert environments (also known as dryland regions, semi-arid and arid lands) [1]. Due to the sparse water availability, deserts are typically devoid of dense vegetation and the spaces between plants may appear barren at first glance. These plant interspaces, however, are often occupied by a microscopic world forming soil surface structures recognized as "biological soil crusts (or biocrusts)" [2]. These biocrusts form at the uppermost millimeters to centimeters of soil and are an aggregation of minerals and microorganisms. In dryland regions, biocrusts can cover up to 70% of the land

surface area [1], including the desert floor of Joshua Tree National Park (JTNP). Within biocrusts, a huge variety of microorganisms can coexist and cooperate as a community. For example, biocrusts can be made up of bryophytes, lichens, eukaryotic algae, cyanobacteria, bacteria, and fungi, which all interact cooperatively to create a protective and productive community on the soil surface (Figure 2.1) [1, 2]. Forming a hot zone of biodiversity at the soil surface, biocrusts can be thought of as a "living skin" on top of the soil, where each microbial member is an essential contributor to the ecology of desert environments [1].

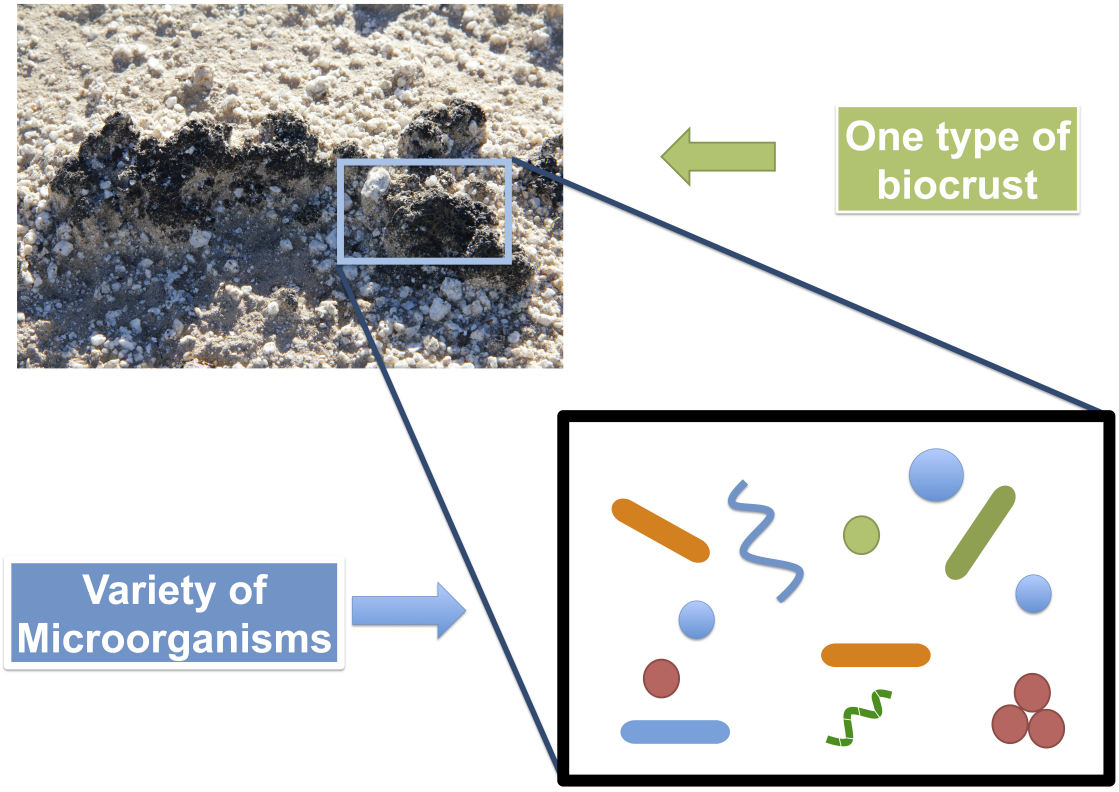

Figure 2.1: Biocrusts are like a "living skin" on top of arid lands soil. They consist of a living community of microorganisms such as bryophytes, lichens, eukaryotic algae, cyanobacteria, bacteria, and fungi. These complex communities contribute to healthy desert ecosystems by promoting nutrient cycling and reducing soil erosion.

Biocrusts play important roles in desert ecosystems. For instance, they facilitate carbon and nitrogen cycling through biochemical processing performed by the microorganisms that inhabit the crusts. They contribute to effective soil nutrient cycles, prevent soil erosion, improve regeneration, and stabilization of vegetation, therefore creating a healthy and stable ecosystem [1–4]. To better understand these complex microbial communities, studies of microorganisms that inhabit and enable the functions of biocrusts are needed.

The goals of this article are threefold: 1) to provide a basic understanding of biocrusts and the microbial diversity found within them; 2) to summarize the findings from previous research conducted in JTNP by a team of scientists over the last two decades and 3) to build upon that knowledge base by presenting additional data collected by the authors of this paper. More specifically, we developed protocols that further investigate the microbial components found in the biocrusts at JTNP such as algae, bacteria, and especially fungi, as this was one of the major knowledge gaps regarding microbial diversity in the park.

2.2 Biocrusts at JTNP

Within the hot Mojave and Sonoran desert biomes, including the area of JTNP, biocrusts can be hard for the untrained eye to notice. These cryptic communities may appear to be bare unconsolidated soil, as they are generally difficult to identify from a distance. However, if we take a minute and look closer at the ground, biocrusts are frequently encountered, especially in the park. A hand lens or magnifying glass can reveal the telltale microscopic structures that are distinctive for many types of biocrusts (Figure 2.2). First, one will notice the consolidated nature of the crust aggregate that will hold the soil

together cohesively as shown in Figure 2.2A. Biocrusts are also characterized by dangling filamentous components that can be seen among the subsurface of biocrust (Figure 2.2A). Those "danglies" represent biological filaments of cyanobacteria, or fungi that can appear like micro-roots; they are often seen with a few soil particles adhering to them.

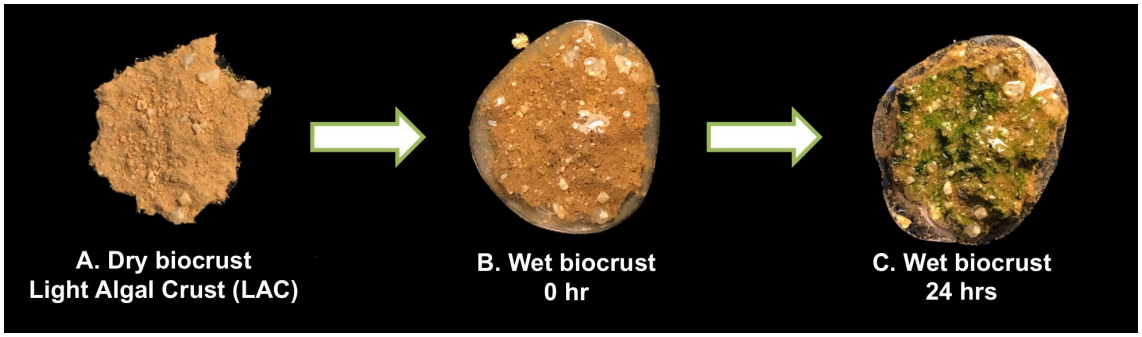

Figure 2.2: Light algal crusts (LAC) are the most common biocrust types in JTNP. They are dominated by cyanobacteria and other algae that quickly turn green once water is added; within 24 hours the algae and cyanobacteria become active (far right), but without the water are invisible to the naked eye (far left).

Biocrusts can be classified based on the dominant photosynthetic microorganisms present in the crust, which are the components that can perform photosynthesis just like plants fixing carbon dioxide from the atmosphere and producing oxygen. Photosynthetic microorganisms include cyanobacteria, other algae, lichens, liverworts or mosses [5]. Light algal crusts (LAC) are the most common biocrust types in hot desert ecosystems, including the Mojave and Colorado Deserts at JTNP, and are dominated by cyanobacteria and other algae that quickly turn green in the presence of water (Figure 2.3) [6, 7]. Another type of biocrust is referred to as lichen crusts (Figure 2.2B), these consist of fungal and algal/cyanobacterial components. A lichen crust may have multiple species of lichen-forming or lichen associated fungi co-occurring within one crust structure (Figure 2.2B and 2.2C).

Lichen crusts can also be found throughout the desert Southwest, but have a more patchy distribution, particularly in JTNP [5]. Two primary types of lichen biocrusts are found in JTNP: 1) a fungus with a cyanobacterial partner (Cyano-Lichen Crusts; CLC) or 2) a fungus with a green algal partner (Green Algal Lichen Crusts; GLC). Finally, moss dominated crusts are much more limited on the landscape, especially in JTNP. They occur preferentially in moist microhabitats, such as under the protective rock ledge of a boulder or along the shady banks of a riparian corridor; anywhere that moisture drips or runs off hard surfaces and can be trapped for longer periods [6, 7].

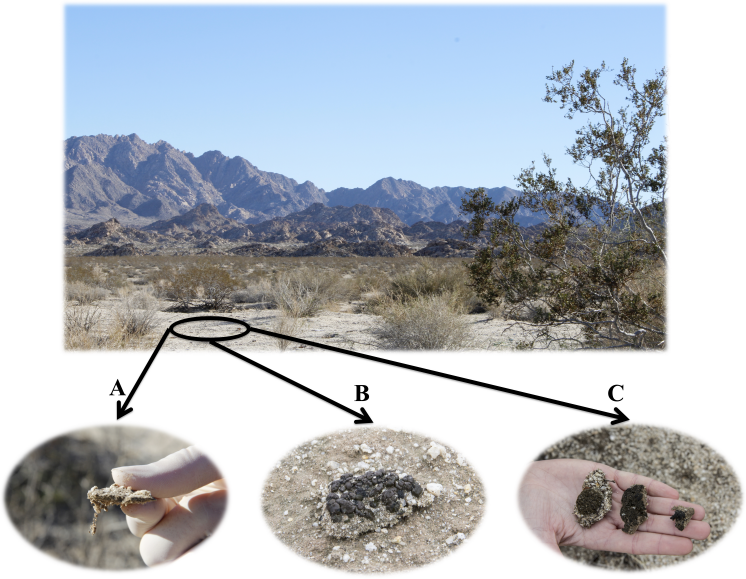

Figure 2.3: Plant interspaces in JTNP contain a variety of biocrusts but are dominated by light algal crusts and lichen crusts, both of which will have characteristic filaments in the subsurface. The filaments look like tiny roots dangling from under the crust (A). Light algal crusts (A) and lichen crusts (B, C) are commonly found in JTNP. The majority of lichen crusts in JTNP are composed of two different fungal species: *Collema coccophorum* (B), which is a cyano-lichen crust and *Clavascidium lacinulatum* (C), which is a green algal lichen crust.

2.3 Algal and cyanobacterial diversity in biocrusts

Most biocrust communities have a basic architecture that includes two main structural components: 1) primary producers that perform photosynthesis, and therefore produce carbohydrates and oxygen; and 2) associated heterotrophic consumers such as fungi and microscopic animals, which then live off the carbon-rich products that the primary producers generate. In JTNP, the most common primary producers in biocrust are cyanobacteria and other algae (Figure 2.3). These algae are making up the majority of the photosynthetic component in the LAC found throughout the park [5–8]. Under certain conditions microalgae may partner with fungi or mosses to create more complex biocrust communities such as lichen or moss crusts.

Algae are very interesting organisms. The term "algae" refers to a very diverse group of organisms spanning across the entire tree of life, including members from the Prokaryotes (Monera) and Eukaryotes (Protista). While "algae" does not represent a technical taxonomic term and there is no agreed upon definition of what most people think of as "algae," it generally refers to organisms that can photosynthesize, but aren't plants. Examples of "algae" include groups of organisms like diatoms, green algae, yellow algae, and blue green algae also known as cyanobacteria (Figure 2.4). The latter are the only Prokaryotes that photosynthesize. Each of these groups of algae represents their own lineage in the tree of life and differ in cell organization and structure, pigmentation, and ecosystem roles. What unites the groups is the presence of the green pigment chlorophyll in their cells which enables the ability to perform photosynthesis while producing oxygen, and the lack of the reproductive organs and vegetative tissues found in multicellular plants [9].

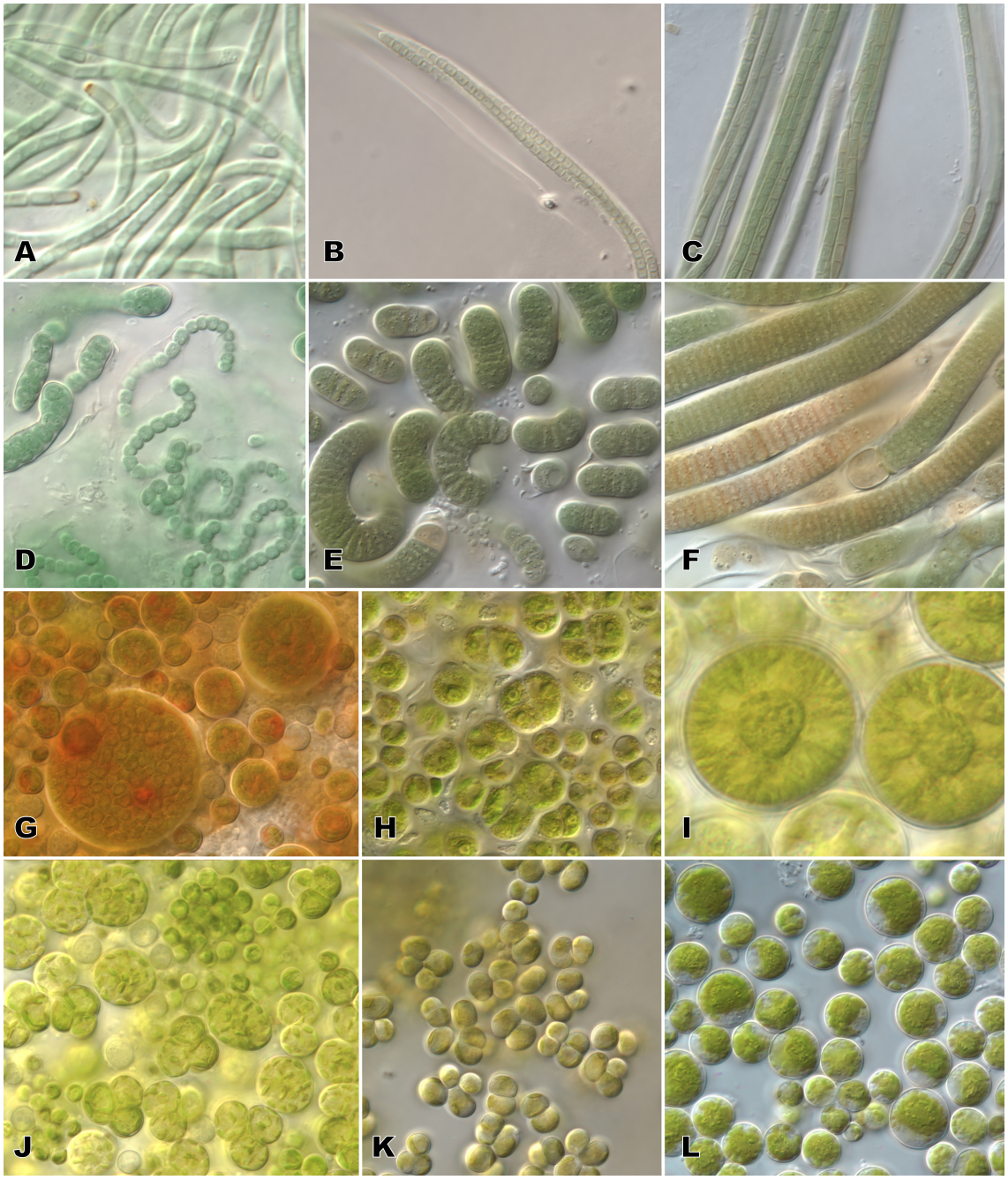

Figure 2.4: Cyanobacterial (A-F) and eukaryotic algal (G-L) diversity of cultures isolated from JTNP biocrusts. Photographs show: A) *Oculatella coburnii* (Synechococcales clade), B) *Trichocoleus desertorum* (Synechococcales clade), C) *Symplocastrum flechtnerae* (Oscillatoriales clade), D) *Nostoc* sp. (Nostocales clade), E) *Spirirestis rafaelensis* (Nostocales clade), F) *Hassallia* sp. (Nostocales clade), G) *Bracteacoccus* sp., H) *Chlorosarcinopsis* sp., I) *Actinochloris* sp., J) *Myrmecia* sp., K) *Stichococcus* sp., and L) *Parietochloris* sp.

Cyanobacteria are one group of algae that are extremely abundant in desert soils. They represent the majority of photosynthetically active biomass in biocrusts. These bacteria play essential roles in the desert environment. For example, cyanobacteria are adept at excreting sugary polymeric compounds around their cells that are very sticky and function as fibrous glue holding soil grains in place [3]. This sticky glue is very important in desert interspaces, where plants are lacking and only a few roots are available to stabilize the seemingly bare soil. The aggregation of soil enabled by the cyanobacteria helps reduce erosion. The stickiness of these cyanobacteria also traps dust, which often contains essential micronutrients that can be incorporated into to the soil [3, 10]. Another important function performed by cyanobacteria is called nitrogen fixation, which brings substantial amounts of nitrogen into the nutrient poor desert soil [5, 11]. Through biochemical reactions performed by cyanobacteria, inert atmospheric nitrogen is incorporated into specialized cell compartments or cell types, where it is assimilated into organic molecules through a process called biological nitrogen fixation. Through these actions cyanobacteria enrich the desert topsoil with essential nutrients and help create a thin, fertile, and biologically active skin of the desert that we call biocrust.

Previous research has laid the foundation for the importance of cyanobacteria in desert soils, but we only have scratched the surface in understanding the breadth of species diversity. Prior studies on the diversity of cyanobacteria in JTNP have discovered many new species [12–16]. Within the last 11 years a team of phycologists under the supervision of Jeffrey Johansen (John Carroll University, OH) and Nicole Pietrasiak (New Mexico State University, NM) described and published two new cyanobacterial genera (Mojavia, Ro-

holtiella) and 5 new species (*Mojavia pulchra*, *Roholtiella mohaviensis*, *Oculatella coburnii*, *Trichocoleus desertorum*, *Symplocastrum flechtnerae*) from JTNP soils (Figure 2.4) using culture dependent methods. Additional assessment of the biocrusts of JTNP, among other desert systems, will certainly be needed to describe all the cyanobacterial members present in desert systems. The application of culture independent assessment using DNA-based description of biodiversity is likely to uncover additional genera and certainly new species of cyanobacteria in JTNP biocrusts. This discovery of taxa then opens the door to unraveling the various functions contributed to the ecosystem by these microorganisms.

Very little is known about the biodiversity of eukaryotic algae, including diatoms, green algae, and yellow algae in biocrusts, not to mention the ecosystem roles of these organisms. The few studies that have been done on eukaryotic algae in biocrust, indicate that the contribution of these groups of algae to biomass is rather low but their diversity is much greater than in cyanobacteria [17]. To date, the only published study investigating the eukaryotic algal diversity of JTNP used a culture dependent approach [8]. In this study, the authors isolated living algal strains by placing soil on culture medium and observing and isolating the organisms which grew. The authors intensively studied 95 algal isolates from 18 locations within JTNP using morphological observations and DNA sequence information from these isolates. This study revealed 28 unique lineages in the families of Chlorophyceae (17), Trebouxiophyceae (7), Xanthophyceae (3) and Eustigmatophyceae (1). Most did not match any described algal species in published taxonomic keys, highlighting the poor knowledge we currently have about these organisms, but more importantly, and their great potential for discovery of new species (unknown to science). In the same year, Fucikova

et al. (2013) [18] included selected JTNP algal isolates in an extensive revision of the green algal genus *Bracteacoccus* and newly described 5 *Bracteacoccus* species from dryland environments. This monographs reports several records of *Bracteacoccus* species from JTNP including: 4 records of the established *Bracteacoccus pseudominor* found at 3 locations within the Colorado desert portion of the park, 1 record of the newly described *Bracteacoccus deserticola* from the Pinto Basin, 1 record of the newly described *Bracteacoccus glacialis* found in the Wonderland of Rocks, and 9 records of the newly described *Bracteacoccus occidentalis* from 5 locations within JTNP. In 2014, a new genus and species of green algae, *Rotundella rotunda*, was discovered on the alluvial fans near Eagle Mountain and named by Fucikova et al. (2014) [19]. Many more discoveries of enigmatic algae from biocrusts can be anticipated in the future.

2.4 Fungi diversity in biocrusts

Studies of biocrusts have primarily focused on algal and bacterial communities as part of understanding the microbiological composition of crusts classified by morphology. Exploration of the fungi in the biocrust systems is still an emerging research area and is needed to better understand the functional roles they play in the morphological properties and nutrient cycling activities of soil surfaces [20, 21]. Currently there are limited published reports on fungal diversity in biocrusts and no previous fungal study in biocrust has been done at JTNP. Most studies were conducted with culture dependent approaches, which underestimate microbial diversity. One study examined fungi in crusts using denaturing gradient gel electrophoresis (DGGE) [22], which is used as a fingerprinting method to esti-

mate environmental microbial diversity. DGGE has only limited utility as it does not allow direct identification of species, only a pattern of sizes of DNA bands run on a gel that can be matched between sample sites to look for similarities. To overcome this, DNA sequence based approaches are applied to estimate diversity and identify fungal species directly from environmental samples of biocrusts [21, 23, 24].

2.5 Mechanisms for discovering microorganisms

Technology advances have enabled and simplified sampling methods for DNA sequencing that allow us to explore microbial diversity found in nature, very little research has focused on conducting inventory type studies of biocrust. There are two ways to assess the biological diversity found within a biocrust. The first approach, called "culture dependent," results from using cultured strains of organisms that were present in the soil sample. This is achieved by plating biocrust soils onto semi-solid microbiological media in order to further isolate life strains and eventually obtain individual isolates of each taxonomic entity. The media consists of nutrients for the organisms to grow and agar to provide a surface to visualize and observe morphologies of the isolates. The nutrient content of the media can be adjusted to favor the growth of one microorganism over another. Specifically, a serial dilution is used to isolate the microbes from the soil (Figure 2.5). This method involves making a soil slurry by suspending the soil sample with increasingly higher proportions of water or media, in order to get a low starting concentration of spores and reduce to manageable counts the number of species growing on the Petri dishes. In order to isolate single organisms, 100 uL of biocrust-water suspensions are spread on a variety of media and the growth

of microbial colonies is scored over the course of a few days. An alternative method used with the serial dilutions is called pour plating, where the crust-water solution can be poured to differentiate among microorganisms based on their oxygen dependence. The top layer obviously favors oxygen-loving microbes, whereas the bottom layer, which is submerged in water, selects for oxygen sensitive strains [25]. Once axenic cultures have been obtained, DNA sequence information can be generated directly from isolated microorganisms from this culture dependent method.

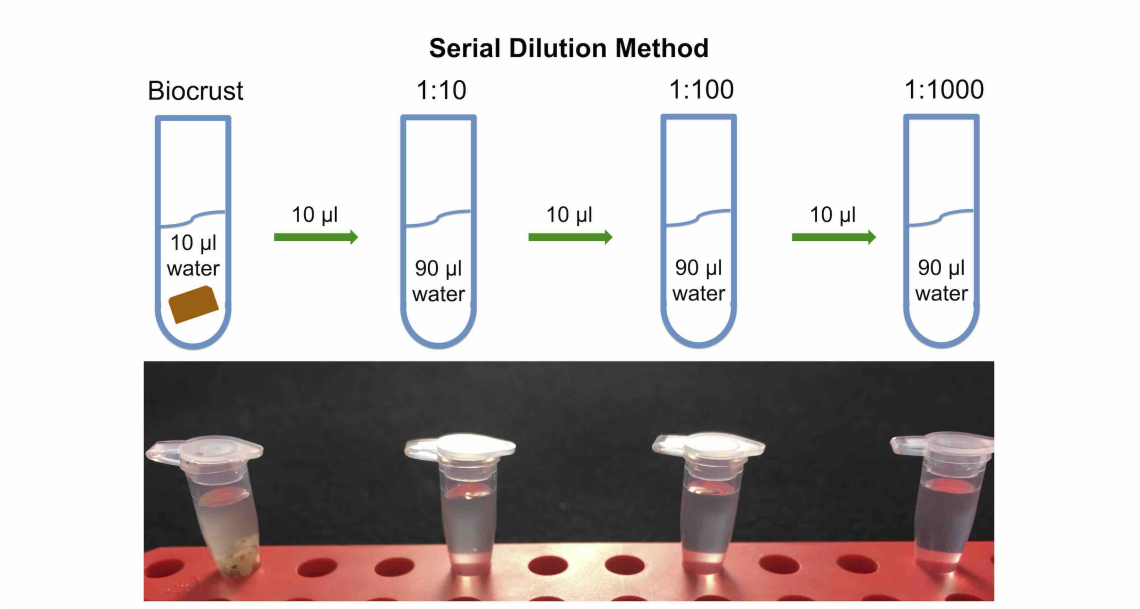

Figure 2.5: Serial dilution method involves a dilution series of biocrust water suspensions are prepared by adding biocrust to 100 ul of sterile water. To dilute the biocrust water suspension, 10 ul of original solution is transferred and combined with 90 ul of sterile water (1:10 dilution). Additional dilutions are created by repeating this step: 1:100 and 1:1000, accordingly.

Although microbial diversity in biocrust can be explored to some extent using a culture dependent method, basic growth media may not provide suitable conditions for the growth of the majority of microorganisms. Direct DNA sequencing from soil samples can be used to capture greater microbial diversity and represent a "culture independent" method for assessing biocrust microbial communities. These molecular and genomic technologies are contributing tremendously to achieve a better understanding of the microbial diversity and composition of many environments from the human body to the open ocean. PCR amplification and Next Generation Sequencing (NGS), are used to assay regions of the genome which are found in all organisms. One of these, the 16S ribosomal RNA (rRNA) gene is used to survey Bacteria and Archaea while the Internal Transcribed Spacer (ITS) regions can be used to identify fungi present in a sample. These molecular markers have proven successful in recovering a broad range of bacterial and fungal diversity in soil microorganisms [26, 27]. Broad sampling and fine scale analysis with NGS can be used to effectively compare biodiversity among biocrusts from different locations or classified as different morphological types. These studies can help identify the core taxonomic composition of biocrusts and indicate key organisms that may play important roles in the formation and ecological functions of biocrust.

2.6 Current research results

We have used NGS sequencing (amplicon sequencing) of the 16S gene on collections we made of LAC and CLC biocrusts in Joshua Tree National Park to test whether there are differences in species that comprise these crust types. DNA samples from both

crust samples were extracted, amplified with Polymerase Chain Reaction (PCR) targeting the 16S gene marker and sequenced using Illumina MiSeq. This sequencing captured a broad range of bacterial diversity and using bioinformatics analyses we focused first on the observed diversity of Cyanobacteria. Comparing these sequences with a collection of previously generated sequences from Cyanobacteria cultures compiled by Drs. Johansen and Pietrasiak, we constructed a phylogenetic tree representing the diversity of the JTNP cyanobacteria (Figure 2.6). Our analysis found that Cyanobacteria from nearly all known described groups (major clades) are present in the biocrust samples. Our work also demonstrates that both culture dependent and culture independent methods can equally recover the broad phylogenetic diversity found in biocrusts for Cyanobacteria (Figure 2.6). Further work on additional groups of Bacteria will help determine the extent of novel taxa which can be observed from the culture independent methods.

We have also assessed the composition of fungal communities using amplicon sequencing of biocrust DNA. The Internal Transcribed Spacer (ITS) 1 ribosomal gene region was amplified with PCR to inventory the fungi present in the biocrust samples using primers [27] that are also being used to identify fungi in the Earth Microbiome Project [28]. The PCR products are sequenced on an Illumina MiSeq to produce sequence fragments 300 bp long. The DNA sequence fragments are compared to each other to collect them into groups which all represent sequences that are mutually similar based on the percentage of DNA bases that match. These clusters of sequences represent a guess of a fungal species or strain that is present in the biocrust sample. To determine what might be the name of this fungus, the sequence is matched against a database of known sequences. A curated

database called UNITE is one of the best references for fungi and contains an enormous library of fungal ITS sequences and corresponding species name [29].

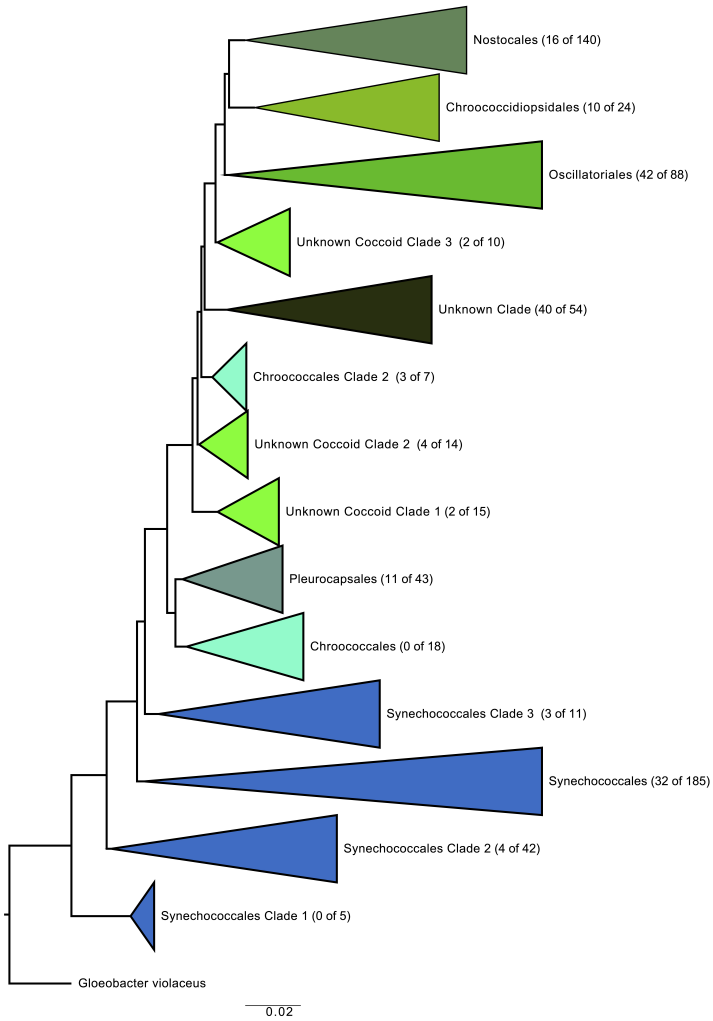

Figure 2.6: Phylogenetic tree of Cyanobacteria with all major clades shown. The number of lineages found in JTNP biocrusts out of the total number of known lineages is shown in parentheses to the right of the clade. These data are based on amplicon sequences and they illustrate that members of nearly all (12 of 14) of the major terrestrial cyanobacteria clades have been recovered from biocrusts in JTNP.

The ITS rDNA marker is sometimes referred to as a "barcode" as the sequence is often different between closely related species so that each species can have its own nearly

unique signature. However, there can still be challenges with the marker as it still may be invariant among some groups of species. It is also difficult to use ITS sequences when they do not match any known Fungi, as it can be difficult to guess if it is a new species or species group not previously seen before. As we have seen in this and many other studies of fungi from the environment, there is vast, unsampled biodiversity that is only now being revealed through amplicon sequencing which leads to many sequenced ITS sequences assigned as an Unknown Fungus.

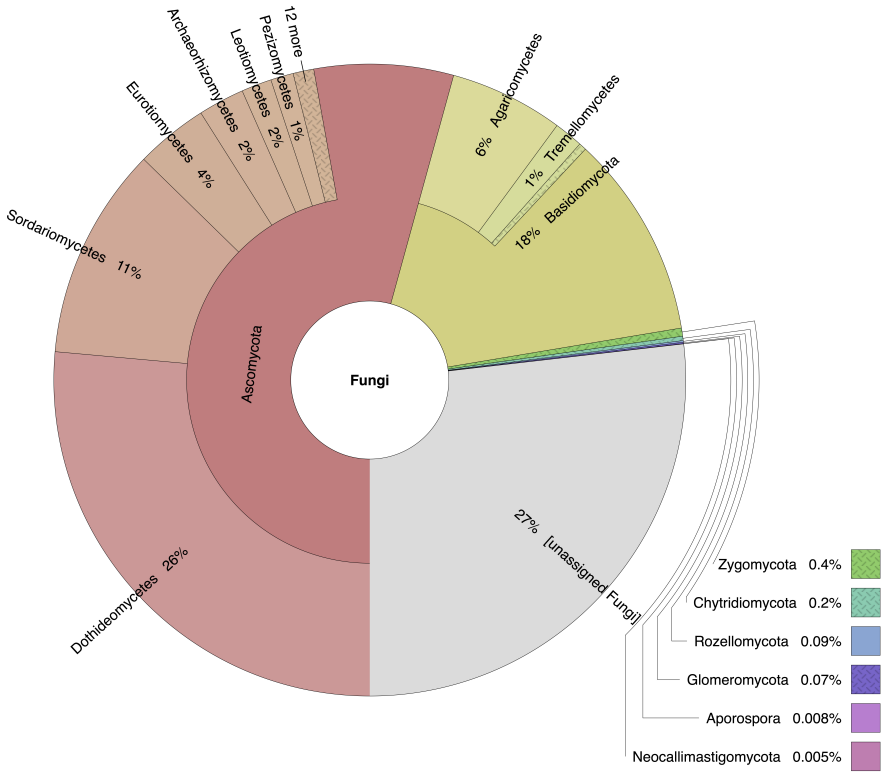

Figure 2.7: Fungal diversity in light algal crust from Joshua Tree National Park. Using Krona for visualization [30], main fungal phyla are Ascomycota and Basidiomycota.

Analysis of our samples identified that biocrusts of different morphological classifications (e.g. LAC and CLC) are comprised of varied fungal taxa that differ at the genera

and family level but are fairly consistent when comparing the presence of major phyla. The observed groups that dominate the crusts include Dothideomycetes, Eurotiomycetes, and Sordariomycetes within the Ascomycota and Agaricomycetes and Tremellomycetes from the Basidiomycota (Figure 2.7). These results are similar to previously reported types of fungi found in biocrusts using other methods [22, 24]. Within the light algal crust (LAC), the three most abundant Ascomycota genera were *Alternaria*, *Phoma*, and *Elasticomyces*; whereas, the top three fungal genera from Basidiomycota were *Coprinellus*, *Cryptococcus*, and *Clitopilus*. While mushrooms do inhabit some arid regions, we did not observe any fruiting in the regions where we sampled and were surprised to see the high abundance of some of these basidiomycetes. It may be that taxa are from spores that have blown in and are dormant awaiting a rain event. Our efforts have provided a high-resolution look at the fungal taxa that can be present in biocrusts and one arid region. The species reported from our study are only examples of some of the most abundant types of fungi living in biocrusts. We expect that many more fungi contribute to the biocrust community (Figure 2.8), but to identify the less abundant species will be undertaken with additional sequencing and robust analyses to confirm the presence of these organisms and compare their abundances across biocrust environments.

Traditional culture dependent methods to isolate fungi generally use a nutrient rich media and are kept at room temperature. Because JTNP represents an extremely dry and cyclically hot environment, we experimented to find optimal growth conditions that might favor the more extremophilic species. We attempted growing the fungi in a range of temperatures, salt concentrations, and pH; we were successful in isolating fungi in the

phyla Ascomycota and Basidiomycota with a range of growth rates (Figure 2.9). Examples of the fungal species we have brought into culture from biocrusts are *Phoma* sp., *Didymella* sp., *Ustilaginales* sp., *Didymella* sp., *Aspergillus* sp., *Alternaria* sp., and *Knufia* sp. Several of these fungal species match high abundance fungal gene markers that we identified from our culture independent amplicon sequencing of DNA from biocrust collected in the field. Using these starting cultures, we can test physiology, enzyme and biochemical properties, and interactions with algae and bacteria to better understand the roles these fungi play in the ecosystems.

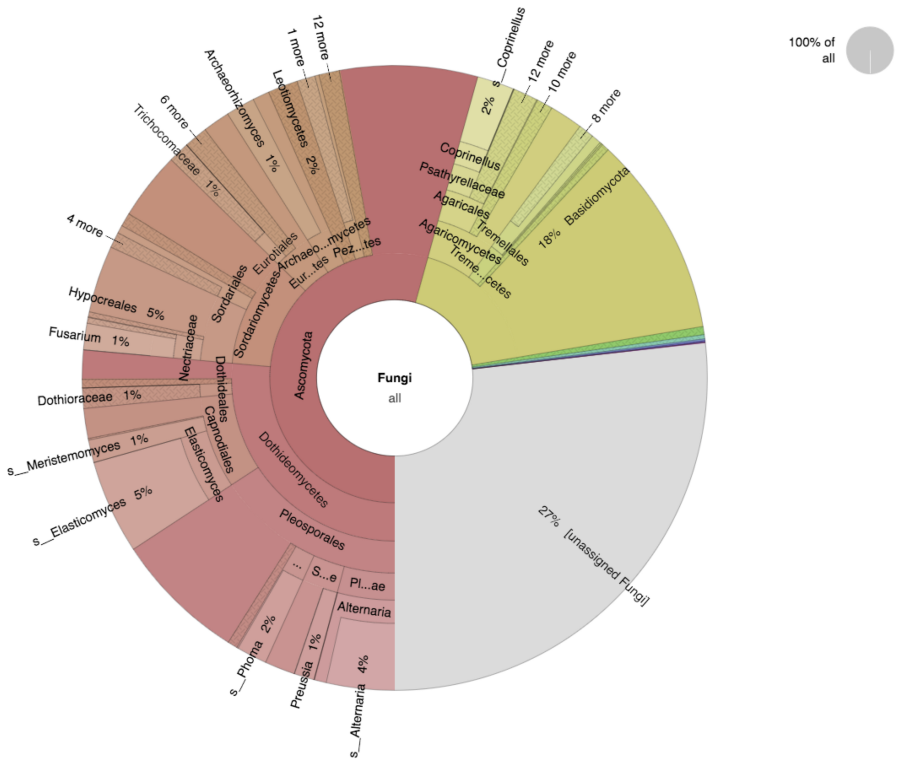

Figure 2.8: Light algal crust (LAC) from Joshua Tree National Park contains a complex fungal composition. A variety of fungal species are represented with several dominant species from Basidiomycota (yellow) and Ascomycota (red).

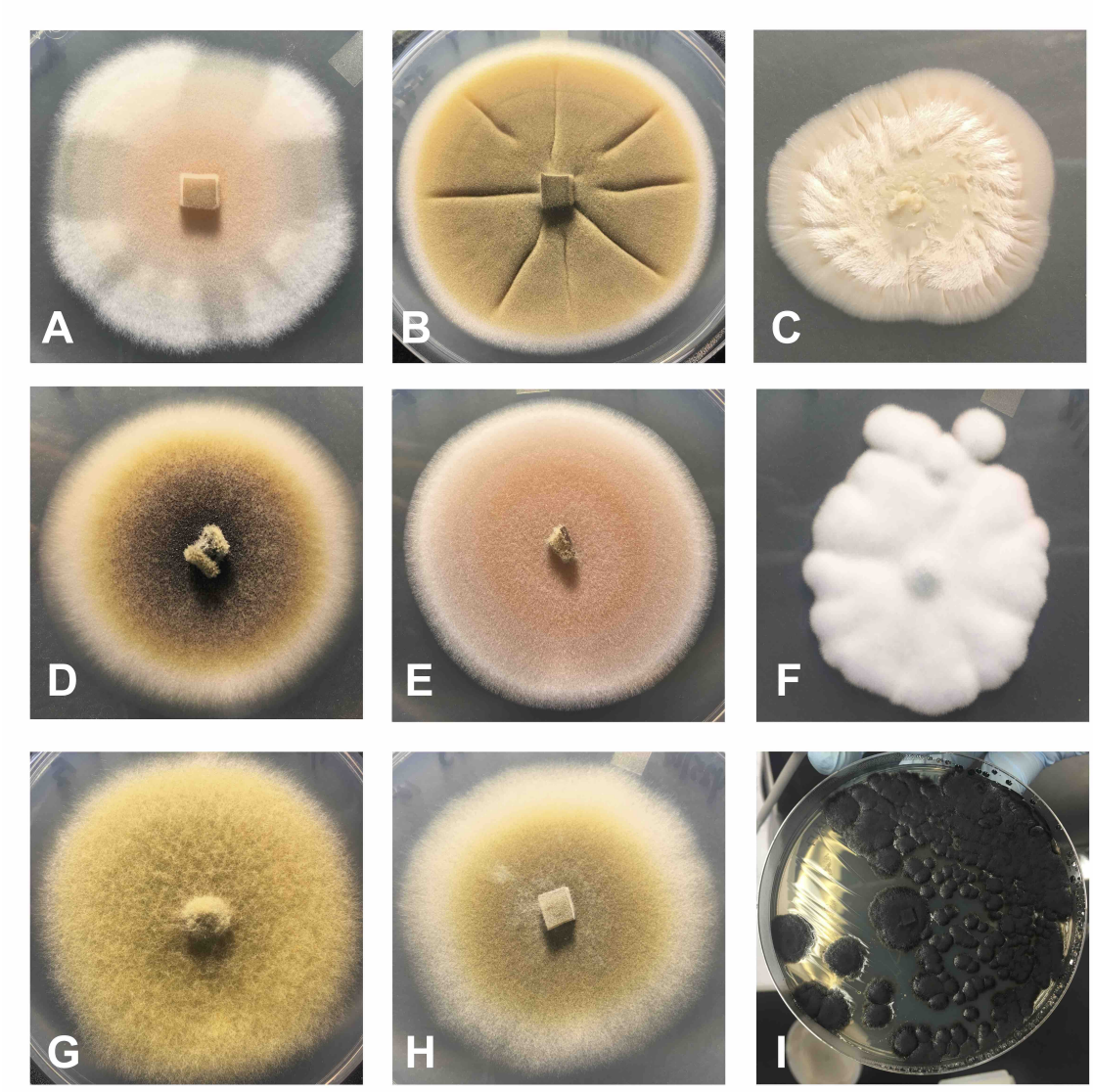

Figure 2.9: Depiction of a small sample of the variety of fungi isolated from the JTNP biocrusts by using a culture dependent method. The genera depicted include: A) *Phoma* sp., B) *Didymella* sp., C) *Acremonium* sp., D) *Didymella* sp., E) *Aspergillus* sp., F) *Aspergillus* sp., G) *Alternaria* sp., H) *Phoma* sp., I) *Knufia* sp.

2.7 Conclusion

We are only in the beginning stages of exploring the microbial diversity of biocrusts at JTNP. Based on the limited research that has been done in the park to date, we know that these microbial communities are very diverse and likely harbor many new species to science across all microbial lineages. In addition to knowing very little about the biological diversity found in these communities, we know even less about the specific adaptations of these microbes or the synergistic roles they play to contribute to ecosystem functions. Culture dependent and independent methods can both yield valuable information in the quest for more information regarding microbial diversity and the basic biology/ecology of these microorganisms. Culture dependent methods enable detailed studies of the biology of cyanobacteria, eukaryotic algae, and fungi found in these arid lands. However, culture dependent approaches are limited to the subset of organisms that can grow on culture media, therefore research that only focuses on these may miss a sizeable fraction of the actual microorganisms living in the soil. New sequencing technologies allow culture independent evaluation of microbial community diversity. Using NGS, we can produce a broader sampling of the microbial community diversity than is possible in the culture dependent approach. For the first time, additional abundant, but unculturable species, can be documented as part of the biocrust microbial community. Before we can hope to understand the function and processes that dominate these biocrusts, we must first document and identify the diversity present. Only then we can begin to quantify or explore how these microorganisms might contribute to ecosystem functions and/or how they respond to different environmental conditions. Finally, a better understanding of the microbial community can

be used to develop management and monitoring strategies to assess the health of desert ecosystems and therefore to develop bioremediation strategies, such as the addition of missing community members. Overall, understanding the interactions and diversity of microbes that support biocrust formation and persistence are key aspects of desert land conservation.

2.8 Future research directions

Our research shows that biocrusts are made up of very complex and unique microbial communities and we have just started to reveal the secret of these communities. Although eukaryotic algae, cyanobacteria, bacteria, and fungi have been found inhabiting biocrusts, their interactions and ecosystem functions are still under investigations. As we learn more about these microorganisms, important species will be revealed along with their functions. However, matching their cooperating microbes and relationships are very challenging at this early stage, since a fraction of microorganisms cannot be isolated using current culture dependent method. By exploring microbial diversity while developing both culture dependent and independent method, microbial interactions experiment can be conducted in the near future and will show how these microorganisms help each other to build biocrust community. Lastly, focusing more on filamentous and mycorrhizal fungi may help us evaluate the connections between biocrust microbial communities and vascular plants in drylands which has already been recognized as the fungal loop hypothesis [31].

2.9 Acknowledgement

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Chapter 3

Insights into dryland biocrust microbiome: geography, soil depth and crust type affect biocrust microbial communities and networks in Mojave Desert, USA

3.1 Abstract

Biocrusts are the living skin of drylands, comprising diverse microbial communities that are essential to desert ecosystems. Despite there being extensive knowledge on biocrust ecosystem functions and lichen and moss biodiversity, little is known about factors struc-

turing diversity among their microbial communities. We used amplicon-based metabarcode sequencing to survey microbial communities from biocrust surface and subsurface soils at four sites located within the Mojave Desert. Five biocrust types were examined: Light-algal/Cyanobacteria, Cyanolichen, Green-algal lichen, Smooth-moss and Rough-moss crust types. Microbial diversity in biocrusts was structured by several characteristics: (i) central versus southern Mojave sites displayed different community signatures, (ii) indicator taxa of plant-associated fungi (plant pathogens and wood saprotrophs) were identified at each site, (iii) surface and subsurface microbial communities were distinct and (iv) crust types had distinct indicator taxa. Network analysis ranked bacteria–bacteria interactions as the most connected of all within-domain and cross-domain interaction networks in biocrust surface samples. Actinobacteria, Proteobacteria, Cyanobacteria and Ascomycota functioned as hubs among all phyla. The bacteria *Pseudonocardia* sp. (Pseudonocardiales, Actinobacteria) and fungus *Alternaria* sp. (Pleosporales, Ascomycota) were the most connected and had the highest node degree. Our findings provide crucial insights for dryland microbial community ecology, conservation and sustainable management.

3.2 Introduction

In vegetation-sparse drylands, plant interspaces are often covered by biological soil crusts (hereafter biocrusts) [1]. Microbial communities form biocrusts by interweaving soil particles as sticky biofilms and biofilaments establishing a living soil aggregate at the soil surface. Evolutionarily diverse organisms such as bryophytes, lichens, eukaryotic algae, cyanobacteria, bacteria and fungi combine to form different types of biocrust distinguished

by their dominant photoautotrophic community member as light (cyanobacterial/algal), dark (cyanobacterial/algal), lichen and bryophyte crusts [1–6]. The complex combinations of microorganisms in biocrusts affect a range of ecosystem functions, such as: mediating soil nutrient cycles, preventing soil erosion and improving soil stabilization, assisting with regeneration of vegetation, as well as fertilizing and transforming subsurface soils [1, 5–8].

While there is an extensive body of literature on biocrust lichen and bryophyte diversity, as well as their roles in dryland ecosystems, studies have only recently begun exploring their less conspicuous community members. The earliest published work employed culture-dependent approaches to survey biocrusts' microbial composition, which likely underestimated microbial diversity as was demonstrated by Amann, Ludwig and Schleifer (1995) [9] and Viaud, Pasquier and Brygoo (2000) [10]. Modern DNA-based procedures greatly improved biodiversity assessment of microbial communities and environmental DNA sequencing approaches were recently introduced to biocrust diversity analysis [6, 11–18]. These amplicon sequencing studies allow general profiles of biocrust bacterial communities to be drawn up. Abundant bacterial phyla in most biocrust systems included Acidobacteria, Actinobacteria, Proteobacteria and Cyanobacteria. In particular, Cyanobacteria are fundamental for biocrust formation. For example, *Microcoleus* is one of the most well-studied cyanobacterial genera in biocrust, functioning both as primary producer in the microbial community and as filament builder essential to the biocrust physical structure [7, 17, 19]. In contrast, our knowledge of biocrust fungal diversity and function is extremely poor. Modern tools such as microbial community network analysis will allow us to investigate how pho-

toautotrophic diversity in biocrusts is associated with nonphototrophic archaea, bacteria and fungi.

Cross-domain microbial network analysis such as SPIECEASI (Sparse InversE Covariance estimation of Ecological Association and Statistical Inference) can provide essential insights into relationships among microbial populations [20, 21], yielding a better understanding of connections between microorganisms as hubs and key connectors. A microbial hub is hereby defined as a microbe that has a high degree of connections to other microorganisms in a community, while a key connector microbe is a bottleneck that serves as essential connection in microbial networks [21]. Cross-domain microbial network analysis has not yet been applied in biocrust systems, but could be crucial to discover biocrust microbial community linkages. Although network analysis presents correlation and not actual causation/interaction, joint surveying of fungal and bacterial/archaeal communities with amplicon sequencing data allows us to investigate the deeper complexity of biocrust microbiome diversity.

Equally underexplored are questions about regional patterning of biocrust diversity, or how diversity changes vertically when comparing the biocrust to the underlying soil. Denaturing gradient gel electrophoresis studies have reported similar major fungal phyla and bacterial phyla in biocrusts from different localities, albeit with different relative abundances [13, 22, 23]. However, sampling was usually conducted over a small spatial scale, and the procedures employed often focused on a single group of organisms. To date, only a handful of studies have reported on geographical patterns of biocrust microbial communities across broader scales in North America [24–27]. Moreover, only a few surveys of biocrusts

have included the underlying subsurface soil microbial community, to investigate the differences between surface and subsurface communities [12, 22, 28]. Vertical heterogeneity not only provides us with additional insights into the ecology of taxa found inside biocrusts, but may also create confounding effects when comparing alpha and beta diversity results from different surveys, especially if standard soil depths are sampled so as to combine surface biocrust material with subsurface soil.

These gaps in our understanding led us to investigate microbial community composition and structure at different levels of complexity: regionally, structurally among biocrust types, vertically by soil depth, as well as across multiple microbial phyla. We surveyed the microorganisms from three domains of life including Archaea, Bacteria and Fungi, using high-throughput amplicon sequencing targeting both the 16S rRNA and ITS1 markers.We collected biocrust samples from four different sites along a north-south axis within the Mojave and at the ecotone of the Mojave and Colorado Deserts, separately collecting surface and subsurface material from five different biocrust types at each site. We hypothesized that: (i) geographical locations do structure biocrust microbial communities: our three central Mojave sites will have similar microbial composition while the Joshua Tree National Park (JTNP) site at the ecotone of the Mojave and Colorado desert will have different microbial composition; (ii) different geographical locations will harbor indicator species that are unique to each site; (iii) soil depth affects fungal and bacterial diversity: light-dependent microbes (Cyanobacteria) have higher abundances on the surface than subsurface soil. Both alpha and beta diversity will distinguish subsurface soil microbial community composition from surface communities; and (iv) biocrust types relate to microbial diversity: more struc-

turally complex assemblages such as lichen and moss crusts will have greater alpha diversity in both fungal and bacterial composition than structurally less complex types such as algal/cyanobacteria crusts.

3.3 Materials and methods

3.3.1 Sampling sites and biocrust sampling

Biocrust samples were collected from four different sites in the Mojave Desert and at its southern edge. Our Joshua Tree National Park site (JTNP, GPS: 34.10 N, -115.45 W) was located at the ecotone of the Mojave Desert with the Colorado Desert, while sites at Granite Mountains (GMT, GPS: 34.78 N, -115.63 W), Kelso Dunes (KELSO, GPS: 34.89 N, -115.69 W) and Cima volcanic field (CIMA, GPS: 35.20 N, -115.87 W) were located further north in the central Mojave Desert (Fig. 3.1D). Using sterile sampling technique, five biocrust types were collected with a spatula. The underlying subsurface soil for each biocrust type was also collected (Fig. 3.1G) by pushing a 5-cm diameter brass core to a depth of 5 cm (or less if subsurface rock was hit at a shallower depth). Light algal/cyanobacterial crust (LAC, Fig. 3.1A), Cyanolichen crust (CLC, Collema spp., Fig. 3.1C) and Green algal lichen crust (GLC, *Clavascidium lacinulatum*, Fig. 3.1B & 3.1E) were collected at all four sites, while rough moss crust (RMC, *Syntrichia* spp., Fig. 3.1F) and smooth moss crust (SMC, *Bryum* spp., Fig. 3.1H) were collected at KELSO, GMT and CIMA (neither type was sufficiently prevalent for collection at the JTNP sampling site). For each type of biocrust, surface versus subsurface soil samples were collected into separate sampling containers. In total, there were 10 soil samples per site collected from KELSO, GMT and

CIMA (1 surface and 1 subsurface samples per crust type), while there were 18 samples from JTNP (3 replicates of 1 surface and 1 subsurface samples per crust type, no moss crusts present). Biocrust samples were stored on ice and transferred to a −80 °C freezer at University of California, Riverside.

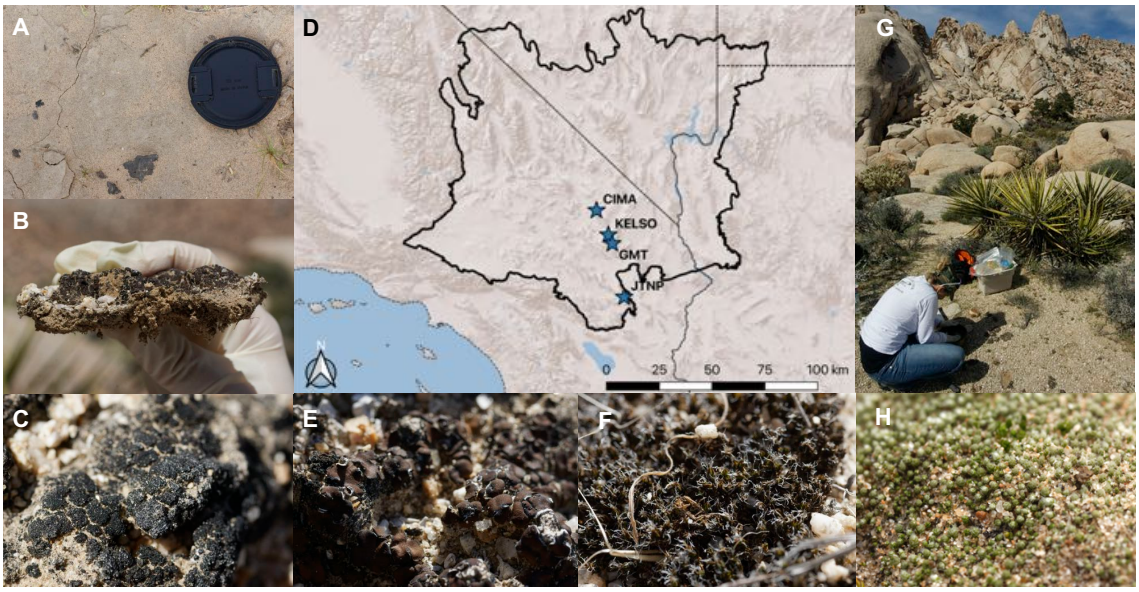

Figure 3.1: Sampling sites and biocrust types. (A) Light algal/Cyanobacterial crust (LAC), (B) dangling filaments underneath GLC, (C) Cyanobacteria lichen crust (CLC), (D) our four sampling sites including Cima Volcanic Flows (CIMA), Kelso Sand Dunes (KELSO), Granite Mountains Research Center (GMT) within the Mojave Desert (black outlined area), and Joshua Tree National Park (JTNP) at the edge of the Mojave and Colorado Deserts, (E) Green algal lichen crust (GLC), (F) Rough moss crust (RMC), (G) crust sampling in Mojave Desert and (H) Smooth moss crust (SMC).

3.3.2 Amplicon sequencing data analysis

DNA extraction was performed with 0.15 g of biocrust using the QIAGEN DNeasy PowerSoil kit (Qiagen, Germantown, MD, USA) following the manufacturer's protocol. The ITS1F and ITS2 primer pair was used to amplify the ITS1 for the fungal communities

according to Smith and Peay's Illumina MiSeq protocol [29]. The 515F and 806R primers were used to amplify the 16S rRNA V4 gene region for bacterial communities following Caporaso et al. (2011) [30]. PCR reactions were processed in 25 μl total volume in three replicates, which included 1 μl of each primer (10 μM), 1 μl of genomic DNA, 12.5 μl of Taq 2X DNA Polymerase (Thermo Fisher Scientific Inc., Waltham, MA, USA) and 9.5 μl of nuclease-free water (Sigma-Aldrich, St Louis, MO, USA). PCR conditions were: initial denaturation at 93°C for 3 min; 35 cycles of denaturation at 95°C for 45 s, annealing at 50°C for 1 min, extension at 72°C for 90 s, and a final extension at 72°C for 10 min using a C1000 thermal cycler (BioRad, Hercules, CA, USA). PCR products from three replicates were combined, purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Hoerdt, France) and pooled to produce equimolar mixture. Pooled libraries were quantified using Qubit dsDNA HS Assay (Life Technologies, Carlsbad, CA, USA) and analyzed using Agilent 2100 Bioanalyzer and Fragment Analyzer (Agilent Technologies, Santa Clara, CA, USA). Then, pooled libraries were sequenced using Illumina MiSeq (San Diego, CA) with the V3 kit to generate paired-end reads in 2×300 bp format, at the Institute for Integrative Genome Biology, Core Facilities, University of California Riverside (http://iigb.ucr.edu). A total of 8 918 345 paired-end sequence reads were produced and submitted to the Sequence Read Archive databases associated with BioProject accession number PRJNA544067.

3.3.3 Bioinformatics

The fungal ITS1 amplicon sequences were analyzed with AMPtk: the Amplicon Toolkit for NGS data (formally UFITS) (v1.2.4) [31] (https://github.com/nextgenusfs/amptk). The demultiplexed paired-end sequences were pre-processed by trimming forward

and reverse reads to a maximum of 300 bp, trimming primer sequences and discarding reads <100 bp in length. The paired-end reads were merged to produce a single long read using USEARCH (v9.1.13) [32] where they could be found to overlap. After pre-processing, a total of 3 040 944 valid paired sequence reads were produced. Sequence quality filtering was performed with the expected error parameter of 0.9 [33], which produced 2 392 561 quality filtered reads. This cleaned sequenced dataset was clustered with UPARSE using a 97% identity parameter, which generated 2 569 operational taxonomic units (OTUs) following the procedure of Palmer et al. (2018) [31]. Chimeric OTUs, sequences produced from PCR amplification of templates or parent sequences, were filtered using VSEARCH (v2.3.2) [34], which removed 65 chimeras after comparison to the database. Finally, taxonomic assignment for 2 504 OTUs was performed with the AMPtk hybrid approach using names from UNITE v8.0 with 97% similarity [35] and functional guilds were assigned using FUNGuild (v1.0) [36].

The 16S V4 amplicon sequences were analyzed using Quantitative Insights into Microbial Ecology version 2 (QIIME2 v2019.1) [37] using bacterial 16S processing workflows. Demultiplexed sequence data (5 757 892 reads) were imported to QIIME2 and the forward reads trimmed of primer sequence, followed by quality control filtering by DADA2 (q2-dada2 plugin) [38]. The sequences were truncated to 250 bp lengths based on quality scores to produce 5 042 292 trimmed reads. DADA2 was further used to produce amplicon sequence variant (ASV) tables from the filtered data. Taxonomy classification was performed using q2-feature-classifier [39] and the SILVA database version 132 with the 16S region 515–806 extracted [40] based on ASV tables and associated sequences, which were well developed

for bacterial data processing through QIIME2 following published protocols [37–40]. Mitochondria and chloroplast sequences were removed from the dataset resulting in 18 564 ASVs. Functional Annotation of Prokaryotic Taxa (FAPROTAX v1.2.2) was used to assign ecological relevant functions to bacterial species [41].

3.3.4 Data analysis

Both fungal and bacterial (including archaeal) data were rarefied to 6 842 reads per sample in fungal data and 37 435 reads per sample in bacterial data, and then analyzed using Phyloseq packages in R version 3.5.1 [42] and Rstudio version 1.1.463 [43] for taxonomic composition, alpha diversity (observed OTUs/ASVs) and beta diversity [44]. Differences in alpha diversity were evaluated for homoscedasticity using Levene's test with the 'leveneTest' function in the 'car' package [45]. Homoscedastic data (location and crust type) were compared using ANOVA with the 'Anova' function and pairwise multiple comparison (Tukey test) was performed with the 'TukeyHSD' function in R. A type = 'III' ANOVA was used to account for unbalanced design when comparing crust type. When data were heteroscedastic (soil depth), Welch correction was performed. Beta diversitywas compared using PERMANOVA with the 'adonis' function in the 'vegan' package in R (Bray–Curtis distance for fungi and Unifrac distance for bacteria, with permutation = 999) [46]. Network analysis was implemented with the SPIEC-EASI package targeting network stability threshold of 0.05 [20] and followed the pipeline procedure for cross-domain analysis using node degree to define hubs and betweenness centrality to examine connected networks [21]. Circular fungal–bacterial networks plotswere generated using the 'circlize' package in R to visualize cross-domain connections [47].We also performed indicator species analysis (func-

tion 'indval' in 'labdsv' package) [48] in R to identify the significant OTUs/ASVs at p <0.05 that are predicted to be part of the structured crust types and sites.

The R scripts used to perform analyses are available at http://github.com/stajichlab/MojaveCrusts2019analysis (DOI: 10.5281/zenodo.3931036).

3.4 Results

3.4.1 Does geographical location structure biocrust microbial communities?

No geographical differences of fungal richness of biocrust microbial communities were identified by analysis of alpha diversity of crust surface in our four sites (ANOVA, $F(3,20) = 1.64$, $p = 0.212$, Fig. 3.2A). There were 38 fungal taxonomic classes observed across all samples (Figure S1, Supporting Information). Although overall fungal alpha diversity analysis did not show significant difference among sites, fungal richness was significantly different among sites for three fungal classes: Leotiomycetes (ANOVA, $F(3,20) = 8.2575$, $p = 0.0009$), Blastocladiomycetes (ANOVA, $F(3,20) = 4.1667$, $p = 0.0191$) and Mucoromycetes (ANOVA, $F(3,20) = 3.1865$, $p = 0.046$) (Figure S1, Supporting Information). GMT had the highest richness among the sites for Leotiomycetes and JTNP had the lowest richness. The chytrid lineages of Blastocladiomycete richness were greater in CIMA and GMT (central Mojave sites) than at JTNP, while Mucoromycetes richness was greatest at CIMA.

Alpha diversity analysis showed significant geographical differences for bacterial and archaeal richness (ANOVA, $F(3,20) = 4.745$, $p = 0.0117$, Fig. 3.2B). At JTNP, bac-

terial species richness was significantly lower than at GMT and KELSO, but not significantly different from the values at CIMA (Fig. 3.2B). Bacterial species richness comparison (for each phylum) by site indicated a variable distribution of richness among 10 bacterial phyla (ANOVA, p <0.05); including Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, Acidobacteria, Planctomycetes, Patescibacteria, Armatimonadetes, Gemmatimonadetes and Verrucomicrobia (Figure S2A and Table S1, Supporting Information). Across the bacteria phyla Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, Acidobacteria and Verrucomicrobia richness was lowest at JTNP, mirroring the pattern observed in the three dominant fungal classes. The Acidobacteria, Planctomycetes, Patescibacteria and Gemmatimonadetes richness was greatest at GMT.

Beta diversity analysis of biocrust fungal communities differed significantly by site (PERMANOVA, p = 0.001, R^2 = 0.178) (Fig. 3.3A). These differences in beta diversity were visualized in principal coordinate analysis (PCoA) plots revealing a geographical pattern: JTNP biocrust fungal composition clustered separately from central Mojave fungal communities (KELSO, GMT and CIMA). Evaluation of bacterial members of the samples found that communitieswere significantly different by site (PERMANOVA, p = 0.001, R^2 = 0.129) (Fig. 3.3B). The distinct clustering of JTNP bacterial communities away from the three sites of the central Mojave resembled findings from the fungal communities.

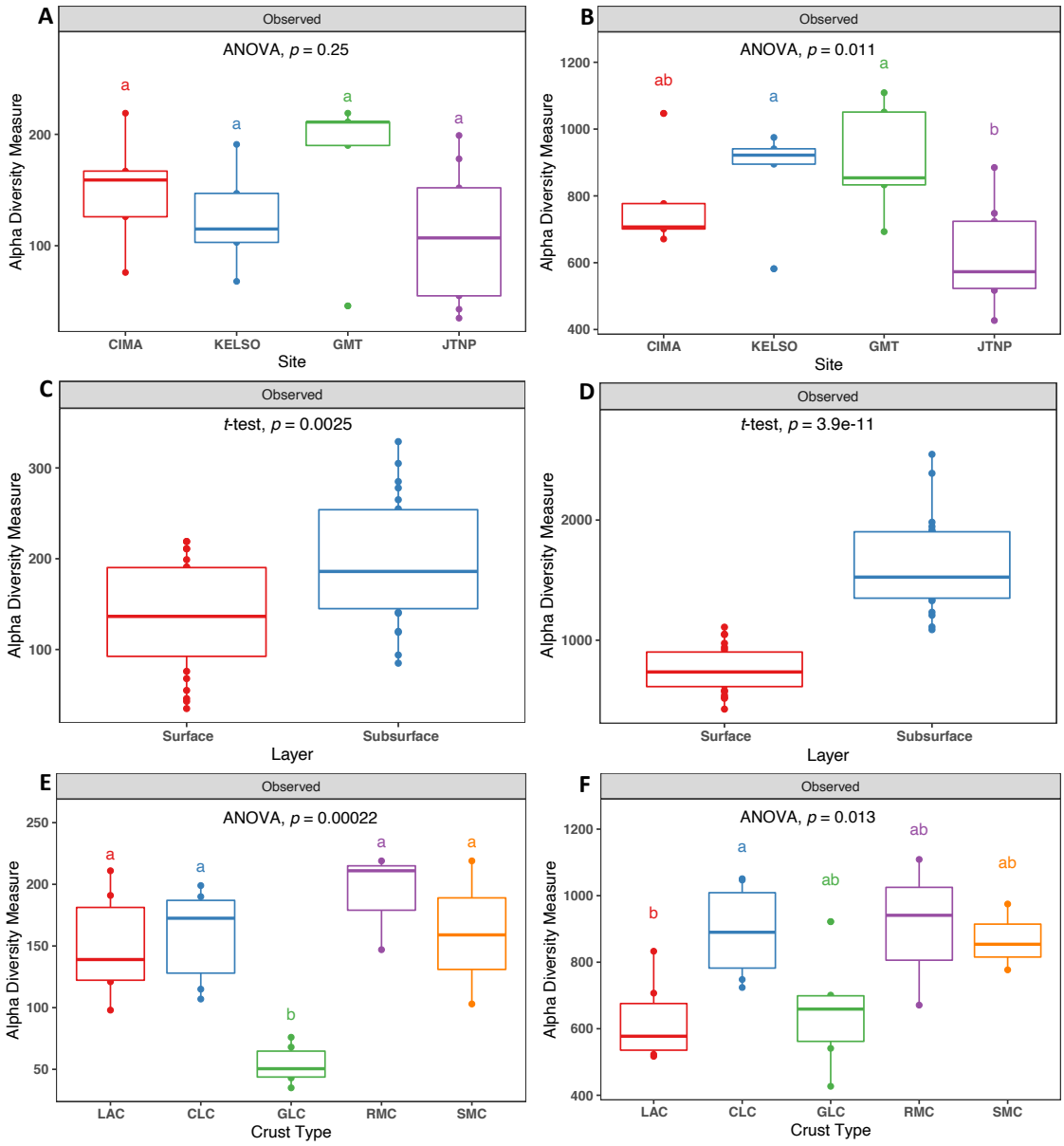

Figure 3.2: Boxplots showing alpha diversity as OTU richness in different site, soil depth and crust type. (A) Fungal alpha diversity of Mojave biocrusts samples by site with rarefaction of 6 842 reads per sample, (B) Bacterial alpha diversity of Mojave biocrust samples by site with rarefaction of 37 435 reads per samples, (C) Fungal alpha diversity by soil depth, (D) Bacterial alpha diversity by soil depth, (E) Fungal alpha diversity by crust types and (F)B acterial alpha diversity by crust type. Boxplots show 25th and 75th percentile while median was shown as lines inside boxes. Error bars show 1st and 99th percentile. Tukey HSD significant differences (p <0.05) are indicated by different letters.

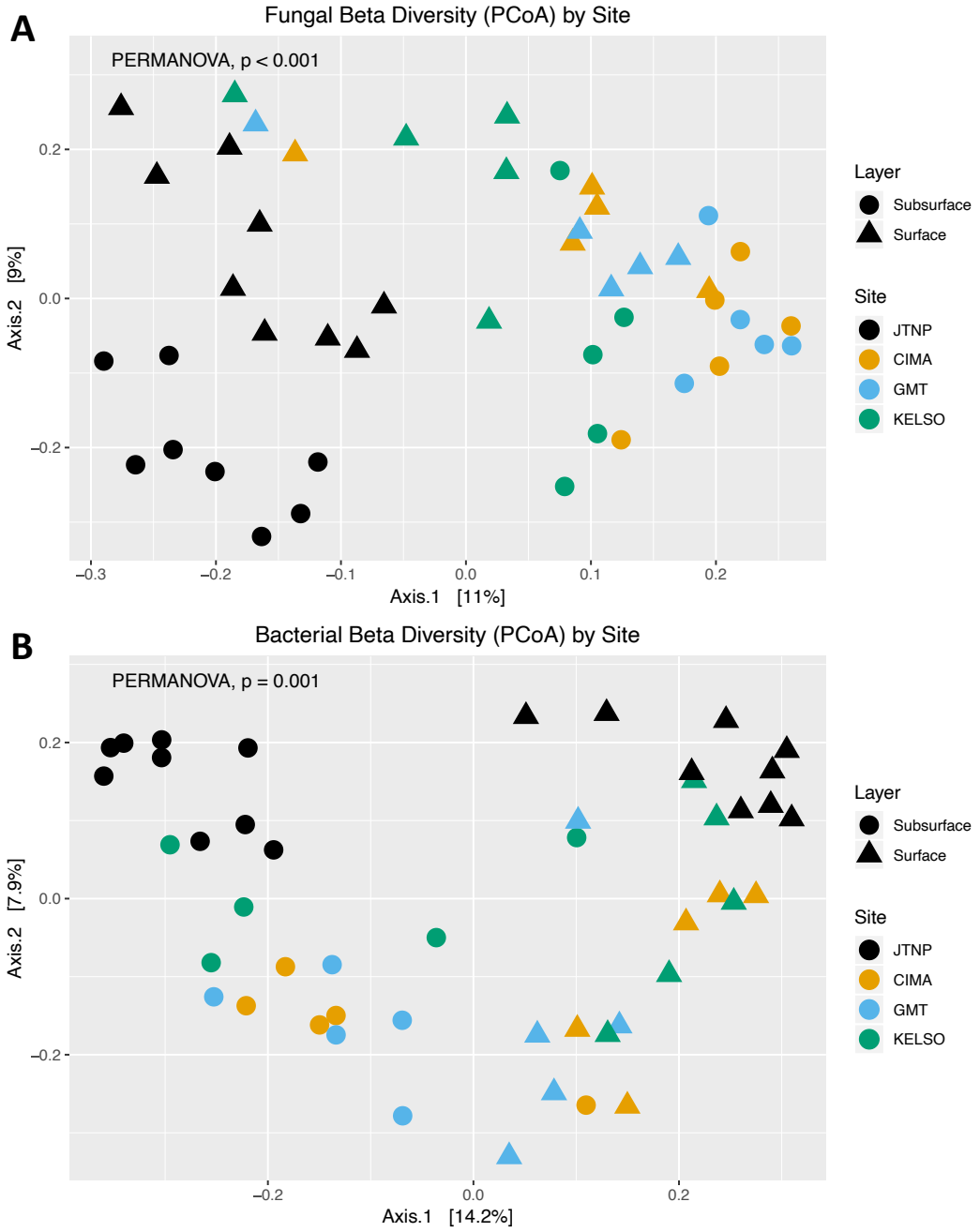

Figure 3.3: Beta diversity analysis of biocrust microbial communities. Dissimilarity of (A) fungal and (B) bacterial community composition in the comparison between site and soil depth (layer) using PCoA. Different colors indicated four sampling sites, including black color for JTNP, yellow color for CIMA, blue color for GMT and green color for KELSO. Circle points showed subsurface samples, while triangle points indicated surface samples. Significant differences (PERMANOVA; p <0.05) were shown on PCoA plots.

3.4.2 Do biocrusts contain indicator microbial species at each site?

Overall, our analysis revealed fewer numbers of fungal indicator taxa for geographic location than bacteria across all surface samples. A total of 11 indicator fungal OTUs were revealed based on sampling site: 2 OTUs for CIMA (closest related taxon: *Catenulomyces convolutus*—with an unassigned functional guild and *Preussia terricola*—a dung saprotroph and/or plant saprotroph); 4 OTUs for JTNP (closest related taxon: *Allophoma labilis*—a plant pathogen; *Curvularia inaequalis*—a plant pathogen; *Entoloma halophilum*—an ectomycorrhizal, fungal parasite and/or soil saprotroph; and /textitPreussia africana—a dung saprotroph and/or plant saprotroph); and 5 OTUs for KELSO (closest related taxon: *Alternaria hungarica*—an animal pathogen, endophyte, plant pathogen and/or wood saprotroph; *Cladosporium herbarum*—a plant pathogen and/or wood saprotroph; *Colletotrichum gloeosporioides*—an endophyte and/or plant pathogen; *Fusarium oxysporum*—a plant pathogen, soil saprotroph and/or wood saprotroph; and *Ulocladium dauci*—a plant pathogen). No fungal indicator species were predicted by the analysis for the GMT site (Table S2, Supporting Information).

Bacterial and archaeal indicator species analysis of biocrust surface samples found 67 ASVs when analyzed by site. Indicator ASVs include 29 indicator ASVs for CIMA (3 classifiable ASVs were most closely similar to bacteroidetes *Segetibacter aerophilus*—with an unassigned functional guild; cyanobacteria *Chroococcidiopsis* sp. BB79.2–with an unassigned functional guild; deinococcus–thermus *Deinococcus maricopensis DSM 21 211*–a nitrate reducer), 15 species/ASVs for GMT (only 1 classifiable species, most closely similar to bacteroidetes *Parahymenobacter deserti*—with an unassigned functional guild), 12

species/ASVs for JTNP (1 classifiable species was bacteroidetes *Hymenobacter rigui*—a nitrate reducer) and 11 species/ASVs for KELSO (closest to proteobacteria *Roseomonas pecuniae*, proteobacteria *Sphingomonas kaistensis*) (Table S3, Supporting Information).

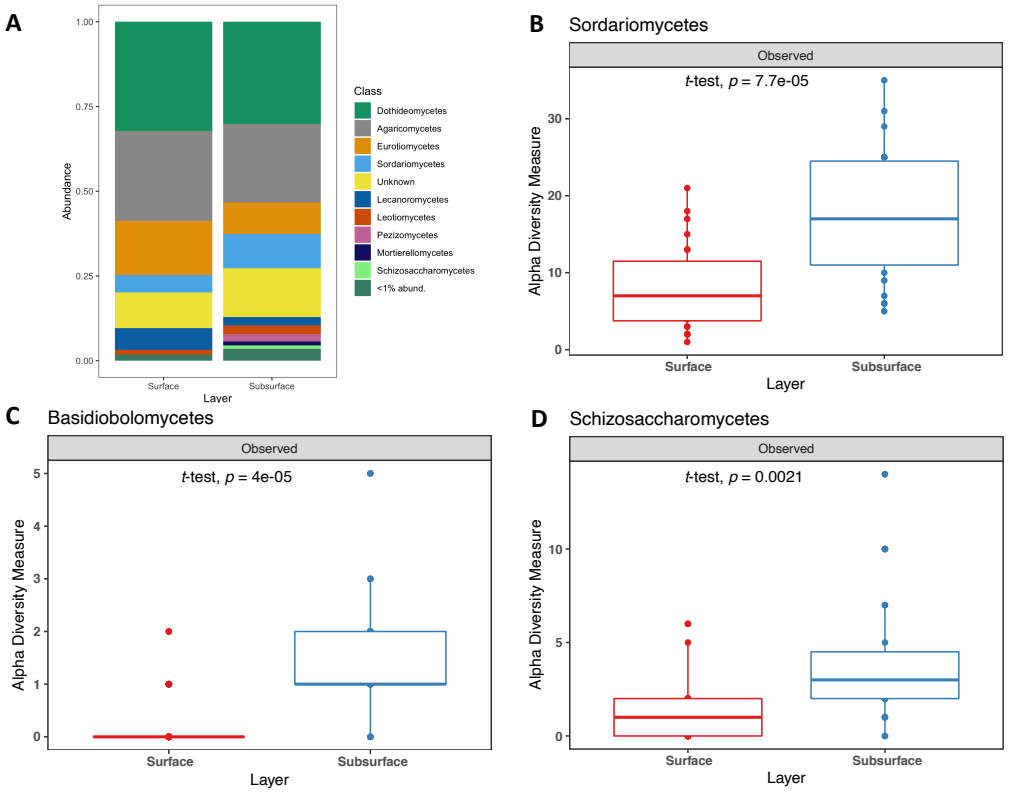

Figure 3.4: (A) Fungal taxonomic composition bar plots at class level by layer (<1% abund. = <1% relative abundance). Top three fungal classes with significantly different alpha diversity by layer, including (B) Sordariomycetes, (C) Basidiobolomycetes and (D) Schizosaccharomycetes. Subsurface soil also had greater species richness than surface biocrust for seven other fungal taxonomic classes (Agaricomycetes,Mucoromycetes, Saccharomycetes, Orbiliomycetes, Entomophthoromycetes, Mortierellomycetes and Pneumocystidomycetes). Boxplots show 25th and 75th percentile while median was shown as lines inside boxes. Error bars show 1st and 99th percentile. Tukey HSD significant differences (p <0.05) are indicated by different letters.

3.4.3 Do biocrust microbial surface communities differ from those in the adjacent soil subsurface?

When comparing alpha diversity by soil depth, biocrust surface samples had significantly lower species richness than subsurface soil samples, both for fungal and bacterial richness (fungal Welch's t-test, $t(43.594) = 3.208$, $p = 0.0025$) (bacterial Welch's t-test, $t(31.587) = 9.84$, $p = 3.856\text{e-}11$) (Fig. 3.2C and 3.2D). Ten fungal classes showed significantly higher richness in subsurface soils (Welch's t-test, $p < 0.05$): Ascomycete classes: Sordariomycetes, Schizosaccharomycetes, Saccharomycetes, Orbiliomycetes, Pneumocystidomycetes; Basidiomycota class: Agaricomycetes; Mucoromycota classes: Mucoromycetes and Mortierellomycetes; and Zoopagomycota classes: Basidiobolomycetes and Entomophthoromycetes (Table S4, Supporting Information; Fig. 3.4B–D).Nevertheless, most fungal OTUs (514 OTUs) were shared between surface biocrust and subsurface samples (Figure S3B, Supporting Information). For bacterial and archaeal communities, distinct differences can already be seen in the relative abundance values in the taxonomic composition bar plot among the 30 prokaryotic phyla (Fig. 3.5; Table S2, Supporting Information). Nineteen phyla showed significant differences between surface vs subsurface samples (Welch's t-test, $p < 0.05$); Proteobacteria, Firmicute, Actinobacteria, Euryarchaeota, Nanoarchaeota, Thaumarchaeota, Acidobacteria, Planctomycetes, Patescibacteria, Elusimicrobia, Armatimonadetes, Chloroflexi, Gemmatimonadetes, Entotheonellaeota, Cyanobacteria, Nitrospirae, FBP, Fibrobacteres and Verrucomicrobia (Fig. 3.5; Table S5, Supporting Information). Nearly all of these phyla showed greater species richness in subsurface soil than in biocrust samples. Cyanobacteria were the only bacterial phylum with significantly greater richness

in biocrust surface samples than in subsurface soil. Similar to the fungal community the majority of bacterial surface ASVs (2883 ASVs)were shared between surface biocrust and subsurface samples (Figure S3A, Supporting Information).

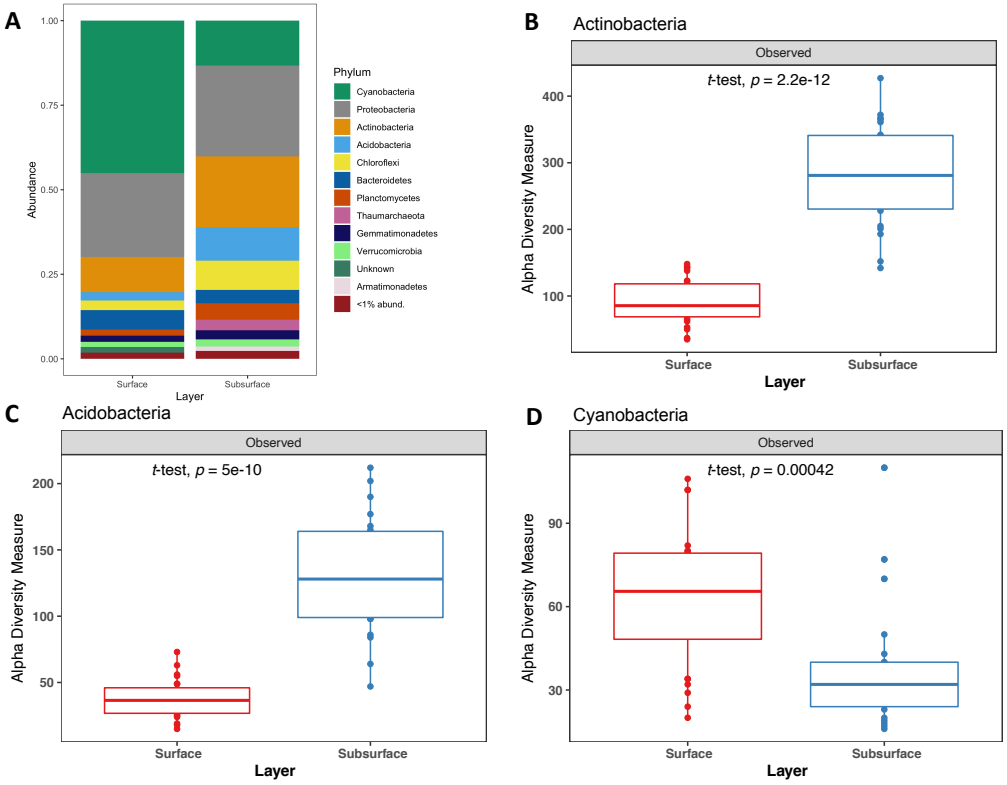

Figure 3.5: (A) Bacterial taxonomic composition bar plot at phylum level by layer (<1% abund. = <1% relative abundance). Top two bacterial phyla inwhich alpha diversity by layer were significantly different including (B) Actinobacteria and (C) Acidobacteria. Same pattern was found in other 16 bacterial phyla in which subsurface soil had greater species richness than surface soil. (D) Cyanobacteria bacterial richness on the soil surface was greater than in subsurface soil. Boxplots show 25th and 75th percentile while median was shown as lines inside boxes. Error bars show 1st and 99th percentile. Tukey HSD significant differences (p ¡ 0.05) are indicated by different letters.

Beta diversity analysis of biocrust fungal communities showed significant difference by soil depth (PERMANOVA, $p = 0.001$, $R^2 = 0.071$) (Fig. 3.3A). These differences in beta diversity were visualized in PCoA plots revealing that JTNP showed the strongest sur-

face–subsurface clustering while central Mojave showed some surface–subsurface clustering, but not as clearly distinct aswe observed in JTNP. Evaluation of bacterial members of the samples found that communities were significantly different by soil depth (PERMANOVA, $p = 0.001$, $R^2 = 0.113$) (Fig. 3.3B). Bacterial communities displayed surface–subsurface patterning: surface samples clustered closer together in the PCoA plots and the majority of subsurface samples were clustered near each other (noting that two subsurface samples clustered with surface samples).

Bacterial networks were the most connected in biocrust surface samples (54% were bacterial–bacterial connections) among all microbial communities both within (bacteria–bacteria, archaea–archaea and fungi–fungi) and across (fungi–bacteria, bacteria–archaea and fungi–archaea) domain networksfor surface community (network stability = 0.047) (Fig. 3.6). The network inferred from the ASV abundances in surface samples indicated that these communities are mostly structured within a single large connected network (in the center of Fig. 3.6) instead of several distinct clusters of networks. Betweenness centrality and node degree analysis showed that microbial hubs of biocrust surface community included Actinobacteria, Proteobacteria, Cyanobacteria and Ascomycota: high node degrees were observed in these four phyla, indicating high numbers of network connections. *Pseudonocardia* sp. was found to have the highest node degree (most connections) while *Methylobacterium* sp., *Microvirga* sp., *Microcoleus* sp. and *Belnapia* sp. also had high node degrees (high connections) representing microbial hubs for biocrust surface network. However, many other microbial hubs were uncultured bacteria and/or unknowns. *Alternaria* sp. had the highest node degree and betweenness centrality in the fungal community

(Fig. 3.7). For biocrust surface samples, overall cross-domain (fungal–bacterial connections, within domain networks removed) links included: (i) Agaricomycetes and Dothideomycetes were linked to Actinobacteria, (ii) Agaricomycetes, Dothideomycetes, Eurotiomycetes, Orbilliomycetes and Sordariomycetes were linked to Cyanobacteria, (iii) Dothideomycetes, Eurotiomycetes and Pezizomycetes were linked to Alphaproteobacteria and (iv) Dothideomycetes, Lecanoromycetes and Sordariomycetes were linked to Blastocatellia (Fig. 3.8). The complete network of microbial connections within and across domains is depicted in Figure S4 (Supporting Information).

Subsurface soil samples showed similar patterns to surface biocrust where bacterial networks were more connected than other microbial community networks (network stability = 0.048) (Figure S5, Supporting Information). Betweenness centrality and node degree analysis showed that microbial hubs of the biocrust subsurface community included Actinobacteria, Proteobacteria, Thaumarchaeota and Ascomycota. *Microvirga* sp. was found to have the highest node degree (most connections) while *Modestobacter* sp. and *Candidatus Nitrososphaera* also had high node degrees and represented microbial hubs for biocrust subsurface network. Similar to the biocrust surface microbial network, many other microbial hubs were uncultured bacteria and/or unknowns. Identical to surface samples, *Alternaria* sp. had the highest node degree and betweenness centrality in subsurface fungal networks (Figure S6, Supporting Information). Although large connected networks were observed as well, a major backbone of multiple fungal–bacterial networks in subsurface communities revealed features different from surface microbial communities. Fungal–bacterial networks in subsurface samples included: (i) Agaricomycetes, Dothideomycetes, Eurotiomycetes and

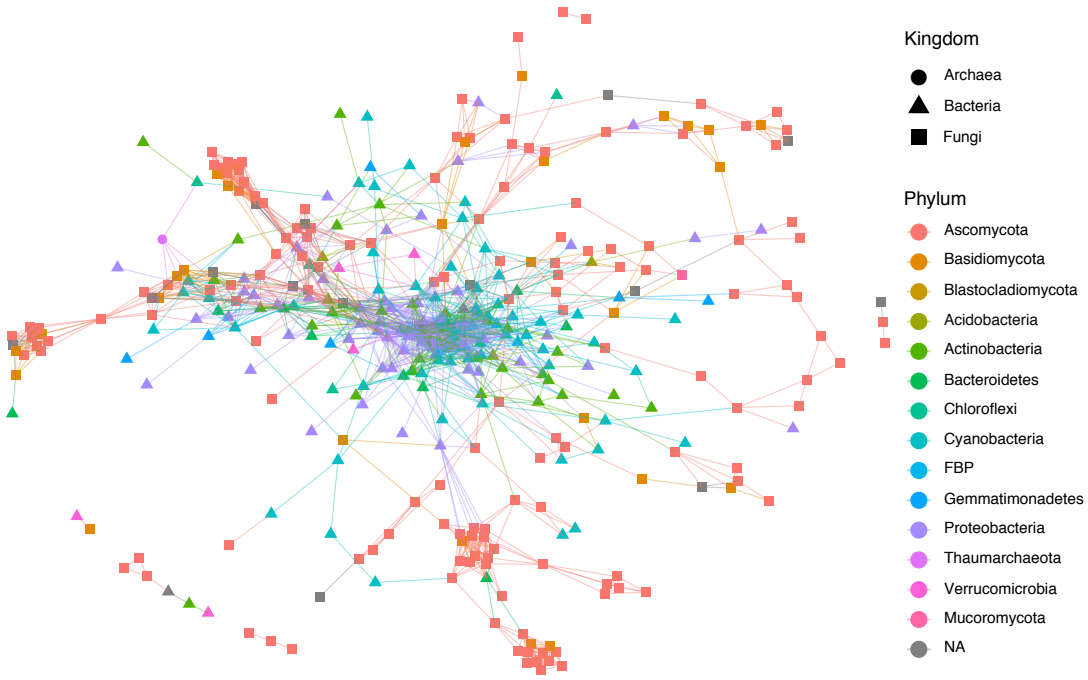

Figure 3.6: Microbial network analysis for biocrust surface samples. Each symbol/point on microbial network plot presents a single OTU. Microbial domains are indicated by different point shapes: archaea by circles, bacteria by triangles and fungi by squares. Microbial networks are shown by line connection between points. Different colors indicate phylum for each point. Major network hubs concentrate at the center of microbial networks.

Lecanoromycetes were linked to Actinobacteria, (ii) Agaricomycetes, Basidiobolomycetes, Dothideomycetes, Leotiomycetes, Orbilliomycetes and Sordariomycetes were linked to Alphaproteobacteria, (iii) Dothideomycetes were linked to Bacteroidia, (iv) Dothideomycetes and Mortierellomycetes were linked to Blastocatellia, (v) Dothideomycetes were linked to Chloroflexia, (vi) Dothideomycetes were also linked to Gammaproteobacteria and (vii) Eurotiomycetes were linked to Rubrobacteria (Figure S7, Supporting Information).

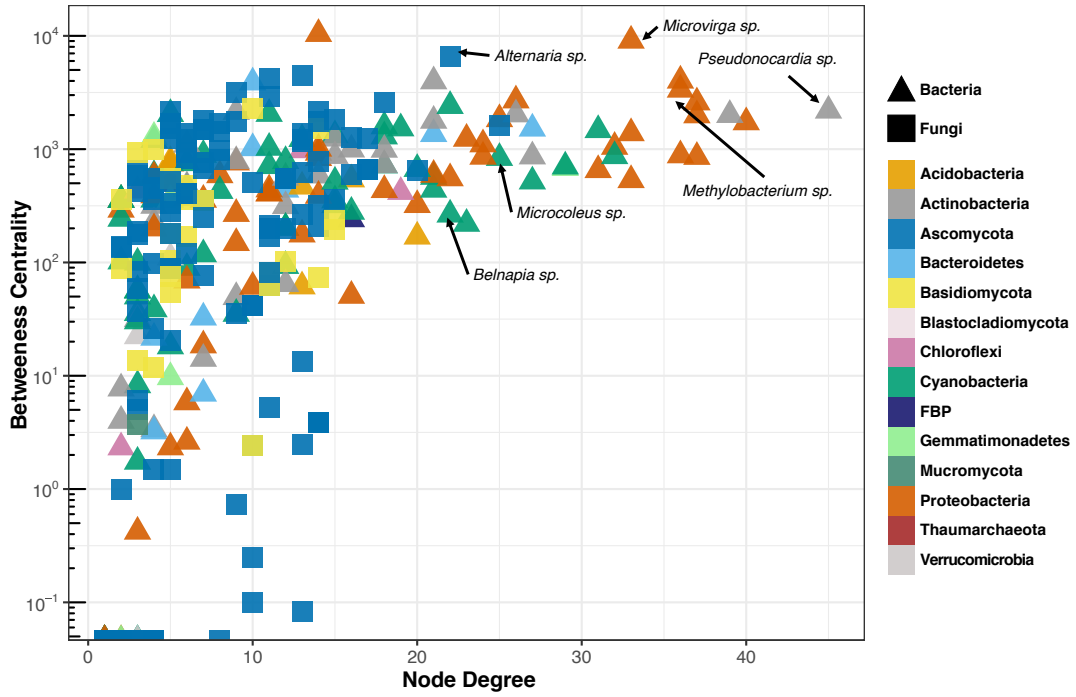

Figure 3.7: Betweenness centrality and node degree analysis of biocrust surface samples. Each symbol/point on the plot represents a single OTU. Microbial domains are indicated by different point shapes: archaea by circles, bacteria by triangles and fungi by squares. High node degree indicates high network connections, which represents microbial hubs. High betweenness centrality specifies key connector for microbial network.When both high betweenness centrality and high node degree are observed, the species/nodes are the major hubs for the networks. Biocrust surface microbial network major hubs are clustered at the top right corner of the plots.

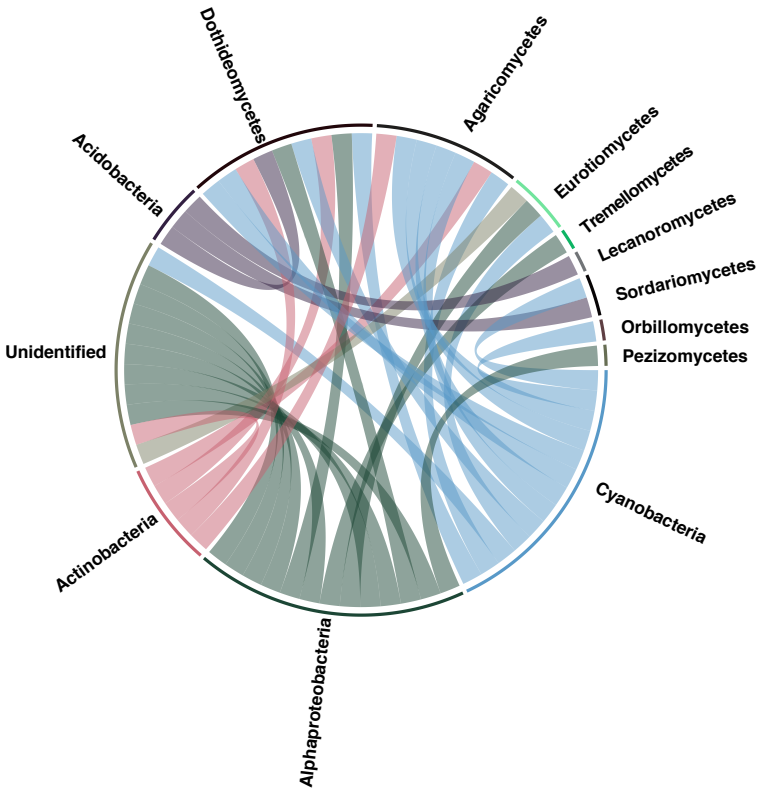

Figure 3.8: Cross-domain fungal–bacterial network analysis for biocrust surface samples. Cross-domain networks are a subset of total microbial networks showing in Fig. 3.6. Each line represents the connection of a fungal OTU to a bacterial ASV. Different colors indicate different bacterial phyla. Cyanobacteria (in blue) had the highest number of connections to fungi. Cross-domain fungal–bacterial network analysis for biocrust subsurface samples is shown in Figure S7 (Supporting Information).

3.4.4 Do biocrust types each have their own unique assemblages of microbes and do characteristic differences in richness exist between them?

Fungal and bacterial richness of biocrust surface samples differed significantly by crust type [fungal ANOVA, $F(4,13) = 5.5869$, $p = 0.007668$ (Fig. 3.2E); bacterial ANOVA, $F(4,12) = 3.9425$, $p = 0.02869$ (Fig. 3.2F)]. The GLC crusts had the lowest fungal species richness among crust types. The LAC samples had bacterial alpha diversity that was significantly lower than CLC, but not significantly lower than in other crust types (Fig. 3.2F). Comparing fungal richness across crust types identified nine fungal classes that differed significantly by crust type (ANOVA, $p < 0.05$), including most of the major classes in Ascomycota: Sordariomycetes, Eurotiomycetes, Lecanoromycetes, Dothideomycetes, Leotiomycetes, Schizosaccharomycetes, Pezizomycetes; and Basidiomycota classes: Agaricomycetes and Tremellomycetes (Figure S1B and Table S6, Supporting Information). GLC generally had lower fungal richness than the other crust types when comparing class-specific richness. Moss crusts (RMC and SMC) had greater richness within the fungal classes Leotiomycetes, Peziozomycetes and Tremellomycetes. For bacterial communities, 10 phyla were significantly different by crust type (ANOVA, $p < 0.05$): Proteobacteria, Acidobacteria, Planctomycetes, Patescibacteria, Armatimonadetes, Deinococcus–Thermus, Chloroflexi, Cyanobacteria, FBP and Verrucomicrobia (Figure S2B and Table S7, Supporting Information). In Proteobacteria, Acidobacteria, Planctomycetes, Patescibacteria and Verrucomicrobia, species richness was greater in moss crusts (RMC and SMC) than in other crust types. Richness of Armatimonadetes was lowest in LAC while Chloroflexi richness was

highest in CLC. Lastly, Cyanobacteria richness was lower in moss crusts than in other crust types, versus highest in CLC.

Beta diversity analysis of biocrust fungal communities showed significant differences by crust type (PERMANOVA, $p = 0.001$, $R^2 = 0.174$) (Figure S8, Supporting Information). These differences in beta diversitywere visualized in PCoA plots. GLC fungal community was distinctly different from other crust types, while the other biocrust types had overlaps. Evaluation of bacterial members of the samples found that communities were also significantly different by crust type (PERMANOVA, $p = 0.001$, $R^2 = 0.181$) (Figure S9, Supporting Information).

Indicator analysis revealed that two fungal OTUs were detected by crust type at all locations. Specifically, for RMC there was one indicator OTU (closest related taxon: *Sporormia subticinensis*, dung saprotroph) and likewise for SMC one OTU (closest related taxon: *Acrophialophora levis*, plant pathogen). No detectable fungal indicator species occurred in CLC, GLC and LAC (Table S8, Supporting Information). Bacterial indicator analysis found 36 indicator ASVs within crust types. There were 9 indicator ASVs for CLC (most closely similar to Proteobacterium *Azospirillum soli*—nitrate respiration), 4 indicator ASVs for LAC (closest to Proteobacterium *Belnapia moabensis*), 6 indicator ASVs for RMC (closest to Proteobacterium *Salinarimonas* sp. BN140002) and 17 indicator species/ASVs for SMC (closest relatives were Deinococcus–Thermus *Deinococcus pimensis DSM 21 231*—nitrate reduction and Cyanobacterium *Calothrix* sp. HA4186-MV5—nitrogen fixer). No bacterial indicator species/ASVs were observed in GLC (Table S9, Supporting Information).

3.5 Discussion

In our study, we identified several distinct patterns structuring biocrust microbial communities in the Mojave Desert. These patterns included: (i) a distinct geographical signal between our three central Mojave sites versus the southern Mojave site, (ii) a soil depth pattern that clearly differentiated biocrust surface diversity from subsurface microbial communities and (iii) a biocrust type pattern that showed differences between algal, lichen and moss crusts.

3.5.1 *Geographical pattern:* Does geographical location structure biocrust microbial communities and reveal unique microbial species?

Many studies have demonstrated biogeographical patterning of bryophyte and lichen biocrusts based on climatic, edaphic, topographic and biotic factors at various spatial scales [49, 50]. Less is known about biogeographical patterns of the microbial taxa that make up biocrusts. We hypothesized that geography would structure biocrust microbial communities and detected distinct geographical patterns within the Mojave Desert in which both alpha and beta diversity differentiated microbial communities in central Mojave (GMT, KELSO and CIMA sites) from southern Mojave (JTNP site). Our results support the findings of other studies in which microbial communities were more similar when collecting sites were in close proximity compared to further away [24, 26, 51]. In addition to spatial autocorrelation relationships these major differences could likely point to environmental gradients based on changes in elevation, temperature and rainfall stretching from central Mojave to the Mojave-Colorado desert ecotone at JTNP, which could contribute to

the separation of southern Mojave microbial communities from central Mojave diversity. In previous research, boundaries between biomes such as the Mojave and Colorado Deserts have been identified using vascular plant community composition. The key species *Prosopis glandulosa var. torreyana* and *Fouquieria splendens* are indicative for the Colorado Desert, which appear in lower elevation and warmer climate while *Yucca brevifolia* is characteristic for higher elevation with winter rain in the Mojave Desert [52]. Microbial communities may similarly respond to the same drivers that can be detected as species turnover when surveying sites along environmental gradients that stretch from the Mojave to Colorado Desert. However, additional studies are needed to specifically identify these drivers.

To our knowledge, this is the first report of geographical fungal indicator taxa obtained from biocrust samples. We hypothesized that different geographical locations will harbor key species that are unique to each site. We also obtained first predictions of putative functional roles of the detected fungal taxa. Indicator taxa were mostly assigned to plant-associated fungi belonging to pathogenic, endophytic and saprotrophic functional guilds. At JTNP all classifiable fungal indicator taxa were plant pathogens while an indicator taxon at CIMA was plant saprotroph. According to these results, certain functional guilds were more confined to colonize at particular sites which is likely because of more living plant availability at JTNP than CIMA versus more plant debris at CIMA volcanic fields than JTNP. The fungal loop hypothesis states that fungi metabolically link plants and biocrusts in drylands [53], yet our results suggest other important plant-biocrust interactions may be mediated through the fungal community found in biocrust. For example, biocrusts' ability to capture seeds [54] could also mean that plant-associated fungal spores trapped by biocrusts

could establish and could interact with the local plant communities in terms of symbiotic or pathogenic relationships. In contrast to the fungal data, most bacterial indicator taxa functions could not be classified in our study and therefore functional patterns cannot be identified. Further study is needed to explore bacterial functional guilds.

3.5.2 *Soil depth pattern:* Do biocrust microbial surface communities differ from those in the underlying soil subsurface?

Because biocrusts are localized to the surface of soils, they are considered a living skin of drylands [8] housing unique microbial communities. One pioneering study comparing microbial communities at the biocrust surface with lower soil layers was conducted on the Colorado Plateau, using a culture-based quantification of viable aerobic copiotrophs and microscopic counts. Bacterial populations were found to be higher in the biocrusts on the soil surface compared to its associated subsurface soil or to soil without crust [12]. Two additional studies sampling the Colorado Plateau and central Mojave demonstrated that bacterial alpha and beta diversity separated samples by soil depth when amplicon sequencing was used, which provided a more comprehensive microbial diversity survey [22, 26]. Yet, only one single study to date has surveyed both the fungal and bacterial communities in biocrusts as well as the soil below, finding that in southern Nevada biocrusts there was lower diversity for both domains compared to the subsurface soil [28].

Based on these studies, we hypothesized that structuring of soil microbial communities is driven by higher richness of light-dependent microbes (Cyanobacteria) on the biocrust surface. Similar to Mueller, Belnap and Kuske (2015) [28],we found lower overall species richness within biocrust samples as compared to their soil underneath. We observed

that cyanobacteria richness was higher in the biocrust consistent with their dependence on light availability for photoautotrophic metabolism [12, 22]. In previous studies, the bacterial phyla Acidobacteria, Actinobacteria, Chloroflexi and Proteobacteria were found to be more diverse in subsurface soil [22, 26] and we observed a similar pattern. In addition, in our work we found 12 additional bacterial and 3 archaeal phyla with significantly greater richness in surface samples than subsurface soil (Table S4, Supporting Information). Our observations found that bacterial communities showed a distinct depth-dependent organization in Mojave Desert biocrust.

Examination of the fungal community revealed a similar pattern and fungal species richness in biocrust subsurface samples was found to be greater than in surface soil. These findings indicated that Sordariomycetes were skewed in a similar vertical distribution as was found in Nevada biocrusts [28], but we also identified nine additional fungal classes with significant soil depth association at our collection sites. Overall, the majority of fungal OTUs were found in both California and Nevada but most did not display significant differences in soil depth in Nevada.

We used cross domain (fungal–bacterial) networks to further explore the soil depth patterns. Incorporating both fungi and bacteria communities in microbial network analysis improved network stability compared to single-domain microbial networks [21]. To better understand the entire microbial network in biocrust systems, bacteria and fungi were jointly analyzed in a single cross-domains network analysis, identifying key, or also known as hub,microorganisms in both domains. Our network analysis showed that Cyanobacteria were key to fungal–bacterial connections for biocrusts (surface), which supports previous

hypotheses of their importance [7, 19]. Cyanobacteria have been inferred to be key taxa in biocrusts due to the high photoautotrophic biomass they contribute to the surface of biocrusts [12, 22, 28]. We also identified Pleosporales (Dothideomycetes) as key to fungal–bacterial connections. Their dominance among fungal taxa in biocrust and semiarid and arid areas in general correspond to their substantial reference database and diverse lifestyle [51, 55]. We also found that Agaricomycetes were another major group of fungal connectors, which fits the abundance of the group as reported in the southern Nevada study [28]. Top OTUs in this group are ectomycorrhizal, mushroom, fungal parasite and/or soil saprotroph which could be essential in plant–microbe and microbe–microbe interaction. Thus, our data suggested two groups of fungi that could potentially be key microorganisms for biocrusts in both southern California and Nevada.

Particular key microbes identified as microbial hub taxa included *Pseudonocardia* sp., *Methylobacterium* sp., *Microvirga* sp., *Microcoleus* sp. and *Belnapia* sp. Specific traits of these microbes could be essential for the functioning and community dynamics, such as structuring biocrust with polysaccharide by *Microcoleus* spp. [7], producing antibiotics against microfungal parasites by *Pseudonocardia* sp. [56, 57], *Microvirga* sp. forming root nodules in plants [58, 59], and *Methylobacterium* sp. facilitating seed germination and plant development [60].

In contrast to surface microbial networks, Cyanobacteria were not present as hubs in our analyses of subsurface soil samples. However, Pleosporales (Dothideomycetes)were still a major connector for fungal–bacterial networks in subsurface soil while Agaricomycetes were also found as a minor key connector. The fungal networks appear to be similar between

surface and subsurface, while bacterial networks were different primarily due to the lack of photoautotrophs. Furthermore, identifiable hubs for subsurface microbial networks seem to drive different functions than networks of the surface community. Only *Microvirga* sp. was found to be similar microbial hubs to the surface network. *Modestobacter* sp. and *Candidatus Nitrososphaera* were the other two major hubs for subsurface soil which are known to inhabit extreme environments (temperature) [61, 62]. *Candidatus Nitrososphaera* is an ammonia oxidizing bacteria which plays a role in the nitrogen cycle while one of the key functions of *Modestobacter* sp. is melanin production [61, 62]. However, the functional roles of these microbial hubs will need to be explored further to better understand these hubs in Mojave Desert soils. Overall, we noted a strong soil depth pattern in our Mojave biocrusts, with greater numbers of bacterial phyla and fungal classes contributing to these patterns than previously reported [12, 22, 28]. Nonetheless, functional guilds could not as yet be identified for both bacteria and fungi that contributed mainly to soil depth patterning.

3.5.3 *Biocrust type pattern:* Are biocrust types linked with microbial diversity?

The classification of biocrusts have been based on a combination of their morphology, aggregation strength, overall functional role and by their dominant photoautotrophs [2–4, 6, 8]. Environmental factors such as temperature, moisture, salinity, soil texture, dust deposition and geomorphology influence the occurrence and abundance crust types at a local to regional scale [8, 63, 64]. Crusts found in different localities are classified as common types such as 'cyanobacterial' or 'lichen crusts' with visibly similar morphologies, but it remains unknown how much the constituent microbial communities vary among

the same crust type found in different locations. We examined and compared the fungal and bacterial communities of five different biocrust types including LAC, CLC, GLC, SMC and RMC. We hypothesized that microbial diversity will be strongly associated with crust type, and structurally complex assemblages such as lichen and moss crusts will have greater alpha diversity in both fungal and bacterial composition than structurally less complex types such as light algal/cyanobacterial crusts. The bacterial species richness was indeed lower in LAC than in the more highly structured lichen and moss crusts, matching previous findings [6, 65]. However, such differences were not mirrored in fungal communities. These observations raise new questions. If fungal communities are more similar to each other based on geographic location but do not differ among crust types, is there substantial crust to crust exchange of fungi with minimal dispersal limitations? Is there a core of fungal taxa required to promote crust establishment that is universal to all types? Alternatively, the patterns of fungal diversity could be explained by other abiotic and biotic factors or just be randomly assembled across biocrust types. Further sampling to test new hypotheses about geographic structure will need to be undertaken to more fully explore these ideas.

Cyanobacteria, which were inferred to be major microbial hubs in our network analysis, were more abundant in LAC, CLC and GLC than in SMC and RMC. The dominance of Cyanobacteria in LAC and lichen crusts, but not inmoss crusts, is indicative of their central role as primary autotrophic community members versus their less prominent role where mosses are dominant. Alpha diversity analysis also differentiated cyanobacterial (LAC) and lichen (CLC, GLC) crusts from moss crusts (SMC and RMC) in their richness of Proteobacteria, Actinobacteria and Acidobacteria with greater alpha diversity in moss

crusts than in cyanobacterial and lichen crusts. Moss crusts have been shown to retain more moisture than light cyanobacterial crust [66] as well as fix carbon at higher rates [4]. Greater microhabitat moisture availability and fertility may increase microbial diversity [67].

In addition, our indicator species analysis also showed that both types of our Central Mojave moss crusts contained fungal as well as bacterial indicator species (Tables S6 and S8, Supporting Information) suggesting that moss crusts may have a very defined core microbiome. However, due to limited sample numbers in this study, this possibility will need validation through geographically extensive sampling efforts in future studies. We were not able to match identical fungal species from sequences with lichen biocrust types as we identified from external morphology, but several OTUs matched Peltigerales (with high abundance in our CLC samples) and could possibly be the fungal symbionts in *Collema sp.* while many OTUs matched Verrucariales (with high abundance in our GLC samples) that might be the symbionts in *Clavascidium sp.* (Figure S1B, Supporting Information). This issue clearly shows that better molecular markers are needed for these lichens. Lastly, due to heterogeneous soil microbial communities and small sample size, increased crust types sampling and replicates are needed in future studies to be able to better understand biogeographical biocrust type patterns and investigate within and between variabilities in community composition and structure.

Lastly, we wanted to compare the microbial communities within the same functional crust type but sampled from different locations. Despite sampling the same functional biocrust types in all four localities, beta diversity and indicator species analysis indicated that central Mojave Desert localities had unique microbial communities in the surveyed

crust types dissimilar from the same crust types sampled in JTNP. Although external morphology was not visibly different, microbial communities differed and indicator taxa were detected for specific locations. If this pattern persists in other desert ecosystems or even other locations within the Mojave Deserts this would mean that we cannot readily assume similarities in microbial community composition when classifying biocrusts by functional groups or morphological community types. Future investigations could focus on a broader more extensive sampling of crust types and exploring the questions of how local some of the microbial communities may be or if communities are rather stochastically assembled.

3.5.4 Implication to conservation and restoration management

Current efforts to restore biocrusts in heavily disturbed landscapes often yield limited success in the field [68–71]. We think that such challenges arise from our lack of a comprehensive knowledge of local and regional community dynamics, dispersal modes, physiological constraints, taxonomic identities, biotic interactions and functional roles of the microscopic community members. Our findings stimulate several new thoughts towards biocrust conservation and restoration management. In our dataset, although limited to five biocrust types from four sampled sites which were 10–50 km apart, microbial communities from the same biocrust type in different locations were not identical. Our results suggest that community heterogeneity could be related to biogeography and ecological processes such as dispersal limitations, competitive exclusion, local-scale microhabitat specializations, etc that could influence biocrust microbial community assembly [72] and can represent an additional drivers of biocrust community composition that has yet to be considered in biocrust research. Followup studies are needed to comprehensively investigate what envi-

ronmental factors impact alpha, beta and gamma diversity of microbial communities. Such knowledge can be highly informative when considering a source of biocrust inoculum for restoration especially between sites that are far apart. More sites and crust types within the Mojave Desert need to be studied to identify the differences in microbial profiles among five crust types in the northern, eastern and western parts of Mojave Desert compared to central and southern locations in our study. Moreover, efforts to implement restoration methods by inoculation should be preceded by combined domain amplicon sequencing surveys like the present work. This is especially true in drylands which lack baseline studies of microbial diversity that can be used to make observations about foundational species important for crust colonization of new soils.

Efforts to establish biocrust restoration using an inoculum based on intact crusts from other sites primarily focus on promoting biomass growth of photoautotrophs, while much less attention is given to other biocrust-associated microorganisms, even though some taxa could nevertheless be important components too. Microbes which are hubs in microbial networks may regulate microbial community functioning and are thus potentially necessary components for growth and sustained health of newly seeded biocrust. We found complex linkages within and between the two surveyed microbial domains. We also demonstrated that hub taxa and indicator species occur in biocrust and are discoverable by the applied methods. However, we have limited information about particular functional guilds and community regulation of these species identified from amplicon sequencing alone. As a result, more research is needed on the functional roles of the vast majority of microbes

including desert soil fungi and how they may affect biocrust microbial communities, to inform effective inoculation experiment designs.

Another aspect still overlooked in biocrust restoration is the risk of potentially distributing pathogens or other types of antagonists. Our results indicated by molecular sequence that several fungi that are typically plant pathogens occur in Mojave biocrusts and could be dispersed as part of restoration procedures such as biocrust transplantation, wetting events and recurring fertilizations. Second, many of the predicted hubs in microbial networks lack much identification beyond a sequence OTU and their functional guild is unknown. The OTUs of these microorganisms were detected in the soil beneath the crust or in both the biocrusts and the subsurface, suggesting that source material for biocrust production or inoculation should incorporate more than simply the surface. This strategy is consistent with previous restoration experiments that observed small shifts in the cyanobacterial community when using local soil/biocrust inoculum [71], in contrast to significant changes in cyanobacterial composition when extraneous inoculum was used. In addition, the temporal variability of biocrust microbial communities remains unknown. A better understanding of how temporal changes and seasonality impact the hub species among microbes of resident biocrust communities will be important to know to inform restoration managers which communities are more or less suited for active microbial inoculation.

3.6 Conclusion

In summary, our findings provide the most extensive characterization of local biocrust microbiota to date from the central and southern Mojave Desert. It is to our

knowledge the first comprehensive biocrust microbial community investigation to reveal geographical, soil depth and crust type diversity patterns when considering both fungi and bacteria microbes. Although identification of biocrust types by their external structural morphology is practical for preliminary observation in the field, we have shown that microbial components within each type can be distinct geographically. Biocrust surface and subsurface communities also have distinct microbial compositions. Our results supported the hypothesis that Cyanobacteria are key microorganisms in biocrust types,with network analysis demonstrating that they are major hubs for cross-domain microbial community connectivity. We also identified Pleosporales fungi as a major hub for fungal–bacterial networks. Our key findings imply that microbial species composition and community dynamics need to be taken into account in future biocrust conservation and management efforts. It is imperative that we improve our understanding of spatial variation in the microbial composition and functioning of biocrusts and improve the taxonomic identification of potentially essential species.Neglecting these differences could possibly lead to counter-effective consequences to both biocrust microbial communities and desert ecosystems, such as risk of pathogen/antagonist spread, potential loss of microbial diversity, altered functioning, introduction of invasive microbial species and conceivably even destruction of any remaining biocrusts.

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Chapter 4

Temporal weather variation influences biocrust bacterial dynamic changes

4.1 Abstract

Biological soil crusts (biocrusts) are major components in drylands ecosystems and harbor diverse microbial communities with various important functions. While microbial communities are highly dynamic in coral reef, phyllosphere, human gut, and airborne microbiomes due to seasonal changes, temporal/seasonal effects on biocrust microbial communities are poorly studied. We surveyed biocrust bacterial communities at Joshua Tree National Park (JTNP) over 12 months with amplicon-based metabarcoding sequencing and showed these communities change substantially throughout the year. Weather factors in-

fluenced microbial composition, with variability depending on biocrust type. In general, increasing temperature, precipitation, humidity and dew point positively affected overall bacterial species richness, while increasing wind speed and atmospheric pressure negatively correlated with overall bacterial species richness. In relatively high temperature and low humidity, *Microvirga* sp. was abundant suggesting it has physiological adaptations especially suitable for dryland conditions. We found that *Microcoleus* sp., *Mastigocladopsis* sp. and *Trichocoleus* sp. could be important contributors to surface stability, especially for biocrust experiencing wind shear, because their abundances expanded as wind speed increased. Beta diversity analysis confirmed that crust type structures biocrust microbial diversity in JTNP. This work highlights the dynamic impact of climatic properties to biocrust microbial community composition as a result of temporal/seasonal effects. We concluded that future comparative biocrust microbiome studies should incorporate collection time and measure weather conditions, in addition to recording metadata such as more static soil/geomorphology parameters.

4.2 Introduction

Biological soil crusts (biocrusts) are one of the major components in the drylands landscape, which can cover up to 70% of the land surface area and contribute to essential ecological functions such as fixing carbon and nitrogen, shielding UV and heat radiation, reducing soil erosion, fertilizing subsurface soil, along with mediating local hydrologic cycles [1–4]. With complex organismal formations and multiple crucial ecosystem functions, biocrusts are now recognized as the living skin of the drylands ecosystems [3]. Biocrust

composition has mainly been studied with regards to macroscopic organisms, and attention has only recently shifted toward the roles and complexities of microbial communities within biocrusts. Cyanobacteria and eukaryotic algae are key microbial components in biocrust microbial communities, playing important roles in establishing biocrusts structural components and acting as primary producers [3]. However, other groups of microorganisms are also found to be abundant in biocrusts such as Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, and Proteobacteria [4–6]. Recent research has focus on identifying biotic and abiotic factors that influence biocrust microbial composition and structure. Some of the major patterns that were observed included (i) biocrust morphology differentiates bacterial communities and affects biocrust carbon and nitrogen concentrations (such as differences between light cyanobacterial/algal crust, dark cyanobacterial/algal crust, lichen crust, moss crust etc.) [7, 8], (ii) photoautotrophic organisms in biocrusts influence their overall microbial abundance and diversity [4], (iii) geography, and (iv) soil depth alter biocrust microbial communities [5, 6, 9]. These studies have not only improved our understanding about what shapes the assemblage of biocrust microbial members, but also inspired us to further investigate other characteristics that possibly structure these microbial communities.

Our understanding of temporal and/or seasonal effects on biocrust microbial communities in drylands remains very limited. However, seasonal effects on microbial communities have been identified in several other systems, for instance (i) seasonal changes associated with winter to spring transition strongly affect a temperate coral species' microbiome diversity and composition shift [10], (ii) phyllosphere microbial succession changed across the growing season of various crops [11], (iii) seasonal variation and diet shifts hu-

man gut microbiome [12] and (iv) fortnightly sampling of a pyrenean mountaintop's airborne microbiome over 7 years revealed seasonal patterns and annual cycles [13]. In biocrusts, many studies have indicated that precipitation and temperature drive biocrust distribution, survivorship, and functions in a particular area [14–16]. For example, altered summer precipitation significantly reduced the abundance of copies of the *nifH* gene (nitrogen fixation) in biocrusts from the Colorado Plateau (Castle Valley, UT) [17] and decreased overall soil surface microbial biomass and cyanobacterial biomass in biocrusts from Porcupine Canyon (Moab, UT) [18]. However, only a few analyses have investigated how temporal variations affect biocrust microbial community composition and sturcture.

Although most studies have focused on how changes in precipitation and temperature affect biocrust microorganisms, additional climatic conditions also may have an impact. Some studies have shown that wind can influence the development and landscape distribution of biocrusts. For example, fungal crusts were found to be more resistant to wind than photoautotrophic crusts [19]. It has also been found that when sufficient biomass of *Microcoleus vaginatus* and *Nostoc* sp. are produced in nascent formation of biocrusts, these can increase resistance to wind erosion. However, the impact of wind on the biodiversity patterns of microbial communities has not been reported. Moreover, there is very limited research on the effects of humidity levels on biocrusts [20] and no study of the impact of whether variation in atmospheric pressure can impact biodiversity. To fill these knowledge gaps, a comprehensive investigation of temporal effects on biocrust microbial dynamics is needed. An evaluation of other climate variables such as air pressure, humidity, dew point,

and wind speed will enrich our understanding of how these complex microbial communities may respond to climate change, while also informing conservation and restoration efforts.

We sampled three different biocrust types and their subsurface soils in Joshua Tree National Park monthly for one year. We examined microbial (Archaea and Bacteria) composition using high-throughput amplicon sequencing of the 16S rRNA marker. Our objective was to test if microbial communities varied on this monthly cycle and if this variation could be explained by changes in measured weather variables including atmospheric pressure, dew point, humidity, precipitation, temperature, and wind speed [21]. We hypothesized that: (i) biocrust microbial richness significantly changes throughout the year, in which weather conditions affect biocrust microbial richness distinctly depending on crust type. We predicted that increasing precipitation, pressure, humidity will positively affect overall biocrust bacterial species richness while increasing temperature and wind speed will negatively affect overall biocrust bacterial species richness; (ii) different microbial groups at varying taxonomic levels respond to weather differently, driving variation of microbial taxonomic composition over time; (iii) crust type is the strongest predictor for biocrust microbial diversity and composition. This work highlights the seasonal changes in bacterial communities. It further indicates that future studies of biocrust microorganisms need to consider collection time and seasonal effects when comparing biocrust microbial communities.

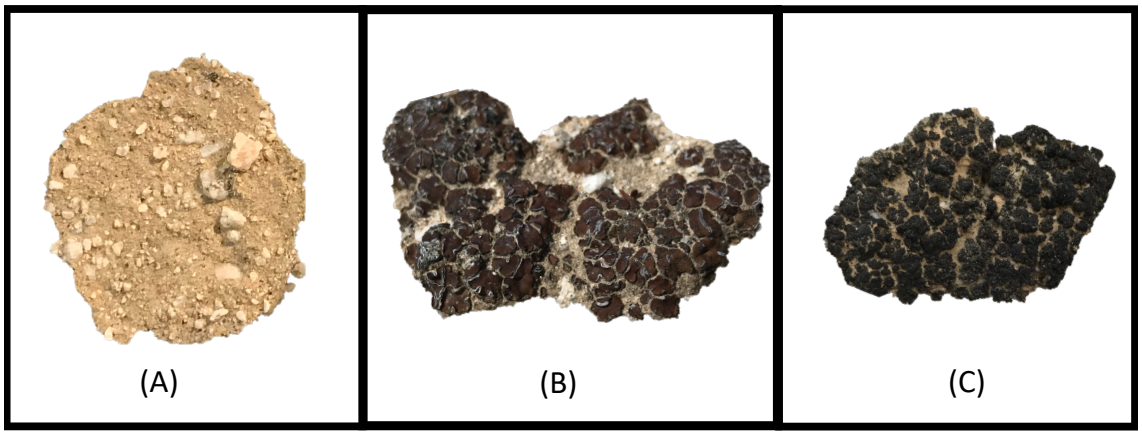

Figure 4.1: Three biocrust types were collected from Joshua Tree National Park. (A) Light algal/cyanobacterial crust (LAC); (B) Green algal lichen crust (GLC) and (C) Cyanobacteria lichen crust (CLC).

4.3 Materials and methods

4.3.1 Sampling site and biocrust sampling

Biocrust samples were collected from a relatively undisturbed site in the north-eastern section of Joshua Tree National Park (JTNP, GPS: 34.10N, -115.45W). Using sterile technique, three biocrust types including Light algal/cyanobacterial crust (LAC), Cyanobacteria lichen crust (CLC, *Collema* sp.), Green algal lichen crust (GLC, *Clavascidium lacinulatum*) were collected and underlying subsurface soil samples for each crust type were collected (Figure 4.1). Underlying subsurface soil samples for each crust type were collected separately. Additionally, bare unaggregated surface sand samples were also collected as a comparison substrate to biocrust and the underlying soil. Sample series were collected once each month for all 12 months of 2017 with 3 replicates for each biocrust type. Our sampling therefore yielded 21 samples each month: 3 LAC, 3 LAC subsurface, 3 CLC, 3 CLC subsurface, 3 GLC, 3 GLC subsurface, and 3 sand samples for a total of 252

samples for the entire study. Samples were kept on ice and transferred to a -80°C freezer at University of California Riverside.

Monthly weather data from the area were obtained from NOAA National Centers for Environmental Information (Lawrimore et al. 2016) [21]: atmospheric pressure, dew point, humidity, precipitation, temperature, and wind speed as recorded at the nearest weather station (GHCND:USR0000CRIC) in Rice Valley California. We used the reported monthly averages for all data except total precipitation which was the accumulated value for the month.

4.3.2 Amplicon sequencing library preparation and data processing

Total DNA was extracted from 0.15g of each soil and sand sample using the QIAGEN DNeasy PowerSoil kit (Qiagen, Germantown, MD, USA) following the manufacturer's standard protocol. Polymerase Chain Reaction was performed on the DNA samples to amplify the 16S rRNA V4 gene region using 515F and 806R primers following the Earth Microbiome protocol [22] (https://www.protocols.io/view/emp-16s-illumina-amplicon-protocol-nuudeww). PCR reactions were performed in 25 μl total volume with three replicates per sample, with 1 μl of genomic DNA, 1 μl of each primer (10 μM), 9.5 μl of nuclease-free water (Sigma-Aldrich, St.Louis, MO, USA), and 12.5 μl of Taq 2X DNA Polymerase (Thermo Fisher Scientific Inc., Waltham, MA, USA). PCR conditions consisted of (i) initial denaturation at 93°C for 3 min; (ii) 35 cycles of denaturation at 95°C for 45 sec, (iii) annealing at 50°C for 1 min, (iv) extension at 72°C for 90 sec, and (v) final extension at 72°C for 10 min using a C1000 thermal cycler (BioRad, Hercules, CA, USA). Three replicates of PCR products were combined, purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-

Nagel, Hoerdt, France) and pooled with per sample volumes selected to produce a mixture with equimolar concentration of each sample. The pooled amplicon concentrations were quantified using Qubit dsDNA HS Assay (Life Technologies, Carlsbad, CA, USA) and further analyzed using Agilent 2100 Bioanalyzer and Fragment Analyzer (Agilent Technologies, Santa Clara, CA, USA). The pooled amplicon libraries were sequenced using Illumina MiSeq system with MiSeq Reagent V3 kit (San Diego, CA), a 2×300 paired-end read format, at the Institute for Integrative Genome Biology, Core Facilities, University of California, Riverside (http://iigb.ucr.edu). A total of 28,421,030 demultiplexed paired end sequence reads were generated and submitted to the Sequence Read Archive (SRA) databases associated with BioProject accession number PRJNA625283.

4.3.3 Bioinformatics

The 16S V4 amplicon sequences were analyzed with AMPtk: the Amplicon Toolkit for NGS data (formally UFITS) (https://github.com/nextgenusfs/amptk) [23]. Demultiplexed paired-end sequences data were pre-processed by trimming forward and reverse reads to a maximum of 250 bp, trimming primer sequences and discarding reads less than 100 bp in length. The paired-end reads were then merged to produce single long overlapping reads using USEARCH v9.1.13 [24]. Sequence quality filtering was performed with the expected error parameter of 0.9 [25], which produced 25,299,858 quality filtered reads. This cleaned sequenced dataset was denoised using UNOISE3 algorithm [26] which generated 45,217 Amplicon Sequence Variants (ASVs) following the procedure of Palmer et al. 2018 [23]. Chimeric ASVs, sequences produced from PCR amplification of templates or parent sequences, were filtered using VSEARCH (v 2.3.2) [27], which removed 4,430

chimeras after comparison to the database. Quantitative Insights Into Microbial Ecology version 2 (QIIME2 v2019.1) [28] was applied using bacterial 16S processing workflows at taxonomy classification step employing the q2-feature-classifier [29], with extracted 515-806 SILVA database version 132 [30] based on ASV table and associated sequences following published protocols [28–30]. Mitochondria and chloroplast sequences were removed from the dataset resulting in 40,789 ASVs.

4.3.4 Data analysis

Filtered microbial ASVs alpha diversity (observed ASVs), beta diversity, and taxonomic composition were analyzed using the Phyloseq package [31] in RStudio version 1.1.463 [32] and R version 3.5.1 [33]. Variances of alpha diversity data were verified for homoscedasticity in R with Levene's test, Bartlett's test and Hartley's Fmax test [34]. Homoscedasticity data (alpha diversity by month) were analyzed with ANOVA using the 'Anova' function and pairwise multiple comparison (Tukey test) was conducted using the 'TukeyHSD' function in R. Unbalanced design comparison among months was accounted for using a type = 'III' ANOVA. Linear regressions between alpha diversity and weather factors were performed using the 'lm' function in R. PERMANOVA with the 'adonis' function in the 'vegan' package in R (Unifrac distance with permutation - 999) was used to compare beta diversity [35].

4.4 Results

4.4.1 Does biocrust microbial species richness change throughout the year? How do weather patterns affect richness?

Significant differences for bacterial and archaeal richness among twelve months were detected in our alpha diversity analysis of LAC (ANOVA, F(11,23) = 3.0947, p = 0.0107, Fig. S1A, Supplemental materials). In LAC, microbial species richness changes throughout the year with lowest values in April and highest values in October with seasonal pattern of low species richness in the spring and high species richness in the summer (Fig S1A). In general, a similar pattern of species richness variation was observed across all biocrust types in which species richness was high in late summer months. Significant species richness variations were also observed in GLC (ANOVA, F(11,23) = 4.8622, p = 0.0006835, Fig. 4.2A) and sand samples (ANOVA, F(10,21) = 3.1664, p = 0.01157, Fig. S2A), while CLC alpha diversity changes showed no significant differences throughout the year (ANOVA, F(11,24) = 1.4757, p = 0.2048, Fig. S3A). In contrast, subsurface samples from underneath LAC and CLC showed no significant differences throughout the year while GLC subsurface species richness showed significant changes in which species richness was highest in September and October while the rest of the year were similar (ANOVA, F(11,24) = 7.1538, p = 3.049e-05).

Linear regression analysis of microbial species richness based on weather conditions showed different correlation patterns among three crust types and sand samples. In LAC, species richness was positively correlated with previous month's dew point (p = 0.002, R^2 = 0.229, Fig. S1C) and previous month's temperature (p = 0.072, R^2 = 0.067, Fig. S1B)

while it was negatively correlated with wind speed ($p = 0.052$, $R^2 = 0.099$, Fig. S1D) (Table 4.1). In CLC, species richness was positively correlated with humidity ($p = 0.031$, $R^2 = 0.104$) and precipitation ($p = 0.006$, $R^2 = 0.175$) (Fig. S3B-E, Table 4.1). In GLC, species richness was positively correlated with temperature ($p = 0.009$, $R^2 = 0.163$, Fig. 4.2B), previous month's temperature ($p = 0.013$, $R^2 = 0.149$, Fig. 4.2C), dew point ($p = 0.001$, $R^2 = 0.248$) and previous month's dew point ($p = 0.0063$, $R^2 = 0.217$, Fig. 4.2D), while negatively correlating with atmospheric pressure ($p = 0.003$, $R^2 = 0.212$, Fig. 4.2E) (Table 4.1). In sand samples, species richness was positively correlated with precipitation ($p = 0.01$, $R^2 = 0.168$) (Fig. S2B, Table 4.1). Weather conditions also affected microbial species richness underneath biocrusts. In LAC subsurface soil, increasing species richness negatively correlated with wind speed ($p = 0.032$, $R^2 = 0.111$) while increasing species richness positively correlated with dew point ($p = 0.027$, $R^2 = 0.12$) and humidity ($p = 0.0496$, $R^2 = 0.09$) (Table S1). In CLC subsurface soil, increasing species richness positively correlated with previous month's dew point ($p = 0.0214$, $R^2 = 0.136$) (Table S1). Lastly, increasing GLC subsurface soil species richness was positively correlated with dew point ($p = 0.0399$, $R^2 = 0.092$) and previous month's dew point ($p = 0.009$, $R^2 = 0.161$) (Table S1).

	Sand	LAC	CLC	GLC
1. Temperature	no effect	no effect	no effect	Positive
2. Previous month temperature	no effect	Positive	no effect	Positive
3. Dew point	no effect	no effect	no effect	Positive
4. Previous month dew point	no effect	Positive	no effect	Positive
5. Humidity	no effect	no effect	Positive	no effect
6. Previous month humidity	no effect	no effect	no effect	no effect
7. Wind speed	no effect	Negative	no effect	no effect
8. Previous month wind speed	no effect	no effect	no effect	no effect
9. Pressure	no effect	no effect	no effect	Negative
10. Previous month pressure	no effect	no effect	no effect	Negative
11. Precipitation	Positive	no effect	Positive	no effect
12. Previous month precipitation	no effect	no effect	Positive	no effect

Table 4.1: Weather effects on overall alpha diversity of three biocrust types and sand samples. Negative = negative linear relationship (eg. high wind speed relates to low richness), Positive = positive linear relationship (eg. high wind speed relates to high richness) and no effect = no linear relationship between weather and richness

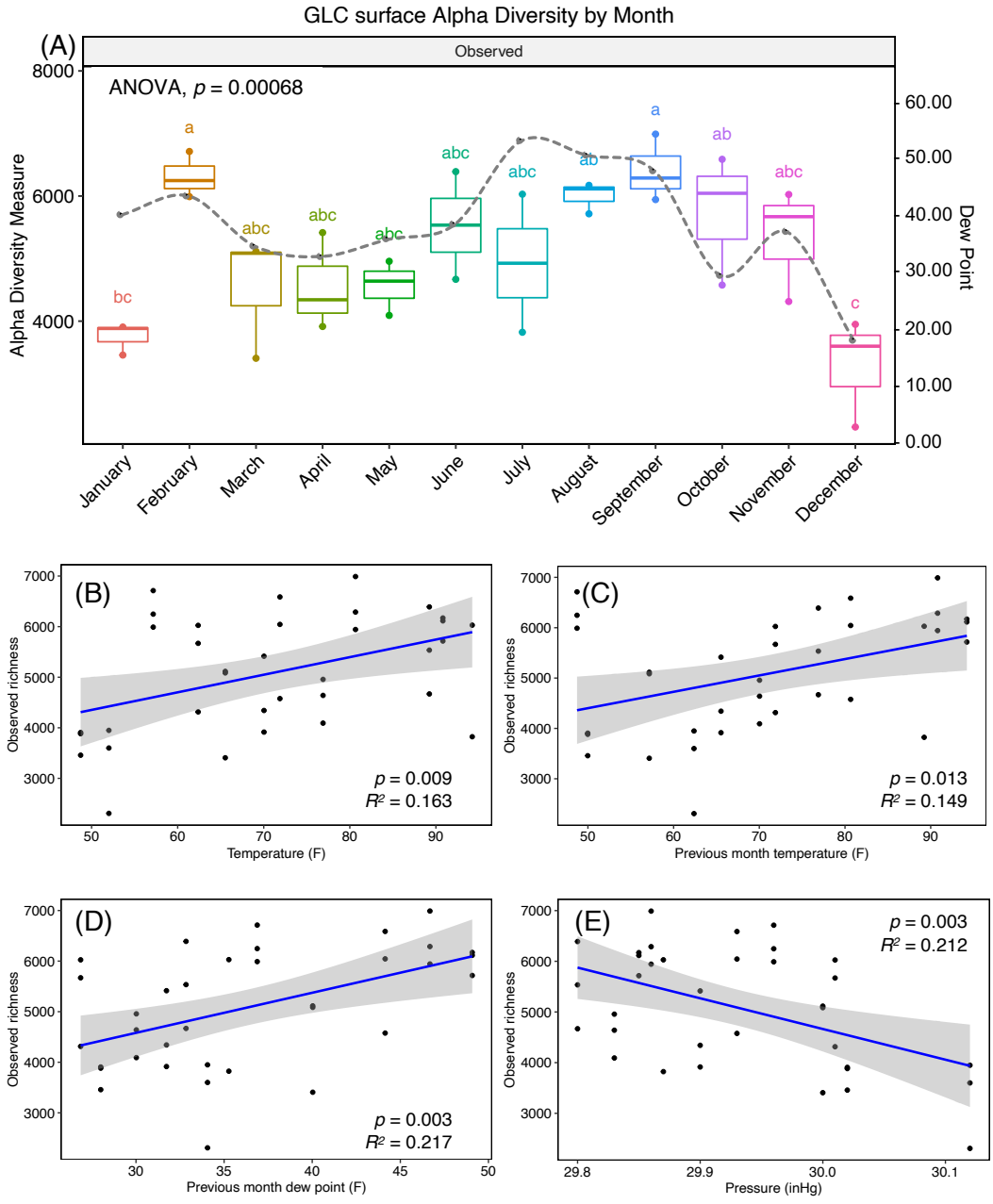

Figure 4.2: Box plots showing alpha diversity as ASV richness in different months and linear regression relationships between observed ASV richness and weather data. (A) Bacterial alpha diversity of JTNP green algal lichen biocrust (GLC) samples by month. Strong correlation between GLC species richness and dew point was observed. Boxplots show 25th and 75th percentile with the median shown as a line inside the box. Error bars show 1st and 99th percentile. Tukey HSD significant differences (p <0.05) are indicated by different letters. Linear regression analyses of bacterial ASV richness in relation to (B) temperature; (C) previous month's temperature; (D) previous month's dew point and (E) atmospheric pressure. 95% confidence interval is shown in grey along regression lines.

4.4.2 Does biocrust bacterial taxonomic composition differ over time?

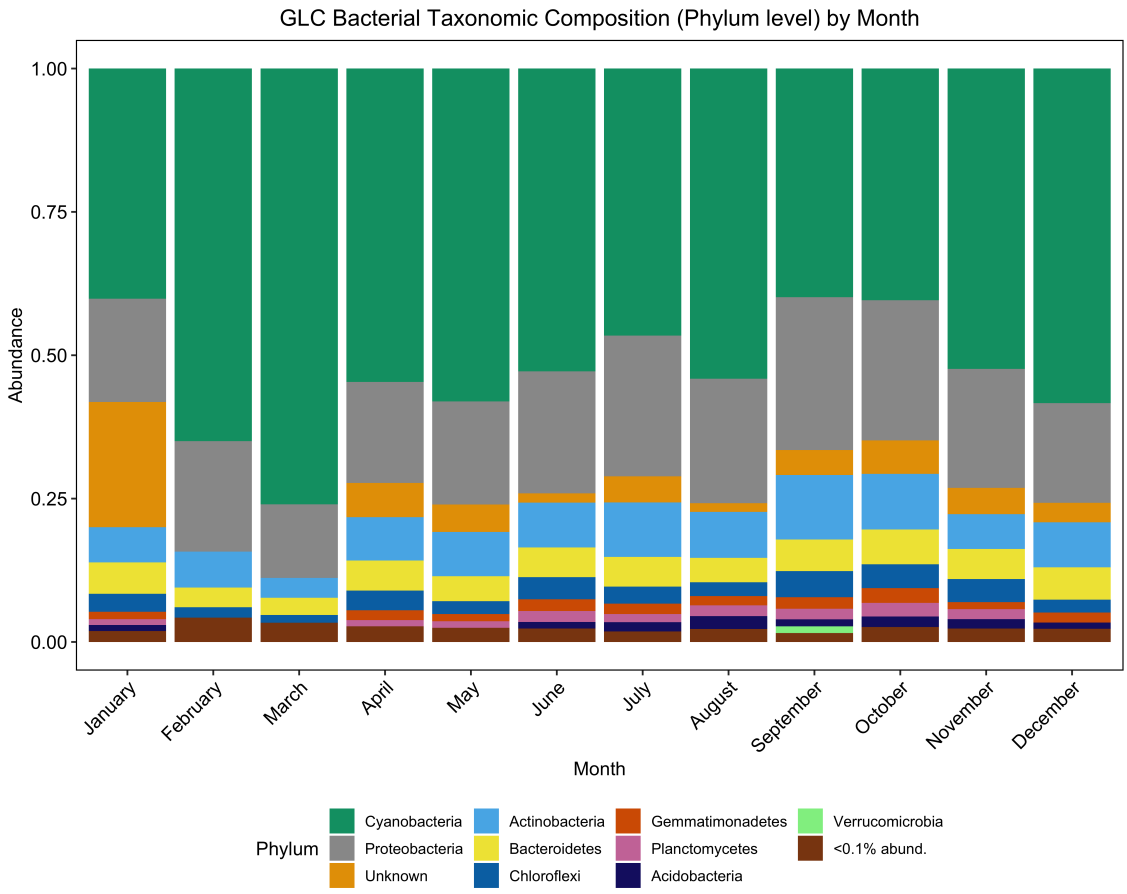

Figure 4.3: GLC bacterial taxonomic bar plots at phylum level by month. 20 bacterial phylum variations were observed in GLC samples throughout the year. Major bacterial phyla included Cyanobacteria, Proteobacteria, Actinobacteria and unknown bacteria.

We aimed to understand which microorganisms changed over time. Microbial taxa variation for each crust type can be observed in the relative abundance values in the taxonomic composition bar plots among prokaryotic phyla. Although major bacterial phyla including Cyanobacteria, Proteobacteria, Actinobacteria, Bacteroidetes, Acidobacteria, and Chloroflexi were found in all crust types throughout the year, distinct differences

can be seen in the relative abundance bar plots (Fig. 4.3, S4, S5, S6). In LAC, five phyla showed significant differences over time (ANOVA, p <0.05); Bacteroidetes, Deinococcus-Thermus, Firmicutes, Planctomycetes, and Proteobacteria. Seven phyla in GLC showed significant differences over twelve months (ANOVA, p <0.05); Actinobacteria, Firmicutes, Patescibacteria, Planctomycetes, Proteobacteria (Fig. 4.4), Chloroflexi, and Gemmatimonadetes. While overall species richness in CLC did not show significant changes, significant differences (ANOVA, p <0.05) did occur over time within phyla Acidobacteria, Deinococcus-Thermus, Euryarchaeota, Gemmatimonadetes, Patescibacteria, and Thaumarchaeota. In sand samples, six phyla showed significant differences over time (generally more abundant in late summer) (ANOVA, p <0.05); Cyanobacteria, FBP, Planctomycetes, Proteobacteria, Thaumarchaeota, and Verrucomicrobia.

Similar changes were also observed in subsurface samples. In LAC subsurface soil, three phyla showed significant differences over time (ANOVA, p <0.05); Armatimonadetes, Patescibacteria, and Thaumarchaeota. In GLC subsurface soil, fifteen phyla showed significant differences over time (ANOVA, p <0.05); Acidobacteria, Actinobcateria, Armatimonadetes, Bacteroidetes, Chloroflexi, Deinococcus-Thermus, Euryarchaeota, FBP, Gemmatimonadetes, Nanoarchaeota, Patescibacteria, Planctomycetes, Proteobacteria, Thaumarchaeota, and Verrucomicrobia. Lastly, in CLC subsurface soil, six phyla changed significantly in richness over time (ANOVA, p <0.05); Armatimonadetes, Chloroflexi, Entotheonellaeota, Euryarchaeota, Planctomycetes, and Thaumarchaeota.

In summary, prokaryotic taxonomic composition changed over time in biocrust and in soil underneath. Firmicutes richness changed significantly over time in surface samples

only, while Armatimonadetes and Entotheonellaeota richness changed significantly over time in subsurface soil only.

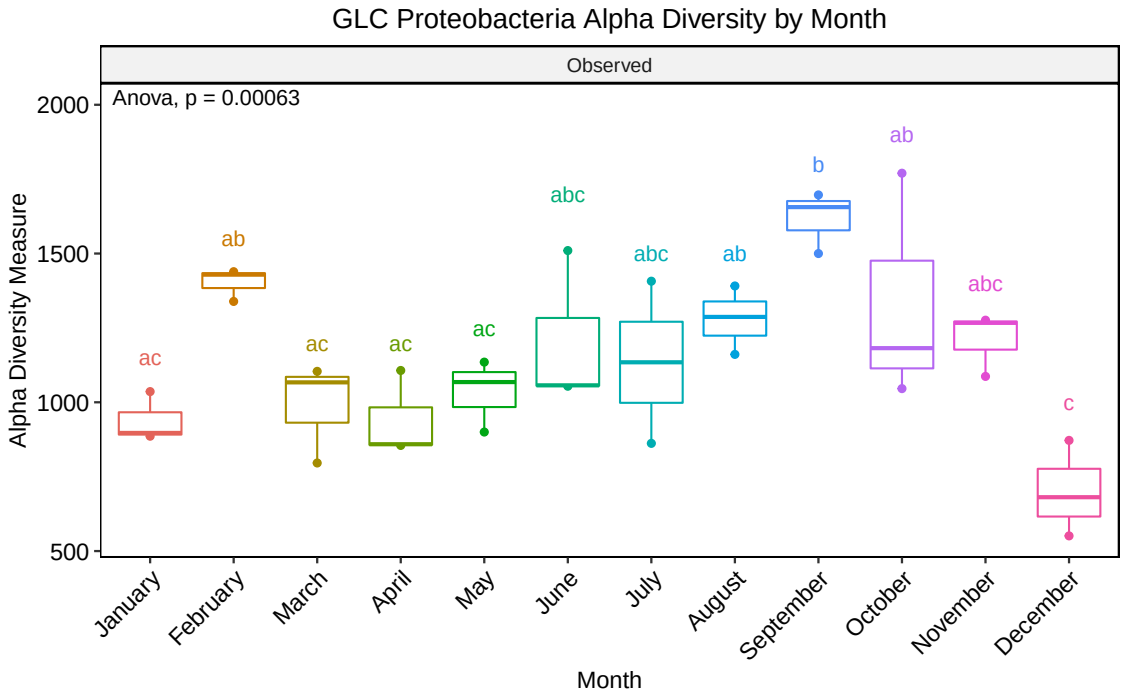

Figure 4.4: Proteobacteria showed significant variation in species richness throughout the year. Additionally, species richness in GLC differed significantly by month in six other bacterial phyla (Actinobacteria, Firmicutes, Patescibacteria, Planctomycetes, Chloroflexi, and Gemmatimonadetes).

For each phylum with more than 1% abundance and/or significant change over twelve months in each crust type, linear regression analysis of species richness on weather conditions was performed and significant linear relationships (p <0.05) were reported (Table S2 with completed details and description). In LAC, Deinococcus-Thermus species richness positively correlated with temperature, 5 phyla species richness positively correlate with dew point, 3 phyla species richness positively correlated with previous month dew point, Bacteroidetes species richness positively correlated with humidity, Firmicutes species rich-

ness positively correlated with wind speed while negatively correlating with Cyanobacteria, Deinococcus-Thermus species richness negatively correlated with atmospheric pressure and previous month atmospheric pressure, and 2 phyla positively correlated with precipitation (Table 4.2 and S2). In CLC, 3 phyla species richness positively correlated with temperature and previous month temperature, Deinococcus-Thermus species richness positively correlated with dew point, Cyanobacteria species richness positively correlated with Cyanobacteria, 9 phyla species richness positively correlated with humidity, Bacteroidetes species richness negatively correlated with previous month atmospheric pressure, and 9 phyla positively correlated with precipitation and previous month precipitation (Table 4.2 and S2). In GLC, 6 phyla species richness positively correlated with temperature, 7 phyla species richness positively correlated with previous month temperature, 9 phyla species richness positively correlated with dew point, 6 phyla species richness positively correlated with previous month dew point, Patescibacteria species richness positively correlated with humidity and previous month humidity, Firmicutes species richness negatively correlated with previous month humidity, Acidobacteria species richness negatively correlated with wind speed, 8 phyla species richness negatively correlated with atmospheric pressure, 6 phyla species richness negatively correlated with previous month pressure, Patescibacteria species richness positively correlated with precipitation and previous month precipitation, and Firmicutes species richness negatively correlated with previous month precipitation (Table 4.2 and S2).

	LAC	CLC	GLC
1. Temperature	1 phylum	3 phyla	6 phyla
2. Previous month temperature	none	3 phyla	7 phyla
3. Dew point	5 phyla	1 phylum	9 phyla
4. Previous month dew point	3 phyla	1 phylum	6 phyla
5. Humidity	1 phylum	9 phyla	1 phylum
6. Previous month humidity	none	none	1 phylum 1 phylum
7. Wind speed	1 phylum 1 phylum	none	1 phylum
8. Previous month wind speed	none	none	none
9. Pressure	1 phylum	none	8 phyla
10.Previous month pressure	1 phylum	1 phylum	6 phyla
11.Precipitation	2 phyla	9 phyla	1 phylum
12.Previous month precipitation	none	9 phyla	1 phylum 1 phylum

Table 4.2: Summary of weather effects on bacterial richness at phylum level. RED = negative linear relationship (eg. high wind speed relates to low bacterial richness), BLUE = positive linear relationship (eg. high wind speed relates to high bacterial richness) and no effect = no linear relationship between weather and bacterial richness

To specifically identify bacterial ASVs that responded to weather, linear regression analysis of the 50 most abundant ASVs for each crust type was performed. Identifiable top ASVs (referred to by their closest related taxon) in LAC that responded to weather included; increasing *Microcoleus* sp. abundance positively correlated with precipitation, previous month's precipitation and atmospheric pressure but negatively correlated with dew point and temperature; increasing *Trichocoleus* sp. abundance positively correlated with previous month's dew point; increasing *Tychonema* sp. abundance positively correlated with previous month's humidity; increasing *Microvirga* sp. abundance positively correlated with humidity, previous month's humidity, precipitation, previous month's precipitation but negatively correlated wind speed; increasing *Geodermatophilus* sp. abundance positively correlated with dew point but negatively correlated with wind speed; increasing *Arthrobacter* sp. abundance positively correlated with humidity, precipitation, previous month's precipitation, and increasing *Rubellimicorbium* sp. abundance positively correlated with previous month's dew point but negatively correlated with atmospheric pressure.

Identifiable top ASVs (referred to by their closest related taxon) in CLC that responded to weather included; increasing *Microvirga* sp. abundance positively correlated with dew point, previous month dew's point, temperature and previous month's temperature but negatively correlated with humidity, previous month's humidity, pressure, previous month's atmospheric pressure, and previous month's precipitation; increasing *Microcoleus* sp. abundance positively correlated with previous month's wind speed but negatively correlated with atmospheric pressure; increasing *Microcoleus paludosus* abundance positively correlated with previous month's humidity; increasing *Symplocastrum* sp. abundance posi-

tively correlated with previous month's dew point; increasing *Trichocoleus* sp. abundance positively correlated with previous month's wind speed; increasing *Rubellimicrobium* sp. abundance positively correlated with previous month's dew point, temperature and previous month's temperature but negatively correlated with humidity, precipitation, previous month's atmospheric pressure; and increasing *Geodermatophilus* sp. abundance positively correlated with previous month's dew point and previous month's temperature but negatively correlated with previous month's precipitation, previous month's atmospheric pressure.

Lastly, identifiable top ASVs (referred to by their closest related taxon) in GLC that responded to weather included; increasing *Microvirga* sp. abundance positively correlated with dew point, previous month's dew point, temperature and previous month's temperature but negatively correlated with atmospheric pressure, previous month's atmospheric pressure; increasing *Microcoleus* sp. abundance positively correlated with humidity, previous month's humidity, and previous month wind speed but negatively correlated with atmospheric pressure; increasing *Nostoc* sp. abundance positively correlated with previous month's dew point and previous month's temperature; increasing *Mastigocladopsis* sp. abundance positively correlated with previous month's humidity and wind speed but negatively correlated with previous month's temperature; increasing *Trichocoleus* sp. abundance positively correlated with temperature; increasing *Geodermatophilus* sp. abundance positively correlated with dew point, previous month's dew point and previous month's temperature but negatively correlated with atmospheric pressure and previous month's atmospheric pressure; *Rubellimicrobium* sp. abundance positively correlated with previous

month's dew point; and increasing *Rubrobacter* sp. abundance positively correlated with previous month's dew point.

4.4.3 Which characteristics influence overall biocrust bacterial beta diversity? Do weather conditions contribute to bacterial beta diversity patterns?

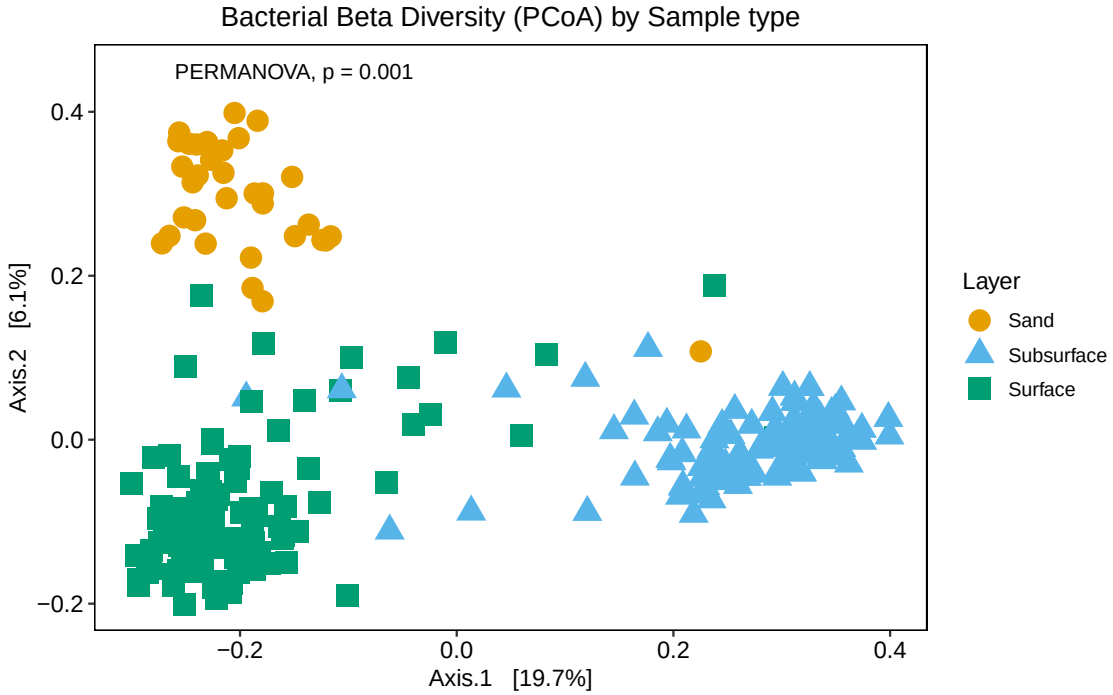

Figure 4.5: Beta diversity analysis of biocrust bacterial communities by sample type using PCoA. Significant differences (PERMANOVA; p <0.05) were shown on PCoA plot.

Beta diversity analysis of overall bacterial and archaeal communities differed significantly by sample type (PERMANOVA, $p = 0.001$, $R^2 = 0.22$) (Fig. 4.5). These differences in microbial beta diversity were visualized in principal coordinate analysis (PCoA) plots, which showed that microbial communities strongly clustered by sample types. Biocrusts

(surface samples) clearly separated from soil underneath biocrust (subsurface samples) and sand samples. To visualize biocrust microbial communities for biocrusts types (surface samples only), a subset of samples including LAC, CLC, and GLC were used in beta diversity analysis which revealed a significant crust type pattern: LAC bacterial composition clustered separately from lichen crusts bacterial communities (CLC and GLC) (PERMANOVA, $p = 0.001$, $R^2 = 0.064$) (Fig. 4.6).

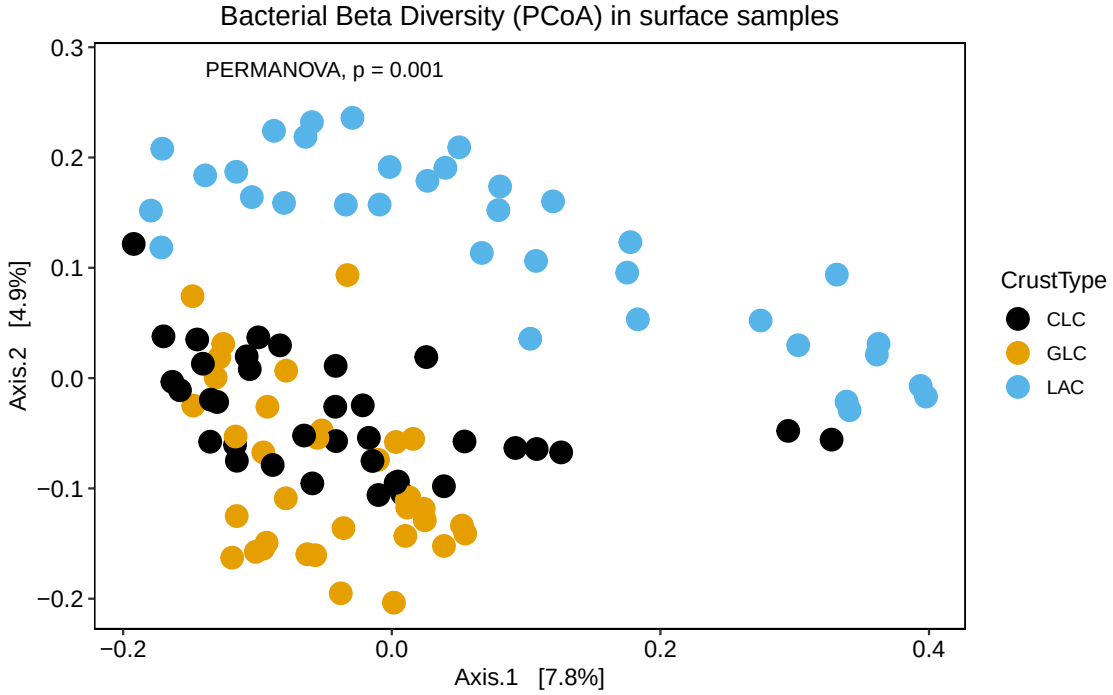

Figure 4.6: Beta diversity analysis of biocrust bacterial communities in surface samples (biocrust only) using PCoA. Significant differences (PERMANOVA; p <0.05) were shown on the PCoA plot.

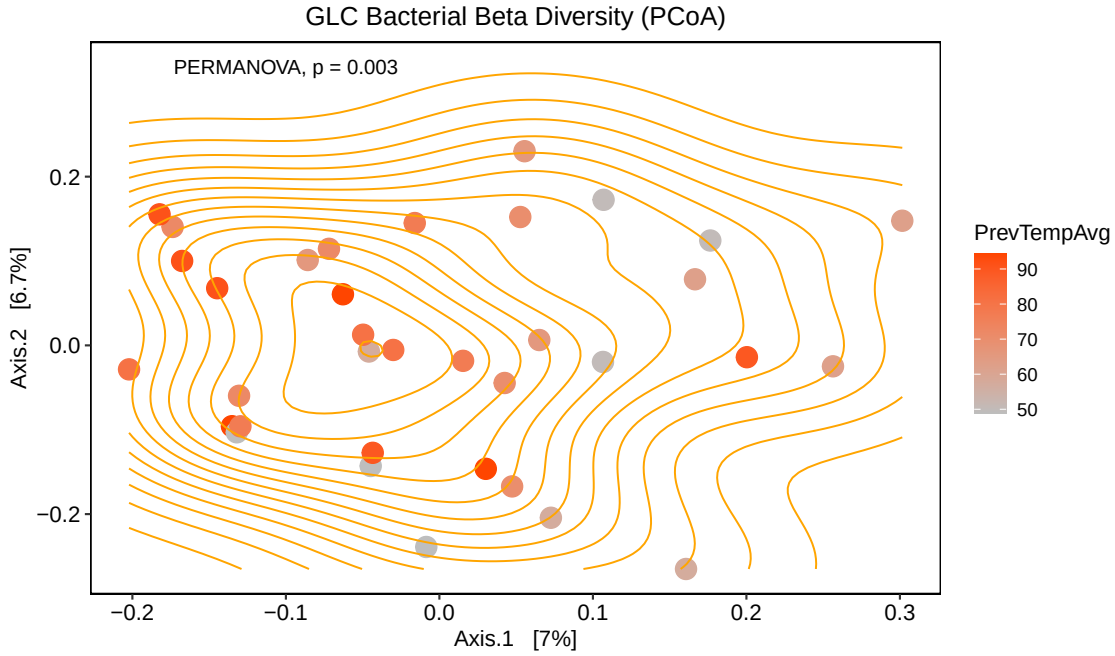

Figure 4.7: GLC Bacterial Beta diversity analysis with density estimation using PCoA. Gradient colors indicate previous month's average temperature from lowest average temperature in the grey color to highest average temperature in orange-red. Density estimation lines show the relationship between GLC bacterial composition and previous month average temperature. Significant differences (PERMANOVA; p <0.05) were shown on the PCoA plot.

Biocrust microbial beta diversity analysis was also performed for each crust type to identify weather conditions that contributed to beta diversity patterns. LAC bacterial and archaeal communities beta diversity pattern was influenced by previous month average temperature (PERMANOVA, $p = 0.017$, $R^2 = 0.047$). CLC bacterial and archaeal communities beta diversity showed significant influence by previous month's average temperature (PERMANOVA, $p = 0.012$, $R^2 = 0.04$), dew point (PERMANOVA, $p = 0.023$, $R^2 = 0.039$), and previous month's atmospheric pressure (PERMANOVA, $p = 0.006$, $R^2 = 0.041$). Lastly, GLC bacterial and archaeal communities beta diversity showed that the

beta diversity pattern is significantly influenced by previous month's average temperature (PERMANOVA, $p = 0.003$, $R^2 = 0.039$) (Fig. 4.7). These differences in microbial beta diversity were visualized in principal coordinate analysis (PCoA) plots revealing the effect of temperature gradients on GLC microbial communities. In addition, GLC beta diversity also showed significant differences by monthly average temperature (PERMANOVA, $p = 0.008$, $R^2 = 0.043$), dew point (PERMANOVA, $p = 0.038$, $R^2 = 0.034$), average wind speed (PERMANOVA, $p = 0.032$, $R^2 = 0.034$), atmospheric pressure (PERMANOVA, $p = 0.047$, $R^2 = 0.033$), and precipitation (PERMANOVA, $p = 0.012$, $R^2 = 0.035$).

4.5 Discussion

Our findings support the prediction that biocrust bacterial and archeal communities at JTNP are dynamic and vary throughout the year. We identified weather conditions that influence microbial diversity changes including: (i) temperature, (ii) precipitation, (iii) wind speed, (iv) atmospheric pressure, (v) humidity, and (vi) dew point. Our results show that these temporal changes influence many bacterial phyla and affect some bacterial species differently, depending on crust type as we predicted. Lastly, crust type is a good predictor of its associated microbial communities and determines how these microorganisms respond to weather conditions. Our findings showed first quantifiable effects of weather conditions on biocrust microbial diversity which have previously been studied as isolated variables rather than combined factors.

4.5.1 Temperature effect

Temperature effect on biocrusts is frequently studied and discussed in the context of climate change [36]. Increasing the temperature by 2-4 °C significantly decreased biocrust cover for lichen and moss crust [37, 38]. Additionally, rising temperature also increases biocrust respiration [39] and carbon loss [40]. These studies motivated us to investigate the combined influence of temporal changes in an actual ecosystem throughout the year, which has not been explored to date. At JTNP, average temperatures range from 9°C in winter to 35°C in summer [21]. Biocrusts at JTNP experience seasonal and daily temperature differences which are much greater than a 2-4 °C increase. However, little is known about how these temperature changes affect biocrust microbial communities over time. Our findings showed that increasing previous month's average temperature positively affects overall bacterial and archaeal richness in LAC, which might be associated with previous studies that showed increasing cyanobacterial/light algal crust cover as temperature increased [37]. However, additional study is needed to link crust coverage and microbial community changes. While it has been reported that increasing temperature reduced lichen crust cover in Spain [38], our lichen data showed that increasing temperature had no effect on CLC bacterial species richness but interestingly increased bacterial richness in GLC suggesting that temperature affects bacterial richness differently depending on lichen species. We also found that some bacterial species rose in abundance showing that high temperatures allow certain species to dominate biocrust microbial communities though further research is crucial to identify whether increasing in abundance is due to growth or better persistent. Interestingly, *Microvirga* sp. which has been identified as a

biocrust microbial hub [6], became more abundant as temperature increases in both CLC and GLC. Similarly, abundance of biocrust pioneer photoautotrophs such as *Nostoc* sp. and *Trichocoleus* sp. [41–43] positively correlated with temperature, which might be associated with previous experiments that showed increasing cyanobacterial crust cover when temperature increased [37]. *Mastigocladopsis* sp. was shown to be an efficient inoculum for biocrust restoration [44], consistent with our finding that its abundance positively correlated with temperature. Taken together, these findings suggested that while high temperatures have different effects on overall bacterial richness and crust coverage depending on crust types, certain bacterial species possibly structure biocrust microbial communities because of their function as biocrust pioneer photoautotrophs, key microbes and microbial hubs.

4.5.2 Precipitation effect

Precipitation effect was another important variable repeatedly studied in the context of climate change and especially the significance of raining (wetting) events in biocrust activation. Precipitation in drylands is infrequent and oftentimes penetrates only the top centimeters of dryland surfaces, where biocrusts are present and microbes are activated [3]. Timing and amount of rain are key climatic determinants of biocrust distribution around the globe [16]. Individual microorganisms are known to be influenced by rain pattern, for example *Microcoleus vaginatus* are more abundant in cool desert with winter precipitation while *Microcoleus steenstrupii* prefer hot areas with summer rain [16]. Similar to the temperature effect, less is known about how rain events throughout the year influence overall microbial community structure. Our findings showed that precipitation affected bacterial communities differently depending on biocrust type. Although previous research has shown

that cyanobacteria were the major bacterial group that responded to precipitation manipulation [18, 45], overall cyanobacterial abundance in our biocrust samples did not change significantly with precipitation over the 12 months sampled at JTNP. However, *Microcoleus* sp., which has been identified as a key microbe and a microbial hub of biocrust microorganisms [1, 6], became more abundant as precipitation increased only in LAC. No precipitation effect on *Microcoleus* sp. was observed in both lichen crust types. *Microcoleus* sp. was the only abundant cyanobacterial species that positively correlated with increasing precipitation. These findings suggest that cyanobacterial communities at UT, USA [18, 45] likely reacted to precipitation differently from cyanobacteria in biocrusts from JTNP, CA, USA likely because manipulated (UT, USA) and natural (CA, USA) precipitation events may have distinct effects on biocrust cyanobacterial communities. In addition, we also found that *Geodermatophilus* sp. (Actinobacteria) abundance in CLC was positively correlated with reducing precipitation, which supports previous research showing that some species in this genus were xerophilic and dryland soil dwelling [46] and were also abundant in other areas [47, 48]. However, the functional roles of *Geodermatophilus* sp. in biocrusts are still unclear and need to be explored further.

4.5.3 Wind speed effect

Increasing Wind speed negatively impacts overall bacterial and archaeal richness in LAC samples, while overall microbial richness in both lichen crusts (CLC and GLC) is not affected Our findings reinforce biocrust functions in drylands, which stabilize soil surfaces and prevent soil erosion [49]. CLC and GLC microbial richness are not affected by wind possibly due to their more well-developed stable crust aggregate structure and thicker

crust than LAC which was supported by previous studies showing that fungal crusts are more resistant to wind than photoautotrophic (Cyanobacterial and algal) crusts [19]. We also investigated bacterial and archaeal species which were strongly influenced by wind and showed that some of the top bacterial species were affected by wind depending on crust type. In previous research, it has been reported that *Microcoleus vaginatus* and *Nostoc* sp., which are normally seen at early biocrust developmental stages, can be resistant to wind erosion when they grow and build up enough biomass [50]. Our findings supported previous research and revealed that *Microcoleus* sp., *Mastigocladopsis* sp. and *Trichocoleus* sp. were abundant at relatively high wind speed confirming that biomass of these bacterial species in CLC and GLC were enough to withstand wind erosion and reproduce at high wind speed likely because of their ability to produce sticky exopolysaccharide. Moreover, lichens are the main structural components of CLC and GLC which potentially help protect these species from wind erosion. In addition, none of these cyanobacterial species were negatively affected by wind speed which indicated that they were able to withstand wind erosion and also confirmed that they were essential components and stable in all stages of biocrust formation. Taken together that *Mastigocladopsis* sp. was shown to be an efficient inoculum for biocrust restoration [44] and observing its abundance increases at relatively high wind speed and high temperature, *Mastigocladopsis* sp. could be another key cyanobacteria to be considered for future biocrust restoration development in an area with wind erosion and high temperature issues. The effects of wind speed on other microbial species, especially non-photoautotrophs, are comparatively limited. In our study, *Microvirga* sp. (Proteobacteria) and *Geodermatophilus* sp. (Actinobacteria) are the only two species in which abundance

was reduced with increasing wind speed. *Microvirga* sp. was previously identified as a key microbial hub in biocrust [6], but little is known about its functional roles in biocrusts.

4.5.4 Atmospheric pressure effect

To the best of our knowledge, this study is the first paper reporting on correlations between atmospheric pressure and biocrust microorganismal abundances. Interestingly, low atmospheric pressure is an essential research area for space habitats such as Mars [51] where microbial survival in low atmospheric pressure and harsh conditions are crucial. Within GLC, our findings indicated that bacterial richness was greater in relatively lower atmospheric pressure suggesting that certain structural support in chlorolichen could help microbes to survive in relatively low atmospheric pressures. Although atmospheric pressure changes at JTNP seem very small and overall bacterial richness of LAC and CLC were not affected, microorganisms are microscopic and slight changes throughout the year might have a substantial impact on individual microbes. For example, *Microvirga* sp. was also able to survive better at lower atmospheric pressure in both CLC and GLC. This indicates that its lifestyle was suitable for harsh condition and did not necessarily follow similar patterns as the overall bacterial communities. In addition, *Microcoleus* sp., a key biocrust pioneer microbe in Mojave and Colorado Desert soils [1] was also found to be more abundant at low atmospheric pressure in GLC but low abundance in LAC under the same condition suggesting that GLC structural support was crucial for both key species and overall bacterial communities. These findings suggested that atmospheric pressure is potentially another important weather factor, which needs to be taken into account for biocrust microorganisms

investigation and may lead us into a new area of biocrust research which has not been explored in the past.

4.5.5 Humidity effect

Humidity can activate green algal lichens biocrust, but authors also suggested that these laboratory results may not have ecological significance [52, 53]. However, it has also been noted that humidity is one of abiotic environmental factors that we have a very limited number of literature, especially in the context of humidity effects on biocrust organisms and their interactions [20]. Therefore, humidity was one of the factors that we focused on to determine the relationship of biocrust microorganisms and humidity. Our findings showed that increasing humidity positively influences CLC microbial community richness but did not have any effect on other crust types. Although we expected that GLC would respond to humidity while humidity will have no effect on CLC as reported in previous studies [52, 53], our results suggested that lichen activation by humidity are likely different from humidity effect on lichen crust microbial communities. Therefore, an additional study is needed to directly compare humidity effects in the field and laboratory experiment. In order to better understand biocrust microorganisms, we have identified top abundant bacterial species and interestingly, *Microvirga* sp. abundance negatively correlated with increasing humidity in CLC, which indicated that its abundance increased as humidity decreased. *Microvirga* sp. has been identified as a biocrust microbial hub [6] which was able to become more abundant at relatively high temperatures and our findings just showed that it is also abundant at low humidity in CLC. Taking these factors into account, *Microvirga* sp. could be a very

important bacterial species for biocrusts because high temperature and low humidity are the key characteristics of drylands yet *Microvirga* sp. was able to thrive in these conditions.

4.5.6 Dew point effect

While dew may be another significant water source for dryland biocrusts [54–56], recent observations and interpretations suggest its effects on different biocrust types can vary widely. If dew and other non-rainfall water hydrates crusts sufficiently for multiple hours during daylight, net carbon gains occur through photosynthesis by the crusts' autotrophs, whereas shorter hydration times or insufficient illumination will cause carbon deficits instead due to respiration and activation costs. Moreover, dew formation on biocrust surfaces and the subsequent retention of absorbed water are both influenced by a wide range of abiotic factors and processes such as dust deposition, burial by shifting sand, surface meso- and microtopography, synergies with preceding or subsequent rainfall, and other confounding influences (see e.g. [57]). The possible relationships between dew point and biocrust distribution/composition are probably just as complex. In a microclimate study of late summer and autumn conditions in a loess valley of the Negev Highlands, Kidron and Kronenfeld (2020a) report that dew formed frequently on cobble surfaces, whose night temperature often dropped below the dew point [58]. However, soil surface temperatures only rarely dropped to dew point level during these same nights, and the authors concluded that dew was much less important for growth of the study site's soil crusts than for its rock lichens at their study site. Moreover, Kidron and Kronenfeld (2020b) found that dew is even less likely to benefit soil crusts in Spain's Tabernas Desert, because its atmospheric relative humidity remains lower than at the Negev site, while night temperature of its soil surface never

dropped to dew point [59]. The scepticism of both authors about the importance of dew to soil crusts also derives from indications that the accuracy of microlysimeter methodologies for measuring dew quantities on soil surfaces is questionable, causing likely overestimations in previous field studies [58]. Their inferences may be premature, however, because they did not take into account other mechanisms of non-rainfall surface moisture deposition besides condensation. An example is vapor adsorption to the bare soil surface, an elusive and poorly understood process that appears to be less dependent on temperature and is thought to be a significant source of surface hydration for moss biocrusts in a sandy study site in the Tennger Desert [57].

Our findings show that average previous month dew point values positively correlated with overall bacterial richness in LAC and GLC, but not in CLC. While we have no direct data on dew formation at our study site, we consider it likely that non-rainfall water does occur in biologically useful amounts, as the sampled soil surface textures consist mainly of sand and granitic gravels and thus are likely to cool more at night than loess surfaces would. Alternatively, it is possible that dew point correlates well with biocrust microbiome diversity in LAC and GLC for less direct reasons: as a variable that specifies condensation thresholds of atmospheric humidity and temperature at solid surfaces, it integrates multiple other interacting factors of a complex physico-biological system in ways that may best capture multifactorial relevances of those other factors to biocrust functioning - even if dew frequency and quantity might not necessarily be key determinants for soil lichen crusts to the same degree as is true for rock lichens.

Although lichen crusts and cyanobacterial/light algal crust have been reported to retain dew deposition at the same level [55], two lichen crusts in our studies exhibited different patterns. These findings indicated that the effects of dew deposition and the correlation with dew point might regulate different pathways and subsequently influence different biocrust organisms as soil lichens at our study. While Chlorolichen (GLC) can be activated by atmospheric humidity and cyanolichen (CLC) are known to depend more strictly on availability of water in liquid form [52, 53], based on our data there may be other mechanisms of lichen crusts found in JTNP possibly contributed to the contrasting findings discussed above. Nonetheless, future biocrust studies that will include these confounding effects will greatly improve our understanding in this system.

4.5.7 Biocrust type effect

Lastly, biocrust types are normally classified by their morphology and their primary photoautotrophs (cyanobacteria, lichen, moss etc.) and sometimes by their developmental/successional stages (early, mid, and late stage) [3, 4, 7], which has been reported to determine microbial communities [4, 6, 7]. In our previous study, we have shown that biocrust microbial communities collected from four different locations in the Mojave Desert were strongly influenced by biocrust types, however, we indicated that more extensive sampling of crust types at a specific site will broaden our understanding of local microbial communities [6]. In this study, we collected three crust types with three replicates in each month throughout the year and our findings supported the crust type pattern as reported in our previous study. Nonetheless, we focused on how weather conditions affect biocrust microbial communities specifically at JTNP, which showed that biocrust communities were

not only determined by crust type but also responded to weather conditions differently depending on crust type. When we investigated further at each phylum and species, changes in species richness were observed in all crust types. However, our finding confirmed that it is unquestionable that GLC and CLC are different in both microbial make up and responses to weather, therefore simplifying them into a single classification as lichen crust or late biocrust successional stage may not be appropriate.

4.6 Conclusions

In summary, this study provides an extensive overview of how weather conditions affected biocrust microbial communities in three different biocrust types at JTNP. While previous studies have shown that some of these weather conditions contributed to biocrust physical structure, to the best of our knowledge, this is the first study, which investigates the relationship between variation in monthly weather conditions and overall biocrust microbial community responses throughout the year. Oftentimes, biocrust microbial communities were compared across studies while weather conditions and time of collection were not taken into account. Our findings highlighted that biocrust microbial communities are vary with season and change throughout the year. Based on our findings, we highly recommend that all factors contributed to these variations should be reported in future biocrust microbial research for consideration and comparison. We demonstrated that weather conditions including temperature, precipitation, wind speed, atmospheric pressure, humidity, and dew point contributed to biocrust microbial variability depending on biocrust type at JTNP. *Microvirga* sp. was abundant in relatively high temperature and low humidity, indicating its

adaptation to dryland conditions. Our results also indicated that *Microcoleus* sp., *Mastigocladopsis* sp. and *Trichocoleus* sp. could be other important biocrust microbes resistant to high wind speed. In summary, our findings showed that it is crucial to consider weather conditions, crust type morphology, time of collection, and location in biocrust ecological research. Acknowledging these factors at specific sites will allow us to better manage and restore biocrust in the near future.

4.7 Author contributions

Conceptualization, N.Po., N.Pi. and J.E.S.; formal analysis, N.Po.; investigation, N.Po. and N.Pi.; writing—original draft preparation, N.Po.; writing—review and editing, N.Po., N.Pi. and J.E.S.; visualization, N.Po. and J.E.S.; supervision, N.Pi. and J.E.S; project administration, N.Po.; funding acquisition, N.Po., N.Pi., and J.E.S. All authors have read and agreed to the published version of the manuscript.

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4.10 Conflicts of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Chapter 5

General conclusions

This dissertation focuses on three main goals including 1) exploring biocrust microbial diversity and verifying/improving our protocols for biocrust microbial diversity studies (Chapter 2), 2) applying next generation high throughput amplicon sequencing to identify archean, bacterial, and fungal communities in Mojave Desert biocrusts (Chapter 3), and 3) identifying temporal/seasonal effects on biocrust microbial communities (Chapter 4). By fulfilling these three main goals, this work has contributed to biocrust microbial diversity research from the beginning when knowledge are very limited up until now when we are able to establish a baseline for microbial diversity and composition in Mojave Desert. Chapter 2, 3, and 4 presented the progress and development of biocrust microbiome studies, determined the important factors that contribute to biocrust microbial communities in the Mojave Desert, and also guided us to improve our understanding in future biocrust microbial research.

Chapter 2 was the beginning of biocrust research in this dissertation in which we have begun to establish some baseline understandings about biocrust microbial diversity at Joshua Tree National Park (JTNP) in the Mojave Desert, CA. Although studies about JTNP biocrusts and the microbial diversity found within them were presented, they were very limited at the time and a summary of previous research was needed to guide the direction of future studies. This work was very valuable since it presented fundamental knowledge about biocrust microbial diversity and exploration which includes: 1) Algal and Cyanobacterial diversity in biocrusts, 2) Fungi diversity in biocrusts, and 3) Mechanisms for discovering microorganisms. This project was one of the very first studies to use next generation amplicon sequencing targeting biocrust microorganisms at JTNP. Previous research using culture dependent methods and current work using culture independent procedures have shown that biocrust microbial communities are complex and very diverse organisms awaiting for science to be discovered. In the past decade, new genera and species of cyanobacteria and algae have been described and we began to understand more about the green algal diversity. However, there are many more species of algae, fungi, and bacteria waiting to be found within biocrusts and drylands environment. Although, Chapter 2 has shown that we are only at the beginning of JTNP biocrusts microorganisms exploration, a combination of culture dependent and next generation culture independent methods that we have developed and modified are promising to improve our understanding about these microbes. Nevertheless, much more work is still needed to identify these biocrust microorganisms and their functions in dryland environments.

Chapter 3 was built upon our baseline understanding about biocrust microbial diversity. This project was an integrated research program on archean, bacterial, and fungal communities in Mojave Desert biocrusts and was the first comprehensive biocrust microorganisms investigation which included all three domains of life to show that microbial patterns were influenced by geographical locations, soil depth, and crust type diversity. While some fundamental knowledge about JTNP biocrust microbial diversity was presented in the previous chapter, this chapter expanded our understanding about biocrust microbes to cover the southern and central Mojave Desert in California and identified key factors that influenced the biocrust microbial communities. In addition to increasing our study area, using next generation amplicon sequencing to target three domains of life also provides us further improvement in biocrust microbial research including microbial networks analysis and functional guilds. To better understand the differences among crust types, five common crust types found in the Mojave Desert were collected including light-algal/cyanobacterial, cyanolichen, green-algal lichen, smooth-moss, and rough-moss crust types. In this study, analyzing microbial sequences using microbial ecology methods, indicator species analysis, and microbial network analysis has deepened our understanding about the microbial patterns and indicates that some key microbial taxa function as the backbone of biocrust microbial communities and networks. Moreover, functional roles of these microbial taxa were also described. Together, these crucial findings in Chapter 3 helped prepare us to develop and improve our interpretation of the biocrust microbial community for future sustainable and conservation management in desert drylands.

While Chapter 3 provided an overall view of biocrust microbial communities in the Mojave Desert, Chapter 4 was conducted to focus on JTNP location and to investigate how temporal and seasonal changes affect biocrust microbial communities throughout the year. Although we have made significant progress in our biocrust microbial community investigation, these microbes represented a snapshot in time, meaning that they represented microbial communities at the time of collection. However, weather conditions such as temperature, precipitation, wind speed, atmospheric pressure, and humidity change throughout the year and these temporal/seasonal variations might influence and affect biocrust microbes. Therefore, a time series collection of biocrust in JTNP was performed to monitor and investigate whether biocrust microbes responded to these changes. By integrating temporal changes into the microbial diversity study, this work helps elucidate the impact of weather conditions on microorganisms and define any temporal changes that significantly affect biocrust key microbial species. In conclusion, Chapter 4 has rigorously completed this dissertation with very detailed findings that will be essential for future biocrust management especially weather conditions which need to be taken into consideration.

Based on these conclusions, our findings can be used as a guide for future biocrust management in several ways. First, throughout this dissertation, we have shown that a combination of culture dependent and independent methods to study microbial communities provided greater insight into microbial diversity than a culture dependent procedure alone. We confirmed that biocrusts in the Mojave Desert were full of diverse microorganisms and should not be neglected when conservation and management are discussed. Second, we described biocrust microbial composition in the Mojave Desert and future research can use

our findings as a baseline. Third, some of the patterns that we found were informative toward conservation and restoration. As we discussed in Chapter 3, biocrust restoration currently relies on certain key species and/or inoculum with very limited success when applied to the actual dryland environment. The geographical pattern that we found in our study likely contributed to as why this was a complicated biocrust restoration issues because key species in particular site could be different in another sites. Although the surface morphology of biocrusts was identical among collection sites, their microbial make up was different. Therefore, future restoration processes and development will need to take this into consideration. Furthermore, some pathogens were identified in our biocrust samples which was another crucial aspect that we need to consider for future restoration methods, in order to not accidentally transport these pathogens to the surrounding area. Lastly, the final chapter, which looked at a time series and showed that biocrust microbial communities are dynamic. In general, our findings indicated that biocrust microbes are more diverse later in the summer than in other seasons. Therefore, time of restoration could also be the key. This dissertation significantly improves our understanding about biocrust microbial diversity in the Mojave Desert and also prepares us for better conservation strategies of biological soil crusts.

Finally, many other aspects of biocrusts microorganisms and microbial community still remains unanswered and will require extensive work in future research. One aspect would be to apply integrative "Omics" approach, which has become the major advancement in next generation microorganism studies, to biocrust microbial research. This dissertation already made progress in metagenomics using amplicon sequencing. By integrating

other omics approaches such as metatranscriptomics, metaproteomics, and metabolomics, we will immensely improve our understanding of the central dogma and molecular ecology of these microbial communities from genome to ribonucleic acid (RNA) to proteins and small molecules from a community level perspective. Some of these biocrust microorganisms identified in this dissertation had potential to be key species. Integrative omics approaches will confirm their importance and provide deeper knowledge of their functions from predicted genes and RNA that were expressed in different conditions. However, biocrust research is an enormously broad interdisciplinary area and there are many other questions and possibilities awaiting the next generation scientists to expand our knowledge on biological soil crust.

Appendix A

Appendix A: Temporal weather effects on biocrust fungal community

A.1 Materials and methods

A.1.1 Biocrust sampling

Light algal/cyanobacterial crust (LAC), Cyanobacteria lichen crust (CLC, *Collema* sp.), Green algal lichen crust (GLC, *Clavascidium lacinulatum*) were collected at Joshua Tree National Park (JTNP, GPS: 34.10N, -115.45W) using sterile technique. Underlying subsurface soil samples for each crust type were collected separately. Sample series were collected once each month for all 12 months of 2017 with 3 replicates for each biocrust type. A total of 252 samples were collected for the entire study. All samples were kept

on ice and transferred to a -80°C freezer at University of California Riverside. Monthly weather data from the area were obtained from NOAA National Centers for Environmental Information [1]: atmospheric pressure, dew point, humidity, precipitation, temperature, and wind speed as recorded at the nearest weather station (GHCND:USR0000CRIC) in Rice Valley California. We used the reported monthly averages for all data except total precipitation which was the accumulated value for the month.

A.1.2 Library preparation and data processing

Total DNA was extracted from 0.15g of each soil and sand sample using the QIAGEN DNeasy PowerSoil kit (Qiagen, Germantown, MD, USA) following the manufacturer's standard protocol. Polymerase Chain Reaction was performed on the DNA samples to amplify the ITS1 region using ITS1F and ITS2 primers following the Earth Microbiome protocol [2]. PCR reactions were performed in 25 μl total volume with three replicates per sample, with 1 μl of genomic DNA, 1 μl of each primer (10 μM), 9.5 μl of nuclease-free water (Sigma-Aldrich, St.Louis, MO, USA), and 12.5 μl of Taq 2X DNA Polymerase (Thermo Fisher Scientific Inc., Waltham, MA, USA). PCR conditions consisted of (i) initial denaturation at 93°C for 3 min; (ii) 35 cycles of denaturation at 95°C for 45 sec, (iii) annealing at 50°C for 1 min, (iv) extension at 72°C for 90 sec, and (v) final extension at 72°C for 10 min using a C1000 thermal cycler (BioRad, Hercules, CA, USA). Three replicates of PCR products were combined, purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Hoerdt, France) and pooled with per sample volumes selected to produce a mixture with equimolar concentration of each sample. The pooled amplicon concentrations were quantified using Qubit dsDNA HS Assay (Life Technologies, Carlsbad, CA,

USA) and further analyzed using Agilent 2100 Bioanalyzer and Fragment Analyzer (Agilent Technologies, Santa Clara, CA, USA). The pooled amplicon libraries were sequenced using Illumina MiSeq system with MiSeq Reagent V3 kit (San Diego, CA), a 2 × 300 paired-end read format, at the Institute for Integrative Genome Biology, Core Facilities, University of California, Riverside (http://iigb.ucr.edu). A total of 12,349,195 demultiplexed paired end sequence reads were generated and submitted to the Sequence Read Archive (SRA) databases associated with BioProject accession number PRJNA705683.

A.1.3 Bioinformatics

The fungal ITS1 amplicon sequences were analyzed with AMPtk: the Amplicon Toolkit for NGS data (formally UFITS) (https://github.com/nextgenusfs/amptk) [3]. Demultiplexed paired-end sequences data were pre-processed by trimming forward and reverse reads to a maximum of 250 bp, trimming primer sequences and discarding reads less than 100 bp in length. The paired-end reads were then merged to produce single long overlapping reads using USEARCH v9.1.13 [4]. Sequence quality filtering was performed with the expected error parameter of 0.9 [5], which produced 10,086,498 quality filtered reads. This cleaned sequenced dataset was denoised using UNOISE3 algorithm [6] which generated 3,142 Amplicon Sequence Variants (ASVs) following the procedure of Palmer et al. 2018 [3]. Chimeric ASVs, sequences produced from PCR amplification of templates or parent sequences, were filtered using VSEARCH (v 2.3.2) [7], which removed 122 chimeras after comparison to the database. Taxonomic assignment for 3,020 ASVs was performed using AMPtk hybird approach with UNITE v8.0 with 97% similarity [8].

A.1.4 Data analysis

Filtered microbial ASVs alpha diversity (observed ASVs), beta diversity, and taxonomic composition were analyzed using the Phyloseq package [9] in RStudio version 1.1.463 [10] and R version 3.5.1 [11]. Variances of alpha diversity data were verified for homoscedasticity in R with Levene's test, Bartlett's test and Hartley's Fmax test [12]. Homoscedasticity data (alpha diversity by month) were analyzed with ANOVA using the 'Anova' function and pairwise multiple comparison (Tukey test) was conducted using the 'TukeyHSD' function in R. Unbalanced design comparison among months was accounted for using a type = 'III' ANOVA. PERMANOVA with the 'adonis' function in the 'vegan' package in R (Unifrac distance with permutation - 999) was used to compare beta diversity [13].

A.2 Preliminary results

A.2.1 Fungal alpha diversity

Significant differences for fungal richness among twelve months were detected in our alpha diversity analysis of LAC (ANOVA, $F(11,19) = 4.94$, $p = 0.00119$, Fig. A.1). In LAC, fungal species richness changes throughout the year with lowest values in December and highest values in July with seasonal pattern of low species richness in the winter and high species richness in the late spring and early summer month. In general, a similar pattern of fungal species richness variation was observed across all biocrust types in which species richness was low in winter months. Significant species richness variations were also observed in CLC (ANOVA, $F(11,20) = 3.685$, $p = 0.00555$, Fig. A.2) and GLC (ANOVA,

F(11,22) = 4.637, p = 0.00109, Fig. A.3). In contrast, subsurface samples from underneath LAC and CLC showed no significant differences throughout the year while GLC subsurface fungal species richness showed significant changes (ANOVA, F(11,21) = 8.683, p = 1.44e-05).

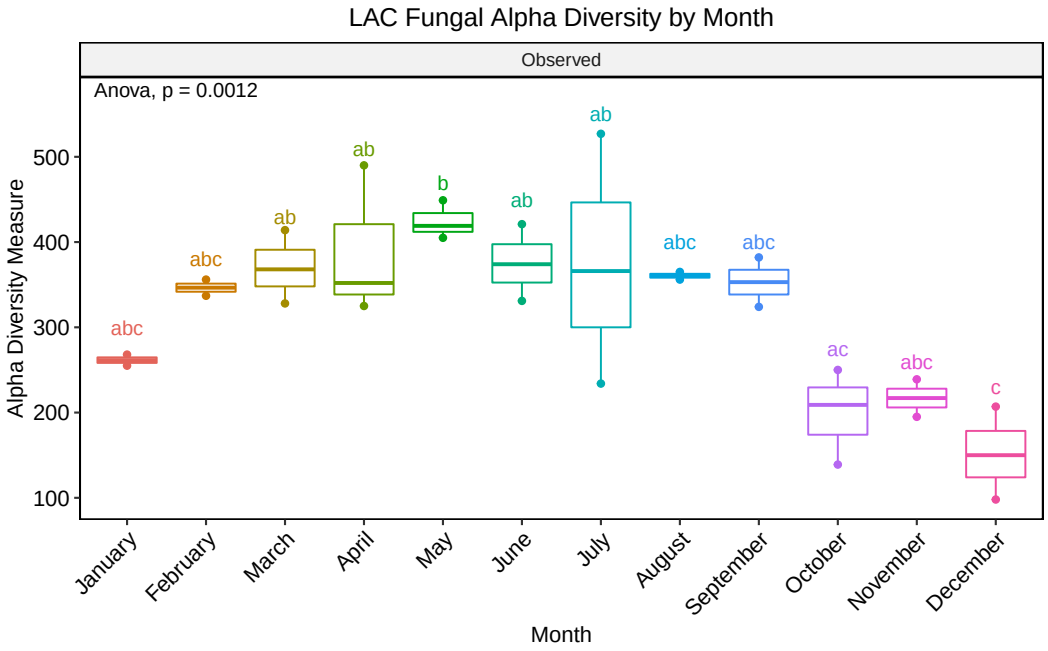

Figure A.1: Box plots showing alpha diversity as ASV richness in different months. Fungal alpha diversity of JTNP light algal biocrust (LAC) samples by month. Boxplots show 25th and 75th percentile with the median shown as a line inside the box. Error bars show 1st and 99th percentile. Tukey HSD significant differences (p <0.05) are indicated by different letters.

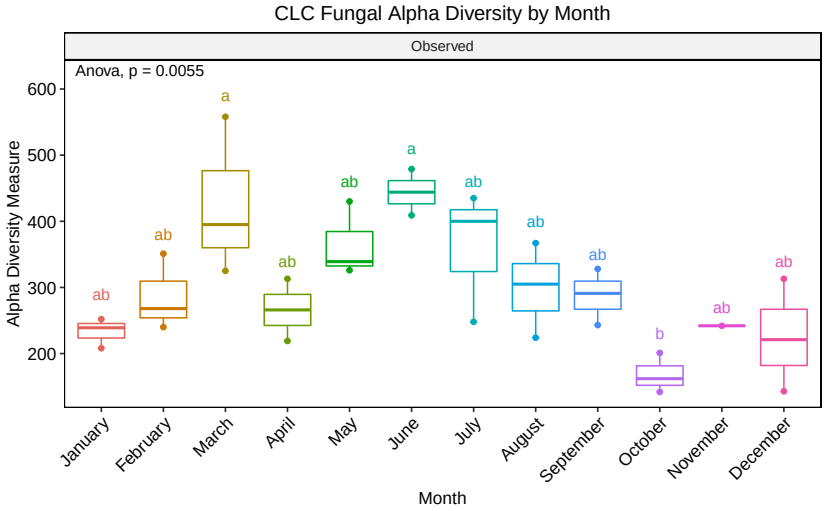

Figure A.2: Box plots showing alpha diversity as ASV richness in different months. Fungal alpha diversity of JTNP cyano-lichen biocrust (CLC) samples by month. Boxplots show 25^{th} and 75^{th} percentile with the median shown as a line inside the box. Error bars show 1^{st} and 99^{th} percentile. Tukey HSD significant differences (p <0.05) are indicated by different letters.

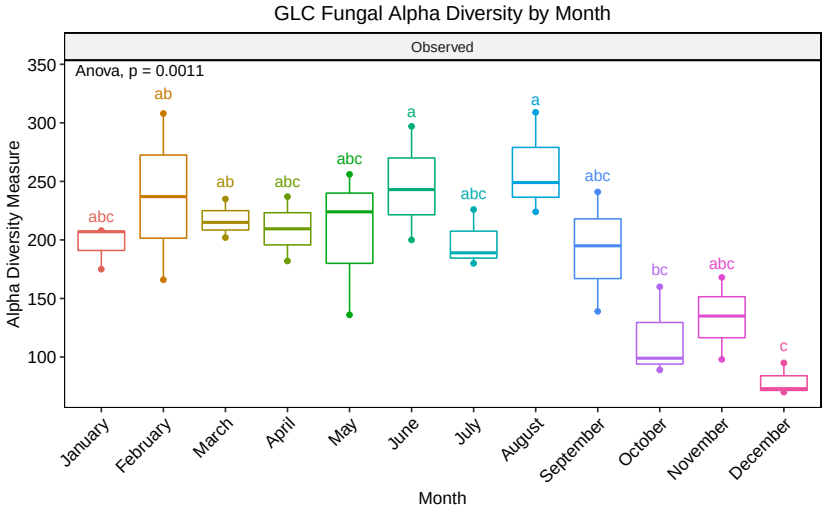

Figure A.3: Box plots showing alpha diversity as ASV richness in different months. Fungal alpha diversity of JTNP green algal lichen biocrust (GLC) samples by month. Boxplots show 25^{th} and 75^{th} percentile with the median shown as a line inside the box. Error bars show 1^{st} and 99^{th} percentile. Tukey HSD significant differences (p <0.05) are indicated by different letters.

A.2.2 Fungal taxonomic composition

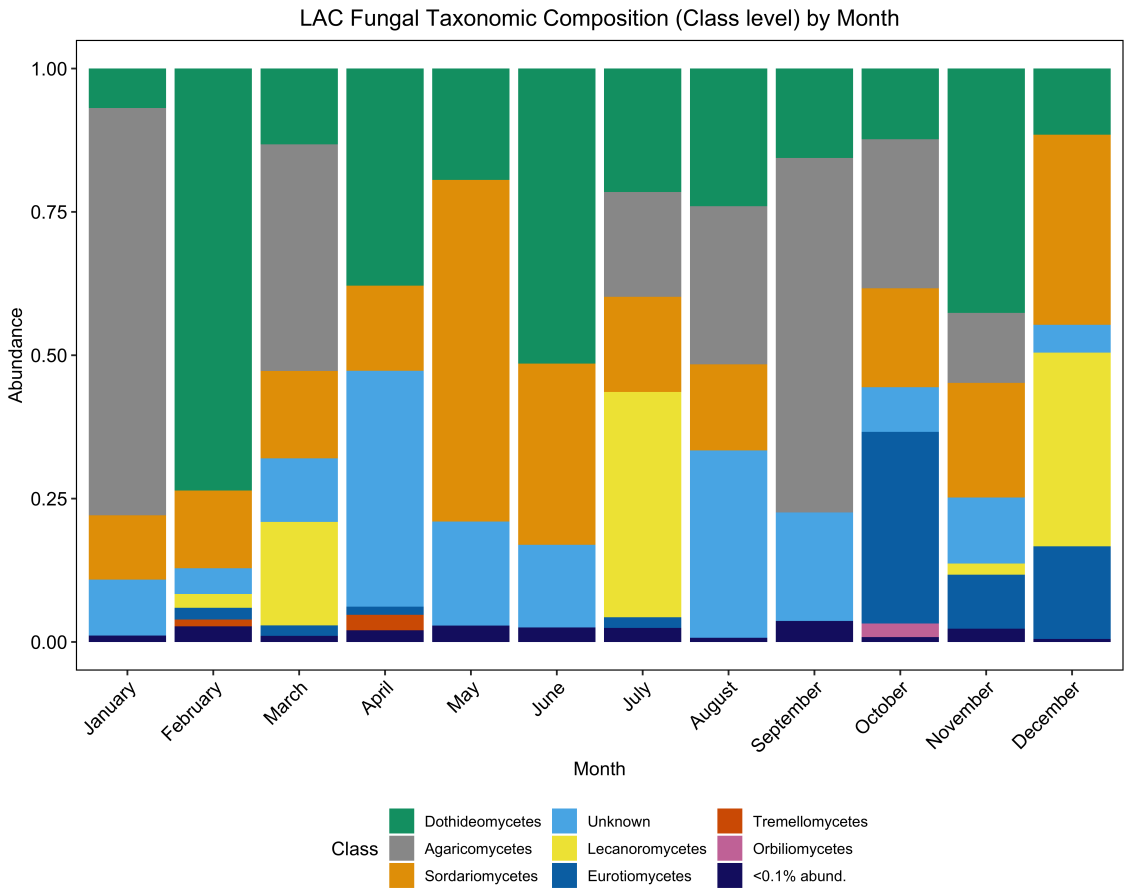

Figure A.4: LAC fungal taxonomic bar plots at class level by month. Seven major fungal class variations were observed in LAC samples throughout the year.

To understand which fungi changed over time, fungal taxa variation barplot for each crust type was generated to observe the relative abundance values in the taxonomic composition among fungal class. Although major fungal class including Dothideomycetes, Agaricomycetes and Sordariomycetes were found in all crust types throughout the year, distinct differences can be seen in the relative abundance bar plots (Fig. A.4). In LAC, nine classes showed significant differences over time (ANOVA, p <0.05); Dothideomycetes,

Eurotiomycetes, Tremellomycetes, Mortierellomycetes, Agaricomycetes, Sordariomycetes, Lecanoromycetes, Pezizomycetes and Leotiomycetes. Seven classes in GLC showed significant differences over twelve months (ANOVA, p <0.05); Dothideomycetes, Eurotiomycetes, Agaricomycetes, Sordariomycetes, Lecanoromycetes, Pezizomycetes and Leotiomycetes. Seven classes in CLC showed significant differences over twelve months (ANOVA, p <0.05); Dothideomycetes, Eurotiomycetes, Agaricomycetes, Sordariomycetes, Lecanoromycetes, Leotiomycetes and Arthoniomycetes. In sand samples, eight classes showed significant differences over time (ANOVA, p <0.05); Dothideomycetes, Eurotiomycetes, Tremellomycetes, Agaricomycetes, Sordariomycetes, Lecanoromycetes, Leotiomycetes and Arthoniomycetes.

Fungal taxonomic variations were also observed in subsurface samples. In LAC subsurface soil, three classes showed significant differences over time (ANOVA, p <0.05); Mortierellomycetes, Agaricomycetes and Lecanoromycetes. In GLC subsurface soil, eight classes showed significant differences over time (ANOVA, p <0.05); Dothideomycetes, Eurotiomycetes, Tremellomycetes, Mucoromycetes, Agaricomycetes, Lecanoromycetes, Pezizomycetes and Leotiomycetes. Lastly, in CLC subsurface soil, four classes changed significantly in richness over time (ANOVA, p <0.05); Dothideomycetes, Eurotiomycetes, Lecanoromycetes and Leotiomycetes.

In summary, fungal taxonomic composition changed over time in biocrust and in soil underneath. Sordariomycetes richness changed significantly over time in surface samples only, while Mucoromycetes richness changed significantly over time in subsurface soil only.

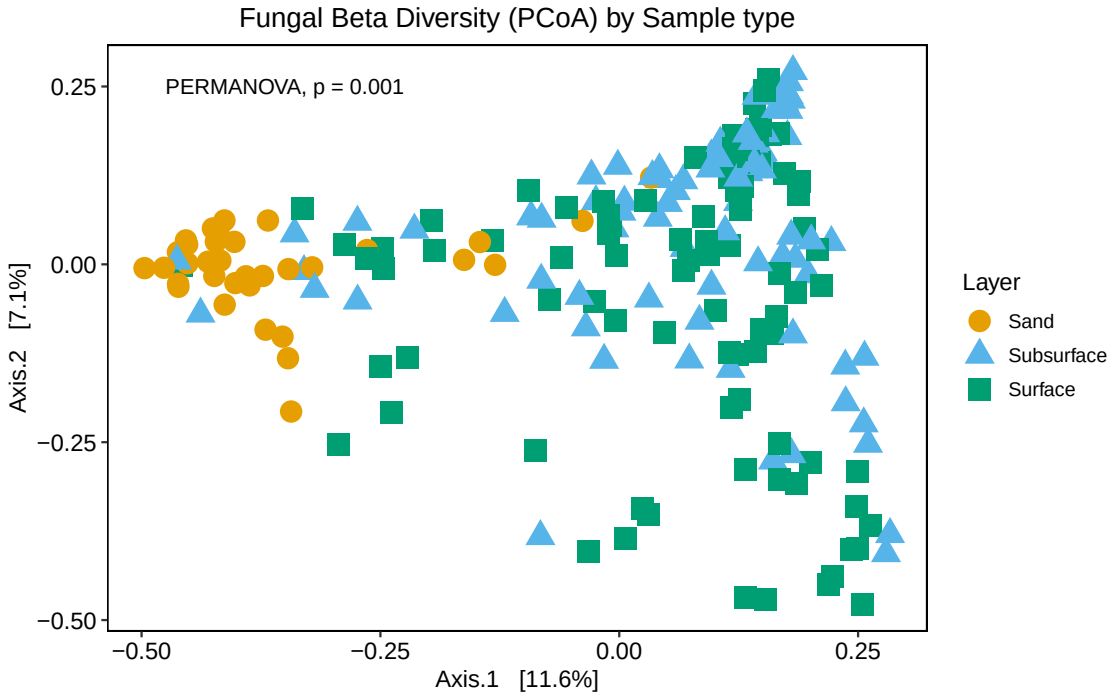

Figure A.5: Beta diversity analysis of biocrust fungal communities by sample type using PCoA. Significant differences (PERMANOVA; p <0.05) were shown on PCoA plot.

A.2.3 Fungal beta diversity

Beta diversity analysis of overall fungal communities differed significantly by sample type (PERMANOVA, $p = 0.001$, $R^2 = 0.0858$) (Fig. A.5). These differences in microbial beta diversity were visualized in principal coordinate analysis (PCoA) plots, which showed that biocrusts (surface samples) and soil underneath biocrust (subsurface samples) separated from sand samples. To visualize biocrust microbial communities for biocrusts types (surface samples only), a subset of samples including LAC, CLC and GLC were used in beta diversity analysis which revealed a significant crust type pattern: lichen crusts fungal communities (CLC and GLC) clustered separately from LAC fungal community (PERMANOVA, $p = 0.001$, $R^2 = 0.0884$) (Fig. A.6).

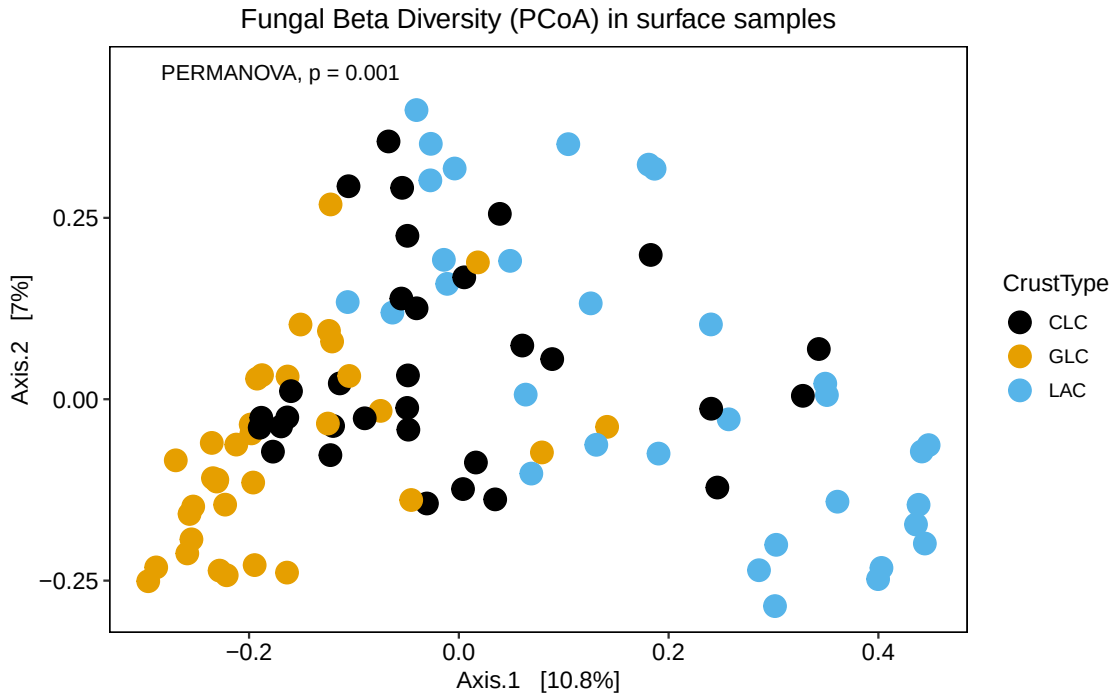

Figure A.6: Beta diversity analysis of biocrust fungal communities in surface samples (biocrust only) using PCoA. Significant differences (PERMANOVA; p <0.05) were shown on the PCoA plot.

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Appendix B

Appendix B: Protocols and SOPs

B.1 Mojave biocrusts DNA extraction

In this dissertation, all biocrust DNA samples were extracted using the DNeasy PowerSoil Kit (formerly known as MOBIO PowerSoil DNA Isolation kit). At the beginning of this project, we used a standard manufacture protocol, but almost all of the DNA samples failed to amplify during the PCR step in amplicon sequencing library preparation (possibly due to PCR inhibitors or chelators in the soil). I have tried using the DNA purification kit and it did reduce PCR amplification issues in some samples. With the assistance from the manufacturing company, I have modified the DNA extraction protocol slightly to solve this issue and almost all biocrust DNA samples succeeded through the PCR step. Steps that were modified are presented below.

- To prepare biocrusts for DNA extraction, each sample must be homogenized thoroughly before starting this protocol. This is a crucial step before starting DNA extraction using the kit.
- Prepare 0.15 g of homogenized biocrust sample.
- Add 0.15 g of biocrust sample to the PowerBead Tube provided, Gently vortex to mix. (Note: this is the first step in the manufacture protocol, but we only use 0.15 g instead of 0.25 g.)
- Steps 2-18 remains the same. The manufacture protocol noted that you can skip the 5 min incubation in many steps, but we maintained all incubation steps.
- Step 19, we only used 40 - 50 μl of Solution C6
- Step 20 remains the same and we keep DNA samples in -20 °C freezer.

Note (1): Modified protocol can be found at the following link https://www.protocols.io/private/F52888457BAD11EBB2400A58A9FEAC2A

Note (2): original manufacture protocol can be downloaded here https://www.qiagen.com/us/resources/resourcedetail?id=5a0517a7-711d-4085-8a28-2bb25fab828a&lang=en

Note (3): if you already have extracted DNA and would like to try cleaning up your DNA samples. I used the following kit and it resolved PCR inhibitors issues for some of my biocrust DNA samples. https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/microbial-dna/dneasy-powerclean-cleanup-kit/

B.2 Amplicon sequencing library preparation

We followed the 16S protocol here https://www.protocols.io/view/emp-16s-illumina-amplicon-protocol-nuudeww and ITS protocol here https://www.protocols.io/view/emp-its-illumina-amplicon-protocol-pa7dihn with some modifications.

1. Instead of using Quant-iT PicoGreen in step 4, we used nanodrop to quantify DNA concentration of all amplicons.

2. Then, we clean up the amplicons using magnetic bead clean up before continuing on to step 5. Please contact Matthew Collin matthew.collin@ucr.edu for the bead clean up protocol.

3. Since we cleaned up all of our amplicons samples after step 4, skip step 6.

4. In step 7, we used Qubit to measure DNA concentration.

Modified protocol can be found at the following link. https://www.protocols.io/private/2C4228B416451B6583D9C6916819CD8C

B.3 Amplicon sequencing data processing

Tutorial:

We will use AMPtk to process amplicon data. A digital (and most updated) copy of this protocol is available at https://stajichlab.github.io/SOP_data/Data_and_Code/AMPtk

We can get sample data from a public dataset stored in NCBI. First let's look at the BioProject page PRJNA379160. This page provides links to the 98 SRA experiments for 16S and ITS amplicon data from Antarctic cryptoendolithic communities.

Although, this study has both 16S and ITS amplicon data, we will perform data processing only on several samples from ITS data. Data are already available on stajichlab UCR HPCC. We will set up our analysis folder using the following instructions.

ITS primers for this project contain unique barcodes for each sample. We usually submit 250 samples per Illumina MiSeq run. After the sequencing process, the barcodes will be used to split sequences into fastq file for each sample.

Setting up analysis

First make a data folder (illumina) in bigdata to begin your analysis, change directory to "illumina" folder, and create a symlink to a subset of ITS amplicon data.

```
cd ~/bigdata/
mkdir -p AMPtk/illumina/
cd AMPtk/illumina/
# create a symlink to the datasets
ln -s ~/shared/projects/Microbiome/data/Amplicon_Pipeline/ITS/illumina/* .
cd ..
```

Now, you should have these 10 data files in your illumina folder and you could verify the previous step by simply use "ls".

```
ls illumina/
```

```
ITS.CC.18A_R1.fastq.gz  ITS.CC.28A_R1.fastq.gz  ITS.CC.8B_R1.fastq.gz
ITS.CC.18A_R2.fastq.gz  ITS.CC.28A_R2.fastq.gz  ITS.CC.8B_R2.fastq.gz
ITS.CC.18B_R1.fastq.gz  ITS.CC.28B_R1.fastq.gz
ITS.CC.18B_R2.fastq.gz  ITS.CC.28B_R2.fastq.gz
```

What does the sequence file look like?

```
zmore illumina/ITS.CC.18A_R1.fastq.gz | head
```

```
@ITS.CC.18A_3 M02457:94:000000000-AMC54:1:1102:9850:1616 1:N:0:1
orig_bc=CTAGTTTTACCA new_bc=CTAGATTTGCCA bc_diffs=2
GTAGGTGAACCTGCGGAAGGATCATTACTGAGAGACGGGCTTTTCTCCCCCCCTCTCTTCTCCC
CTCTCTTCTTTACCCTCTTTCTTTCTCCCTTTTTTGCTTTTTCCCTCCCGGTTCTCTTCCCCGC
CTTCTCCCCCTCTCCCCTCCCCTGCTCGCCTATCTCCCTTTCAACTCTCTTCTTATTTTCTTTT
TTCCTTCCTCTCCCTTTCTACACTTTTTCATTTCTTATTTTTTTCTTCTTTTCTCTTTTTTCTT
CCCTCCCTCTATATTTTCTCTTCTCTTTTTCCTTCTTTTTCTTGT
+
-868@CC,,CDE-FF>++7;8CF8FF9-C9,,,,,,-,++8,,6,6C,,8@+++,,:CEE9,CC
,C,,,,,<C,,,:9C,,C<<,C9E,6,,,,,,,:++,,<,9?,5,,48++++++:,,5<,,,+6
+++,5,:38+3+,,,,+6@+++>+,@B++@++,,,77@<A,,,,,36,,,33,,,,,3,,,7,6
61,,,,,,,,,,,,,,,2,,,,,61,,,,+++++++++3+++1+++2;+2++312+:++)(++
*****/********0*/0*)+**.-/.):***)))1)))).66).
```

AMPtk data processing steps

Now, I will create a bash script containing all the general steps from processing Illumina reads to generating an OTU table and assigning their taxonomy. We will call this pipeline script `01_AMPtk_ITS.sh`. We will practice a similar set up as we have done before by keeping all the scripts in the pipeline folder and log files in the logs folder.

```
#create pipeline folder for scripts and logs folder for log files
mkdir -p pipeline/ logs/
```

You should now check your AMPtk folder to make sure that you have these folders. Once, we have all the data and folders, we can begin STEP1.

```
ls -F

illumina/ logs/ pipeline/
```

STEP 1. Pre-processing

There are several different file formats that Illumina MiSeq sequencing (or sequencing centers) can generate. We will focus on demultiplexed PE reads in which all the sequences were split into separated fastq files for each sample. The general workflow for Illumina demultiplexed paired end (PE) reads is the following:

- Merge PE reads (use USEARCH or VSEARCH)
- Filter reads that are phiX (USEARCH)
- Find forward and reverse primers (pay attention to the `{require_primer argument)`
- Remove (Trim) primer sequences
- If the sequence is longer than `{trim_len`, truncate sequence

You can use the terminal nano editor to simply create `01_AMPtk_ITS.sh` script by copying the following command to `nano` and save the script to the pipeline folder.

```
nano pipeline/01_AMPtk_ITS.sh
```

The beginnings of this script will be listed here. You will copy all 4 steps into `01_AMPtk_ITS.sh` and run the script.

Note: In this tutorial, we used 10 input files including the forward reads (`_R1`) and reverse reads (`_R2`). After the run is completed, we will have one big file combining all of the samples with `_R1` and `_R2` merged.

```
#!/usr/bin/bash
#SBATCH -p short -N 1 -n 8 --mem 8gb --out logs/AMPtk_ITS.%a.log

CPU=$SLURM_CPUS_ON_NODE

if [ ! $CPU ]; then
 CPU=2
fi

#AMPtk needs to be loaded in miniconda2 for UCR HPCC
#We'll need to unload miniconda3 and load miniconda2 before load AMPtk
module unload miniconda3
module load miniconda2
module load amptk/1.4.0

#Set up basename for all the output that will be generated
BASE=AMPtkITS

#Change this to match your data folder name
INPUT=illumina

#Pre-preocessing steps will use 'amptk illumia' command for demultiplexed PE reads
if [ ! -f $BASE.demux.fq.gz ]; then
 amptk illumina -i $INPUT --merge_method vsearch -f ITS1-F -r ITS2 \
 --require_primer off -o $BASE --cpus $CPU --rescue_forward on -l 250
 fi
```

STEP 2. Clustering

This step will cluster sequences into Operational Taxonomy Units (OTUs), then generate representative OTU sequences and an OTU table. OTU generation pipelines in AMPtk uses UPARSE clustering with 97% similarity (this can be amended to a similarity of your choosing).

Note (1): At the clustering step, we used the merged sequences from STEP1 as the input to generate clustered sequence files and the OTU table. Note (2): Denoising approaches such as DADA2 and UNOISE are also available. Therefore, ASV table can be generated by using denoising approach instead of clustering method.

```
if [ ! -f $BASE.otu_table.txt ];  then
 amptk cluster -i $BASE.demux.fq.gz -o $BASE --uchime_ref ITS \
 --usearch usearch9 --map_filtered -e 0.9 --cpus 8
fi
```

Checking OTU table

```
head AMPtkITS.otu_table.txt
```

```
#OTU ID ITS.CC.18A ITS.CC.18B ITS.CC.28A ITS.CC.28B ITS.CC.8B
OTU1 2301 2871 353140 11034 14929
OTU10 0 2580 2 0 0
OTU100 0 3 0 0 0
OTU101 0 0 6 0 0
OTU102 1 0 3 0 1
OTU104 1 0 1 0 0
OTU105 0 2 33 0 0
OTU106 1 0 5 0 1
OTU107 1 0 5 1 1
```

STEP 3. Taxonomy Assignment

This step will assign taxonomy to each OTU sequence and add taxonomy to the OTU table. This command will generate the taxonomy based on the ITS database. (Note:

at the Taxonomy Assignment step, we will use clustered sequence files and the OTU table for taxonomy assignment from the ITS database.)

```
if [ ! -f $BASE.otu_table.taxonomy.txt ]; then
 amptk taxonomy -f $BASE.cluster.otus.fa -i $BASE.otu_table.txt -d ITS
fi
```

When the taxonomy assignment is completed, we can check the taxonomy file which will be `AMPtkITS.cluster.taxonomy.txt`

```
head -5 AMPtkITS.cluster.taxonomy.txt

#OTUID taxonomy USEARCH SINTAX UTAX
OTU1 GS|100.0|GU074436|SH1524733.08FU;k:Fungi,p:Ascomycota,c:Lecanoromycetes
OTU3 US|0.9077|KF823589|SH1564421.08FU;k:Fungi,p:Basidiomycota,c:Tremellomycetes
OTU4 SS|1.0000|LN810767|SH1614717.08FU;k:Fungi,p:Ascomycota,c:Lecanoromycetes
OTU5 SS|1.0000|LN881898|NA;k:Fungi,p:Ascomycota,c:Lecanoromycetes,
```

STEP 4. FUNGuilds Assignment

We can also assign Fungi Functional Guilds for each taxonomy using FUNGuilds.

```
if [ ! -f $BASE.guilds.txt ]; then
 amptk funguild -i $BASE.cluster.otu_table.taxonomy.txt --db fungi -o $BASE
fi
```

Checking `AMPtkITS.guilds.txt` result

```
cut -f11 AMPtkITS.guilds.txt | sort | uniq -c

     60 -
      2 Animal Endosymbiont-Animal Pathogen-Endophyte-Plant Pathogen
      2 Animal Pathogen-Endophyte-Plant Pathogen-Wood Saprotroph
      1 Animal Pathogen-Fungal Parasite-Undefined Saprotroph
      1 Animal Pathogen-Plant Pathogen-Soil Saprotroph-Undefined Saprotroph
      5 Animal Pathogen-Plant Pathogen-Undefined Saprotroph
      1 Animal Pathogen-Undefined Saprotroph
      1 Dung Saprotroph-Plant Saprotroph
      1 Ectomycorrhizal-Fungal Parasite-Plant Pathogen-Wood Saprotroph
      1 Fungal Parasite-Plant Pathogen-Plant Saprotroph
      7 Fungal Parasite-Undefined Saprotroph
```

```
 1 Guild
15 Lichenized
 2 Plant Pathogen
10 Undefined Saprotroph
```

STEP 5. Run `01_AMPtk_ITS.sh`

We have learned all four main steps for NGS amplicon data processing. Now, we will add all the steps together and run as a bash script `01_AMPtk_ITS.sh`

```
sbatch pipeline/01_AMPtk_ITS.sh
```

B.4 Amplicon sequencing data analysis

We used the phyloseq package to analyze our data. To learn how to use this tool, please follow tutorials at https://joey711.github.io/phyloseq/ and example of our codes that were used in this dissertation can be found at http://github.com/stajichlab/MojaveCrusts2019analysis (DOI: 10.5281/zenodo.3931036)