RESPONSES TO BIOCRUST RESTORATION AND THE ROLE OF CYANOBACTERIAL EXOPOLYSACCHARIDES IN DRYLAND ECOSYSTEMS

by

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Responses to biocrust restoration and the role of cyanobacterial exopolysaccharides in dryland ecosystems

Thesis directed by Associate Professor Nichole N. Barger

ABSTRACT

Anthropogenic land use and changes in climate can cause severe degradation in dryland ecosystems, leading to the loss of ecosystem functioning and services. Thus, restoring ecosystem function in these already low productivity areas is critical. Biological soil crusts (biocrusts) are soil surface communities of cyanobacteria, lichens, mosses, and other microorganisms that play key roles in dryland ecosystem functions, making them excellent targets for dryland restoration. In this study, we addressed ecosystem barriers to successful biocrust rehabilitation - propagule scarcity, resource limitation, and soil stability - with treatments of inoculation, shading, and an artificial polyacrylamide soil stabilizer in the Great Basin Desert. Combinations of these treatments were also implemented to address the possibility of multiple barriers. All experimental treatments were implemented on clay and sandy clay loam (SCL) soils and monitored annually for three years. We found that overall biocrust recovery was much faster on clay soil, and that soil type influenced treatment efficacies. On clay soil, the combination of shade+inoculum was the most successful in promoting biocrust recovery, while on SCL soils, a low inoculation level was sufficient. Although identifying effective restoration treatments is a critical step in biocrust restoration, mechanisms underlying ecosystem functions and what functional groups of microorganisms contribute to these functions also need to be better understood. To try to understand this, we explored how cyanobacterial exopolysaccharides (EPSs) affected soil stability and hydrology to see if EPSs could be used as proxies for these functions. We found that EPSs were more important to soil stability on less stable SCL soils than on clay soils, implying that other contributing factors need to be considered on finer soil types. Finally, we observed shifts in microbial communities over time following an inoculum restoration treatment to more abundant later-successional, dark cyanobacteria species, reflecting patterns seen in natural recovery. However, microbial

communities of restored plots were still distinct from mature crusts after three years. Findings from this study can be applied to dryland ecosystem management. Biocrust restoration efforts should focus on coarser soil types since finer soil types recover faster naturally. However feasibility, cost, and time to implement restoration treatments should be carefully considered when scaling up to a landscape level. Biocrust disturbance mitigation should also be prioritized as biocrusts take a long time to recover, even following the most effective restoration methods.

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CHAPTER 1
BIOCRUST RESTORATION STRATEGIES AND THE ROLE OF CYANOBACTERIAL EXOPOLYSACCHARIDES IN DRYLAND ECOSYTEMS

1.1 ABSTRACT

Biological soil crusts (biocrusts) are comprised of cyanobacteria, lichens, mosses, and other microorganisms that play key roles in dryland ecosystem functions, and are thus excellent targets for dryland restoration. Biocrust communities differ spatially in their structure and functions, therefore site-specific considerations need to be addressed in biocrust rehabilitation. In this study, we addressed possible ecosystem barriers of propagule scarcity, resource limitation, and soil stability with specific restoration techniques: inoculation, shade structures, and a polyacrylamide soil stabilizer (PAM), respectively. We also considered combinations of these restoration techniques in the case that multiple barriers were present. We implemented restoration treatments on two soil types, clay and sandy clay loam (SCL), to account for differences in biocrust colonization on different soil textures. Over three years post-treatment, we observed faster biocrust rehabilitation on the finer clay soil and treatment differences between soil types. On clay soil, the combination of shade+inoculum resulted in the greatest overall biocrust recovery; however, this was not uniform across all biocrust recovery metrics tested. In the SCL soil, the addition of a low level of inoculum was sufficient to increase biocrust recovery across some recovery metrics relative to the natural recovery plots. Mechanistically, cyanobacteria in biocrusts can contribute to ecosystem functions such as soil stability and hydrology through exopolysaccharides (EPSs) exudates, which they secrete for protection from abiotic stressors. Within the framework of the study, we examined how EPSs contribute to

overall soil stability and hydrology. We considered two fractions of EPSs: tightly-bound EPSs (TBEPSs) associated with filamentous cyanobacteria and colloidal EPSs (cEPSs), which are exuded as slime. We found that EPSs affected ecosystem functions differently on the two soil types. EPSs did not affect stability or hydrology on clay soils, but weakly increased overall stability on less stable SCL soils. Thus, considering EPS amounts as proxies for ecosystem functions may be more useful when evaluating coarser soil types, but other factors need to be considered when analyzing overall soil stability and hydrology. Findings from this study can be used toward dryland ecosystem management by focusing biocrust restoration efforts on coarser soil types with slower natural recovery and implementing shade+inoculum treatments on finer soil types.

1.2 INTRODUCTION

1.2.1 Disturbance in dryland ecosystems and to biological soil crusts

Dryland ecosystems comprise the largest biome on the planet (~45% of Earth's terrestrial surface) and are home to more than 2 billion people (Schimel, 2010; Davies et al., 2012; Pravalie, 2016; Weber, Büdel, and Belnap [Editors (Eds.)], 2016). Drylands face many anthropogenic stressors such as land use and land cover change that may be further exacerbated by changing climates (Pravalie, 2016; Zaady et al., 2016), resulting in declines of productivity. Dryland land degradation or "desertification" is the greatest threat to the ecological functioning of these areas; an estimated 3.5 billion hectares of drylands are currently experiencing some level of degradation, and this area is rapidly increasing (Gilbert, 2011; Davies et al., 2012).

Biological soil crusts (biocrusts) are a critically important functional component of drylands, at times covering up to 70% of plant interspaces in these ecosystems (Belnap and Lange [Eds.], 2001; Davies et al., 2012; Doherty et al., 2015; Pravalie, 2016). Biocrusts are comprised of communities of cyanobacteria, algae, fungi, bryophytes, and lichen species that colonize the top few millimeters of soil surfaces. Biocrust communities strongly influence ecosystem functions such as soil stability, hydrology, fertility (fixing C and N), and nutrient cycling; all of which are essential to sustaining production in these low-productivity ecosystems (Belnap and Lange [Eds.], 2001; Warren, 2001; Chamizo et al., 2012; Rossi et al., 2012; Colica et al., 2014; Kidron et al., 2015; Weber, Büdel and Belnap [Eds.], 2016; Faist et al., 2017).

Soil surface disturbance due to human activities such as livestock grazing, recreational activities, and energy development impairs biocrust functions by destabilizing the soil surface.

Unstable soils can slow natural recovery of biocrust communities by burying photosynthetic organisms that reside at or near the soil surface (Weber et al., 2016). Soil surface disturbance and the subsequent loss of the biocrust communities increase soil erosion, which can cause airborne dust. Airborne dust has consequences on human health and can also increase the rate of snowmelt (Painter et al., 2010), impacting regional hydrological processes (Pointing and Belnap, 2014). Once disturbed, biocrusts are slow to naturally recover. Natural recovery begins with large, filamentous cyanobacteria of the genus *Microcoleus* that are the first colonizers on bare soil. Smaller, pigmented cyanobacteria (*e.g. Nostoc*) and green algae follow. These early colonizers fix C and N, contributing to soil fertility, as well as create stable surfaces for later successional lichen and moss species. Generally, early colonizing cyanobacteria species are seen at most disturbed sites; however, mid- to late successional species are determined by site-specific characteristics such as climate, soil type, and other abiotic and biotic factors (Weber, Büdel, and Belnap [Eds.], 2016). Depending on the nature and severity of disturbance, recovery on time scales of decades to even centuries (Weber et al., 2016). Therefore in highly degraded dryland soils, biocrust rehabilitation strategies are critically needed to restore soil stability, fertility, and other ecosystem functions.

1.2.2 Restoration of biological soil crusts

Past biocrust restoration efforts to accelerate biocrust recovery have been met with mixed success (Bowker, 2007). This is likely due to ecosystem barriers limiting effective rehabilitation (Bowker, 2007; Mager and Thomas, 2011) including propagule scarcity, resource limitations (e.g., water and nutrients), and lack of soil stability in actively eroding soils (Bowker,

2007). Depending on the severity of disturbance and the level of soil degradation, one or more of these barriers may exist; thus a combination of biocrust restoration techniques may be needed (Bowker, 2007; Zhao et al., 2017).

To overcome propagule scarcity, inoculation-assisted techniques such as using field-collected inoculum (Belnap and Gardner, 1993; Scarlett, 1994; Chiquione et al., 2016) or lab-grown biocrust organisms (Johansen and St. Clair, 1993, 1994; Buttars et al., 1998; Chen et al., 2006; Antoninka et al., 2017) have been successful. The addition of inoculum has been found to increase soil stability of moving sand dunes (Chen et al., 2006) and help later successional moss and lichen species to establish (Chiquione et al., 2016). While field-collected inoculum can be salvaged with foresight of disturbed areas (Chiquione et al., 2016), this technique is not sustainable on a large-scale because it requires a "sacrifice area" for collection (Bowker, 2007). A more sustainable source of inoculum is grown in the lab (Davidson et al., 2002; Chen et al., 2006; Bowker, 2007; Velasco-Ayuso et al., 2016). However, while most of the *ex situ* cultivation inoculum thrived in laboratory environments, they often failed to survive under harsher field conditions (Buttars et al., 1994).

To alleviate resource limitations under field conditions, the addition of nutrients, water, and UV reduction strategies have also been implemented (Davidson et al., 2002; Bu et al., 2014; Chiquoine et al., 2016; Velasco Ayuso et al., 2016). Water addition has been shown to generally result in positive biocrust growth in drylands (Chen et al., 2006; Bu et al., 2014; Velasco Ayuso et al., 2016). Nutrient additions, however, have shown mixed success and are highly dependent on climate, soil type, and whether lichen or cyanobacterial establishment is the restoration goal (Davidson et al., 2002; Antoninka et al., 2016; Velasco Ayuso et al., 2016). Shading reduces

abiotic stresses such as temperature and UV, while increasing soil moisture (Kidron and Benenson, 2013). Shade has had varied effects on biocrust growth in one laboratory study (Bu et al., 2014), but positive effects in another (Velasco Ayuso et al., 2016). Another study found that moderate light intensity (300-700µE/m^2s) and temperature (20-30°C) were optimal for photosynthesis in *Microcoleus vaginatus* (Chen et al., 2006). As field conditions are much more intense than these optimal ranges, these findings further indicate that shading can be beneficial to biocrust growth. However, while enhancing resources has been successful, altering resource conditions by shading or water additions on a large scale are both costly and labor intensive to implement at the landscape scale, and thus must be carefully considered before they are administered.

In highly disturbed, actively eroding soils, addition of biocrust inoculum and resource additions are likely to be unsuccessful because biocrust organisms are unable to establish (Zhao et al., 2016). As a result, stabilizing actively eroding soils is critically important to restore biocrust communities. A broad range of artificial soil stabilization techniques such as straw checkerboards (Li et al., 2006; Qiu et al., 2014; Zhao et al., 2016) and application of polyacrylamide (PAM) (Davidson et al., 2002), have shown varied success. Straw checkerboards have been especially effective in stabilizing mobile sand dunes and establishing early successional cyanobacteria (Chen et al., 2006). While the addition of PAM has been successful in assisting early successional biocrust species, it has had limited or even negative effects on later successional moss and lichen establishment, perhaps due to competition between biocrust organisms (Davidson et al., 2002). In order to implement large-scale artificial soil stabilization techniques, sufficient labor and incentive for funding are needed (Bowker, 2007).

In addition to ecosystem barriers, other site-specific abiotic and biotic factors such as soil texture (Rozenstein et al., 2014), climate, biocrust composition, and natural disturbances present (Zhao et al., 2016) can complicate biocrust restoration and must be considered.

1.2.3 Biological and ecosystem functions of cyanobacterial exopolysaccharides (EPSs)

Restoration strategies have historically focused on establishing early successional species such as cyanobacteria, as they are fairly easy to cultivate and can provide soil stabilization for later successional biocrust species to utilize (Chen et al., 2006; Bowker, 2007). Cyanobacteria are early colonizers of disturbed soils and can withstand dessication, high temperatures, and ultraviolet (UV) exposure (Weber, Büdel, and Belnap [Eds.], 2016). To provide protection from these stressors, cyanobacteria secrete exopolysaccharides (EPSs) that aid in water retention and assist in cell adhesion and locomotion (Wingender, Neu, and West [Eds.], 1999), both of which are important for establishment and colonization (Belnap and Gardner, 1993). Substrate grain size also affects cyanobacteria establishment, as large pore sizes associated with larger grain sizes hinder EPSs from spreading, and consequently, cyanobacterial establishment (Rozenstein et al., 2014). Although other organisms such as fungi, archaea, and algae also produce EPSs (Wingender, Neu, and West [Eds.], 1999), cyanobacterial EPSs comprise the majority of EPSs in biocrusts (Zheng et al., 2011).

Cyanobacterial EPSs are primarily polysaccharides comprised of two fractions: tightly-bound EPSs (TBEPSs) and colloidal EPSs (cEPSs). These fractions have essential biological and ecological functions, such as increasing water retention and soil stability (Van den Ancker et al., 1985; Williams et al., 1995; Mazor et al., 1996; Wingender, Neu, and West [Eds.], 1999; Belnap

and Lange [Eds], 2001; Bowker et al., 2008; Chen et al., 2014; Belnap and Büdel, 2016). TBEPSs are comprised of high molecular weight sheaths and capsules, through which filamentous cyanobacteria such as *Microcoleus spp.* can migrate toward the surface when moisture and light are available (Belnap and Büdel, 2016) (Figure 1-1a). TBEPSs envelop and organize soil particles, creating soil macroaggregates and structural pores, preventing compaction, and giving the soil tensile strength, which reduces soil loss in runoff (Belnap and Gardner, 1993; Warren, 2001; Chen et al., 2014). Soil stability from TBEPSs creates stable soil aggregates for later biocrust successional species, as well as collects fine soil particles and nutrients (Van den Ancker et al., 1985; Williams et al., 1995; Mazor et al., 1996; Belnap and Lange [Eds], 2001). The collection of fine soil particles promotes faster cyanobacterial colonization and more soil stability, as filamentous TBEPSs traverse smaller pore sizes more easily (Rozenstein et al., 2014). Sheath materials from *Microcoleus* species have even been used for erosion prevention in the field, where they helped cyanobacteria establishment (Davidson et al., 2002; Xu et al., 2012).

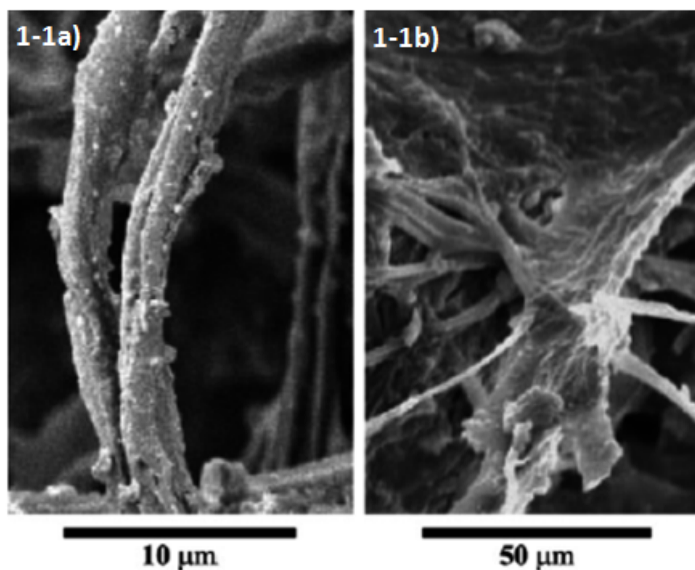

Figure 1-1: Scanning electron microscope images of 1-1a) tightly-bound exopolysaccharide (TBEPS) filaments and 1-1b) colloidal exopolysaccharide (cEPS) slime secreted by biocrust cyanobacteria (from Mager and Thomas, 2010).

cEPSs are loosely-bound, lower-molecular weight “slime” exudates secreted in copious amounts (Figure 1-1b). cEPSs can comprise up to 75% of the labile C in soil (Mager, 2010) and are important C sources for many heterotrophic soil microorganisms (Wingender, Neu, and West [Eds.], 1999; Chen et al., 2014; Baran et al., 2015). Chemical affinities to clay and calcium compounds allow cEPSs to create microaggregates with soil particles, assisting in soil stabilization (Belnap and Gardner, 1993). cEPSs have high absorption capacities to retain precious water; however, their effects on infiltration and runoff are varied. Wetting causes EPSs to swell, which can block soil pores and increase runoff while decreasing infiltration (Rossi et al., 2012; Colica et al., 2014; Chamizo et al., 2016; Faist et al., 2017). As important contributors to soil stabilization and moisture retention in drylands, EPSs are strong indicators of biocrust function, though many other abiotic and biotic factors can also influence overall stabilization and hydrology (Brock, 1975; Kidron et al., 1999; Williams et al., 1999; Warren, 2001; Rossi et al., 2012; Pointing and Belnap, 2012; Colica et al., 2014; Chamizo et al., 2015; Chamizo et al., 2016; Belnap and Büdel, 2016).

Hydrological function of EPSs can change as EPSs increase over time. As biocrusts develop and hydrophobic EPSs accumulate, biocrust thickness and runoff increase (Kidron, 2015). However, as cyanobacterial EPSs increase soil stability, later successional moss, bryophyte, and lichen species can colonize and increase in abundance (Mager and Thomas, 2011). Each functional group has its own contribution to infiltration and runoff. For example, mosses absorb copious amounts of water relative to lichens, which tend to be more hydrophobic and are associated with higher runoff (Belnap and Gardner, 2003; Belnap et al., 2008; Rodriguez-Caballero et al., 2015; Rossi and De Philippis, 2015, Kidron, 2015).

Furthermore, biocrust thickness and development can result in microtopographic features on the soil surface (Kidron, 2015). Microtopography can redistribute or pool water, as well as capture nutrients and fine soil particles that decrease infiltration (Chamizo et al., 2016). Additionally, soil aggregation can increase and stabilize macropores that enhance infiltration (Malam Issa et al., 2009). Abiotic factors such as soil type and physical crust cover can also contribute to hydrological function (Chamizo et al., 2016).

1.2.4 Purpose and hypotheses of study

The purpose of this study was to address ecosystem barriers to biocrust restoration and provide insight on how EPSs influence soil stability and hydrologic function. The specific objectives of this study were to 1) determine the most effective restoration treatments or combination of treatments to overcome propagule scarcity, resource limitation, and actively eroding soils to restore ecosystem functionality and 2) determine the relative roles of EPSs in driving variation in soil stability and hydrological function. To achieve these objectives, we implemented active restoration treatments designed to overcome each barrier, as well as looked as a combinations of restoration treatments. To overcome the propagule scarcity barrier and determine how much inoculum was needed, we added field-collected inoculum in low, medium, and high amounts. We addressed the resource limitation barrier by erecting shade structures, which ameliorate harsh UV rays and high temperatures while retaining moisture. A combination shade+inoculum treatment was also implemented to address the possible coexistence of both propagule scarcity and resource limitations. To address actively eroding

soils, we applied a polyacrylamide (PAM) artificial soil stabilizer, as well as the combination of PAM+inoculum in the case that propagule scarcity also existed.

We hypothesized that restoration treatments targeting these barriers to biocrust recovery would enhance the growth of early successional cyanobacteria biomass due to the ultimate release from ecological constraints. Furthermore, increases in cyanobacteria biomass would, in turn, increase EPS production at a faster rate than sites experiencing natural recovery of the biocrust community. Treatments addressing the barrier(s) most hindering biocrust rehabilitation at the site would be the most effective. We also predicted that restoration responses would vary between soil types, and that biocrust colonization would be faster on clay, a finer soil type, compared to sandy clay loam (SCL), a coarser soil type. Finally, we expected to see strong correlations between EPS amounts and observed soil stability, but varied responses between EPS amounts and hydrological response, as numerous and contrasting factors can determine overall hydrologic function.

1.3 METHODS

1.3.1 Site description

The project site is located in the Great Basin Desert, a cool desert system that receives an average of 200mm (100mm-500mm) of precipitation each year (Figure 1-2). The bulk of precipitation occurs in the spring from March-May, with hot, dry summers having mean monthly temperatures (MMT) of 34°C and cold winters with MMT of 3°C. The site is located near the Bonneville Salt Flats and contains highly saline soils. Two locations within the overall site were chosen for their distinct soil types: 1) Skumpah-Yenrab Complex and 2) Amtoft/Dynal-

Tooele Complex (Soil Survey Staff NRCS, 2017) (Figure 1-2). Soil texture classification on the top 1cm of soil using the hydrometer method identified the soil type at the Skumpah-Yenrab Complex to be clay (28% sand, 29.8% silt, 41.4% clay), and at the Amtoft/Dynal-Tooele Complex as sandy clay loam (SCL) (46.4% sand, 26% silt, 27.5% clay). Plots were established on both soil types to account for possible differences in biocrust colonization and recovery in different grain sizes (Rozenstein et al., 2014; Weber, Büdel, and Belnap [Eds.], 2016).

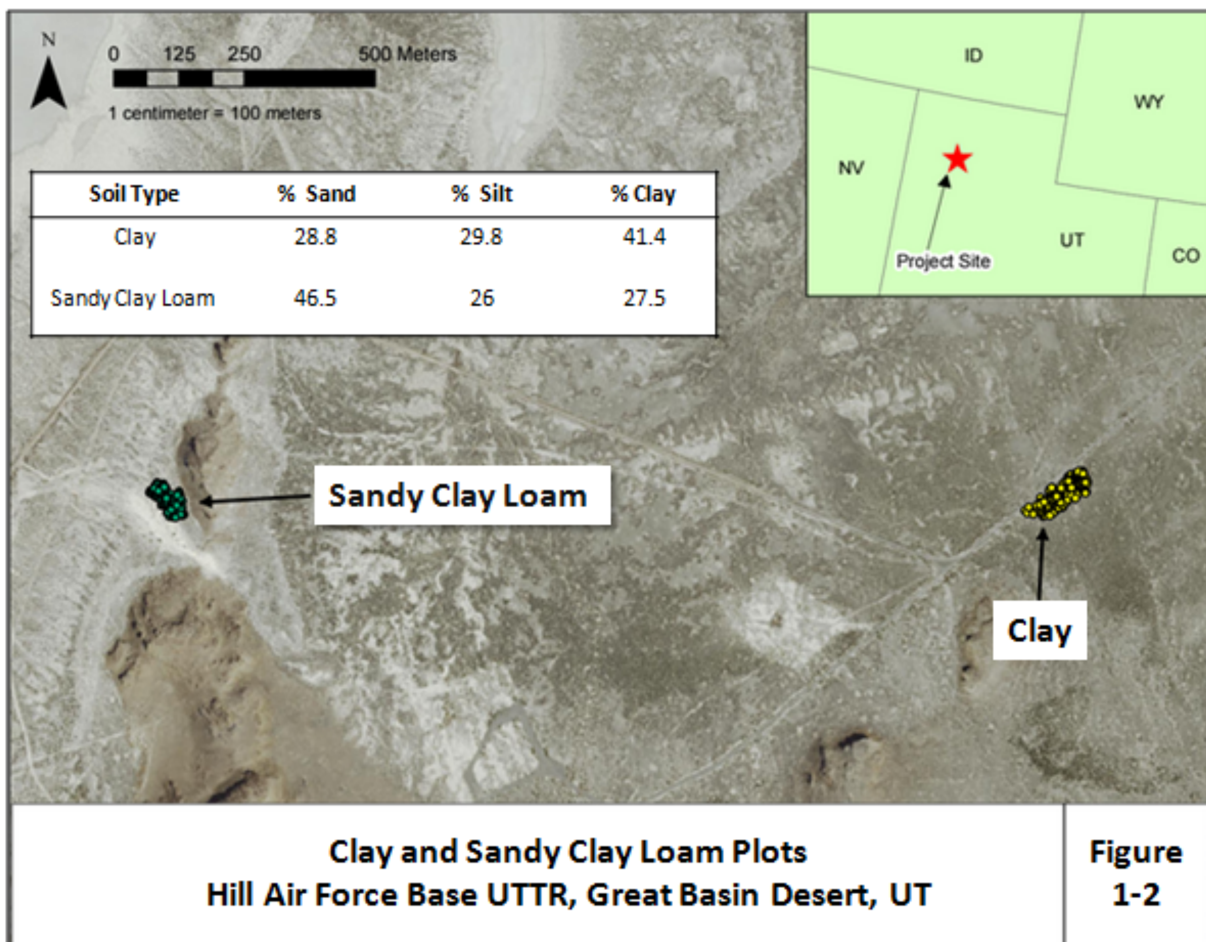

Figure 1-2: Hill Air Force Base Utah Test and Training Range (UTTR) project location, displaying plot locations on clay and sandy clay loam soil types.

Three experiments – inoculum, shade, and PAM addition - to address each potential ecological barrier to biocrust establishment were implemented at Hill Air Force Base, Utah Test

and Training Range (41°00’00”N 113°15’00”W) in June 2013 (Figure 1-2). At the start of each experiment, biocrusts were completely removed from the soil surface by scraping biocrusts to 2cm below ground surface (bgs). Removed biocrusts were collected and crumbled into pea-sized fragments to be used as field-collected inoculum. Each treatment was implemented on clay and in SCL soils. We monitored the plots annually for three years following restoration treatments.

1.3.2 Inoculum experiment

To address the barrier of propagule scarcity and to determine the amount of inoculum needed, 0.25m² plots were constructed through the removal of the top 2cm of the soil surface and inoculated with low (500cc, 10% cover), medium (1000cc, 20% cover), and high (2000cc, 40% cover) cover of field-collected biocrust inoculum. Natural recovery (NR) plots had no inoculum added and tracked natural boicrust recovery without restoration. Each inoculum treatment (NR, low, medium, high) was replicated in 5 plots across an approximately 2.5-acre area in each soil type (clay and SCL) (Table 1-1).

Table 1-1: Restoration treatment experiments corresponding to ecosystem barriers to biocrust restoration success as identified by Bowker et al., 2007. The dotted line separates passive treatments (above) from active restoration treatments (below). Each experiment was conducted on clay and sandy clay loam soils.

Ecosystem Barrier to Biocrust Recovery	Experiment	Type of Restoration Treatment	Restoration Treatment	Inoculum Level
Time	--	None	Natural Recovery	None
Propagule Scarcity	Inoculum	Inoculation	Inoculum Addition	None, Low, Medium, High
Resource Limitations (+ Propagule Scarcity)	Shade	Habitat Modification	Shade (± Inoculum)	None, High
Actively Eroding Soils (+Propagule Scarcity)	PAM	Artificial Soil Stabilization	PAM (± Inoculum)	None, High

1.3.3 Shade experiment

To address resource limitations using habitat modification, 0.25m² plots were scraped and then shaded. Shade structures that reduced 50% of incoming UV were placed 15cm over the scraped plots for 24 months (June 2013-June 2015). Shade structures were removed during the winter months to avoid damage from heavy snowfall. NR plots were left unshaded. To address the possibility of propagule scarcity and resource limitations, plots were shaded and had a high amount of field-collected inoculum (2000cc, 40% cover) added. Each treatment (NR, shade only, shade+inoculum) was replicated in 5 plots on each soil type (Table 1-1).

1.3.4 Polyacrylamide (PAM) experiment

To overcome actively eroding soils, biocrusts were scraped off 1m² plots. A photodegradable water soluble polyacrylamide (PAM) (Dirtglue Enterprises® Salem, NH), was sprayed in a 1:8 PAM:water dilution with and without inoculum, the former to address actively eroding soils in combination with propagule scarcity. NR plots did not have any PAM or inoculum added. Each treatment (NR, PAM, PAM+inoculum) was replicated in 7 plots on each soil type (Table 1-1).

1.3.5 Annual monitoring of experimental plots

Plots were monitored one (June 2014), two (June 2015), and three (May 2016) years after treatment (hereafter referred to as Years 1, 2, 3, respectively). During the final year of monitoring in Year 3, field measurements and soil samples were also taken from randomly generated freshly scraped (scraped control) and intact plots (intact control) within each site to

be used as reference points to evaluate biocrust recovery progress after three years. To determine biocrust recovery and EPS levels, monitoring assessed level of development (LOD), soil stability, hydrology, quantification of chlorophyll a (a proxy of photosynthetic potential), cEPSs, TBEPSs.

1.3.6 Biocrust response to treatments

Level of Development

Level of development (LOD) in each 0.25m² plot was determined by splitting each plot into quadrants, and then ranking each quadrant from 1-6 (1=least developed, 6=mature and undisturbed) as determined by parameters specified in Belnap et al., 2008. The four LOD scores from each quadrant were averaged to get the overall LOD of the plot. LOD is largely determined by biocrust color, which indicates the presence of darker pigmented later-successional cyanobacteria, mosses, and lichen, as well as the development of biocrust thickness and microtopography (Belnap et al., 2008).

Soil Stability

The shear strength of the soil surface was determined by hand-held torsional vane shear tester (Forestry Suppliers, Inc.) while soil compressibility was determined by a pocket penetrometer (Forestry Suppliers, Inc). Both shear strength and soil compressibility tests were only conducted in Year 3. Soil aggregate stability (Herrick et al., 2001) was measured in all three years following treatment and was determined using three soil surface samples collected from standardized locations on each plot.

Water Drop Penetration Time (WDPT)

The water drop penetration time (WDPT) field method was used to measure water repellency of soil (Bisdom et al., 1993). Three drops of water were placed on a dry soil surface, and the time for the water to be absorbed was recorded. Three WDPT readings were taken per plot, and then averaged. A soil is considered water-repellant if it takes >5 seconds for the water to completely absorb. WDPT was only measured in Year 3.

Unsaturated Hydraulic Conductivity

Unsaturated hydraulic conductivity (HC) of soil is a measure of how easily water moves through dry soil pore spaces or fractions. A Mini Disk Portable Tension Infiltrometer (Decagon, Inc.) with a 3.1cm diameter was used to measure HC at a suction rate of 2.0cm. Infiltration measurements were recorded every 3 minutes for 15 minutes. HC was calculated using the Van Genuchten parameters for the 12 soil classes (Carsel and Parrish, 1988; Zhang, 1997). HC was only taken in Year 3 on intact, scraped, NR, low, medium, and high inoculum plots on both soil types. Negative HC values have no biological value and were removed from the dataset.

Chlorophyll a and EPS sample preparation

Three soil samples were collected from each experimental plot and pooled. Samples were collected dry using a 1.5mm diameter centrifuge tube to a depth of 10mm. Upon field collection, samples were stored in the dark at -20°C. Samples remained frozen until processing. Plant litter and rocks were manually removed from samples before weighing. Samples were homogenized with a mortar and pestle and were portioned into approximate 1g and 50mg subsamples using the cone and quarter method (Gerlach et al., 2002) for chlorophyll a and EPS analyses, respectively.

Chlorophyll a

Chlorophyll a (chl a) quantification is an indicator of biocrust photosynthetic potential. One gram of soil was ground with a mortar and pestle in 3mL of 90% acetone for 3 min. The sample and solvent were transferred into a 15mL centrifuge tube, and the total volume brought up to 10mL with 90% acetone. The sample was vortexed at maximum for 2 min and incubated in the dark at 4°C for 24 hours. Following incubation, the sample was centrifuged for 12 min at 4,000 RPM at 15°C. The supernatant was retained and read using an Ocean Optics CHEMUSB4-VIS-NIR Spectrophotometer (400-950nm) at 663nm for chl a content and at 1000nm for noise. Absorbance at 1000nm was subtracted from absorbance at 663nm to account for noise. The adjusted absorbance at 663nm and soil sample mass were used to determine chl a content for cyanobacteria extracted in 90% acetone using calculations outlined in Ritchie, 2006.

EPS extraction

cEPSs and TBEPSs fractions were extracted from a single 50mg soil sample. 50mg of soil sample and 400µL DI water were combined in a 1.5mL centrifuge tube, vortexed briefly to suspend the solids, then placed on a shaker at room temperature (RT) for 15 min. Samples were centrifuged at 8,000 x g for 6 min. 200µL of supernatant containing cEPSs was used for analysis using the phenol-sulfuric acid assay. The remaining supernatant was discarded, and the resulting soil pellet retained for TBEPS analysis (de Brower and Stahl, 2001). 500µL of 100mM Na_2EDTA was added to the remaining soil pellet. The sample was vortexed briefly to suspend the solids, then placed on a shaker for 16 hours at RT. Samples were centrifuged at 8,000 x g for 6 min. 200µL of supernatant containing TBEPSs dissolved in EDTA was used for analysis using the phenol-sulfuric acid assay (Dubois, 1956).

Phenol-Sulfuric Acid Assay

The phenol-sulfuric acid assay (Dubois, 1956) was used with a glucose standard to determine EPS amounts. 200μL of each EPS fraction was combined with 200μL of 5% w/v phenol and 1mL of sulfuric acid. The reaction was vortexed and incubated for 45 min at RT before being read by an Ocean Optics CHEMUSB4-VIS-NIR Spectrophotometer (400-950nm). Absorbance at 490nm was taken, with a background reading at 1000nm subtracted as noise. Absorbances of EPS fractions were compared to the glucose standard curve to calculate μg glucose/g soil.

1.3.7 Data analyses

Experimental restoration treatment effects

All data analyses were performed using the statistical program R (R Core Team, 2016). For normal or transformed variables that met ANOVA assumptions (confirmed using the Anderson Darling test), multiple-way ANOVAs were used to look at the effects of soil type, year, and treatment on response variables and to see if there were interactions among factors. In the inoculum level experiment, a 3-way ANOVA (soil, year, inoculum level) was used; for the shade and PAM experiments, 4-way ANOVAs were used (soil, year, inoculum, shade or PAM, respectively). If no interactions between predictor variables were present but soil type and/or year were significant predictors, each soil type or year was evaluated separately with corresponding one-way ANOVAs and post-hoc Tukey honest significant difference (HSD) pairwise tests. If the data were unable to be transformed to meet assumptions of normality, non-parametric data were analyzed by linear mixed effect (lme) models (R packages: lme4 [Bates et al., 2015], and MuMIn [Bartón, 2016]), using soil type, year, and treatment (i.e.,

inoculum level, shade+inoculum, or PAM+inoculum) as fixed effects and plot number as random effects. Fixed effects and interactions between them were tested with the lme model. We used Akaike's Information Criterion (AIC) to select the best of several candidate models, using a $\Delta AIC \leq 2$ to determine the best models. Corresponding Kruskal-Wallis and Dunn's Tests were run for pairwise comparisons of non-parametric data.

Impact of EPS amounts on soil stability and hydrology

To create a summary variable representing biocrust stability (i.e., soil aggregate stability, torvane, penetrometer) response in Year 3, we ordinated sampling events using a data-reduction non-metric multidimensional scaling (NMDS) method based on Bray-Curtis dissimilarities with the community ecology "vegan" package in R (Okasanen et al., 2017). We explored the significance of EPS amount effects (i.e., cEPSs and TBEPSs) on stability using a non-parametric permutational analysis of variance (PERMANOVA) approach (Anderson, 2001). To evaluate overall hydrology, we created a hydrology variable of the relative proportion of hydrophobicity:infiltration. We then used regression tests to explore correlations between cEPSs and TBEPSs and hydrology. Finally, we compared cEPSs and TBEPSs with each individual stability and hydrological response variables using linear regression tests.

1.4 RESULTS

1.4.1 Inoculum experiment

Overall, the addition of inoculum assisted in some aspects of biocrust recovery; however, higher levels of inoculum did not correspond to increased biocrust recovery. Year since treatment was the greatest driver in increasing overall recovery, displaying that natural

recovery was fairly quick. Soil type also affected chl a and EPSs amounts, with clay soils recovering faster overall than SCL soils. Over three years post-treatment, chl a in both soil types increased over time (approximately 2-fold in successive years), but there were no effects of inoculation level (Figure 1-3a) on chl a amounts. Chl a was influenced by treatment with inoculated plots seemingly having higher chl a amounts than NR plots, but this result was confounded by a soil type*year interaction (Table 1-2). This interaction made it difficult to draw overarching treatment patterns with all of the predictor variables, so we focused on treatment effects after three years of recovery. We compared intact and scraped control plots to NR and inoculum-treated plots after three years of recovery on each soil type separately. On clay soil, chl a in the intact control, NR, low, and high inoculum plots exceeded that of the scraped control, and intact controls contained more chl a than the NR or medium plots (Figure 1-3a; Table 1-3). Surprisingly, increases in inoculum level did not correspond to increases in chl a. On SCL soil, a similar phenomenon was seen. All inoculated treatments contained more chl a than the scraped control and NR plots (Figure 1-3b; Table 1-3); however, increases in inoculation did not correspond to increases in photosynthetic potential. These results suggest that propagule scarcity seems to be a small restoration barrier on SCL but not clay soils. However, a low inoculum amount is sufficient in accelerating photosynthetic potential recovery after 3 years (Figure 1-3b; Table 1-2).

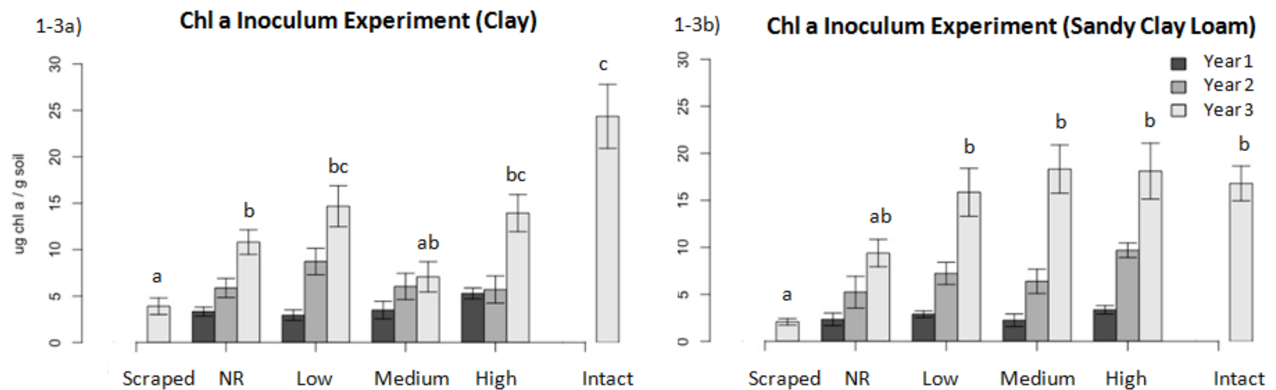

Figure 1-3: Chl a amounts averaged across natural recovery (NR), low, medium, and high inoculum plots in each year following restoration treatment. Intact and scraped controls were taken in Year 3 only. 1-3a) Averages of chl a between treatments and years on clay soil. In Year 3, there was significantly more chl a in the intact control than in NR or medium inoculum treatments ($p<0.05$). 1-3b) Averages of chl a between treatments and years on sandy clay loam soil. In Year 3, medium and high inoculum treatments resulted in more chl a than NR plots ($p<0.01$). Time was the driving factor in chl a amounts ($p<0.001$) on both soil types. Letters indicate significance between treatments in Year 3 ($p<0.05$), error bars represent ±1 SE.

Table 1-2: Repeated measures ANOVA or lme results for selected response variables. The F value is followed by the p value in parentheses. Bolded values indicate **p<0.05**. Asterisks indicate that response variable could not be normalized, and that a non-parametric lme model was used. For lme models, AIC values are reported and bolded if they had a ΔAIC<2, indicating the best model fit. Chl a reported in (µg chl a/g soil), cEPSs and TBEPSs reported in (µg glucose/g soil).

Inoculum Experiment (Predictor Variables)	*chl a*	*cEPSs**	*TBEPSs*
Inoculum Level	5.568 **(0.001)**	1261.2	4.032 **(0.001)**
Soil	0.896 (0.3)	1307	6.343 **(0.002)**
Year	97.460 **(<0.0001)**	1262.6	4.127 **(0.04)**
Inoculum Level*Soil	2.155 (<0.1)	1313	1.225 (0.3)
Inoculum Level*Year	0.662 (0.7)	1268.4 (Inoculum Level+Year)	0.413 (0.7)
Soil*Year	4.895 **(0.009)**	**1255.4 (Soil+Year)**	2.61(0.08)
Inoculum Level*Soil*Year	2.343 **(0.04)**	1261.2 (Soil+Inoculum+Year)	0.925 (0.5)

Shade Experiment (Predictor Variables)	*chl a*	*cEPSs*	*TBEPSs*
Soil	14.113 **(0.0003)**	15.345 **(0.0002)**	6.863 **(0.01)**
Inoculum	28.328 **(<0.0001)**	14.732 **(0.0002)**	13.817 **(0.0004)**
Shade	14.399 **(0.0003)**	9.963**(0.002)**	4.376 **(0.02)**
Year	184.866 **(<0.0001)**	48.456 **(<0.0001)**	4.733 **(0.03)**
Soil*Inoculum	5.399 **(0.02)**	8.025 **(0.006)**	2.23 (0.1)
Soil*Shade	0.317 (0.6)	3.351 (0.07)	1.869 (0.2)
Soil*Year	0.199 (0.08)	13.962 **(<0.0001)**	0.352 (0.7)
Inoculum*Year	0.237 (0.8)	3.515 **(0.03)**	0.168 (0.7)
Shade*Year	1.535 (0.2)	3.815 **(0.03)**	0.0944 (0.4)
Soil*Inoculum*Year	1.009 (0.4)	1.519 (0.2)	1.078 (0.3)
Soil*Shade*Year	1.013 (0.4)	1.974 (0.1)	0.996 (0.4)

PAM Experiment (Predictor Variables)	*chl a*	*cEPSs**	*TBEPSs*
Soil	6.807 **(0.01)**	1414.3	46.568 **(<0.0001)**
Inoculum	7.654 **(0.001)**	1407.8	0.279 (0.6)
PAM	0.378 (0.5)	1408.2	1.854 (0.2)
Year	95.244 **(<0.0001)**	1388.1	0.757 (0.5)
Soil*Inoculum	0.85 (0.4)	1406 (Soil+Inoculum)	0.351 (0.6)
Soil*PAM	3.009 (0.08)	1406.4 (Soil+PAM)	0.125 (0.7)
Soil*Year	0.319 (0.7)	1385.3 (Soil+Year)	1.925 (0.2)
Inoculum*Year	0.362 (0.7)	1389.9 (Inoculum+Year)	1.593 (0.2)
PAM*Year	0.046 (1)	1390.3 (PAM+Year)	1.152 (0.3)
Soil*Inoculum*Year	0.206 (0.8)	1387.2 (Soil+Inoculum+Year)	1.108 (0.9)
Soil*PAM*Year	1.288 (0.3)	1387.6 (Soil+PAM+Year)	0.418 (0.7)
Year+Soil+Year*Soil		**1370.2**	
Year+Inoculum+Soil+Year*Soil		**1372**	

Table 1-3: Averages and standard errors of chl a, cEPSs, and TBEPSs amounts in Year 3 of each experimental treatment compared to intact and scraped controls on clay and sandy clay loam soils. Chl a reported in (µg chl a/g soil), cEPSs and TBEPSs reported in (µg glucose/g soil).

			Chl a		cEPS		TBEPS	
Experiment	Treatment	Soil Type	Avg	SE	Avg	SE	Avg	SE
--	Intact Control	Clay	24.361	3.448	178.286	30.282	285.695	36.662
	Scraped Control	Clay	3.9002	0.891	93.571	17.574	190.949	11.037
Inoculum	NR	Clay	10.814	1.324	177.486	48.782	236.443	56.346
	Low	Clay	14.679	2.208	114.922	20.817	248.453	19.654
	Medium	Clay	7.066	1.627	178.591	25.365	215.905	46.397
	High	Clay	13.937	1.998	179.601	10.518	275.944	50.7115
Shade	NR	Clay	10.814	1.324	177.486	48.782	236.443	56.346
	Shade	Clay	17.3133	1.714	170.694	25.213	225.997	16.345
	Shade + Inoc	Clay	25.571	1.697	176.548	14.86	256.653	19.455
PAM	NR	Clay	9.318	0.698	148.706	74.145	171.811	12.717
	PAM	Clay	9.324	1.775	84.991	14.174	181.046	11.41
	PAM + Inoc	Clay	11.34	2.412	106.513	12.97	170.34	25.947
--	Intact Control	Sandy Clay Loam	16.803	1.848	73.854	11.5576	183.845	17.569
	Scraped Control	Sandy Clay Loam	2.075	0.343	48.172	7.3623	108.805	4.065
Inoculum	NR	Sandy Clay Loam	9.392	1.448	67.692	10.499	168.229	10.439
	Low	Sandy Clay Loam	15.863	2.559	78.703	11.901	237.737	23.181
	Medium	Sandy Clay Loam	18.33	2.564	64.294	4.583	176.018	8.4101
	High	Sandy Clay Loam	18.115	2.976	60.714	10.959	215.324	10.939
Shade	NR	Sandy Clay Loam	9.392	1.448	67.692	10.499	168.229	10.439
	Shade	Sandy Clay Loam	11.453	2.154	84.283	12.942	207.379	38.392
	Shade + Inoc	Sandy Clay Loam	17.635	2.316	72.508	13.208	217.748	22.339
PAM	NR	Sandy Clay Loam	7.789	1.79	68.767	13.123	133.982	6.335
	PAM	Sandy Clay Loam	9.912	1.602	61.933	9.928	167.465	14.07
	PAM + Inoc	Sandy Clay Loam	8.988	1.044	86.179	10.475	140.725	12.346

Treatment did not affect cEPSs amounts, but the interaction between soil type*year did (Table 1-2). At the clay site, cEPS increased by 2.7-fold from Year 1 to Year 2, and 2.3-fold from Year 2 to Year 3 (Figure 1-4a). However at the SCL site, cEPSs were the greatest in Year 2 (63% greater than Year 1 and 8% greater than Year 3) (Figure 1-4b). In Year 1, cEPSs at the SCL site were greater than those at the clay site. However by Year 3, there was 3x the amount of cEPSs

in the clay soil compared to SCL soil (Figure 1-4a,b). After three years, there were no significant differences in cEPSs between inoculum treatments and scraped or intact controls (Table 1-3).

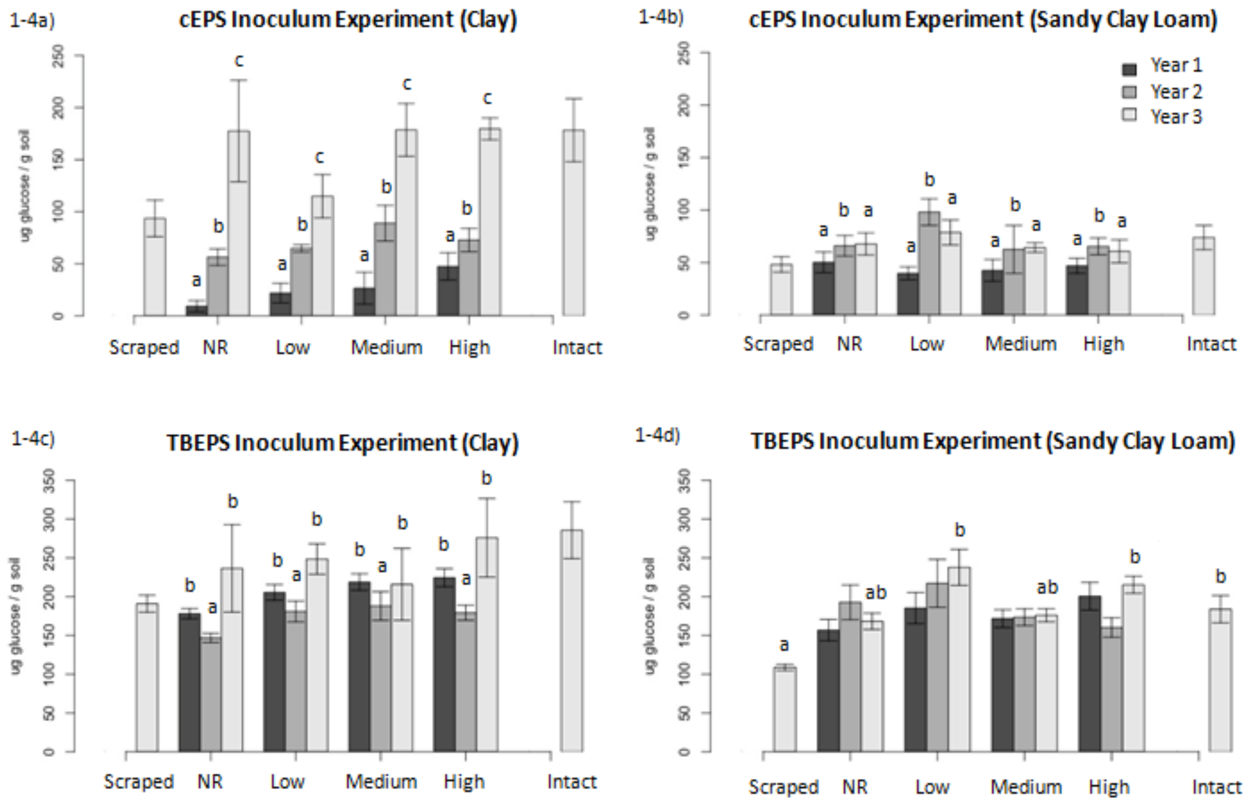

Figure 1-4: EPS amounts from inoculum experiment averaged between years and treatments. Scraped and intact controls were only taken in Year 3. 1-4a) cEPSs on clay soil, separated by year and treatment. There were no differences between treatments, but cEPSs increased by year ($p<0.001$). 1-4b) cEPSs on sandy clay loam soil, separated by year and treatment. There were no differences between treatments, but cEPS amounts in Year 2 were greater than in Years 1 and 3 ($p<0.05$). 1-4c) TBEPSs on clay soil, separated by year and treatment. There were no differences between treatments, but Year 2 had less TBEPSs than Years 1 and 3 ($p<0.01$). 1-4d) TBEPSs on sandy clay loam soil, separated by year and treatment. In Year 3, low and high inoculum plots contained more TBEPSs than NR plots ($p<0.05$). Letters indicate significance between years (1-4a,b,c) and treatments in Year 3 (1-4d) ($p<0.05$). Error bars represent ±1 SE.

TBEPSs levels differed by inoculum level, soil type, and year (Table 1-2). At the clay site, there were no differences in TBEPSs between treatments, but TBEPSs were significantly lower in Year 2 than in Years 1 and 3 (19% and 41%, respectively) (Figure 1-4c). At the SCL site, low inoculum plots contained 24% more TBEPSs than the control plots and surprisingly, 23% more

than medium inoculum plots, but there were no differences between years (Figure 1-4d). In Year 1 following inoculation, TBEPSs in the high inoculum treatment exceeded those in NR by 26%; however, by Year 3, that difference disappeared (Table 1-3). In Year 3, TBEPSs in experimental plots were the same as in the intact control and surprisingly, scraped control on clay soil (Table 1-3), indicating that treatment did not increased TBEPSs after 3 years. After 3 years on SCL however, low inoculum plots had 41% more TBEPSs than the NR plots, and all plots (i.e., intact control, NR, low, medium, high) had significantly more TBEPSs than scraped control plots (Figure 1-4d; Table 1-3). Thus, low inoculum level was the most effective restoration treatment for TBEPSs on SCL.

Soil aggregate stability varied by soil type, but not by treatment. On both clay and SCL soil types, soil aggregate stability increased from Year 1 to 2 (23% and 28%, respectively) and then maximally stabilized.

1.4.2 Shade experiment

Shade increased chl a on clay soil, and the shade+inoculum treatment resulted in the greatest chl a amounts across years on clay soil (Figure 1-5a,b). Shade had a strong but unsignificant (p=0.052) effect on increasing chl a at the SCL site (Figure 1-5b). Soil type, inoculum, shade, year, and the interaction between soil*inoculum affected chl a levels in the overall model. When intact and scraped plots were compared to treatments in Year 3, shade+inoculum plots resulted in the highest amount of chl a, almost 50% higher than in shade only plots on clay soil (Figure 1-5a, Table 1-3). The shade+inoculum treatment was the only treatment across all experiments, including the inoculum and PAM experiments, that resulted

in chl a amounts equal to that of the intact control in Year 3 (Table 1-3). Shade+inoculum also appeared to be the greatest on SCL; however, the difference in chl a was not significantly different than that of shade only plots (Figure 1-5b). On both soil types however, shade+inoculum resulted in almost 2x the amount of chl a amounts than in NR plots. This result demonstrates that shade+inoculum is an effective treatment that increases photosynthetic potential at both sites, particularly on clay soils.

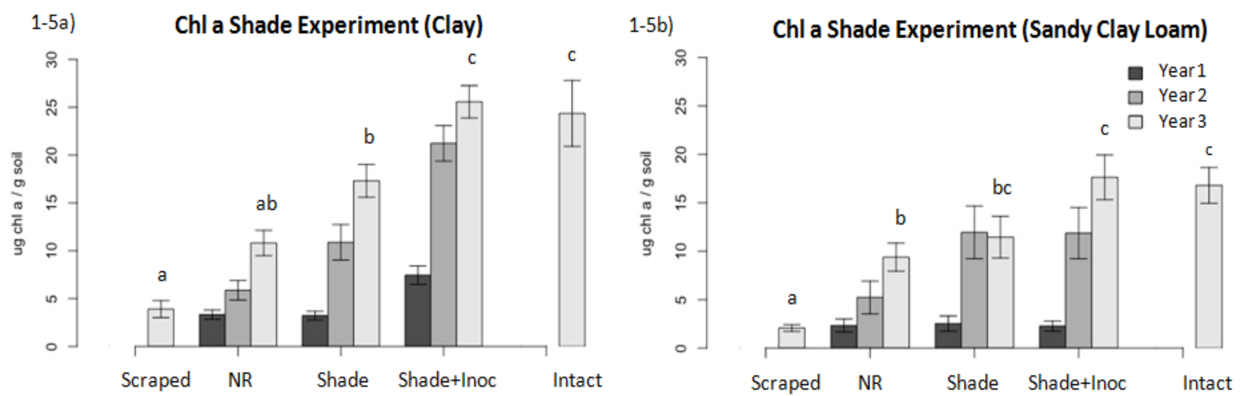

Figure 1-5: Chl a amounts averaged across natural recovery (NR), shade, and shade+inoculum plots in each year following restoration treatment. Intact and scraped controls were taken in Year 3 only. 1-5a) Averages of chl a between treatments and years on clay soil. Shade+inoculum was the most successful treatment in increasing chl a, and the presence of shade increased chl a compared to NR plots ($p<0.01$) overall. 1-5b) Averages of chl a between treatments and years on sandy clay loam soil. Shade+inoculum resulted in greatest amount of chl a compared to other treatments ($p=0.052$); however, the margin was not as striking as the same treatment on clay soil. Letters indicate significance between treatments in Year 3 ($p<0.05$), error bars represent ±1 SE.

cEPS amounts were affected by soil type, inoculum, shade, year, and interactions between soil*inoculum, soil*year, inoculum*year, and shade*year (Table 1-2). The many interacting variables made it difficult to parse apart individual fixed variable effects on cEPSs (Figure 1-6a,b; Table 1-2). When cEPSs in shade, shade+inoculum, and NR plots were compared to scraped and intact plots in Year 3 on each soil type, there were no significant differences

between any treatments (Figure 1-6a; Table 1-3). However in Year 3, clay soils contained 130% higher cEPSs than SCL (Figure 1-6a, b).

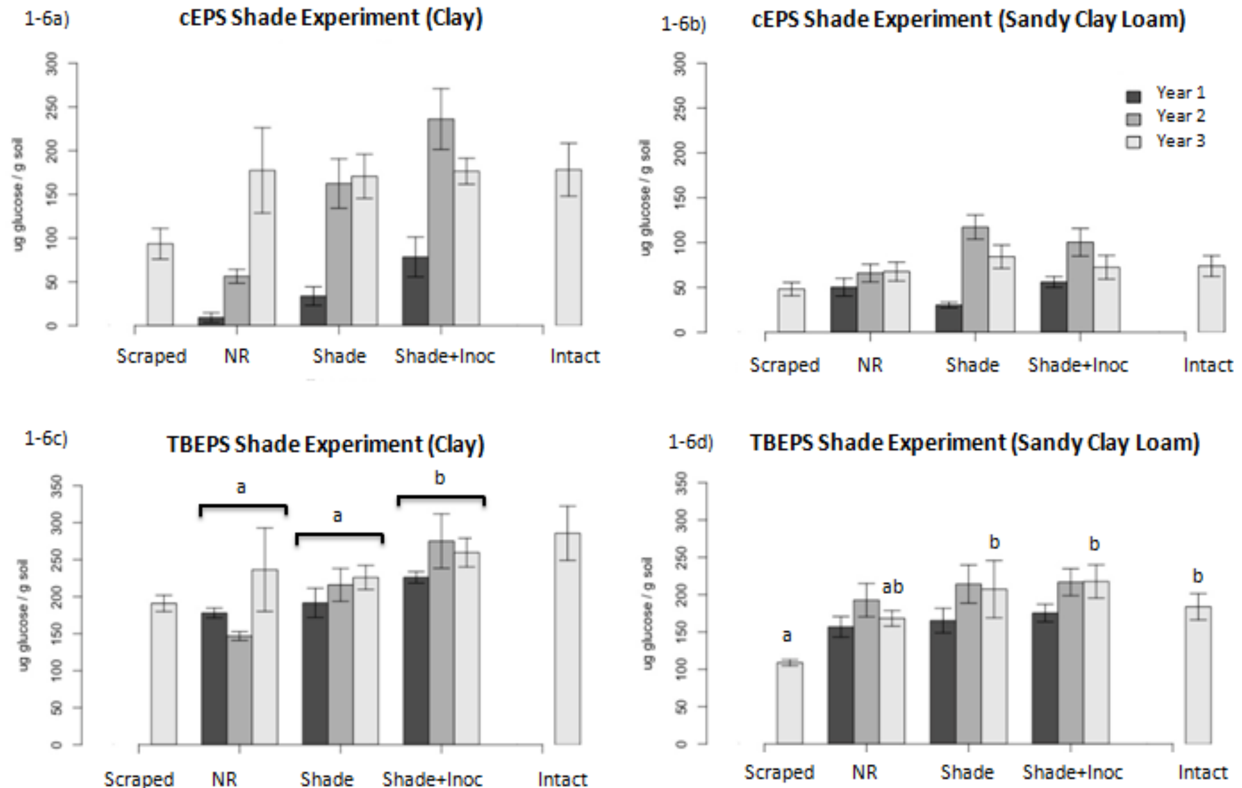

Figure 1-6: EPS amounts from inoculum experiment averaged between years and treatments. Scraped and intact controls were only taken in Year 3. 1-6a) cEPSs on clay soil, separated by year and treatment. There were too many interacting factors between soil type, year, and treatment to determine any overall patterns. 1-6b) cEPSs on sandy clay loam soil, separated by year and treatment. There were too many interacting factors to determine any overall patterns. 1-6c) TBEPSs on clay soil, separated by year and treatment. Shade+inoculum treatment significantly increased TBEPSs compared to NR and shade only plots ($p<0.001$). 1-6d) TBEPSs on sandy clay loam soil, separated by year and treatment. In Year 3, shaded plots contained TBEPSs than scraped plots ($P<0.05$), but not NR plots. Letters indicate significance between overall treatments (1-6c) and treatments in Year 3 only (1-6d) ($p<0.05$). Error bars represent 1 ±SE.

Shade+inoculum treatment also resulted in the highest amounts of TBEPSs (36% more than TBEPSs in NR plots) on clay soil (Figure 1-6b, Table 1-2). We did not see any shade or inoculum effects on TBEPSs on SCL across years; however, shade and shade+inoculum

treatments did increase TBEPSs in Year 3 (Figure 1-6d; Table 1-3). Overall, clay soils contained 39% more TBEPSs than SCL soils.

Similar to patterns observed in the inoculum experiment, soil aggregate stability increased by 18% from Year 1 to Year 2, and then maximally stabilized. There were no differences between soil types or treatments.

1.4.3 Polyacrylamide (PAM) experiment

The addition of PAM did not affect chl a, cEPSs, or TBEPSs on either soil type (Figure 1-7, Table 1-2). The overall model of chl a response showed that inoculum presence, finer soil type, and year following treatment increased the amount of chl a across plots (Table 1-2); however after running post-hoc pairwise tests, soil type and inoculum did not turn out to be significant drivers. Thus, year was the main driver in increasing chl a 3.2-fold from Year 1 to Year 2, and by an additional 63% from Year 2 to Year 3 (Figure 1-7; Table 1-2). In Year 3, intact control plots contained significantly higher amounts of chl a than NR, PAM, and PAM+inoculum plots, but the scraped control plots contained less chl a than these plots (Figure 1-7; Table 1-3).

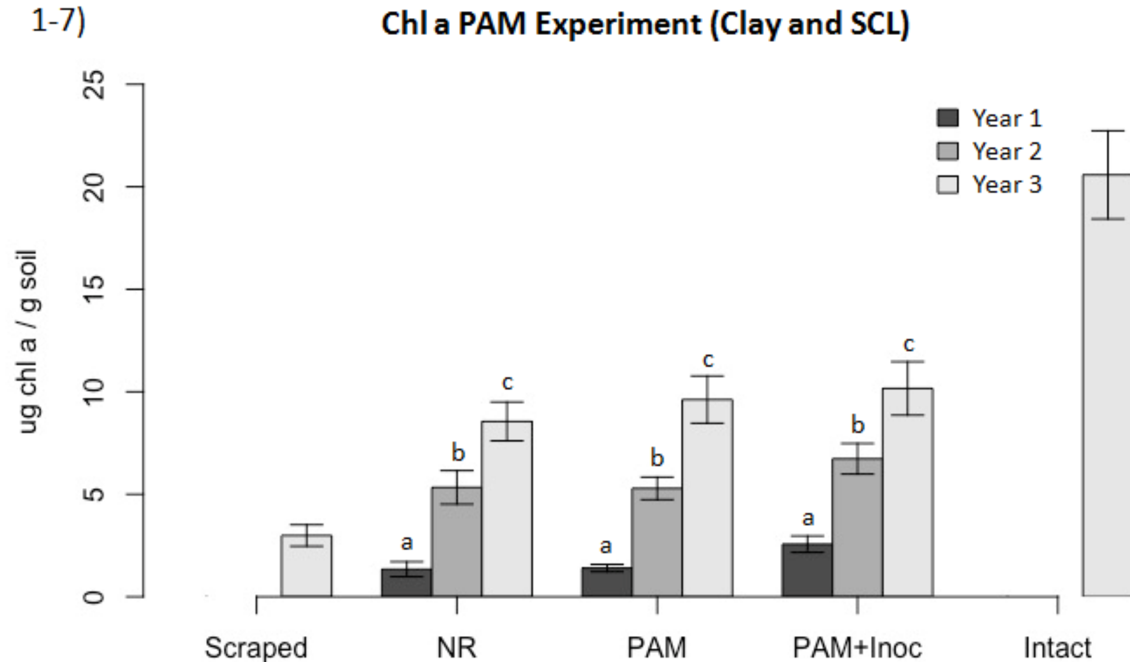

Figure 1-7: Chl a amounts averaged across natural recovery (NR), PAM, and PAM+inoculum plots in each year following restoration treatment on clay and sandy clay loam. Intact and scraped controls were taken in Year 3 only. PAM and PAM+inoculum treatments did not increase chl a amounts. Time was the driving factor in chl a amounts between year ($p<0.001$). Letters indicate significance between chl a amounts between year ($p<0.05$), error bars represent ±1 SE.

cEPSs were only affected by time (Table 1-2). On clay soil, cEPSs increased from Years 1 to 2, while on SCL, cEPSs increased between Years 2 and 3. TBEPS amounts did not change between year or treatment, but were almost 30% higher in clay soils than in SCL.

Soil aggregate stability increased in each successive year following treatments (33% from Year 1 to Year 2, 8% from Year 2 to Year 3); however, there were no differences between treatments or soil types.

1.4.4 Impact of EPS amounts on soil stability and hydrology

Overall Year 3 soil stability (shear strength, compressibility, and soil aggregate stability) was influenced by soil type, cEPSs, and TBEPSs, so we looked at stability on separate soil types.

On clay soil, cEPSs and TBEPSs did not affect stability at all (Table 1-4a). On SCL, EPSs significantly affected stability, but only very weakly (Table 1-4a). Regression analyses on clay soil between cEPSs and TBEPSs by individual responses of soil aggregate stability, shear strength, and compressional strength showed that both EPS fractions were not related to soil stability (Table 1-5). Although the same analyses on SCL soil revealed significant relationships between EPSs and stability measurements, the relationships were extremely weak (Table 1-5).

Table 1-4: Results of cEPS and TBEPS effects on l stability and hydrology. 1-4a) PERMANOVA results using Bray-Curtis dissimilarity distances, evaluating the effects of cEPSs and TBEPSs on soil stability on each soil type (soil aggregate stability, shear strength, and compressional strength). cEPSs and TBEPSs weakly influenced soil stability on sandy clay loam only. Bolded values indicate **p<0.05**. 1-4b) ANOVA results of hydrology (hydrophobicity:infiltration) as predicted by cEPSs, TBEPSs, soil type, and interactions between predictors. F statistics and p values are reported. There were no significant effects of predictor variables on hydrology, although the interaction between TEPS*soil type strongly influenced hydrology (p=0.053).
1-4a)

		PERMANOVA results		
Soil Type	Predictor	F	R^2	p
Clay	cEPSs	2.5	0.04	0.1
Clay	TBEPSs	1.25	0.004	0.6
Clay	cEPSs*TBEPSs	0.02	0.0002	0.9
SCL	cEPSs	4.75	0.08	**0.02**
SCL	TBEPSs	6.97	0.09	**0.007**
SCL	cEPSs*TBEPSs	8.6	0.11	**0.006**

1-4b)

Hydrology (Predictor Variables)	F	p
cEPS	1.7	0.2
TBEPS	0.22	0.6
Soil	0.13	0.7
cEPS*TBEPS	2.5	0.1
cEPS*soil	1.9	0.2
TBEPS*soil	4.0	0.05
cEPS*TBEPS*soil	2.0	0.2

Table 1-5: Linear regression analyses between cEPSs and TBEPSs with individual metrics of soil stability (i.e., soil aggregate stability, shear strength, compressional strength) and hydrology (i.e., unsaturated hydraulic conductivity, hydrophobicity). SCL=sandy clay loam. F-statistics, R^2 values, and p-values are reported for each test. Bolded values indicate **p<0.05**.

Response Type	Soil Type	Variables compared	F	R^2	p
Soil Stability	Clay	cEPS*soil aggregate stability	3.2	0.03	0.08
	Clay	TBEPS*soil aggregate stability	2.5	0.02	0.1
	Clay	cEPSs* shear strength	2.1	0.02	0.12
	Clay	TBEPSs*shear strength	0.5	-0.008	0.5
	Clay	cEPSs*compressional strength	3.6	0.04	0.06
	Clay	TBEPSs*compressional strength	0.81	-0.003	0.4
	SCL	cEPS*soil aggregate stability	5.5	0.07	**0.02**
	SCL	TBEPS*soil aggregate stability	15.6	0.2	**0.0002**
	SCL	cEPSs* shear strength	4.3	0.05	**0.04**
	SCL	TBEPSs*shear strength	0.2	-0.01	0.7
	SCL	cEPSs*compressional strength	4.6	0.05	**0.04**
	SCL	TBEPSs*compressional strength	9.1	0.11	**0.003**
Hydrology	Clay+SCL	cEPSs* hydraulic conductivity	0.37	0.009	0.5
	Clay+SCL	TBEPSs* hydraulic conductivity	0.6	-0.001	0.4
	Clay+SCL	cEPSs*hydrophobicity	10	0.07	**0.002**
	Clay+SCL	TBEPSs*hydrophobicity	8.5	0.05	**0.004**

An ANOVA of hydrological function, measured by the ratio hydrophobicity:unsaturated hydraulic conductivity showed that cEPSs, TBEPSs, soil type, and combinations of these predictor variables did not affect hydrological function (Table 1-4b). However, the interaction between TBEPSs*soil type had a strong effect on determining hydrology (p=0.053) (Table 1-4). Because the overall ANOVA showed that soil type was not a significant predictor in overall hydrology (Table 1-4b), single response variable regression analyses looked at both soil types together. Results from these analyses showed that both cEPSs and TBEPSs were significantly but very weakly correlated to hydrophobicity, and were not correlated to unsaturated hydraulic conductivity (Table 1-5).

1.5 DISCUSSION

1.5.1 Inoculum experiment

Inoculation of soils with field-collected biocrusts did not significantly enhance biocrust recovery, suggesting that scarcity of propagules was a weak limiting factor to biocrust rehabilitation. After three years of recovery, NR plots were all equivalent to intact controls in soil stability, photosynthetic biomass, and EPSs amounts. As demonstrated in other studies, early filamentous cyanobacteria such as *Microcoleus spp.* are able to colonize quickly and enhance ecological functions (Belnap et al., 2003; Zheng et al., 2011), especially on fine soil types (Garcia-Pichel et al., 2001; Rozenstein et al., 2014). This suggests that restoration efforts should be prioritized on sandier soils vs. soils with smaller particle sizes.

Although time was the most important factor driving overall recovery, we observed some treatment differences after three years, namely that low inoculum cover slightly increased chl a recovery on both clay and SCL soils. The determination of what inoculum amount is sufficient for biocrust rehabilitation is important for creating biocrust restoration plans. Because field-collected inoculum is dependent on a “sacrifice area” from which inoculum is collected, it is encouraging to see that only a modest amount of inoculum was needed to see positive effects. However, we did observe positive edge effects of biocrust colonization from the sides, especially on clay soils (*T. Chock, unpublished observation*.) This necessitates testing the low inoculum treatment on larger plots to minimize edge effects and to work toward scaling up to a landscape scale. Encouragingly, studies that have attempted large scale inoculation of cultivated (Chen et al., 2006) and field-salvaged inoculum (Chiquione et al., 2016) indicate that large-scale inoculation efforts across large disturbed areas have shown success.

However, using field-collected inoculum may not be the most sustainable land management practice because of the “sacrifice” areas that are created by collection (Bowker, 2007). Thus, cultivating biocrust organisms under laboratory conditions is an alternative strategy when landscape-scale rehabilitation projects are implemented.

Inoculation did not result in increased cEPSs, but finer soil type did. This is consistent with previous studies that have found cyanobacterial crusts colonize fine-textured soils much faster than soils with larger particle sizes, as cyanobacterial trichomes are able to migrate and bridge smaller spaces between soil particles (Rozenstein et al., 2014). In addition, soils with smaller particle sizes retain more moisture, further facilitating cyanobacterial establishment (Rozenstein et al., 2014).

In clay soil, cEPSs showed the same trend as chl a, accumulating and increasing in each year following treatment. In contrast, TBEPS amounts at the same site were surprisingly lower in Year 2 than in Years 1 and 3. This could possibly be attributed to the heavy rainfall (140mm) in May 2015, the month before monitoring was performed (Supplementary Table S1) (Utah Climate Center, 2017). This heavy rainfall could have reduced moisture stress on *Microcoleus*, thus production of TBEPSs against desiccation was less needed (Wingender, Neu, Flemming [Eds.], 1999). We did not observe depressed TBEPS amounts in Year 2 on SCL soils (Supplementary Table S1), perhaps because these soils drained faster and were unable to retain as much moisture as the clay soil.

On SCL soil, the low inoculum treatment resulted in greater TBEPSs than NR. This suggests that inoculation may be more helpful on coarser soil types, where more cyanobacteria result in greater EPS production that can fill the larger spaces between soil particles and assist

in colonization (Rozenstein et al., 2014). cEPSs were greatest in Year 2, perhaps positively affected by high precipitation in 2015 (Supplementary Table S1) (Utah Climate Center, 2017) and possible increases in C:N ratios (Wingender, Neu, Flemming [Eds.], 1999; Austin et al., 2004).

Soil aggregate stability only took two years to recover to its original level. Although EPS turnover is probably occurring (Chen et al., 2014), overall amounts of EPSs stabilized after 3 years and contributed to soil stability. Positive feedback between biocrust development with microtopography and catchment of fine soil particles could also have contributed to increasing the rate of biocrust recovery (Campbell, 1979; Zheng et al., 2011; Weber, Büdel, and Belnap [Eds.], 2016).

1.5.2 Shade experiment

Shade structures resulted in significant increases in chl a in both soil types, indicating that photosynthetic potential at the site is likely limited by environmental factors such as UV, water, and temperature stress. The combination of shade+inoculum was clearly the most effective biocrust rehabilitation treatment on clay soil, and shade also helped biocrust rehabilitation on SCL. These results suggest that shade may have succeeded in creating more optimal biocrust growth conditions in the field. In the greenhouse, shading has had varied effects on biocrust growth, depending on how much light intensity and temperature are shifted from their optimums (Bu et al., 2014). Other studies attempting to grow biocrust inoculum have also found shade to have positive effects on biocrust growth by increasing soil moisture and reducing UV stress (Velasco Ayuso et al., 2016). For our experiment, the increase in soil

moisture content could have been better retained in the finer clay soil, contributing to the positive effect of shade on biocrust growth in the clay soils. Furthermore, moisture availability has been shown to stimulate cyanobacterial EPS production, possibly creating positive feedback between EPS amounts and moisture retention, resulting in more EPSs (Austin et al., 2004).

We observed increased TBEPS amounts with decreased UV, temperature, and moisture stress in clay soils with the shade+inoculum treatment. Shade did not have as much of an effect on SCL soil, which contained less TBEPSs overall than in clay soils. Interestingly, we did observe differences in TBEPSs amounts between soil types across years. In Year 1, TBEPSs were greater in clay soil than SCL soil, again indicating that early successional cyanobacteria were able to colonize the smaller particle sizes quicker. By Years 2 and 3, however, TBEPS amounts were similar across soil types and did not increase by year. This could have been due to either quick establishment of *Microcoleus spp.* in the first year following treatment, or remnant sheaths present after the original scraping. Sheaths have been observed as deep as 10cm in undisturbed biocrusts (Belnap and Gardner, 1993), and only 2cm of the top surface was scraped at the commencement of our experiment. TBEPSs seemed to level off at about 200μg/soil, and then did not increase with time. This threshold is consistent with other studies looking at amounts of TBEPSs in induced biological soil crusts 4,6, and 8 years post-restoration treatment (Chen et al., 2014), suggesting that there may be a threshold of filament amounts.

Shade treatments did not have any effects on cEPS amounts; however, we again observed higher cEPS accumulation in the clay soil. Although shade proved to be an effective treatment in increasing chl a and TBEPSs amounts, land managers must consider the feasibility and cost of this option if they want to implement this treatment on a landscape scale. In

addition, shading finer soil types that can better retain moisture from reduced temperature and UV stress should be targeted over coarser soil types that have higher rates of evaporation.

1.5.3 Polyacrylamide (PAM) experiment

PAM had no effect in increasing chl a, cEPSs, or TBEPSs, or soil stability on either soil type. However, PAM did not inhibit chl a amounts, as it is had been found to do in previously on sandy soils in the field (Davidson et al., 2002). Another artificial soil stability method using straw checkerboards paired with broadcast inoculation has been highly successful on moving sand dunes in extremely arid environments (Li et al., 2006; Qiu et al., 2014; Zhao et al., 2016). However, actively eroding soils do not seem to be a barrier at Hill AFB, most likely due to the fine soil particle size.

1.5.4 Impact of EPS amounts on soil stability and hydrology

EPSs effects on soil stability differed by soil type. On clay soils, neither EPS fraction influenced overall soil stability or any individual soil stability metrics. However on SCL, both cEPSs and TBEPSs had significant, but very weak effects on overall soil stability and were only marginally correlated positively to individual soil stability measurements. Finer soil types are inherently more stable than coarser soil types due to their higher surface area to volume ratio, which increases their ability to bind to minerals and organic matter (NRCS, 1996). Organic matter, in conjunction with clay content, has also been shown to increase soil aggregate stability (Chenu et al., 2000). This may be the reason why EPSs contributed more to stabilization on coarse soils than fine soils in our experiment. Because of the strength of TBEPSs and their ability to bind individual soil particles, we had expected that TBEPSs amounts would be more

strongly correlated with stability. However, an increase in TBEPS amounts may have been the result of increased sheath thickness rather than surface area, the latter which is more important in physical aggregation of soil particles (Bowker et al., 2008).

We had also expected that EPSs would have an influence on overall hydrology, but did not observe this. Previous studies have showed that soil texture is the paramount factor in determining the overall speed of water infiltration (Brady and Weil, 1996) over biotic factors such as biocrusts (Rossi et al., 2012); however, we did not observe differences in infiltration on soil type. Our conflicting observations may be due to the fact that hydrological function is a very complex process. Soil type, biocrust composition and development, the scale of measurement, microtopographic features, and even time following the wetting event can be contributing and confounding factors to infiltration and runoff (Brock, 1975; Kidron et al., 1999; Williams et al., 1999; Warren, 2001; Rossi et al., 2012; Pointing and Belnap, 2012; Colica et al., 2014; Chamizo et al., 2015; Chamizo et al., 2016; Belnap and Büdel, 2016). Thus, EPSs need to be considered along with more of these hydrological factors when trying to determine which mechanisms are underlying biocrust hydrology.

1.6 CONCLUSION

The results from this study can be used to direct future biocrust restoration efforts to regain ecosystem functions in widespread and expanding dryland ecosystems. This study suggests that barriers to biocrust restoration should first be identified and then addressed with appropriate restoration techniques, with special attention given to soil type. Limiting barriers observed at the Hill AFB are likely propagule scarcity and moisture and UV stress limitations.

Biocrusts on clay soils responded very postively to the shade+inoculum treatment, while the addition of field-collected inoculum at a low level emerged as the most generalizable restoration strategy on SCL. PAM did not demonstrate any perceivable effects on biocrust recovery on either soil type. Overall natural recovery on clay soils was faster than that on SCL soils, indicating that restoration treatments should be focused on less stable soils. Land managers, conservation organizations, and other stakeholders can use these data to consider tradeoffs between the amount of ecosystem function gained and the feasibility and sustainability of these biocrust restoration strategies at a larger scale, considering cost, effort, and time. If time is not a restricting factor, natural recovery may be the most parsimonious option, especially if soil stability is a main restoration goal.

This experiment also provides insight into mechanisms of ecosystem functions under natural conditions. We demonstrated that amounts of cEPSs and TBEPSs have no correlation with overall soil stability and hydrology on clay soil types, but do slightly influence hydrophobicity and overall soil stability on coarser soil types. Therefore, cyanobacterial exudates may better estimate overall soil stability on sandier soil types. However, if biocrust restoration strategies are to be tailored to augment these specific ecosystem functions, other factors such as soil type, scale, and biocrust heterogeneity need to be considered, especially on finer soil types.

More research is needed to address scaling up these restoration techniques to the landscape level. However, the results of this study provide valuable insight into potential issues (e.g., barriers to restoration, soil type, time) that need to be addressed when considering biocrust rehabilitation. We also now have a deeper understanding of how EPSs contribute

mechanistically to ecosystem functions to help tailor restoration efforts to specific ecosystem functions.

CHAPTER 2:
BIOCRUST MICROBIAL COMMUNITY COMPOSTION FOLLOWING INOCULUM RESTORATION

2.1 ABSTRACT

Biological soil crusts (biocrusts) are comprised of soil surface microbial communities of cyanobacteria, lichens, mosses, and other microorganisms that play key roles in dryland ecosystem functions, and are thus excellent targets for dryland restoration. One biocrust restoration strategy is to use field-collected inoculum following disturbance. We wanted to see if biocrust microbial community richness increased with time following an inoculum restoration treatment on bare soil, and if biocrust microbial community composition reflected macroscopic level of development (LOD) patterns of natural biocrust recovery. We found that biocrust microbial richness (defined by number of operational taxonomic units [OTUs]) did not change over time, perhaps because field-collected inoculum was collected from mature biocrusts with rich microbial communities. We also found that microbial community composition reflected macroscopic LOD patterns and changed in each year following restoration treatment, driven by increases in relative abundances of later successional dark cyanobacteria *Nostoc* and *Scytonema*. After three years of recovery, recovering biocrust communities were still distinct from nearby intact control communities, indicating that biocrust microbial community recovery is a slow process.

2.2 INTRODUCTION

Biological soil crusts (biocrusts) contribute to dryland ecosystem functions such as soil stability, biogeochemistry, and hydrology (Weber, Büdel, Belnap [Editors (Eds.)], 2016). Rehabilitation and restoration of biocrusts to try to restore these ecosystem functions are currently being explored (Bowker, 2007). In order to successfully restore biocrusts and their contributions to ecosystem functioning, we need a better understanding of the microbial communities that comprise biocrusts. In this study, we wanted to determine how biocrust microbial communities change through time following an inoculation restoration technique. We also wanted to see if microbial community changes reflect macroscopic observations of level of development (LOD) in natural recovery.

Biocrust inoculation is geared at mimicking natural succession and restoring ecosystem functions such as soil stability. Natural biocrust succession is initiated by large, filamentous light cyanobacteria of the genus *Microcoleus* (Zheng et al., 2011; Weber et al., 2016). *Microcoleus* secrete exopolysaccharides (EPSs), which create filaments that hold individual soil particles together and provide a stable surface for dark cyanobacteria, moss, lichen, and other later successional colonizers (Campbell 1979; Belnap and Gardner, 1993; Mazor et al., 1996; Zheng et al., 2011; Mager and Thomas, 2011). Dark cyanobacteria containing scytonemin sunscreen pigments (e.g., *Scytonema*) and that fix nitrogen (e.g. *Nostoc*) are mid-successional colonizers, while lichen and moss species are the last to colonize. As biocrusts age and mature, they contain a greater array of monosaccharides, and sucrase and hydrogenase enzymes, suggesting that EPSs are degraded by heterotrophic microorganisms into various carbon forms (Chen et al., 2014). This diverse array of metabolites supports the idea of exometabolite niche partitioning,

as byproducts from one microorganism's metabolism are often used as another's metabolite, and biocrust microbes seem to be fairly selective in the metabolites that they can utilize (Baran et al., 2015). The large array of exometabolites and microbial metabolic specialization may drive the high levels of soil biota diversity typically seen in soil (Baran et al., 2015).

A macroscopic way to quickly assess biocrust development is by level of development (LOD), a visual field assessment using the presence and amount of broad types of biocrust species present (e.g., lichen, light cyanobacteria, moss) and microtopography (Belnap et al., 2008). Early successional biocrusts following disturbance are dominated by light cyanobacteria, have little variability in microtopography, and are thus characterized as an LOD of 1. As LOD progresses, biocrusts become pigmented with dark cyanobacteria such as *Scytonema* and *Nostoc*, thicker, and have more dramatic surface microtopography. Biocrusts with the highest LOD, 6, contain a high cover of mosses and lichens and have dramatic microtopography, at times showing pinnacles and troughs from frost-heaving (Belnap et al., 2008; Weber, Büdel, and Belnap [Eds.], 2016). As LOD increases, ecosystem functions such as soil aggregate stability and water retention increase as well (Weber, Büdel, and Belnap [Eds.], 2016; Faist et al., 2017). Although much is known about broad levels of biocrust taxonomy throughout succession, only few studies have characterized detailed microbial community operational taxonomic unit (OTU) composition of biocrust throughout natural succession following recovery (Yeager et al., 2004, Kuske et al., 2012, Nejidat et al., 2016).

The few studies that explore biocrust microbial community composition throughout natural recovery have shown that microbial communities reflect LOD characteristics. In natural recovery, poorly developed biocrusts were dominated by *Microcoleus vaginatus,* and later

transitioned into increased *Nostoc* and *Scytonema* abundances with development (Yeager et al., 2004). Soil types and characteristics may select for specific cyanobacteria species, playing an important role in overall biocrust community structure (Garcia-Pichel et al., 2001). Mature biocrusts have been shown to have approximately 10 times the amount of nitrogenase activity than less developed biocrusts, mainly attributed to the presence of nitrogen-fixing *Nostoc spp.* (Yeager et al., 2004). In addition, mature crusts have been shown to have less soil erosion and C and N concentrations than trampled biocrusts (Kuske et al., 2012). To date however, there have not been any studies that specifically try to determine if microbial community composition following restoration follows the same patterns of natural succession under natural conditions.

The main question addressed in this study is: does microbial community composition of biocrusts change in a predictable manner with biocrust succession after restoration efforts? To answer this question, we looked at both whole-community composition as well as diversity of the most abundant group in biocrusts, cyanobacteria. Samples were taken from plots restored with inoculum addition one, two, and three years post-inoculation treatment. We also took intact control samples from nearby in the third year post-treatment to compare how similar restored biocrust communities were to those of intact controls after three years. We hypothesized that in the years following inoculation, LOD would increase and microbial community composition would follow that of natural recovery, displaying corresponding shifts in community composition through increases in later-successional dark cyanobacteria such as *Nostoc* and *Scytonema*. We also predicted that species richness would increase with LOD, as there would be more available N forms and varied carbon sources, creating more niches for specialized soil microorganisms (Chen et al., 2014; Baran et al., 2015).

2.3 METHODS

2.3.1 Sample collection

Samples were collected from the Utah Test and Training Range at Hill Air Force Base, Utah, in the Great Basin Desert. A biocrust restoration project was implemented in April 2013, when 0.25m² plots were physically scraped to remove all biocrust from the surface (top 2cm). The scraped biocrust was saved and crumbled into pea-sized fragments to be used as field-collected inoculum. Approximately 100cc (10% cover) of this biocrust inoculum was sprinkled on the scraped plots, with a total of 5 replicate plots within a 200m² area. Restoration progress 1, 2, and 3 years after treatment was assessed by collecting biocrust samples in June 2014, June 2015, and May 2016, respectively. Five replicate intact biocrust controls were taken only in May 2016 as an intact biocrust reference. For each 0.25m² plot sampled, three sub-samples using a 1.5 cm diameter core were taken to 1 cm depth and pooled. The combined biocrust sample from each plot was homogenized with a mortar and pestle and further mixed using the cone and quarter method (Gerlach et al., 2002). Samples were collected from each of the five inoculum plots in 2014, 2015, and 2016 (hereby referred to as Year 1, 2, and 3, respectively), and five samples from intact control plots in 2016. 20 samples were analyzed in total.

2.3.2 DNA Extraction, PCR, and Sequencing

Bacterial DNA from biocrust samples was extracted using the MoBio PowerSoil® DNA Isolation Kit with modifications from approximately 0.2g of soil for each sample (Fierer et al., 2008). Extracted DNA was amplified by PCR (Bates et al., 2011; Lauber et al., 2013; Emerson et al., 2015). Soil bacterial communities were identified using barcoded Illumina MiSeq sequencing

of the V4 region in bacterial and archaeal 16S rRNA genes. Extracted genomic DNA was amplified by PCR using barcoded 515F/806R primers. All samples were diluted to equimolar concentrations, cleaned with the UltraClean PCR Clean-up Kit (MoBio Inc., Carlsbad, CA, USA), and pooled. Pooled barcoded PCR product samples were analyzed on the Illumina MiSeq machine at the University of Colorado at Boulder Next Generation Sequencing Facility.

2.3.3 Sequence processing

Resulting sequences were processed as described using the UPARSE pipeline (Edgar, 2013). Sequences were demultiplexed, and paired end reads were merged. Remaining sequences were quality filtered at a rate of 0.5%. Singleton sequences were identified and removed. A *de novo* database and subsequent OTU table were created with the remaining filtered sequences at 97% similarity level. Taxonomic classifications were assigned to each OTU using the Ribosomal Database Project taxonomic classifier with the GreenGenes database. Chloroplast and mitochondria sequences were removed, and the remaining OTUs were rarefied to a depth of 12,000. There were 2,744 distinct OTUs represented in the rarefied data set.

2.3.4 Cyanobacterial OTUS

We extracted cyanobacterial OTUs to consider this most abundant biocrust functional group on its own. Cyanobacterial OTUs were rarefied to a depth of 4,130. The same sequencing processing and statistical analyses described above were performed on cyanobacteria only. The most abundant cyanobacterial OTUs were identified using BLAST alignment searches and a cyanobacterial phylogenetic tree based on individual scrutiny (Velasco-Ayuso et al., 2016).

2.3.5 Statistical analyses

The vegan package (Okansen et al., 2017) in R (R Core Team, 2016) was used for all statistical analyses on the rarefied dataset. Principle coordinate analyses (PCoA) using Bray-Curtis and Jaccard dissimilarity matrices were used to visualize differences in microbial composition between samples. We used permutational multiple analysis of variance (PERMANOVA) to determine which factors (i.e., year, treatment) accounted for differences in community composition between samples. We also explored which taxa were responsible for in driving differences in community composition between sample groups.

2.4 RESULTS AND DISCUSSION

2.4.1 Richness

Across samples, OTU richness was very high (Figure 2-1), as is commonly observed when sampling whole soil microbial communities. There were no obvious differences in OTU richness between years (Figure 2-1). This was a bit surprising, as we were expecting to observe more taxa in successive years post-restoration with the highest diversity in the intact control; a pattern seen in previous studies of natural biocrust recovery (Yeager et al., 2004, Kuske et al., 2012, Nejidat et al., 2016). However, the field inoculum that we used was taken from mature biocrusts, so perhaps propagules from well-developed biocrusts were able survive and colonize the bare soil substrate. In addition, environmental factors such as moisture and temperature varied between years (Supplemental Table S1) (Utah Climate Center, 2017), which could have affected microbial richness and overshadowed increases in richness from niche partitioning created by greater variability of carbon sources (Chen et al., 2014; Baran et al., 2015). We found

that the intact control samples collected in 2016 had higher OTU richness than those from restoration plots in Year 3, suggesting that after three years following restoration treatment, recovering biocrusts were not as diverse as intact biocrusts. If we had collected paired intact samples from Years 1 and 2 post-restoration, we could have had intact reference points for each year and analyzed restoration plots in relation to these.

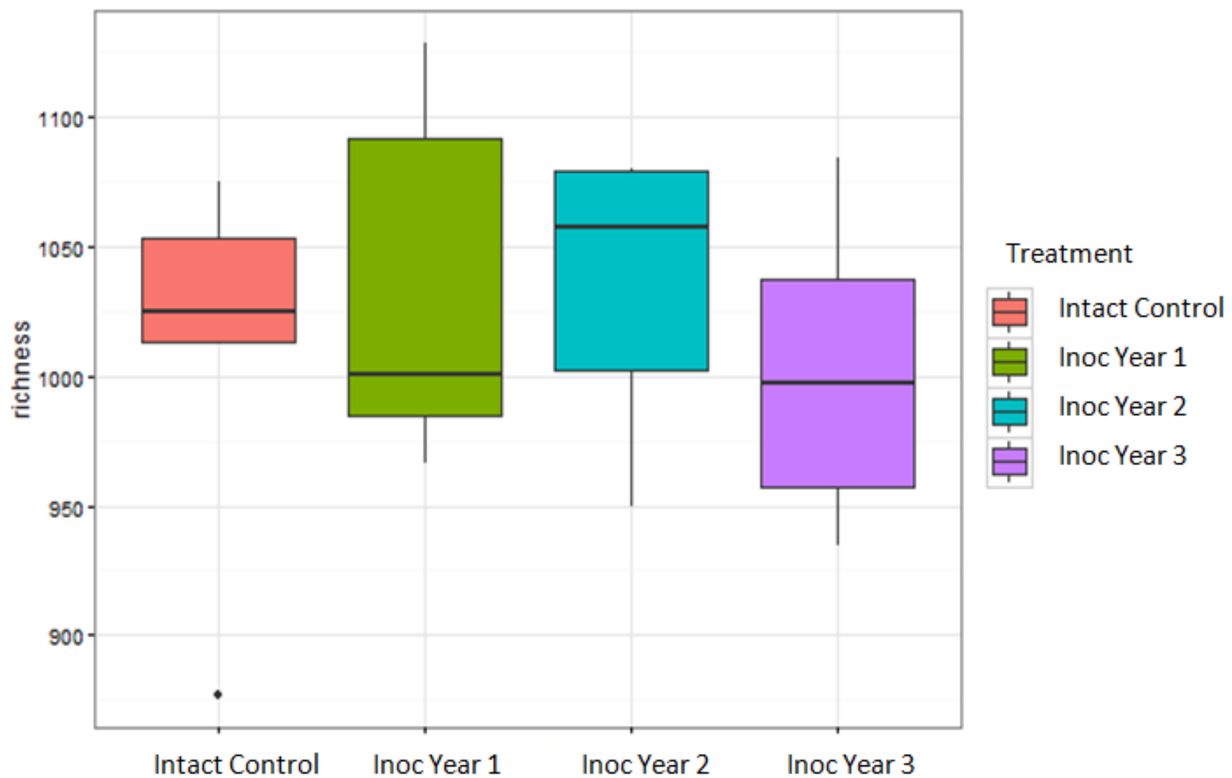

Figure 2-1: Box and whisker plot displaying richness of microbial communities in four sample types: intact control collected three years after restoration treatment (Intact Control), inoculum-treated plots collected one year post-treatment (Inoc Year 1), inoculum-treated plots collected two years post-treatment (Inoc Year 2), and inoculum-treated plots collected three years post-treatment (Inoc Year 3) (rarefaction depth = 12,000).

2.4.2 Microbial community composition

We found that the most relatively abundant phyla across samples were Cyanobacteria, Bacterioides, Proteobacteria, and Acidobacteria, which were consistent with other studies

across the globe that have characterized biocrust communities (Steven et al., 2013; Kuske et al., 2012; Nejidat et al., 2016) (Figures 2-2a, 2-2b). Cyanobacteria and Proteobacteria have been shown to be significantly higher in biocrusts (samples taken from 0-1cm bgs) than in paired samples taken below the biocrust (2-5 cm bgs) in three different soil types (Steven et al., 2013). These phyla were also the dominant OTUs in all soil types; however, their relative proportions differed among soil types (Steven et al., 2013). This suggests soil type is a key factor in microbial community composition, and so the same restoration treatment on different soil types may differ in its efficacy.

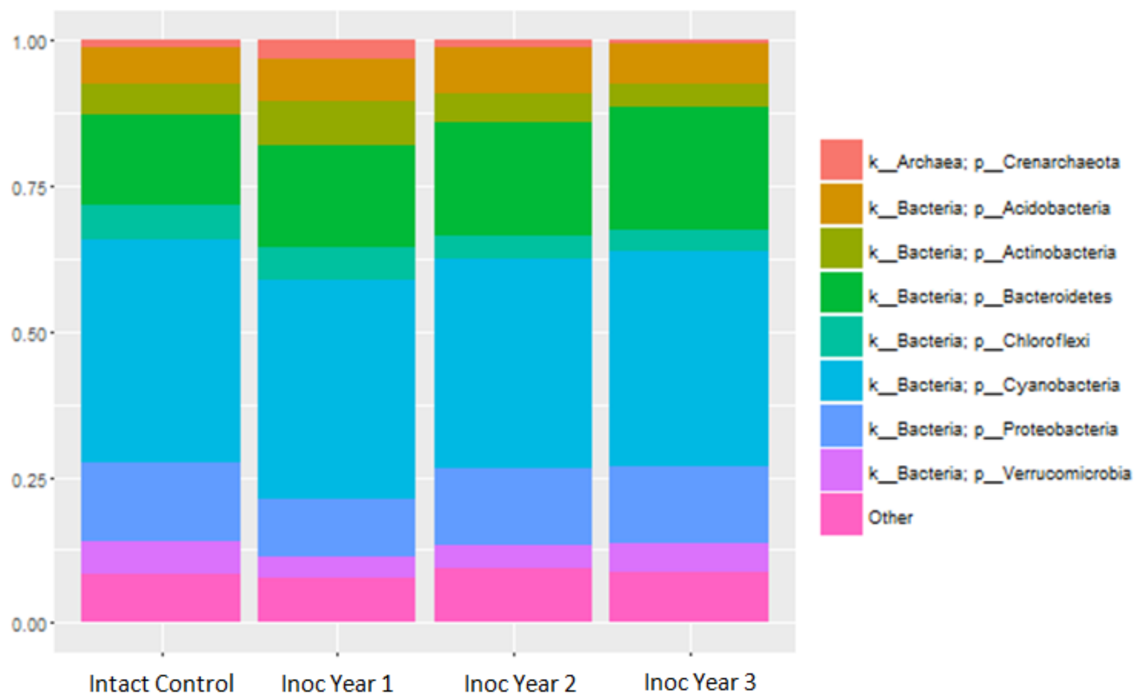

Figure 2-2a: Most abundant phyla in sample types intact control collected three years after restoration treatment (Intact Control), inoculum-treated plots collected one year post-treatment (Inoc Year 1), inoculum-treated plots collected two years post-treatment (Inoc Year 2), and inoculum-treated plots collected three years post-treatment (Inoc Year 3) (rarefaction depth = 12,000).

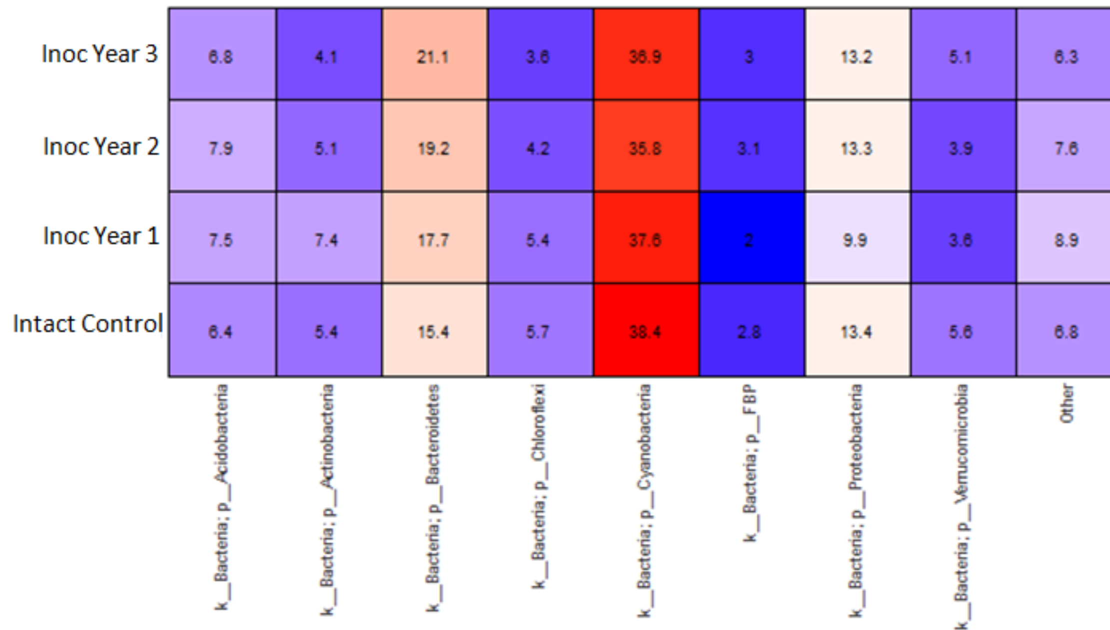

Figure 2-2b: Heat map showing distribution of most abundant phyla across sample types, showing relative evenness of the most abundant phyla.

Principle coordinate analyses (PCoA) ordinations of samples using Bray-Curtis and Jaccard dissimilarity matrices grouped inoculated samples distinct from intact control samples, and generally by year in ordination space (Figures 2-3, 2-4). We found that there were significant temporal changes in communities following inoculation (PERMANOVA, p=0.005) and that there were differences in community composition between intact control and inoculum restoration plots after three years post-treatment (PERMANOVA, p=0.001). This suggests that although biocrusts were recovering, they still had not reached their intact community state after three years. This observation was supported with field LOD observations, where the LOD of inoculated plots was only 4 after three years of treatment, while the LOD of intact controls was 6 (Figure 2-5). While inoculum restoration has been shown to be able to quickly recover some ecosystem functionality such as soil stability (Wei et al., 2005; Chen et al., 2006; Chiquione et al., 2016), full recovery of the microbial community may take much longer.

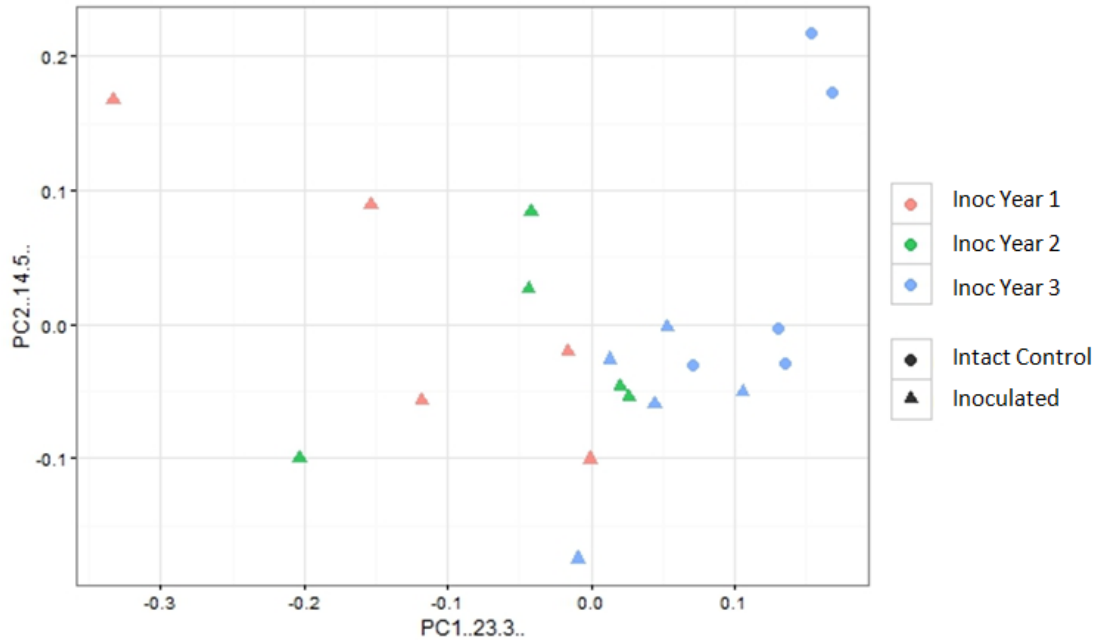

Figure 2-3: Principle coordinate analysis (PCoA) of whole microbial communities using a Bray-Curtis dissimilarity matrix (measure of variability in community composition and abundances) showing the distribution of biocrust communities distinguished by year collected post-treatment and by intact control vs. inoculated plots in ordination space.

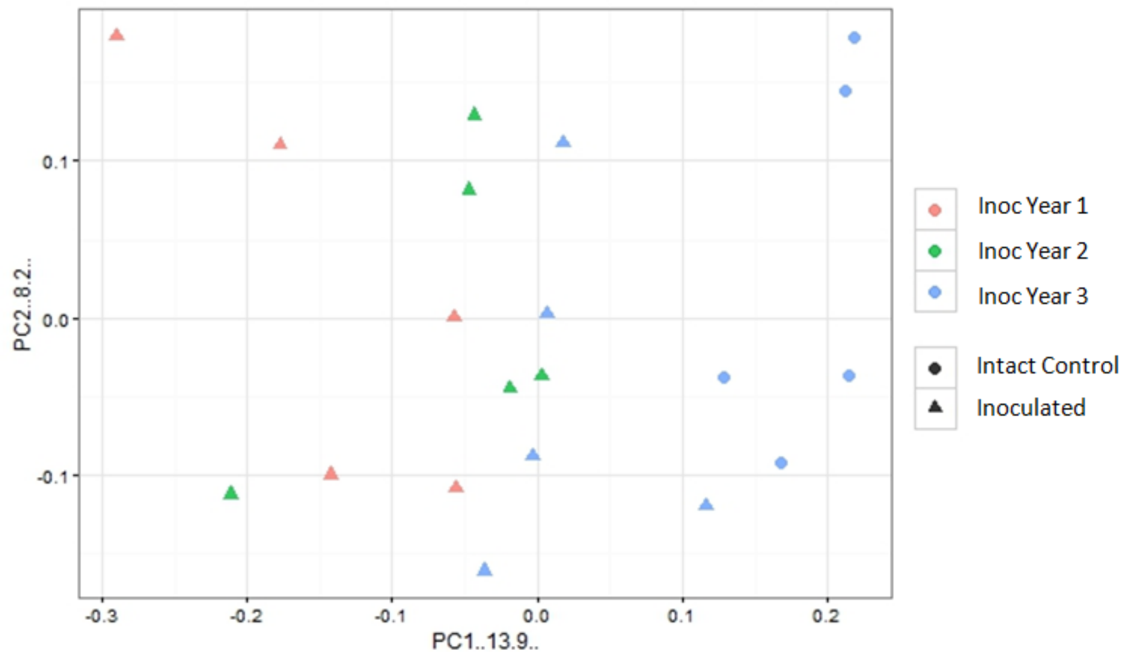

Figure 2-4: Principle coordinate analysis (PCoA) of whole microbial communities using Jaccard dissimilarity matrix (measure of variability in community composition using presence/absence of taxa) showing the distribution of biocrust communities distinguished by year collected post-treatment and by intact control vs. inoculated plots in ordination space.

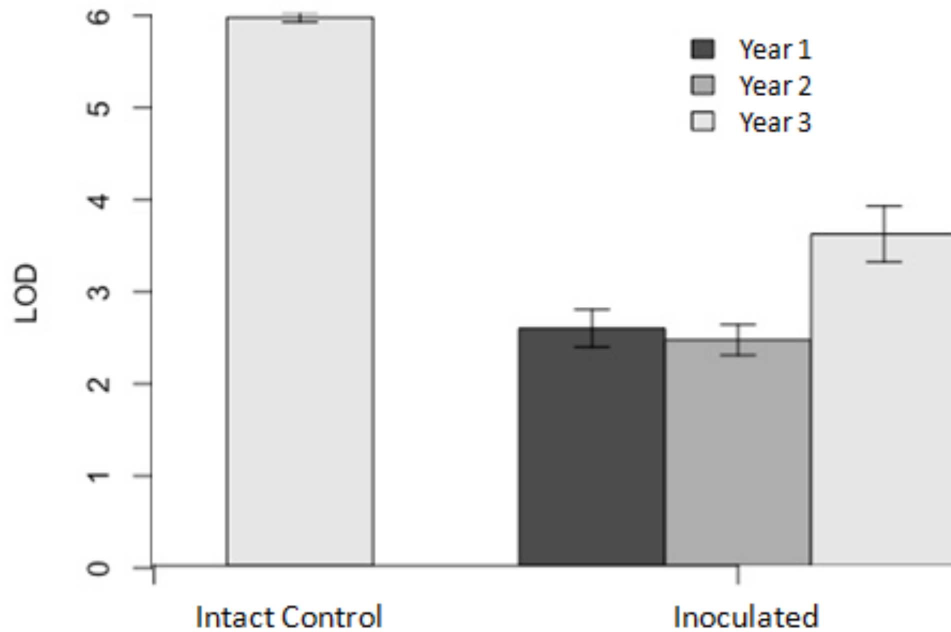

Figure 2-5: Level of development (LOD) characterizations of intact control and inoculated plots, by year following inoculum restoration treatment. Bars represent ±1 standard error (SE).

This study focused on comparing the community composition of 10% cover inoculum plots in each year following inoculation for three years. The taxa most responsible for driving community differences between years (focusing only on taxa in ≥1% abundance at the genus level), were Nostocaceae, Sphingomonas, *Candidatus Nitrosophaera*, Chloroflexi, Cytophagacae, Rubrobacter, Chthoniobacteraceae, Scytonematacae, and FBP ($p<0.05$ for all uncorrected p-values) (Table 2-1). As predicted, cyanobacteria from the family Nostcaceae, a common mid-successional dark cyanobacteria biocrust species that fixes N, approximately doubled in each successional year following inoculation treatment (Table 2-1). Correspondingly, there was also a steady increase of another common dark biocrust cyanobacteria associated with mid-succession, Scytonemataceae, which contains a sunscreen pigment called scytonemin that is responsible for its dark color (Weber, Büdel, and Belnap [Eds.], 2016) (Table 2-1). We also observed a steady decrease in the archaea *Candidatus Nitrosophaera* with time. Previous

studies have identified *Candidatus Nitrospaera* as an ammonia-oxidizing archaea species (AOA) (You et al., 2009; Bates et al., 2011) (Table 2-1). The increase of this AOA could have been an indirect result of the rise in *Nostoc* and other N-fixers, which fix available N into nitrate, therefore leaving less N in the ammonia form for AOAs. We observed a steady decrease in Chloroflexi from Year 1 to Year 2. Chloroflexi have been shown to be more abundant in trampled biocrusts than intact biocrusts (Kuske et al., 2012), and thus our observations reflect biocrust community recovery from the initial scraping event. Finally, there were positive correlations between year and heterotrophic OTUs, with an increase in the family Cytophagaceae, which has been shown to break down plant matter and organic C (McBride et al., 2014) (Table 2-1). Accumulations of cyanobacterial EPSs and photosynthesis products observed in studies looking at EPSs through time following restoration events (Chen et al., 2014) could also have contributed to increase C sources for Cytophagacae. Accumulations of EPSs due to biocrust recovery may have also resulted in the increase observed of an OTU from the family Chthoniobacteraceae, a heterotrophic bacteria that has been shown to break down various types of organic carbon (Kant et al., 2011) (Table 2-1).

Table 2-1: Abundance of the OTU taxa sorted by the genus level that are responsible for microbial community differences in inoculum treatment plots in 1, 2, and 3 years post-restoration efforts (Inoc Year 1, Inoc Year 2, Inoc Year 3, respectively). Known functions of the genus, relative abundances of the taxa for each year, and uncorrected p-values are shown.

OTU (Genus level)	Known Function	Relative Abundance Inoc Year 1	Relative Abundance Inoc Year 2	Relative Abundance Inoc Year 3	P-value (uncorrected)
k__Bacteria; p__Cyanobacteria; c__Nostocophycideae; o__Nostocales; f__Nostocaceae; g__	Dark cyanobacteria, N-fixing (Weber, Büdel, and Belnap [Editors (Eds.)], 2016)	0.006866667	0.013366667	0.02335	0.00919
k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae; g__Sphingomonas	Can use PAHs as energy, often found in contaminated soils, metabolically versatile, chemoheterotrophic, produce quinone (Balkwill et al., 2006)	0.019616667	0.0298	0.031116667	0.011333
k__Archaea; p__Crenarchaeota; c__Thaumarchaeota; o__Nitrososphaerales; f__Nitrososphaeraceae; g__Candidatus Nitrososphaera	Ammonia-oxidizing archaea, tolerant of extremely high levels of ionizing radiation, thermophilic (You et al., 2001; Bates et al., 2011)	0.0311	0.012516667	0.007516667	0.011796
k__Bacteria; p__Chloroflexi; c__Chloroflexi; o__AKIW781; f__; g__	Found in biocrusts in Negev, Israel 0.12% (Hagermann et al., 2014)	0.024533333	0.017266667	0.017116667	0.012778
k__Bacteria; p__Bacteroidetes; c__Cytophagia; o__Cytophagales; f__Cytophagaceae; g__	Chemoheterotrophic, Digest polysaccharides, proteins, cellulose (McBride et al., 2014)	0.012783333	0.018283333	0.020783333	0.013982
k__Bacteria; p__Actinobacteria; c__Rubrobacteria; o__Rubrobacterales; f__Rubrobacteraceae; g__Rubrobacter	Radiotolerant and thermophilic (Albuquerque and da Costa, 2014)	0.024983333	0.01785	0.010983333	0.018500
k__Bacteria; p__Verrucomicrobia; c__[Spartobacteria]; o__[Chthoniobacterales]; f__[Chthoniobacteraceae]; g__	Breakdown of organic C in soil (Kant et al., 2011)	0.015083333	0.0156	0.025383333	0.026252
k__Bacteria; p__Cyanobacteria; c__Nostocophycideae; o__Nostocales; f__Scytonemataceae; g__	Dark cyanobacbateria contains sunscreen pigments (Weber, Büdel, and Belnap [Eds.], 2016)	0.00575	0.011	0.02025	0.027052
k__Bacteria; p__FBP; c__; o__; f__; g__	n/a	0.02055	0.031266667	0.030666667	0.03652

2.4.3 Cyanobacterial OTUS

Cyanobacteria richness stayed stable between years and was similar between the intact control and inoculated plots across years (Figure 2-6). Cyanobacteria composition in successive years reflected the expected LOD pattern of initial colonization and high abundance of light cyanobacteria such as *Microcoleus*, and then the transition to darker cyanobacteria as succession progressed (Belnap et al., 2008; Weber, Büdel and Belnap [Eds.], 2016). Unsurprisingly, the most abundant cyanobacterial OTU in all samples types was *Microcoleus vaginatus,* which is widely documented as the most abundant species in biocrusts around the world (Belnap and Gardner, 1993; Weber, Büdel and Belnap [Eds.], 2016) (Figure 2-7). *Microcoleus steenstrupii*, another common sheath-forming cyanobacteria, was the next most abundant OTU present across samples (Figure 2-7). We observed increases in *Nostoc* and *Scytonema*, two dark N-fixing cyanobacteria known establish following light cyanobacteria in biocrust succession among inoculated plots with time (Belnap et al., 2008; Weber, Büdel and Belnap [Eds.], 2016) (Figure 2-7). The increase in *Nostoc* corresponds to an increase in observed *Collema tenax* lichen in Years 2 and 3 (*A. Antoninka and T. Chock, unpublished observation*), as *Nostoc* is *Collema*'s autotrophic symbiont. A PCoA using a Bray-Curtis dissimilarity matrix showed that cyanobacteria communities grouped together by year and treatment in ordination space (Figure 2-7). There was some overlap in ordination space of microbial communities in Year 1 and 2, but Year 3 microbial communities were more distinct (Figure 2-8). Furthermore, intact communities grouped toward the bottom of the PCoA plot (Figure 2-8). This corresponds with LOD field observations, where we observed an LOD of approximately 3 in Years 1 and 2 post-inoculation treatment and an LOD of 4 in Year 3 post-inoculation treatment (Figure 2-5).

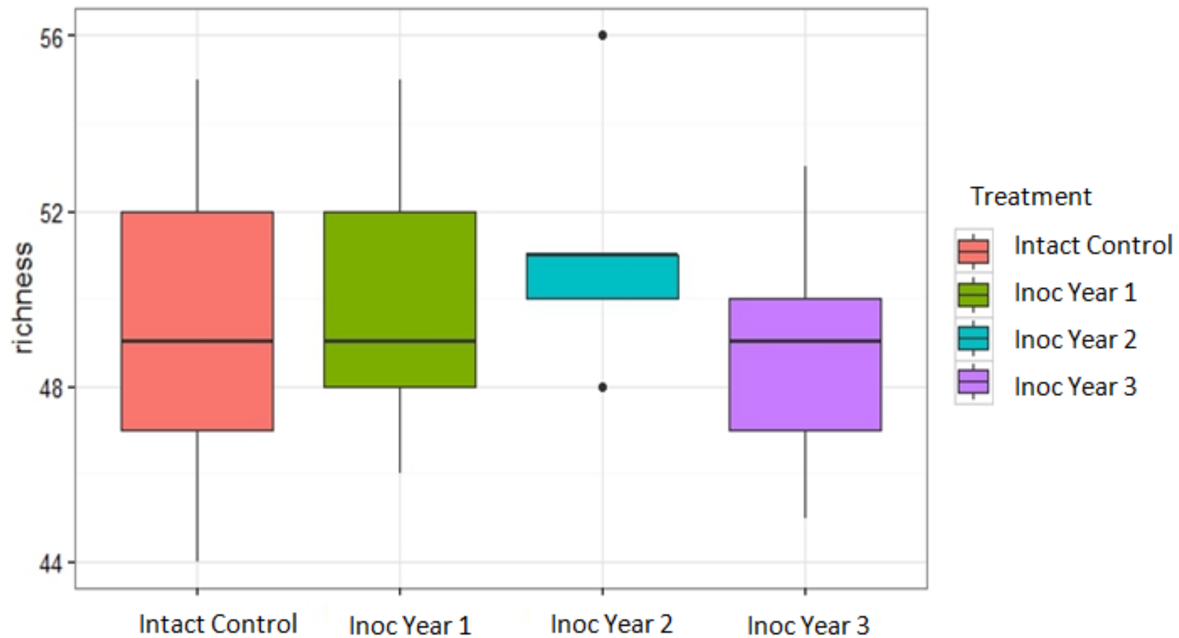

Figure 2-6: Box and whisker plot displaying richness of cyanobacterial communities in four sample types: intact control collected three years after restoration treatment (Intact Control), inoculum-treated plots collected one year post-treatment (Inoc Year 1), inoculum-treated plots collected two years post-treatment (Inoc Year 2), and inoculum-treated plots collected three years post-treatment (Inoc Year 3) (rarefaction depth = 4,130).

Figure 2-7: Most abundant cyanobacterial OTUs in sample types: inoculum-treated plots collected one year post-treatment (Inoc Year 1), inoculum-treated plots collected two years post-treatment (Inoc Year 2), and inoculum-treated plots collected three years post-treatment (Inoc Year 3) (rarefaction depth = 4,130).

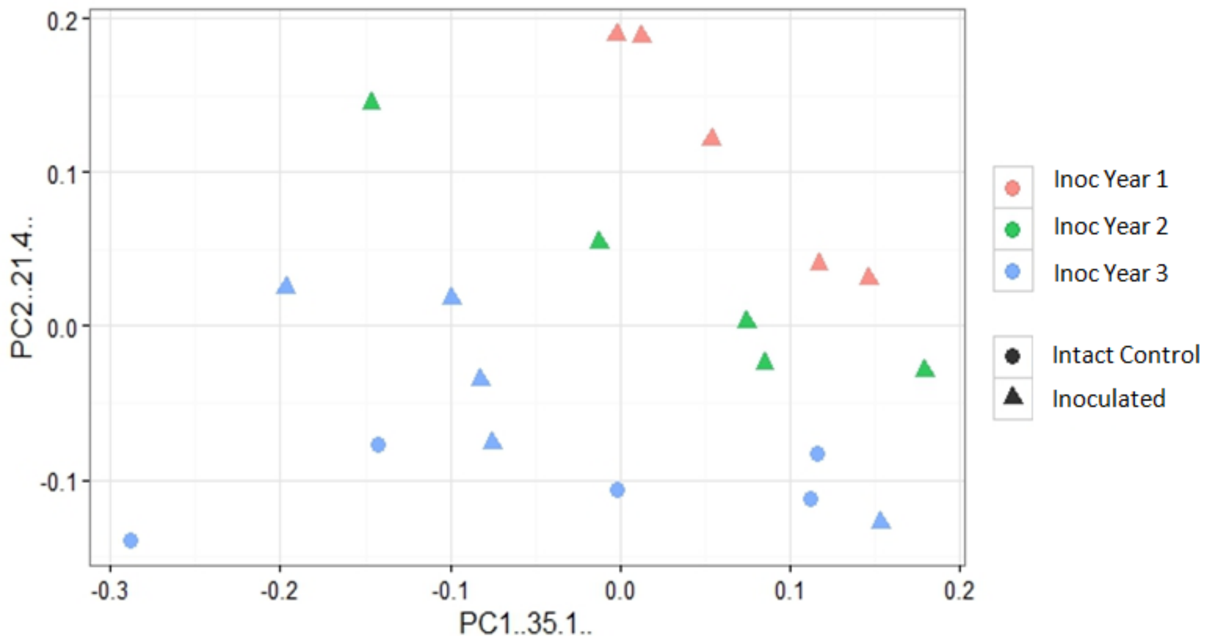

Figure 2-8: Principle coordinate analysis (PCoA) of cyanobacterial communities using a Bray-Curtis dissimilarity matrix (measure of variability in community composition abundances) showing the distribution of biocrust cyanobacteria communities distinguished by year collected post-treatment and by intact control vs. inoculated plots in ordination space.

2.5 CONCLUSION

Overall, biocrust community composition following an inoculation restoration treatment changed in patterns also seen in natural recovery. Richness of microbial communities following field-collected inoculum restoration did not change between years, although community composition did. This result is encouraging for restoration efforts using field inoculum, because it suggests that there is sufficient diversity of biocrust organisms early in succession, allowing the present propagules to flourish under favorable conditions. We saw shifts in community composition between years in the inoculum restoration treatment, most notably in increases in later-successional dark cyanobacteria, N-fixing *Nostoc* and *Scytonema*. We also observed a decrease in the AOA *Candidatus Nitrososphaera*, and increases in heterotrophic taxa

(Cytophagaceae and Chthoniobacteraceae) as time following the inoculum treatment progressed. However, after three years of restoration, microbial community composition in the restored plots was still distinct from intact controls, displaying that restoration of biocrust communities is a long process. Thus, prevention of disturbance and biocrust conservation programs are needed alongside restoration efforts to preserve biocrust communities and functions.

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APPENDIX

Supplementary Table S1: Precipitation data from Hill Air Force Base, Utah Test and Training Range in 2013, 2014, 2015, 2016. Experiments commenced in June 2013, and annual monitoring was performed in June 2014 (Year 1), June 2015 (Year 2), and May 2016 (Year 3) (highlighted). Data provided by the Utah Climate Center at the Utah Test Range, Station ID USC00428978 (Lat: 41.0497, Long:-112.937, Elevation 1353.3m).

Month	Year	Precipitation (mm)	Snow Depth (mm)	Snow Fall (mm)	Min Temp (°C)	Max Temp (°C)	Mean Temp (°C)
January	2013	19.3	2975	204	-14.3	-6.09	-10.2
February	2013	19.8	2644	279	-11.3	-0.248	-5.76
March	2013	2.3	0	0	-1.70	11.2	4.73
April	2013	17.5	0	0	0.404	16.2	8.29
May	2013	21.1	0	0	6.56	23.0	14.8
June	2013	0	0	0	12.3	31	21.6
July	2013	25.4	0	0	18.1	34.7	26.4
August	2013	13.2	0	0	15.9	33.6	24.7
September	2013	65.5	0	0	11.1	25.4	18.2
October	2013	18.7	0	0	0.78	16.09	8.44
November	2013	2.8	0	3	-3.39	10.1	3.35
December	2013	31.8	1399	267	-14.0	-2.06	-8.04
Annual Total	2013	237.4	7018	753	1.63	16.0	8.82

Month	Year	Precipitation (mm)	Snow Depth (mm)	Snow Fall (mm)	Min Temp (°C)	Max Temp (°C)	Mean Temp (°C)
January	2014	15.1	557	76	-10.2	1.35	-4.42
February	2014	8.9	0	3	-2.88	8.09	2.61
March	2014	31.8	0	0	-1.43	13.8	6.2
April	2014	29.8	0	0	1.34	17.0	9.16
May	2014	19.3	0	0	7.22	24.0	15.6
June	2014	10.2	0	0	11.2	29.1	20.1
July	2014	8.6	0	0	17.4	34.7	26.0
August	2014	24.4	0	0	14.2	30.3	22.2
September	2014	57.9	0	0	11.5	27.9	19.7
October	2014	0	0	0	3.73	20.9	12.3
November	2014	11.5	0	0	-4.53	9.88	2.68
December	2014	7.9	0	0	-4.84	6.27	0.719
Annual Total	2014	225.4	557	79	3.61	18.7	11.1

Month	Year	Precipitation (mm)	Snow Depth (mm)	Snow Fall (mm)	Min Temp (°C)	Max Temp (°C)	Mean Temp (°C)
January	2015	43.4	0	0	-5.9	4.04	-0.932
February	2015	1.3	0	0	-2.98	11.5	4.26
March	2015	10.2	0	0	-1.18	16.3	7.56
April	2015	23	0	0	1.44	18.5	10.0
May	2015	140	0	0	8.02	20.5	14.2
June	2015	6.9	0	0	14.2	32.0	23.1
July	2015	17.2	0	0	14.3	31.7	23.0
August	2015	27.2	0	0	14.8	32.5	23.7
September	2015	24.9	0	0	9.77	28.1	18.9
October	2015	33.1	0	0	5.86	20.6	13.2
November	2015	5.9	25	25	-5.47	7.29	0.912
December	2015	63.5	327	127	-9.25	2.45	-3.4
Annual Total	2015	396.6	352	152	3.71	18.9	11.3

Month	Year	Precipitation (mm)	Snow Depth (mm)	Snow Fall (mm)	Min Temp (°C)	Max Temp (°C)	Mean Temp (°C)
January	2016	28.2	227	101	-7.65	1.12	-3.27
February	2016	6.6	253	25	-4.29	6.04	0.875
March	2016	36.6	0	5	-0.742	13.9	6.60
April	2016	57.6	0	0	3.28	17.8	10.5
May	2016	18.9	0	0	5.25	22.1	13.7
June	2016	8.9	0	0	12.8	32.4	22.6
July	2016	0	0	0	16.0	34.9	25.4
August	2016	0	0	0	13.5	32.7	23.1
September	2016	46.2	0	0	8.43	25.6	17.0
October	2016	20.6	0	0	4.60	19.2	11.9
November	2016	6.6	0	0	-2.82	11.6	4.37
December	2016	43.5	532	116	-10.1	0.0323	-5.05
Annual Total	2016	273.7	1012	247	3.30	18.2	10.8