FINAL REPORT

Achieving Dryland Restoration Through the Deployment of Enhanced Biocrusts to Improve Soil Stability, Fertility and Native Plant Recruitment

SERDP Project RC-2329

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Biological soil crusts ('biocrusts') are communities of microorganisms that develop on soil surfaces and are a critically important functional component of dryland systems of the globe. Due to the functional importance of biocrust communities to the ecological functioning of dryland ecosystems there is keen interest in restoring these communities. The overarching research objective in this project was to facilitate the recovery of degraded arid and semi-arid Department of Defense lands by restoring biocrust communities.

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CONTENTS

LIST OF TABLES……..…….…….3

LIST OF FIGURES…….…….…...4

LIST OF ACRONYMS…….……...5

KEYWORDS…….……..5

ACKNOWLEDGEMENTS……..……...5

ABSTRACT…….……6

OBJECTIVES……..……....7

TECHNICAL APPROACH…….…...8

RESULTS AND DISCUSSION……13

CONCLUSIONS TO DATE……..23

LITERATURE CITED……...……...24

LIST OF TABLES

Table 1. Objectives and hypotheses…….....…….....3

Table 2. Fractional factorial design for LB inoculum…….....5

Table 3. Task 1 LB fractional factorial experiment statistical results.....13

Table 4. Task 2 Habitat modification statistical results.....26

Table 5. Task 2 Soil stability statistical results.....27

LIST OF FIGURES

1. Objective 2 Hardening experiment design…….....11

2. Objective 3 Multifactorial field experiment design …….....12

3. Objective 1 LB fractional factorial results.....…….....14

4. Objective 1 LB soil microbial community composition.....……...…15

5. Objective 1 LB soil microbial community dissimilarity indices ...…….....15

6. Objective 1 Photos of LB inoculum nursery……...……..…16

7. Objective 1 LB inoculum chl *a*…….....…….....17

8. Objective 1 LB time series cold desert.....…….....18

9. Objective 1 LB time series hot desert.....18

10. Objective 1 MI community composition…….....……...19

11. Objective 1 Photos of MI cyanobacterial culture…….....….....20

12. Objective 1 *Microcoleus* growth curves….....21

13. Objective 1 MI inoculum composition.....21

14. Objective 1 *Microcoleus* fog chamber …….....….....22

15. Objective 2 Moss growth in response to fertilization and hydration.....22

16. Objective 2 Biocrust community response to hydration…….....23

17. Objective 2 *Nostoc* growth in response to fertilization and hydration.....21

18. Objective 2 Inoculum amount field trials…….....25

19. Objective 2 Habitat modification trials …….....26

20. Objective 2 Soil stability trials - biocrust cover….....28

21. Objective 2 Soil stability trials - soil stability analysis.....28

22. Objective 3 Soil aggregate stability UTTR …….....….....29

23. Objective 3 Soil aggregate stability JER …….....30

24. Objective 3 Soil chlorophyll a UTTR …….....30

25. Objective 3 Soil chlorophyll a JER …….....31

26. Objective 3 Soil sheer strength..... …….....31

27. Objective 3 Soil moisture …….....32

LIST OF ACRONYMS

ASU – Arizona State University
UCB – University of Colorado at Boulder
DoD – Department of Defense
FB – Fort Bliss
FC – Field collected inoculum
JER – Jornada Experimental Range
LB – Local biocrust inoculum
MI – Mixed isolate inoculum
NAU – Northern Arizona University
USDA – United State Department of Agriculture
USGS – United States Geological Survey
UTTR – Utah Test and Training Range

KEYWORDS

biological soil crust, biocrust, soil ecology, soil restoration, dryland, Great Basin, Chihuahuan Desert, Utah Test and Training Range, Fort Bliss

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ABSTRACT

Objectives

Biological soil crusts ('biocrusts') are communities of microorganisms that develop on soil surfaces and are a critically important functional component of dryland systems of the globe. Due to the functional importance of biocrust communities to the ecological functioning of dryland ecosystems there is keen interest in restoring these communities. Our overarching research objective in this project was to facilitate the recovery of degraded arid and semi-arid Department of Defense (DoD) lands by restoring biocrust communities.

Technical Approach

In this project, we: 1) *established* a biocrust nursery as an inoculum testing and supply center for biocrust restoration 2) *identified* successful field application methods of biocrust inoculum in a series of field trials 3) *evaluated* soil and plant responses to biocrust restoration in multi-factorial field experiments and 4) *shared* knowledge of biocrust restoration success and challenges with DoD and federal land managers. In years 2013-2015 of the project, cultivation of inoculum was completed and delivered to field experiments in both our hot and cold desert sites. A broad range of experiments have continued over the past two years to optimize inoculum cultivation under greenhouse and laboratory controlled environments. Our project team implemented multi-factorial field experiments at our two research sites, Utah Test Training Range (UTTR) and the Jornada Experimental Range(JER) in June and September of 2015. We monitored the biocrust response to three types of inoculum; field collected (FC), lab grown local biocrust (LB), and mixed isolates (MI) using two soil stabilization strategies (straw borders and polyacrylamide 'DirtGlue').

Results

Our research has yielded effective methods to grow biocrust inoculum both from small field collected samples and cultured isolates of early successional cyanobacteria, mosses, and lichens. We have shown that inoculation of soils with lab and greenhouse growth biocrusts enhance biocrust recovery. Barriers and challenges still exist in biocrust recovery with inoculation under field settings and this is likely due to resource limitation to biocrust growth and recovery and more specifically water availability. We did show that irrigation and shading likely alleviate resource constraints and UV stress resulting in enhanced biocrust recovery over a short period of time.

Benefits

We have developed novel approaches to developing biocrust inoculum for restoration of degraded dryland ecosystems. Biocrusts play a functional important role in dryland ecosystems influencing soil stability, nutrient availability, and hydrology. Thus, rehabilitation of these biotic communisms will benefit these ecosystems and the services they provide. Our future challenge is scaling these approaches to larger landscape scale restoration approaches.

OBJECTIVES

Department of Defense (DoD) military installations cover nearly 30 million acres, 70% of which are located in dryland regions of the western U.S. These installations provide critical pre-deployment training ground and —as these training centers are located in deserts— they have increased in importance over the last decades with the Iraq and Afghanistan wars. Many training activities result in significant disturbance on these lands, which are well known to have a limited capacity for recovery, even over longer time scales. Low and highly variable precipitation in conjunction with the common occurrence of infertile soils create significant challenges to restoration of DoD lands across this region. DoD depends upon this land base for sustaining future training activities, while tasked with maintaining the long-term ecological functioning of these ecosystems. When disturbed, dryland soils may become a significant source of airborne atmospheric dust. Atmospheric dust may have both ecosystem and public health effects, often times at locations far from the source. In many dryland ecosystems affected by soil degradation (e.g., Phoenix Metro area) atmospheric dust is the main pollutant. Because of this, there is broad societal interest in stabilizing dryland soils in order to protect not only the functioning of local ecosystems but also human populations that reside in surrounding communities. Our research project met these scientific needs by addressing these important issues: 1) evaluating the functional role that biocrust communities play in assisting the recovery of degraded dryland ecosystems and 2) developing management strategies to meet DoD's natural resource management challenges.

Our overarching research objective was to facilitate the recovery of degraded arid and semi-arid Department of Defense (DoD) lands by restoring biological soil crust communities (henceforth biocrusts). Biocrusts are communities of organisms such as cyanobacteria, lichens, and mosses that develop on soil surfaces, which in turn support populations of heterotrophic bacteria and fungi. Biocrusts colonize the top few millimeters of surface soils in dryland ecosystems, and create a mesh of biological and mineral conglomerates. Biocrusts are an essential functional component of dryland systems of the globe. They are often associated with increased soil nutrient and water retention—resources that are highly limiting to plant productivity in these ecosystems. But most importantly, biocrusts stabilize soil surfaces against wind and water erosion. We predicted that effective biocrust restoration across dryland DoD installations will enhance resistance to erosion, soil fertility, and hydrologic function.

In order to achieve *our primary research objective to facilitate the recovery of degraded arid and semi-arid lands by restoring biocrust communities,* we outlined four sub-objectives to be accomplished over the life of the project:

1) *Establish* a biocrust nursery as an inoculum testing and supply center for biocrust restoration.
2) *Identify* successful field application methods of biocrust inoculum in a series of field trials.
3) *Evaluate* soil and plant responses to biocrust restoration in multi-factorial field experiments.
4) *Share* knowledge of biocrust restoration success and challenges with DoD and federal land managers.

Table 1 summarizes the objectives and related hypotheses for research objectives 1-3 across the life of the project (Table 1).

Table 1. Research objectives and associated hypotheses that were addressed during the project.

Objectives and Hypotheses	
Objective 1 *Establish* a biocrust nursery as an inoculum testing and supply center for biocrust restoration.	
	H1. Local field-collected biocrust (LB) and lab-reared mixed isolate (MI) growth are limited by suboptimal temperature, light, nutrient and moisture regimes.
	H2. LB and MI biocrust populations will increase significantly during incubations in a controlled environment with increased water availability and a softening of the environmental conditions under which they develop in the field.
Objective 2: *Identify* successful biocrust field application methods	
	H4. Overcoming propagule limitation by inoculating soil surfaces with biocrust organisms will result in higher biocrust recovery relative to sites that remain uninoculated or inoculated at low levels.
	H5. Biocrust recovery will increase when inoculation is followed by additions of limiting resources to boost early growth and subsequent establishment under field conditions.
	H6. Modifying habitat characteristics in a way that decreases stress (i.e. water and UV manipulation) and increases soil stability and resource retention (i.e. water and nutrients) will enhance recovery of biocrusts under field conditions.
Objective 3: *Evaluate* soil and plant responses to biocrust restoration in multi-factorial field experiments	
Soil response	H7. Inoculation of previously disturbed soils with native biocrust organisms that produce soil binding exogenous compounds will result in increases in soil surface stability.
	H8. Inoculation of disturbed soils with biocrust organisms will increase water infiltration and decrease runoff.
	H9. Inoculation of disturbed soils with native N-fixing biocrusts organisms will result in increases in nutrient availability.
Plant Response	H10. Enhanced soil stability and fertility with biocrust inoculation will increase plant-limiting resources (i.e. water and nutrients), resulting in successful native plant establishment.
	H11. Even if biocrust organisms establish successfully, native plant establishment is limited by propagule availability. Thus, seeding with native plants species in a manner that promotes germination through proper seed placement and burial will enhance the recovery of these populations.
	H12. Biocrust restoration will reduce germination of exotic species by reducing seed water potential of these species.

TECHNICAL APPROACH

Site Description and Characterization

We chose two research sites that represented areas with contrasting arid climates, one hot and one cold desert to conduct our field trials and experiments. Our sites were located at Hill Air Force Base-Utah Test and Training Range (UTTR)and Jornada Experiment Range (JER), sites that represent areas with contrasting cold and hot desert climates, respectively. Our cold desert was located in the Basin and Range Physiographic Province but is in the Great Basin Desert. Soils in this region is greatly influenced by ancient Lake Bonneville, with most all of the installation at elevations below the high-water mark. The climate is characterized by a cool moist spring; hot dry summers; and cold and dry falls and winters. MAP is 200 mm with approximately 33% occurring March-May. Mean monthly maximum temperature ranges from 3 °C in January to 34 °C in July. Biocrusts, including cyanobacteria, lichen, and mosses, are widespread in undisturbed sites on both installations. However, UTTR has much higher cover and diversity of lichens and mosses than JER. Our hot desert study area, extends from West Texas into southern New Mexico. JER is in the northern reaches of the Chihuahuan Desert and southeastern corner of the Basin and Range Physiographic Province. The climate is characterized by a warm dry spring; hot and wet summer; warm wet fall; and cold dry winter. Mean annual precipitation (MAP) is 282 mm, with 64 % of it occurring between June and September. Mean monthly maximum temperature ranges from 14 °C in January to 36 °C in July. At each site, we selected two soil types that contrast in inherent soil stability and natural resilience to disturbance, one on coarser texture soils (generally less resilient) and one on a fine textured soil (generally more resilient).

Objective 1- Establishment of a Biocrust Nursery

In the first two years of the project (2013-2015) the Arizona State University (ASU) team established two biocrust nursery facilities. The facility to grow hot desert biocrust inoculum was located at ASU in Tempe, AZ and the cold desert inoculum was grown in the cooler and higher elevation location of Northern Arizona University (NAU) in Flagstaff, AZ. During this time two types of biocrust inoculum were developed to support field experiments on both of the chosen sites. During years three and four of the project (2015-2017) the Arizona State University (ASU) team dedicated its efforts to optimizing biocrust inoculum growth, which serves as the inoculum supply for biocrust restoration. Two lines of inoculum development were local field-collected biocrust (LB) and lab-reared mixed isolates (MI). Trade-offs in costs and analytical expertise exist with developing different approaches to inoculum development. The LB methods of biocrust inoculum development requires harvesting small amounts of existing biocrust from the research sites and then increasing the biomass under controlled greenhouse conditions. This method would be well suited for land managers and restoration professionals since it requires very little microbiology expertise. The trade-off in the LB method is that biocrusts must be harvested from the field resulting in impacts to an undisturbed site. The benefit of the MI approach is that a very small amount of biocrust is used to culture different strains of biocrust organisms. Thus, there is no risk of overharvesting biocrust organisms from the field. These strains are then scaled up to create larger quantities of inoculum. The trade-off is that this

approach requires significant expertise in microbiology. This approach, however, holds more promise in future commercial production of biocrust inoculum.

Inoculum 1, Local Biomass (LB) Inoculum— LB inoculum was obtained by harvesting small amounts of existing crust from the research sites. LB inoculum was developed from these small field-collected samples in a multi-step process through several experiments to determine the key factors limiting biocrust growth and strategies to alleviate growth limitation while maintaining biocrust community composition. In initial trials, we conducted two parallel experiments with the objectives to a) enhance biocrust biomass in greenhouse facilities to provide artificial inoculum for degraded soils and b) develop inoculum that was similar in microbial community composition to field collected biocrusts. For each site and soil type, we performed a fractional factorial experiment (Table 2), to test the effects of seven factors with two levels per factor on the growth of biocrusts in a greenhouse setting.

Table 2. *Treatments for greenhouse incubations used in the fractional factorial design experiments (first phase experiments), which were combinations of independent factors (P, addition of phosphorus; N, addition of nitrogen; M, mosaic-like inoculum; S, slurry-like inoculum, high (+) or low (-) watering; ambient (+) or shaded (-) illumination, and additions (+) or not (-) of calcium and metals.*

Treatment	Watering	Light	Nutrients	Inoculum	Calcium	Metals
1	+	+	P+N	M	+	+
2	+	+	N	S	+	-
3	+	-	N	M	-	+
4	+	+	P	M	-	-
5	+	-	P	S	+	+
6	-	-	N	M	-	+
7	-	-	P+N	S	-	-
8	-	+	P	S	-	+
9	+	-	P	M	+	-
10	-	+	P	M	-	-
11	-	+	P+N	M	+	+
12	+	+	N	S	-	+
13	+	-	P+N	S	-	-
14	-	-	P+N	S	-	-
15	-	+	N	S	+	-
16	-	-	P	S	+	+
17	+	+	P+N	M	+	+
18	-	-	N	M	+	-

The water frequency factor (W) had two levels: high frequency (+, where crusts samples were watered every 3 days for hot desert sites and every 2 days for cold desert sites), and a low frequency (−, crusts were watered every 9 and 4 days, respectively). The frequency of watering

per location was arrived at based on local rainfall records, after calculating average rainfall event frequencies. In each watering event, crusts samples received an amount of water through mist emitters designed to attain ca. 80% of the water holding capacity of the soil, and allowed to dry naturally thereafter. The light intensity (illumination) factor (L) had also two levels, a high light intensity (+, exposed to full greenhouse sunlight) and a low light intensity (−, crusts were covered with a black cloth that blocked approximately 60% of sunlight). The inoculum factor consisted in two types: mosaic (M), where 15 discrete fragments of appropriate biocrust, 0.4 cm diameter and 1 cm deep, were directly transplanted on top of the bare soil, in a mosaic pattern, and slurry (S), where 15 discrete fragments of biocrust, 0.4 cm diameter and 1 cm deep, were slurred and then spread over the bare soil. The nutrient factor had three levels, P (addition of a mix of KH_2PO_4 and K_2HPO_4, to a final concentration of 75 µg P g soil^{-1}), N (addition of NH_4NO_3, to a final concentration of 150 µg N g soil^{-1}) and P+N (addition of both P and N); all nutrients were prepared in fresh, autoclaved, double-distilled water, and added as a unique pulse on day 1 of the experiments. The calcium factor had two levels, a high content in calcium (+, addition of Ca as calcium pellets, to a final concentration of approximately 40 µg Ca g soil^{-1}) and a low content in calcium (−, no addition of Ca). Finally, the trace metal factor had two levels, a high content in trace metals (+, addition of the trace metal solution of the BG11 medium (41), final concentration 2 µg metal solution g soil^{-1}) and a low content in essential metals (−, no addition of this metal solution); the metal solution was prepared in fresh, autoclaved, double-distilled water, and added as a unique pulse on day 1 of the experiments.

After 4 months, the chlorophyll *a* content was measured in all the treatments as a proxy for autotrophic biomass. Microbial community composition was analyzed only in those treatments showing significant biomass responses using a 16S rDNA pyrosequencing approach. A screening model with chlorophyll *a* data as the independent variable was carried out in order to select the factors that best determine the growth of biocrusts. Bray-Curtis dissimilarity indices were used to determine community composition distances (bacterial phyla for the microbial community composition and organism level for the cyanobacteria) between the initial inoculum and the selected treatments in each site.

Once we developed a protocol to enhance biocrust growth while maintaining community composition, we then conducted time series experiments to evaluate the minimum amount of time to maximize LB biomass. A paper describing this approach was recently published by our team (Velasco Ayuso et al., 2017). Inoculum growing time for this protocol ranged between eight to 12 weeks, which is likely longer than necessary. In an effort to reduce LB inoculum production time, we set up a greenhouse experiment that followed the same protocols as described in Velasco et al. (2017). In that experiment, we monitored weekly biocrust biomass growth in order to identify the minimum amount of time that was needed to obtain adequate biocrust biomass and whether adequate biomass could be achieved in less than 12 weeks. In this new experiment, we included biocrust growth from all of our initial research sites. For the cold desert: UTTR sandy soil, UTTR silty soil; and for the hot desert: JER silty soil and Fort Bliss sandy soil. Chlorophyll *a* (chl *a*) determinations were used as a proxy for phototrophic biomass growth. DNA sequencing was performed to determine when optimal cyanobacterial community structure was reached.

Inoculum 2-Mixed Isolate (MI) Inoculum Development—The development of mixed isolate (MI) inoculum was a second approach to inoculum development. The overall approach in MI development was to isolate specific species from biocrust communities from each of our sites

and soil types and to scale up the biomass of these pedigreed biocrust organisms under laboratory conditions. In the first step to developing MI inoculum, bacteria and cyanobacterial community structure and relative abundance were determined across research sites and soil types using 16S rDNA pyrosequencing analysis. The key biocrust forming cyanobacteria from native biocrust communities of our two research sites and two soil types were isolated. Traditional isolation techniques were used to obtain the cyanobacteria cultures as described in Andersen (2005) (e.g. enrichments cultures, single-cell (and bundle) isolation by micropipette and streaking cell across agar plates). All isolates were then identified based on 16S rDNA amplification by PCR using cyanobacteria specific primers (Nübel et al., 1997). Sequences were used to reconstruct cyanobacteria phylogeny, for each of the field locations. Phylogenetic relationships were used to select the specific isolates to be used MI inoculum development. Isolates that were phylogenetically similar to field-collected cyanobacteria were selected for the inoculum production step.

In a following step, efforts were then focused on scaling up the biomass of each of the selected cultures of the main biocrust forming cyanobacteria at the cold desert sites. Traditional scaling up methods from the biofuel and biomedical industry gave good outcomes when growing some of the target cyanobacteria (*Nostoc* sp., *Tolypothrix* sp. and *Scytonema* sp.). By implementing traditional techniques (Sharma et al., 2014), the selected cultures of the cyanobacteria *Nostoc* sp., *Tolypothrix* sp. and *Scytonema* sp. were scaled up from 50 ml incubation flasks up to 20 L carboys, under natural light and field temperatures in a greenhouse environment.

When growing *Microcoleus vaginatus* and *M. steenstrupii* (the main biological component of biocrusts) biomass yields using traditional scaling up methods were very low. Following this, an alternative approached was developed to scale up the remaining two targeted cyanobacteria (*M. vaginatus* and *M. steenstrupii*). A detailed protocol is presented in Appendix A.1. By implementing this new approach, we were able to obtain exponential and rapid growth of the biocrust pioneers *M. vaginatus* and *M. steenstrupii*. Once all the biomass production was achieved for the isolates, our delivery strategy consisted of introducing isolates at a relative abundance that was similar to field collected samples to sterile native soil. This mixture of cultured biocrust organisms in native soils was then conditioned to dry-wet cycles and increasing light exposure. During this 'hardening' treatment, cyanobacterial biomass was conditioned to increasing light (from culture room to full outdoor sunlight conditions), and 14 wet-dry cycles. A detailed protocol of the hardening process is described in Appendix A.2.

The dominant filamentous cyanobacteria (*Microcoleus* spp.) are not suitable for traditional scaling up techniques in liquid media. Following this, we developed a technique of plating on to filter paper that allowed us to effectively produce the inoculum we needed for the multifactorial field experiments (Giraldo Silva et al., submitted to Restoration Ecology). However, the technique is time and labor-intensive which is a significant barrier to scaling up the production of cyanobacteria. As a result of the investment of time and labor, we are working toward developing alternative methods to growing biocrust pioneer cyanobacteria, which are described below.

In a first experiment, we developed a fog based watering system using distilled water to grow *Microcoleus* spp. Sterilized native soil was placed into petri plates with drainage holes, and saturated with BG11 medium (only once). A homogenized liquid *Microcoleus* sp. culture was added to the surface of the soil and subjected to multiple dry and wet cycles over ~24 days. Chl *a*

was used as a proxy for phototrophic biomass growth. Microscopy was performed at the end of the growing time to ensure the desired morphotype was the cyanobacterium present in the grown biomass.

When growing *Microcoleus* spp. in liquid medium, it tends to aggregate into large clumps and the cells in the center of these clumps tend to die. We believe that this aggregation is potentially the factor that is preventing us from successfully grow these filamentous cyanobacteria in liquid medium. To prevent cells from aggregating, we introduced shredded KimWipes into liquid medium to de-aggregate clumps that *Microcoleus* spp. cells. We evaluated *Microcoleus spp.* growth under two levels of KimWipe mass and two levels of inoculum in four experimental treatments: 1) 0.32g (1 KimWipe) of shredded KimWipes and 5 mL of inoculum 2) 0.32g of shredded KimWipes and 15 mL of inoculum. 3) 4.8 g (15 KimWipes) of shredded KimWipes and 5 mL of inoculum 4) 4.8g of shredded KimWipes and 15 mL of inoculum. Three replicates of each treatment were incubated for ~16 days on a shaker at 120 rpm. This process was repeated twice using *M. vaginatus* strains HSN003 and FB020 as inoculum. Visual evaluation was used to determine cultures clumping state and growth.

In conjunction with the cultivation work occurring at ASU, the team at NAU has also made strides in developing a cultivation technology that works for the later successional species such as mosses and lichens. Our first effort was to develop an experimental cultivation system which is described in detail in Doherty et al. (2015). We targeted mosses from the genus *Syntrichia* because they are common and abundant in biocrusts around the western U.S. and provide unique ecosystem services such as desiccation tolerance (Stark et al. 2012), water absorption (Eldridge et al. 2010, Xiao et al. 2011, Chamizo et al 2012), soil stability (Bowker et al. 2008, Chaudhary et al. 2008, Li et al. 2004) and nitrogen inputs by harboring nitrogen-fixing cyanobacteria (Rousk et al. 2013). Mosses in particular are highly suitable for biocrust restoration due to the fact that any vegetative tissue of a moss is a propagule that may grow into new plants (totipotency), propagules can be stored and retain viability for decades to centuries in the right conditions (Stark et al. 2004), and mosses are highly tolerant to dessication.

In a second experiment, we worked to determine how best to cultivate cold desert mosses from the genus *Syntrichia* by manipulating water and nutrients. Mosses (*Syntrichia caninervis* and *S. ruralis*) were collected from the Utah Test and Training Range (UTTR) are stored dry in the dark at room temperature. Mosses and lichens were gently broken up, soil removed, and cleaned with water over a two-mm mesh sieve to remove the majority of mineral soil particles. Washing was followed by gentle shaking for 10 minutes in water. Washing and shaking was repeated five times. Mosses and lichens were then carefully and slowly dried by gently patting them and spreading them on slotted trays over paper towels. The trays were placed in closed fume hoods to allow for maximal air flow, and lights were kept dim. After drying, lichens and mosses were broken into small fragments by "grating" over a two-mm mesh sieve. Using our automated greenhouse experimental cultivation system (Doherty et al. 2015), we filled individual 1.4 L round (16 cm diameter) containers with 800 ml of autoclaved sand. Sand was sourced from a dune near Moab, Utah because it has properties favoring rapid capillary action, is relatively infertile compared to finer soils, and contains little calcium carbonate which could interact with added nutrients. In this experiment, we manipulated: 1) moss species (*S. caninervis* or *ruralis*), 2) hydration length (5, 4, 3, or 2 days of continuous hydration followed by dry down events of 2, 3, 4, or 5 days) and 3) number of fertilizer events (biweekly, monthly, one time addition of a dilute solution of all macro and micronutrients in a full factorial experiment). Each treatment combination was replicated five times. Un-inoculated controls were used to determine

the extent of moss, algae, cyanobacteria and fungal recruitment from air, water, or fertilizer additions.

Once a month we assessed percent cover of all detectable taxa following watering using a circular gridded quadrat frame with each square equivalent to two percent cover. Species identity was verified using a dissecting microscope. At the same time, we also collected repeat natural light and infrared images based on the methods of Fischer et al. (2011). This allowed us to calculate the Normalized Difference Vegetation Index (NDVI) total "green" cover in the pots. NDVI is a remote sensing technique which is commonly used as a proxy for productivity and has successfully been applied to biocrusts. After 180 days of growth, we measured N_2 fixation and then harvested the cores for chl *a* analysis.

Objective 2 - Identify successful biocrust field application methods - Hardening Experiment— Field trials in Years 1 and 2 informed the larger multi-factorial experiments in Years 3-5. In Year 1 we implemented field experiments at UTTR and JER to test techniques to stimulate biocrust growth by varying levels of inoculum, modifying habitat to enhance water capture and retention, and stabilizing soils. In Objective 2 we identified successful field application methods of biocrust inoculum. We targeted a coarse and fine textured soil at the UTTR and JER sites and replicated experiments at all four locations. Experiments were established to coincide with moisture conditions favorable to biocrust growth. At UTTR, experiments were established in April of 2013, and at JER in November of 2013. Data was collected on all experiments 14 months post-establishment. All experiments involved scraping the top 2 cm of soil and biocrust from the surface to remove biocrust propagules and create a homogeneous surface for treatment. The biocrust from the top 0.5 cm was saved and crumbled into pea-sized fragments and homogenized for later use in experiments. We established four experiments to answer the following questions: 1) How much inoculum is needed to maximize field survival and establishment? 2) What habitat modifications will maximize biocrust survival and establishment in the field? 3) How can we simultaneously stabilize the soil surface while promoting biocrust recovery? and 4) How does adding biocrust inoculum affect seed establishment?

Experiment 1- Inoculum Amount Trials—At each site we scraped the surface of 20 25 cm x 25 cm plots, and randomly assigned one of the following treatments (replicated 5 times): control (no inoculum), 10%, 20%, or 40% soil surface cover. Inoculum was delivered by volume (calculated from the amount of inoculum scraped from a plot surface), and dispersed evenly over the plot.

Experiment 2-Habitat Modification Trials—In this experiment, we created 25 cm x 25 cm plots in all possible combinations of the following (5 replicates each, N = 80): 1) inoculum (control or 40% cover), 2) surface roughening (control or roughened), 3) shade (control or 50% shade cloth) and 4) deionized water addition (control or 500 ml added at establishment). *Surface roughening* was created by making ~2cm troughs diagonally across plots every 5 cm at a NNE-SSW direction. *Shading* was created by building ½ inch PVC frames 50cm x 50 cm on a side, and covering with cut shade cloth that reduced light by 50%. Shade cloth was attached using a fabric stapler. Shades were centered over a plot, and attached to 3 ft. rebar posts 15cm above the soil surface. *Water addition* was achieved with pump sprayers. Through testing, we established that a 30 second spray was equivalent to 500ml of water at the lightest spray setting. Water addition was timed, and water was added evenly over the soil surface. The order of treatments was as follows: roughening, inoculation, water and then shade.

Experiment 3- Soil Stability Trials—For this experiment we created $1m^2$ plots to test a variety of soil stabilizing methods. Treatments were replicated 7 times. Treatments were as follows: 1) control (0 or 40% inoculum), 2) straw border with no inoculum added, 3) one of three polyacrylamides diluted to 1:8 ratio with water, and added with or without inoculum (inoculum added post spray), 4) surface roughening + polymer + shade, with and without inoculum addition. *Straw borders* were created by placing a thin layer of straw on the soil surface bordering a plot, and inserting a flat bladed shovel through the center to ~15 cm. This left a standing border of straw ~2 cm wide and 5 cm high. *Polyacrylamides* were selected based on the following criteria: 1) documented use on DoD lands, 2) documented use and effectiveness at soil stabilization in the peer reviewed literature, 3) UV and biodegradability and 4) variety in chemical composition. As a result, we chose the following polymers: 1) Dirt Glue (aqueous acrylate polymer emulsion), 2) SoilTac (vinyl copolymer emulsion) and 3) TerraLoc (polyvinyl alcohol). Treatment 4 was created in the following order: surface roughening, polymer, inoculation (in any), followed by shade addition. This original experiment included 77 original plots (7 replicates). Based on early success with biocrust growth in the straw border experiment, we established an additional 14 straw-bordered plots with and without inoculum a few months later (UTTR: September 2013, JER: March 2014).

Experiment 4-Seed Establishment Trials—We targeted three management-relevant grasses from each site. At UTTR we used seed wild collected from the region by Kelly Memmont with the FS Utah Shrub Lab: *Leymus cinvereus* (Basin wild rye), *Elymus elymoides* (squirreltail grass) and *Sporobolus cryptandrus* (sand dropseed). At JER, we were limited in choices because recent drought conditions had reduced available supply. Seeds were purchased from Curtis and Curtis in Las Lunes, NM. We selected: *Bouteloua eryopoda* (black grama), *Sporobolus cryptandrus* (sand dropseed), and *Sporobolus airoides* (alkali sacaton). To approximate high density (~ 440 seeds per species per plot) and low density (~ 100 seeds per species per plot) seeding, we counted and weighed batches of seed from all species to come up with a standard volume approximating our desired density. We established 40, 25 cm by 25 cm plots (5 replicates of all treatment combinations), which were treated as follows: 1) seed addition (high or low density), 2) inoculum (40% cover native soil or 40% cover biocrust crumbles over seed bed), and 3) burial (seeds placed on the surface or under soil or inoculum addition).

Fourteen months from treatment implementation, we measured and sampled all plots. Using the point intercept method, we quantified biocrust and plant cover at 20 points per plot. We also composited 5 randomly selected soil cores (1cm depth by 1.5 cm diameter) to quantify chlorophyll *a* (a proxy for biocrust biomass) and scytonemin (a pigment present in cyanobacteria and some lichen photobionts, indicative of later successional elements in biocrust development). We used multiple methods to measure soil stability. First, we used soil aggregate stability (slake) kits to sample four pedons per plot to determine the water stable aggregate stability. Next, we used a paired torvane and penetrometer test (one per plot in small plots and 3 per plot in large plots) to measure the wind shear threshold. These best candidate treatments were then used in multi-factorial field experiments in Objective 3.

Once the best candidate methods for successful biocrust field applications were identified, our team continued to explore whether exposing inoculum to increasingly more stressful conditions or "hardening" would result in more successful biocrust colonization in the field. We hypothesized that biocrust inoculum that had been grown under optimal greenhouse

conditions, were likely to survive harsh field conditions of high UV and low water availability if the inoculum was increasingly exposed to increasing UV and water stress.

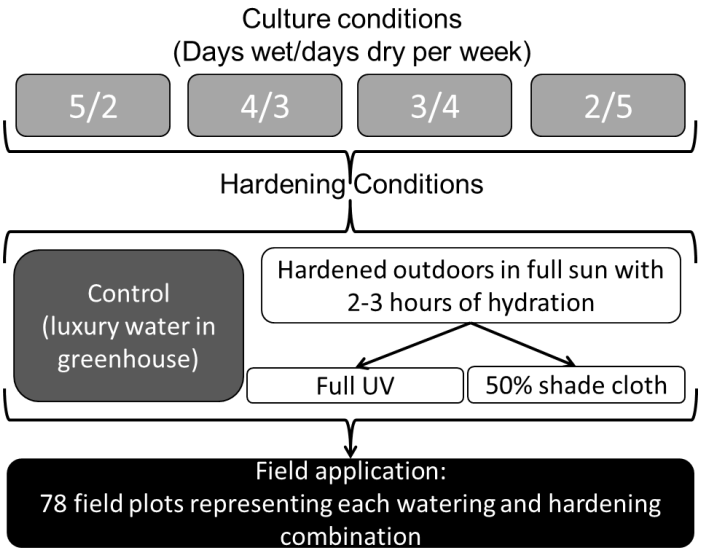


Figure 1. A schematic depicting the phases of the biocrust inoculum hardening experiment. Initial culture conditions included a range of continuous hydration each week for 6 months, followed by three hardening conditions and introduction to the field.

We tested this hypothesis by using greenhouse cultured biocrusts that were grown under reduced UV and milder climate conditions than they would experience in the field. The biocrust experimental trays of greenhouse-cultured material (Antoninka et al. 2016) were allowed to slowly dry in the greenhouse for one week. We then harvested biocrusts and put them through a 2mm sieve and homogenized the material by gently mixing. We placed 1 cm of autoclaved sand into each of 12- 0.4m^2 plastic basins (3 x 4 watering treatments) with 16- 0.3cm holes drilled in the bottom and covered with cotton cloth to allow for drainage and to keep the sand in place. We then sprinkled 400ml of inoculum evenly over the surface of each basin.

We applied three hardening conditions to the four inoculum types: 1) no hardening: kept in the greenhouse and provided luxury water, 2) moderate hardening: kept outside with 50% of natural UV and given low water conditions, or 3) severe hardening: kept outside with full UV and low water conditions (Fig. 1). This resulted in 12 separate inoculum treatments (i.e., four initial watering conditions and three subsequent hardening conditions). The unhardened treatment units (control) were placed in basins in the greenhouse, and hydrated daily with DI water using a pump sprayer from above to achieve full hydration of the biocrust organisms lasting 24 hours per day. This was achieved by timed spraying equivalent to ~2L water per day. We placed the remaining units outside adjacent to the greenhouse in an area that receives no natural shading. In both cases, we hydrated basins for 2-3 hours per day by watering from above until the surface was moist with a timed spray, resulting in ~0.5L per unit per day. We created the "moderate" treatment by covering the basins with a shade cloth that removes 50% of incoming solar radiation to separate the effects of exposure to short hydration periods and the effect of UV light exposure. We applied all treatments for 21 days, and allowed three days for complete drying before we harvested and homogenized as described above.

We located our experimental plots adjacent to where the inoculum material was initially collected. We designated 78, 50cm x 50cm plots in October 2015 that were level, free of vascular plant vegetation, and no closer than 1m to the nearest shrub. We scraped the surface and removed all biocrust materials. In the center of each plot, we designated a 25cm x 25cm area, surrounded by a 12.5cm x 12.5cm buffer area, marked on the corners with nails. The buffer areas were intended to decrease biocrust colonization from the plot edge. We randomly assigned treatments and created six replicate plots for 12 treatment types (four watering by three hardening combinations, plus controls). Each inoculated plot received 125ml of crumbled

inoculum to cover ~10% of the surface area. Constituents of the greenhouse-grown inoculum varied, depending on the watering treatment under which they were grown, but in all cases, they were strongly dominated by dark pigmented cyanobacteria and contained a mix of early-, mid- and late-successional biocrust organisms.

We monitored the experiment at six months (April 2015) and 12 months (October 2015) after inoculation. We assessed each plot for biocrust cover, biomass, and stability. We used the point intercept method with 20 points to estimate biocrust cover (Jonasson 1983). Species not captured by the points were noted at 2.5% cover. We assessed the biocrust level of development (LOD) using methods described in Belnap et al. (2008). This method correlates well with biocrust maturity on a scale of 1-6, where 1 represents an early successional light cyanobacteria crust, and 6 represents a fully developed, mature biocrust dominated by dark cyanobacteria, lichens, and mosses. Species richness was calculated by summing the number of cyanobacteria, moss and lichen species recorded in each plot. We used chl *a* concentrations as a proxy for phototrophic biomass. From each plot, we collected and pooled five soil cores (1cm diameter by 0.5cm depth) from the randomly selected points. We extracted chl *a* using the methods of Castle et al. (2011). We measured soil aggregate stability using a field-based test kit based on immersion and wet sieving (Herrick et al. 2001).

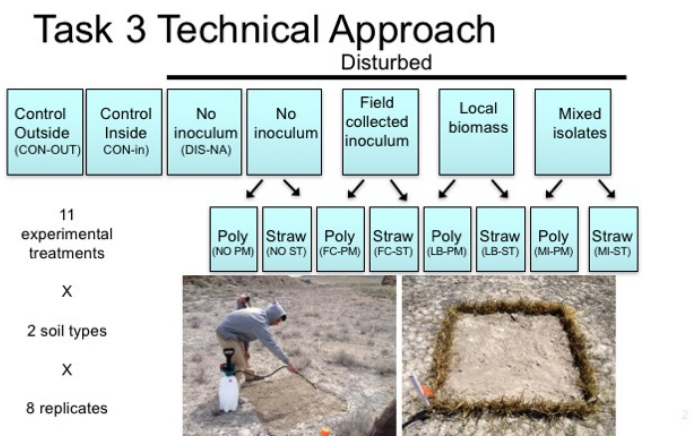


Fig.2. Experimental design for Objective 3. Poly = polyacrylamide ('DirtGlue'), Straw = straw borders. These experiments were installed at UTTR in April 2015. JER experiments were installed in September 2015. Treatment codes within boxes are used in later data reporting.

Objective 3-Evaluate soil and plant responses to biocrust restoration —Results of the lab and field trials in Years 1 and 2 (Objectives 1 and 2) informed the full factorial field experiments to evaluate soil and plant responses to biocrust restoration in years 2 through 5. To test our hypotheses that biocrust inoculation increases soil stability and fertility in addition to enhancing native plant establishment, in each of our research sites we implemented 10 experimental treatments on 2 soil types at UTTR in April 2015. Soils were disturbed by removing (i.e. scraping) the top 5 mm of the soil, which was then followed by foot trampling to further disturb the soil surface horizons. Trampling disturbance was conducted in a 5 x 3 m area. 3 x 1 m experimental plots were then located within this area. We then applied one of three types of inoculum: 1) LB inoculum 2) MI inoculum and 3) field collected (FC) inoculum (Fig. 2). LB and MI plots were inoculated with biocrust organisms cultivated in the biocrust nurseries described in Objective 1. The soil scraped from the disturbance plots was collected and then crumbled into smaller aggregates. These soils served as the FC inoculum. Each of the disturbance plots were then assigned one of two soil stabilization strategies which showed some of the strongest biocrust recovery responses in Objective 2. The first soil stabilization method was a thin application of a polyacrylamide (PM) to the soil surface. The second soil stabilization strategy was the use of straw borders (ST). Straw was inserted vertically around the perimeter of the plot.

The three types of inoculum were spread evenly across the plots after application of the soil stabilization treatments. In addition to the disturbance plots, we set up intact controls (CON-IN, CON-OUT). Uninoculated plots were also created to monitor natural recovery without soil stabilization (DIS-NA), with polyacrylamide (NO PM) and straw checkerboard (NO ST). Each of the plots was replicated 8 times for a total of 160 plots. Two weeks after treatment implementation soils were collected for chl *a* and texture analysis. In addition, soil stability was measured using soil aggregate stability, torvane, and pocket penetrometer tests. Plots were monitored one year after treatment in 2016 and again after two years (2017).

RESULTS AND DISCUSSION

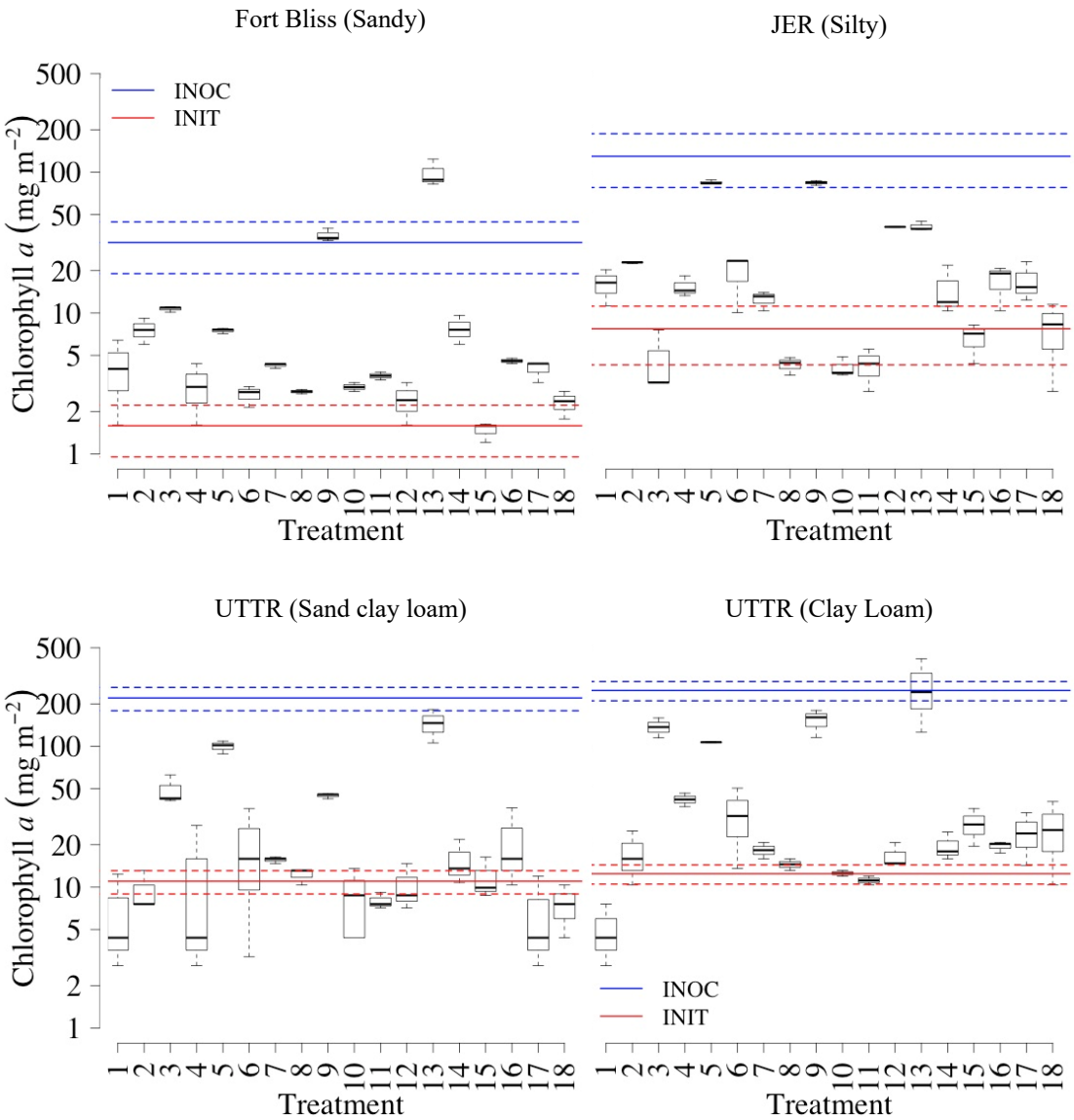
Objective 1- Establishment of a Biocrust Nursery

LB Time series experiment— In initial LB trials we conducted two parallel experiments with the objectives to a) enhance biocrust biomass in greenhouse facilities to provide artificial inoculum for degraded soils and b) develop inoculum that was similar in the microbial community composition of biocrusts. Results of the first experiment on UTTR soils screening 7 factors revealed that a high watering frequency and a low light intensity promoted the growth of biocrust biomass in all sites (Fig. 3, Table 3). Similarly, the addition of nutrients enhanced the yield of biocrust growth in hot desert sites: P+N in FB, but only P in JER.

Table 3. *Results of linear models for the effect of selected factors, as obtained after the preliminary screening process for each of the four sites, on chlorophyll a, chl a, and Bray-Curtis dissimilarity index, BC, as an estimate of community composition shift based on bacterial phyla and cyanobacteria. In parenthesis, levels of factors that maximized production of biomass (chl a) or minimized changes in community composition (BC based on bacterial phyla or cyanobacteria) according to LS-means tests ($p \leq 0.05$) (P, addition of phosphorus; N, addition of nitrogen; S, slurry-like inoculum)*

	JER (Silty)				JER (Sandy)				UTTR (Sandy clay loam)				UTTR (Clay Loam)			
	Factor (level)	**df**	***F***	***p***	**Factor (level)**	**df**	***F***	***p***	**Factor (level)**	**df**	***F***	***p***	**Factor (level)**	**df**	***F***	***p***
Chl *a* content	Water (+)	1	4.14	0.039	Water (+)	1	11.03	0.005	Light (-)	1	8.99	0.009	Water (+)	1	10.84	0.005
	Light (-)	1	3.97	0.042	Light (-)	1	5.56	0.034	Water (+)	1	6.24	0.024	Light (-)	1	9.92	0.006
	Nutrients (P+N)	2	3.87	0.047	Nutrients (P)	2	3.94	0.041								
BC (bacterial phyla)	Inoculum (S)	1	6.41	0.024	Inoculum (S)	1	5.49	0.041	Water (+)	1	6.16	0.027	Nutrients (P+N)	2	11.78	0.001
	Nutrients (P)	2	4.81	0.026	Nutrients (P)	2	3.97	0.040	Light (-)	1	5.95	0.029	Inoculum (S)	1	10.43	0.008
									Nutrients (N)	2	3.88	0.047	Calcium (+)	1	8.83	0.012
													Water (+)	1	7.81	0.017
													Light (-)	1	6.63	0.023
BC (cyanobacteria)					Inoculum (S)	1	3.99	0.048	Water (+)	1	6.65	0.021	Inoculum (S)	1	9.65	0.008
					Calcium (-)	1	5.85	0.029	Light (-)	1	6.18	0.025	Water (+)	1	6.07	0.028
													Nutrients (P+N)	2	4.84	0.026

Figure. 3. Boxplots for final phototrophic biomass (as areal chl a content) obtained after greenhouse incubation of native soils from 4 sites (each panel shows a site) inoculated with natural biocrusts from their respective site under 18 different treatments. Boxes denote lower and upper quartiles (with median values depicted as black, solid lines) and whiskers denote lower and upper extremes (n = 3). Blue lines indicate chl a content of field biocrust samples used as inoculum (INOC), red lines indicate initial chl a content in the inoculated soils (INIT) (color solid lines indicate mean, color dashed lines standard deviations of n = 3)



The second objective in this experiment was to evaluate whether microbial community composition of biocrusts grown under these conditions of enhanced water and nutrients and reduction of light remains relatively stable. We specifically were interested in whether weedy, opportunistic species such as fungi or green algae were responding to the altered water, nutrient and light conditions. To test whether microbial community composition shifted during the

cultivation of samples for the first experiment, we analyzed samples using 16S rDNA pyrosequencing analyses at the phyla level for bacteria and at genus level for cyanobacteria.

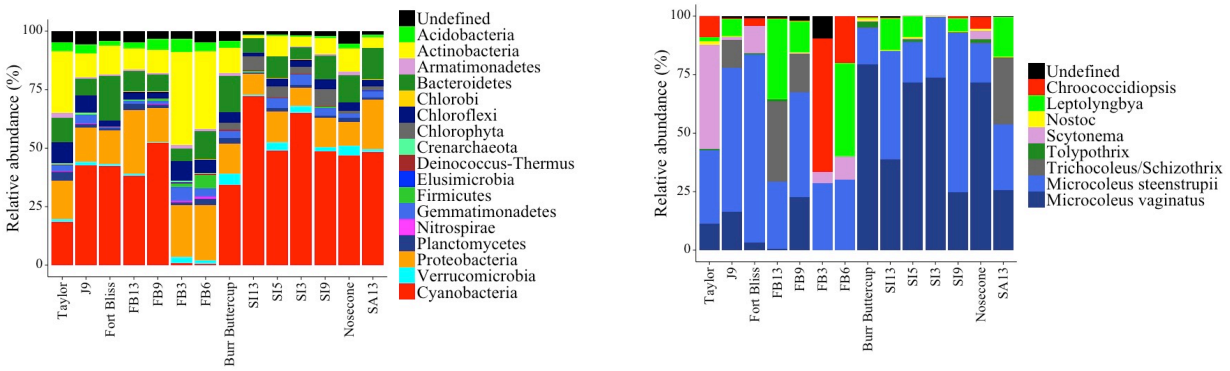


Fig. 4. *Relative abundances of bacterial phyla (left panel) and cyanobacteria organisms (right panel). Field collected abundance is denoted by Taylor = JER silty, Fort Bliss = FB sandy, Burr Buttercup = UTTR silty and Nosecone = UTTR sandy. The Greenhouse grown LB inoculum for each of these sites is denoted by J9, FB13, FB9, FB3, FB6, SI13, SI5, SI3, SI9 and SA13. We were unable to obtain field inoculum from JER sandy sites due to regional drought. Thus, we obtained inoculum from Fort Bliss in this first experiment.*

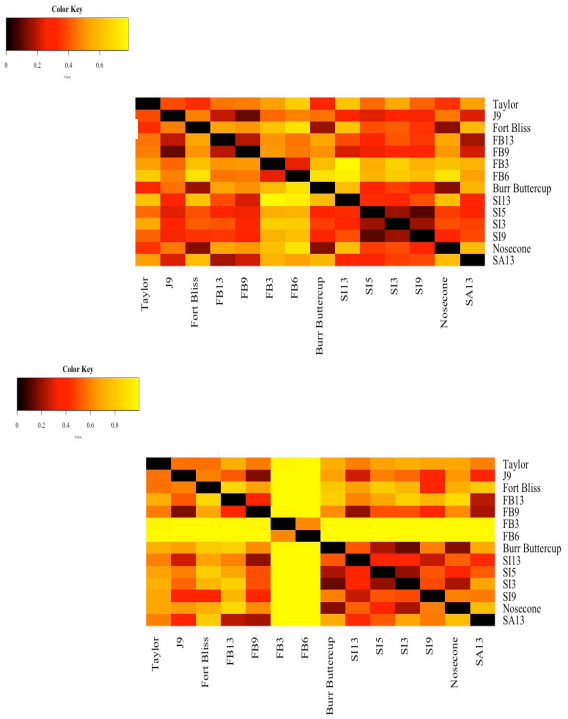


***Fig. 5**. Bray-Curtis dissimilarity matrices for bacterial phyla (top panel) and cyanobacteria organisms (lower panel).*

Similar relative abundances of different bacterial phyla were found in the analyzed treatments when comparing field-collected biocrusts to those grown under greenhouse conditions. Two samples (FB3 and FB6) did, however, show significant changes in microbial community composition from the field collected samples (Fig. 4). When cyanobacterial communities were compared, all treatments, except FB3 and FB6, showed a high proportion of organisms considered to be pioneers and important structural components to favor the establishment of a functional crust (principally *Microcoleus* species) (Fig. 4). With the exception of FB3 and FB6, in several treatments *Microcoleus* species and other important cyanobacteria organisms present in biocrust, such as *Nostoc* or *Scytonema*, comprised more than 50% of all the sequences analyzed in the samples (Fig. 4).

To examine the similarity between field collected biocrust and LB inoculum, we calculated Bray-Curtis dissimilarity distances. In Fig. 5, light yellow denotes dissimilar communities and black denotes similar communities. Again, with the exception of FB3 and FB6, we did not observe significant changes in bacterial phyla or cyanobacteria composition. In the cases in which we observed significant differences in community composition, important pioneer cyanobacteria organisms

were still present in high numbers. Overall, we are confident in our ability to optimize biocrust growth condition in the greenhouse without significantly changing the community composition. Results of FB3 and FB6 clearly show that some sites diverged in microbial community composition, which suggests that additional monitoring of microbial community composition should be performed while biocrusts are being grown in the greenhouse.

Using information from these small-scale screening experiments, LB inoculum was then cultivated in the greenhouse on a larger scale (Fig. 6). It took approximately 4 months for

Fig. 6. *Local biomass (LB) biocrust nursery for cold desert samples in Flagstaff, AZ (left panel) and hot desert samples in Tempe, AZ (right panel)*

containers inoculated at 5 and 20 % soil surface cover to reach 100% biocrust cover. During this time chlorophyll *a* as well as the microbial community composition was monitored. Chlorophyll *a* concentrations of LB inoculum was 5 to 20-fold higher in the cold desert sites and 13 to over 100-fold higher in the hot desert sites compared to initial field collected biocrust samples (Fig. 7). These large and highly significant increases in chlorophyll a content shows that high quality inoculum may be grown in the greenhouse in a relatively short period of time (~ 4 months).

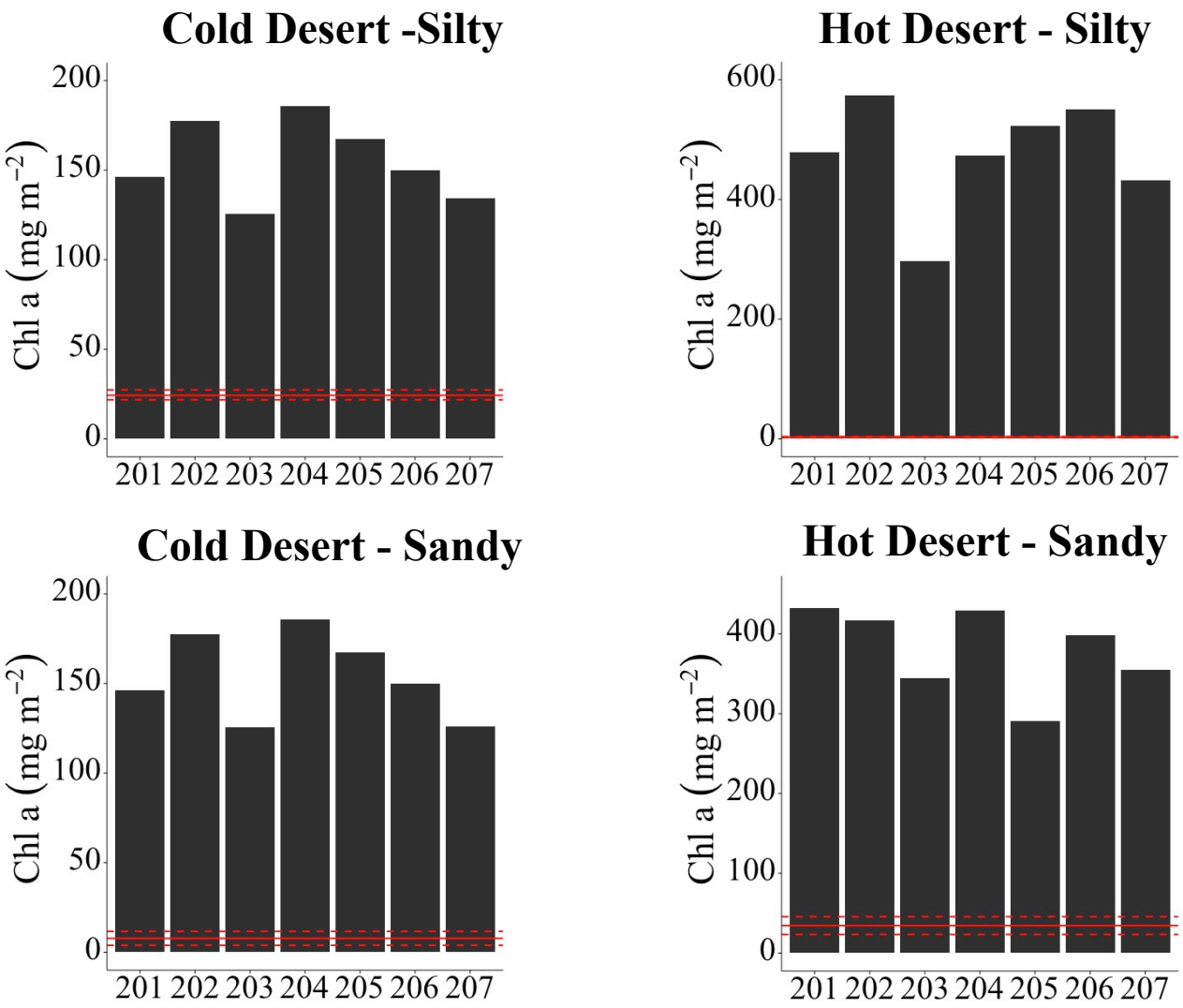


Fig. 7. *Chlorophyll* a *contents of inoculum developed for the cold and hot desert sites. Samples from 7 different bins are represented here. Red lines indicate chlorophyll* a *content of field-collected biocrusts.*

Our time series studies of biocrust growth revealed that chl *a* may be highly variable over time and by soil texture. Maximum average biomass yield for LB inoculum in cold desert locations was highly dependent on soil textures (Fig. 8). Biocrust chl *a* levels were similar to those of intact field collected samples after three weeks on sandy soils and 8 weeks on silty soils. However, growth on silty soil was much more heterogeneous than that of sandy soil and did not reach field biomass levels consistently across all plots (Fig. 8).

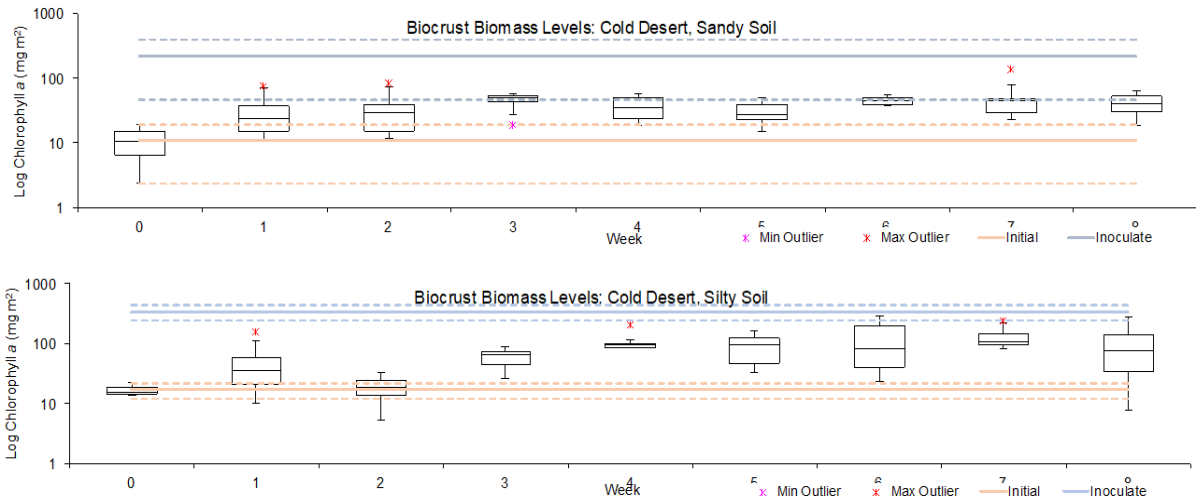


Fig. 8. Boxplot for the final phototrophic biomass (as aerial chl a content) obtained after greenhouse inoculation of native soils from UTTR sandy and silty soil (cold desert location). Boxes denote the lower and upper quartiles (with median values depicted as black solid lines), and whiskers, denote lower and upper extremes (n=3). Blue lines indicate the chl a content of field biocrust samples used as inoculum, and red lines indicate initial chl a content in the inoculum (color solid lines indicate mean, and color dashed lines indicate standard deviations of n=3).

Maximum average LB inoculum biomass yield in hot desert locations was also highly dependent on soil texture. Biocrust chl *a* levels were similar to those of intact field collected samples after 12 weeks. However, growth on sandy soils were more heterogeneous than that of silty soils (Fig. 9). Chl *a* content of sandy soils was highly variable over time with some evidence for a steep drop in chl *a* content at 8 weeks with a subsequent recovery by 12 weeks.

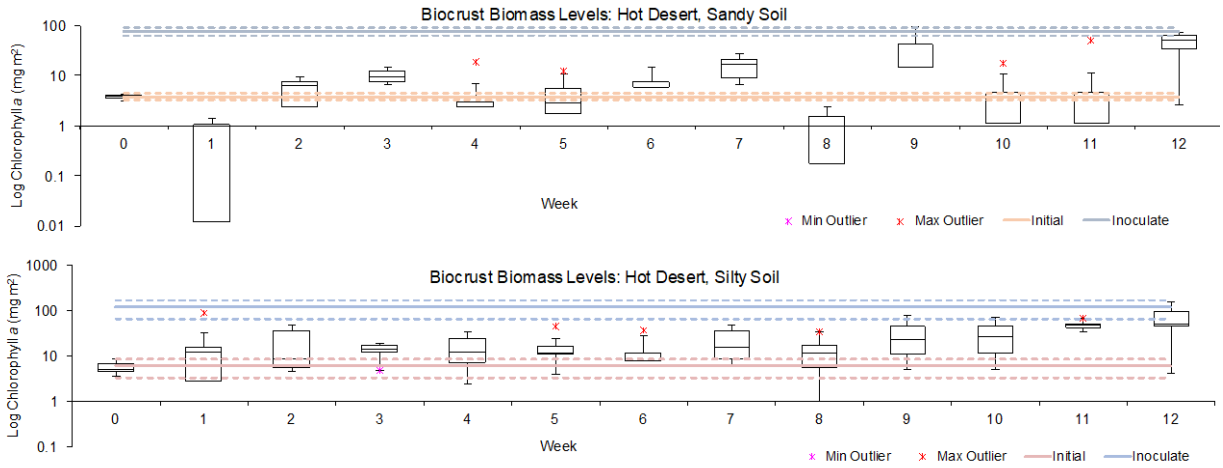


Figure 9. Boxplot for the final phototrophic biomass (as aerial chl a content) obtained after greenhouse inoculation of native soils from Fort Bliss sandy and silty soil (hot desert location). Boxes denote the lower and upper quartiles (with median values depicted as black solid lines), and whiskers, denote lower and upper extremes (n=3). Blue lines indicate the chl a content of filed biocrust samples used as inoculum, and red lines indicate initial chl a content in the inoculum (color solid lines indicate mean, and color dashed lines indicate standard deviations of n=3).

Inoculum 2-Mixed Isolate (MI) Inoculum Development — Microbial community structure at each of the field sites and soil types was similar to those previously reported for biocrust ecosystems in the southwest of United States, with the phylum cyanobacteria as the main microbial component (Fig. 10).

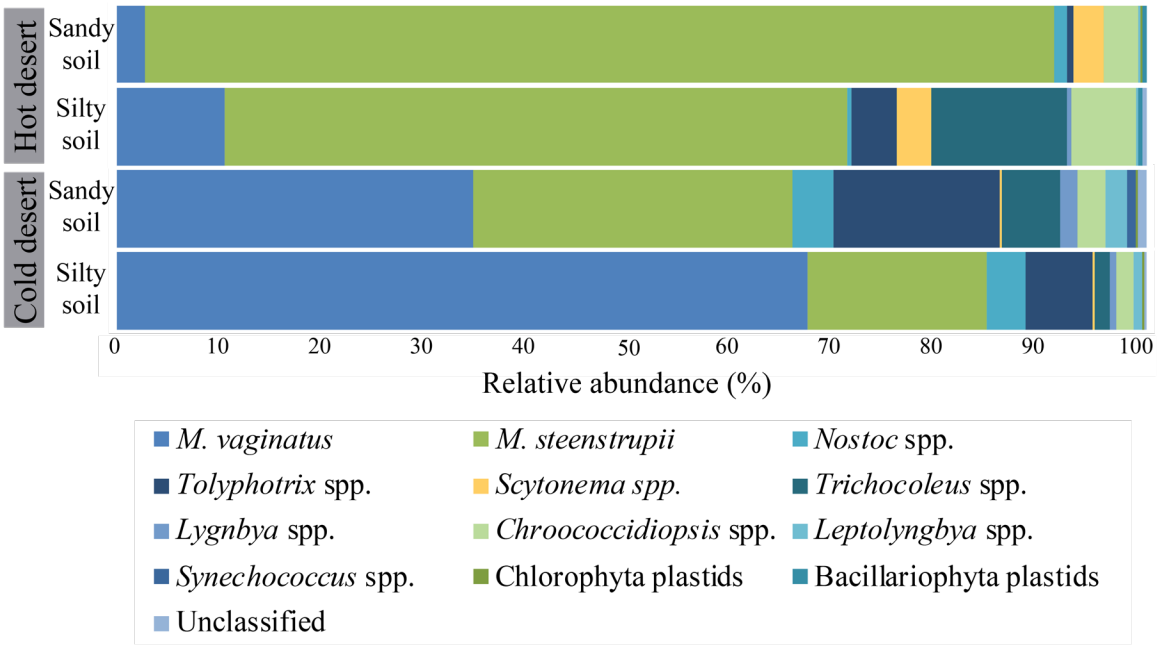


Fig. 10. Bacteria and cyanobacteria community structure and relative abundance for each of the field locations. Cold desert location: UTTR (Cold desert - silty) and UTTR (Cold desert - sandy). Hot desert locations: Fort Bliss (Hot desert – Sandy) and JER (Hot desert – silty). For all locations, cyanobacterial community structure follows the expected pattern: > 50 % of the relative abundance belongs to the cyanobacteria *Microcoleus vaginatus* and *Microcoleus steenstrupii*. Other important biological soil crust forming cyanobacteria as *Nostoc* sp., *Tolypothrix* sp., *Scytonema* sp. and others, account for the rest of the community. Burr buterrcup (Cold desert - silty) and Nosecone (Cold desert - sandy). Hot desert locations: Fort Bliss (Hot desert – Sandy) and JER– Taylor (Hot desert – silty).

Obtaining the cyanobacterial community structure and the cyanobacteria relative abundance for each of the field locations (Fig. 10), was the first step to get a general idea of the final cyanobacteria cultures amount needed to scale up. This was a crucial step in our inoculum producing process, since our restoration strategy is based on introducing a community that is similar in composition to biocrust communities at our field sites. More than 150 cultures were obtained from biocrust samples. Of these cultures, isolates were then selected for scaling up based on phylogenic similarity to reference sequences from sequence libraries (Fig. 11). A total of 10 cultures were scaled up for the cold desert. The scaling up process is described in detail in Appendix A.1. To create the inoculum for the cold desert sites, a total of 3708 plates were produced in a six-month time period.

The next step was to develop feasible approaches to produce enough biomass to support field rehabilitation efforts. All isolates belonging to *Nostoc* spp., *Tolypothrix* spp. and *Scytonema* spp. (non-motile, N_2-fixing cyanobacteria) could be easily scaled-up with standard liquid cultures, in batches of up to 15 L. All of the 32 isolates exhibited robust growth in liquid cultures

in standard incubation chambers. Twelve out of 12 strains that were tested in a greenhouse setting also showed robust growth. For *Nostoc* spp. strains, doubling time ranged from 6 to 11 days, for *Tolypotrhix* spp. from 8 to 15 days, and for *Scytonema* spp. from 8 to 18 days. The final yield of these scaled-up cultures was in the range 0.8 to 1.2 mg Chl *a* per liter, so that principally 1 L of scaled-up inoculum would suffice to inoculate 5-50 m^2 of soil at a 5% of the biomass typically found in the biocrusts of origin.

In contrast, when isolates of *Microcoleus* spp. were submitted to a liquid-culture based scale-up approach, we invariably observed either low yields or no growth at all, even when we used variations in incubation conditions that included light exposure, temperature, nutrient concentration, shaking intensity, or adding glass beads. In our experiment, all 33 *Microcoleus* spp. isolates tended to rapidly clump together into an irregular mass that ceased to grow. In most cases, these clumps still contained viable filaments on the surface for months, but exhibited no further growth unless they were actively removed. The mass in the core was typically bleached and non-viable. Because of this, we developed fundamentally different approaches for *Microcoleus* strains. Among those, we found that evenly inoculating an artificially homogenized stock culture on cellulose tissue support followed by incubation floating on the medium (subaerially, as opposed to submerged in it) resulted in fastest growth (see Fig. 5 A and B). The method is explained in detail in the materials and methods. Similarly, positive results were obtained with various *Microcoleus* strains from all of our locations (Fig. 5 C and D). Under these conditions, for example, *M. steenstrupii* HS024 grew at exponential rates of 0.31 d^{-1}, and *M. vaginatus* HSN003 at 0.47 d^{-1}. More importantly however, the yield of this incubation approach was high, with biomass fully covering the entire surface within 8-14 days of incubation, which was dependent on the strain. Cultures reached peak biomass rapidly and the population would conspicuously turn yellow and crash rather quickly if it was not harvested soon after this point. Typical maximal yield of this procedure was in the range of 0.20 to 0.64 mg Chl *a* per Petri dish. At this yield, a single plate would suffice to inoculate between 0.2 to 3.3 m^2 of soil (strain dependent) at 5% Chl *a* concentrations of those typical for biocrusts in the field.

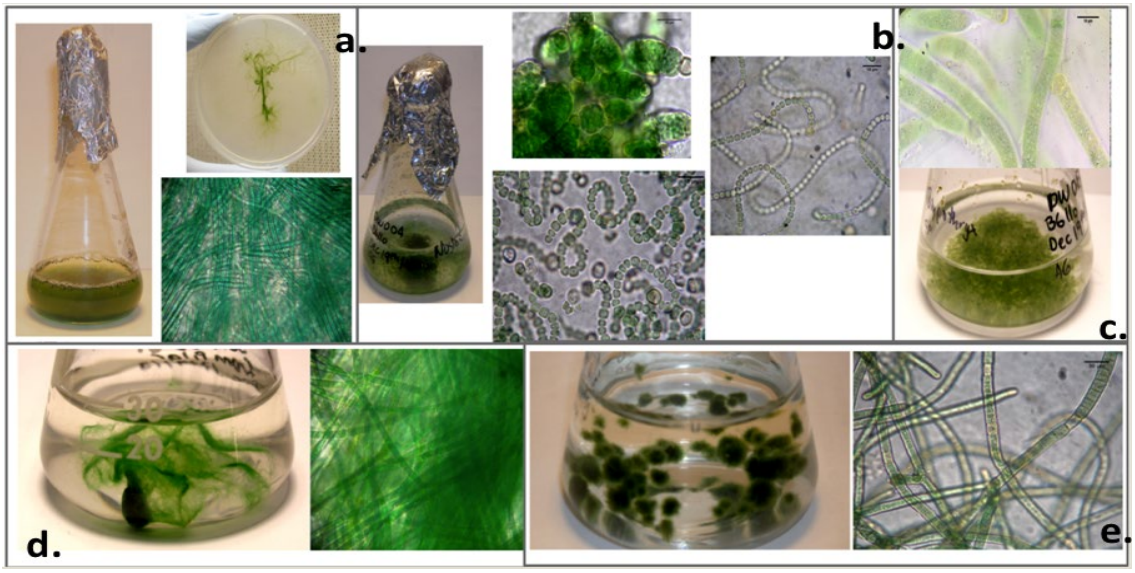


Fig. 11. Cyanobacteria cultures of the main biological soil crust forming cyanobacteria. **a.** *Microcoleus vaginatus*, **b.** *Nostoc* sp., **c.** *Tolypothrix* sp., **d.** *Microcoleus steenstrupii* and, **e.** *Scytonema* sp.

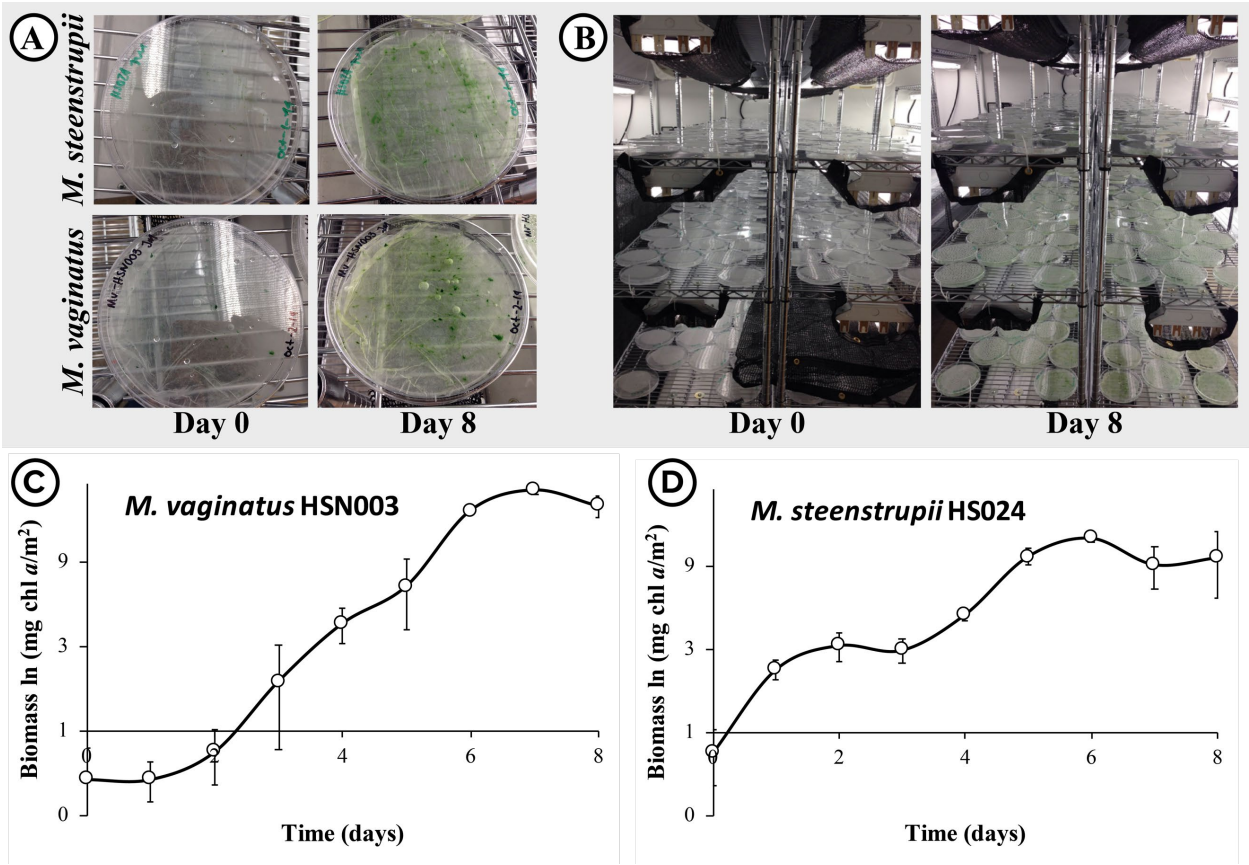


Fig. 12. A novel approach was needed to grow M. vaginatus and M. steenstrupii and a floating cellulose tissue technique was developed. A: visual aspects of set up and growth. B: scale-up. C and D: growth dynamics showing exponential growth and maximum yields.

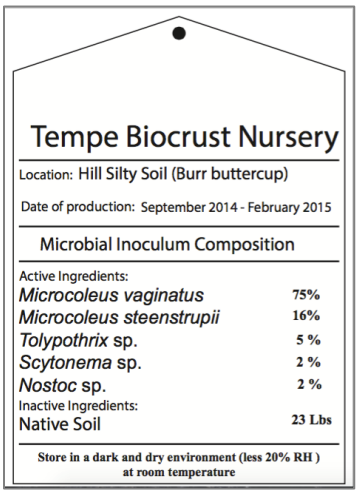


***Figure 13**. Tempe biocrust nursery label shows the microbial inoculum composition of the final isolate mixed inoculum. (Label corresponds to one of the two locations in the cold desert).*

The final isolate mixed inoculum was conditioned under field like conditions. The hardening process is described in detail in Appendix II. The inoculum formulation was based on pedigreed

laboratory cultures that match the cyanobacterial relative abundance of the original sites (Fig. 10), and additionally, have been conditioned to dry-wet cycles and increasing light exposure, with the goal of increasing field adaptation and survival rates. This inoculum was delivered to Task 3 multi-factorial experiments in 2015.

We continued to develop novel techniques to growing mixed isolates under laboratory conditions. Two mixed isolate strains of *M. vaginatus* and two strains of *M. steenstrupii* have been succesfully grown by using the fog chamber (Fig. 14).

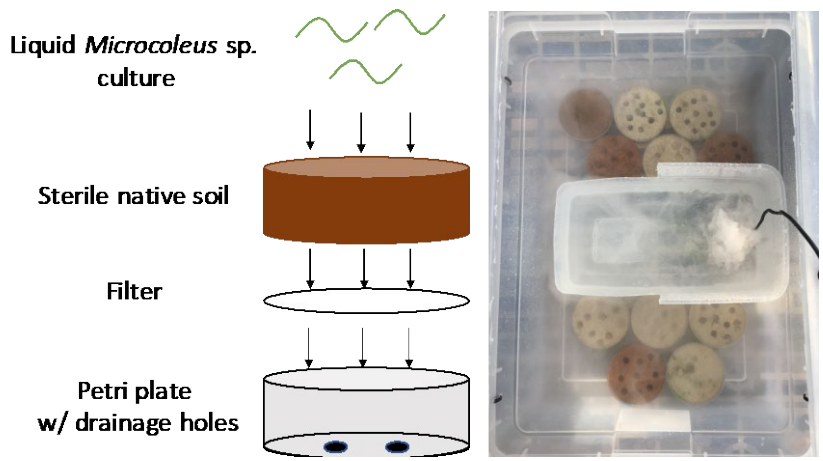


Figure 14. Microcoleus spp. Fog chamber

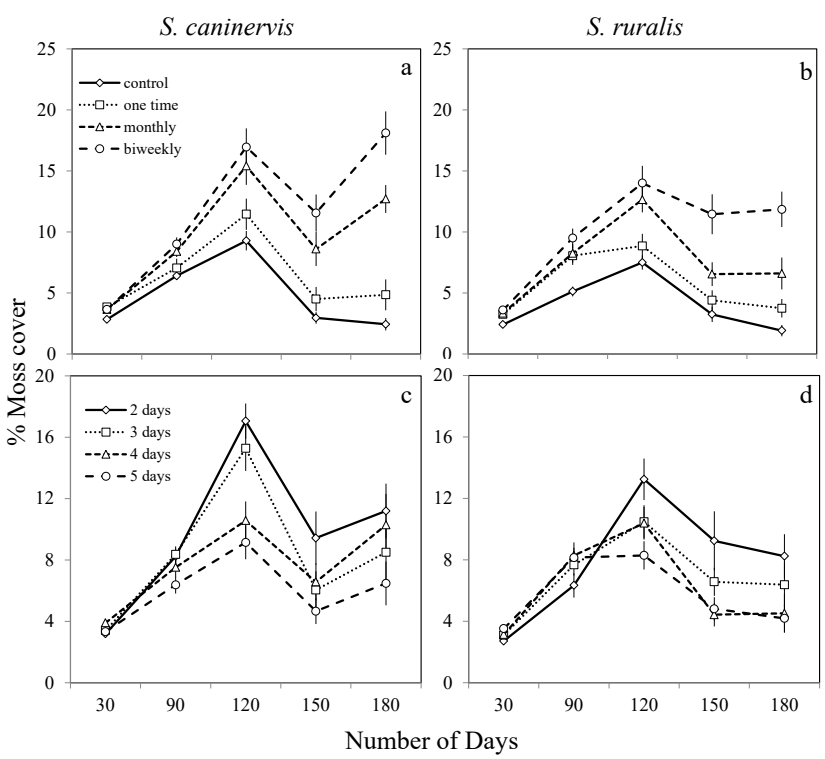


Fig. 15. The change in moss cover in time (days), by: fertilizer treatment (top, a & b) and hydration period (bottom, c & d) for S. caninervis (left, a, c) and S. ruralis (right, b, d). Symbol legends are given in the top right of each graph. Error bars represent ±SE.

The fog chamber method gave similar MI inoculum biomass yields (~20 mg chl *a*/m^2), compared to the current method (paper tissue). Although the time to grow this biomass more than doubled compared to the current method (25 vs. 9 days), the advantage of this method is that biomass is directly grown in its native soil which then eliminates several time-consuming steps in the inoculum cultivation process. As a result, the overall time to produce inoculum with the fog chamber is reduced as compared to the current filter paper method. Our next step is to scale up mixed isolate inoculum biomass using the fog chamber method.

Moss and Lichen Cultivation—Fertilizer and minimal watering promote biocrust moss growth. Both moss species increased from the initial cover of ~ 4% to a maximum of 23 % cover after 120 days of growth (Fig. 15). Both species declined in cover after 120 days. For both moss species, the best weekly cultivation environment was two or three days hydration with biweekly fertilizer addition. Other biocrust species, especially cyanobacteria, incidentally colonized pots with moss fragments. Control soils remained uncolonized by any dominant biocrust organisms over 180 days, although some control pots did show some colonization by green algae. In contrast, all pots receiving moss inoculum had biocrust composition characteristic of the collection site and of biocrust communities in general. In particular, we documented: *Volvox spp*. (a green algae present in Great Basin Desert biocrusts); light cyanobacterium, *Microcoleus sp*.; dark pigmented nitrogen-fixing cyanobacteria, *Nostoc sp*. and *Scytonema spp*.; and lichen species, *Collema spp.* (Fig. 16). This phenomenon led to biocrust cover greater than 100% of the surface area (where organisms overlapped one another other) in the most productive treatments, particularly where 3+ days of weekly hydration was coupled with monthly or biweekly fertilization (Fig. 16). *Nostoc* spp. made up the majority of cover in all treatment combinations, with moss second in abundance (Fig. 17). Although green algal contamination was a concern before the experiment, algae had the least cover, indicating that contamination was not a major issue in the pots receiving biocrust inoculum (Fig. 16). *Nostoc sp*. cover was examined separately because of its prominence, and important contributions to biocrust function. Unlike mosses, which declined between 120 and 180 days, *Nostoc sp.* cover steadily grew over time throughout the course of the experiment (Fig. 17). *Nostoc sp*. cover was affected by moss species, water treatments, fertilizer, time, and interactions among time and treatments. At 180 days *Nostoc sp*. cover was no longer different between moss species, but was still affected by hydration period, fertilizer and the interactions of water × fertilizer and species × fertilizer.

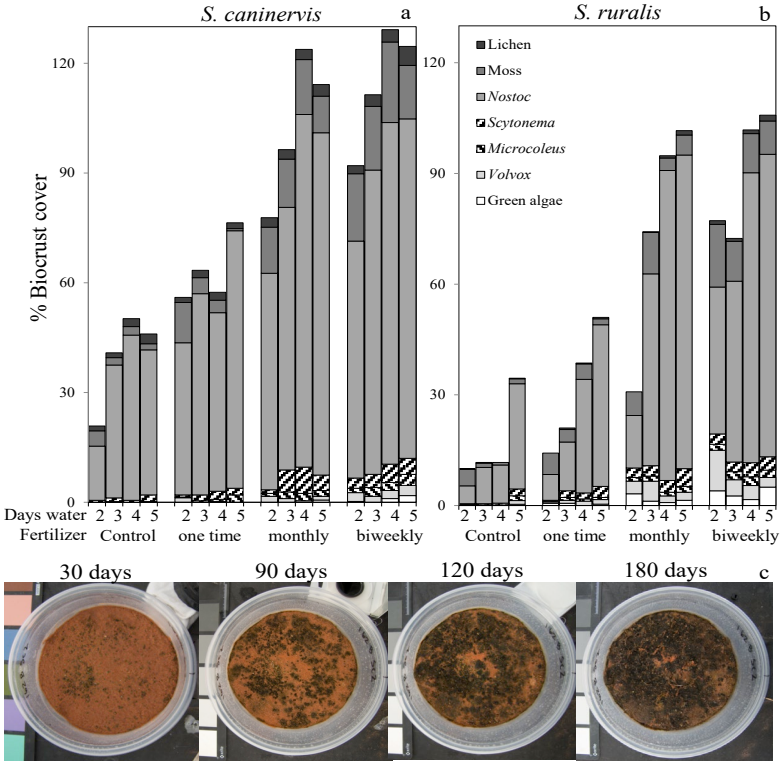


Fig. 16. Stacked bar graphs of total biocrust cover for a) S. caninervis and b) S. ruralis. Error bars represent standard error of the mean. Symbol legend is given in the top right portion of panel b. Panel c gives repeat images of a representative unit over time.

Objective 2 - Identify successful biocrust field application methods - Hardening Experiment

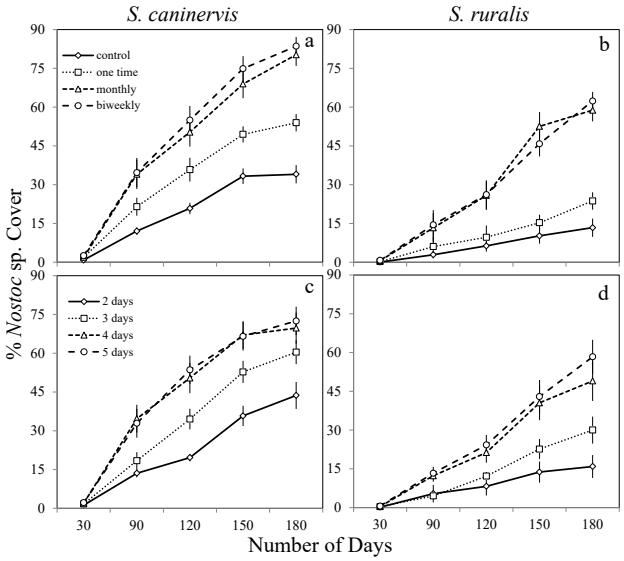


Figure 17. The change in Nostoc sp. cover in time (days), by: fertilizer treatment (top, a & b) and hydration period (bottom, c & d) for S. caninervis pots (left, a & c) and S. ruralis pots (right, b & d). Symbol legends are given in the top right of each graph. Error bars represent ±SE.

We hypothesized that exposing greenhouse grown LB biocrust inoculum to increasing water and UV stress or "hardening" would promote lower mortality and higher growth responses after soils were inoculated. However, our hypothesis was not supported and there was little response of biocrust growth to hardening conditions. The exception to this was late successional cover (the sum of dark pigmented cyanobacteria, lichens and mosses), which responded to an interaction of time, culture conditions and hardening. The highest late successional cover was observed with two or three-days continuous hydration during cultivation and moderate hardening (outdoor with 50% shade and low water), compared to the lowest cover with three-days continual hydration with no hardening, or extreme hardening with two or five days of continuous hydration during cultivation.

The rationale behind “hardening” is to condition organisms to a harsher environment than the one in which they were cultivated. Field conditions have higher UV, more variation in temperature and relative humidity, and a lower frequency and predictability of water. Our three hardening conditions were chosen in an effort to maximize feasibility for land managers, and offer conditions that might benefit different groups of biocrust organisms. Different biocrust organisms are known to have variable sensitivities to environmental conditions (e.g., Grote et al. 2010), and thus biocrust populations may require different hardening treatments to achieve optimal establishment and growth. In addition, we know that some mosses require a period of “dehardening” where plants are given luxury conditions in order to build up all of their protective systems to minimize damage caused by desiccation events (Stark et al. 2012.). This suggests that mosses might establish best in the field when cultured with long hydration periods and treated to luxury greenhouse conditions, or no hardening. Dark pigmented cyanobacteria and the dominant lichens of our study system have protective UV pigments that are inducible by UV exposure (Gao and Garcia-Pichel 2011). We also know that lichens, mosses and dark pigmented cyanobacteria are sensitive to warming, and particularly warming with water stress (Belnap et al. 2006, Escolar et al. 2012, Ferrenberg et al. 2015). This might suggest that these late successional groups could benefit by hardening to temperature fluctuation and water stress, as given with shorter hydration culturing and exposure to outdoor conditions. Light pigmented cyanobacteria without UV-protective pigments have different strategies to avoid stress, retreating under the soil surface for protection from UV, and to track moisture (Garcia and Pringault 2016). It is possible that light pigmented cyanobacteria need no hardening because of their avoidance strategy, but instead, would benefit from being added to the field with the physical cover of soil, another substrate, or dark pigmented, later successional biocrust organisms.

Objective 2-Identify successful field application methods of biocrust inoculum in a series of field trials— Objective 2 was to identify successful field application methods of biocrust inoculum in a series of field trials. The time period for our field trials yielded very different weather patterns at the two research sites that had impacts on our experiments. At UTTR, we saw favorable conditions, with above average precipitation and distribution of rain events. At JER, we saw a recovery from a twelve-year drought, which resulted in extreme weather events with freezing rain, high winds, and heavy monsoon rains. The majority of the plots at both sites experienced some effects of either overland flow or saltation, leading to difficulty in establishing treatment patterns. Results of experiments are given below.

Experiment 1- Inoculum Amount Trials—At UTTR, we found that 10% inoculum addition maximized biocrust establishment and recovery (Fig. 18). At JER, most treatment responses were masked by soil movement into the plots, although a soil-texture difference can be seen, with higher colonization on fine soils (Fig. 18). There was no result of inoculations on any stability measures at either site (p>0.05). Table 4 gives the statistical results of the ANOVA model for biocrust cover at the two research sites.

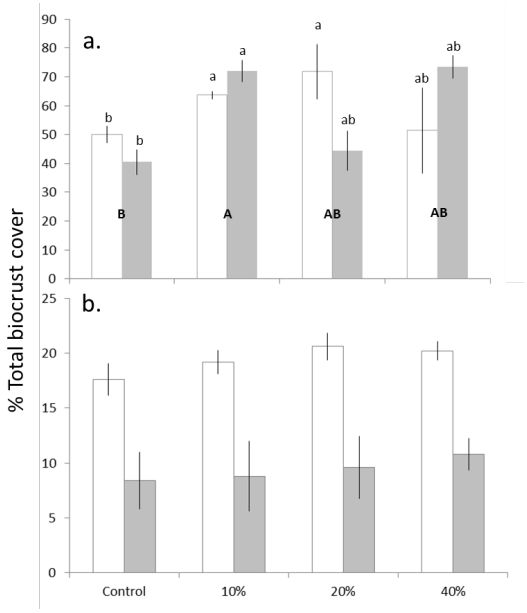


***Fig. 18**. Total biocrust cover as measured by point intercept at a.) UTTR and b.) JER. Open bars indicate fine textured soils and shaded bars indicate coarse textured soils. Letters above a bar indicate differences at the p=0.05-level using a post-hoc Student's T-test. Different uppercase letters on the bars indicate an overall difference among inoculation levels at the p=0.05-level using a post-hoc Student's T-test.*

Experiment 2-Habitat Modification Trials—The results from this complex experiment are nuanced, but informative. At UTTR, the strongest main effects on total biocrust were soil type and inoculum addition. At JER, again, treatment signals were masked by soil movement. To tease apart the differences more carefully, we chose to sum only the late-successional members of the biocrust community (i.e. dark cyanobacteria, lichens and mosses). When we do this, we see that soil type and inoculum addition are still important at UTTR (soil type is also important at JER), but shade, and combinations with shade are also important (Table 4, Fig. 19). Soil stability was most affected by soil type at both sites (Table 4), but inoculation, water, surface roughening, and combinations of soil by water or roughening impacted soil stability at UTTR. Again, JER treatments were affected by soil movement, masking most treatment differences, but shading reduced soil movement at both sites, and we saw higher soil stability in these plots (Table 4).

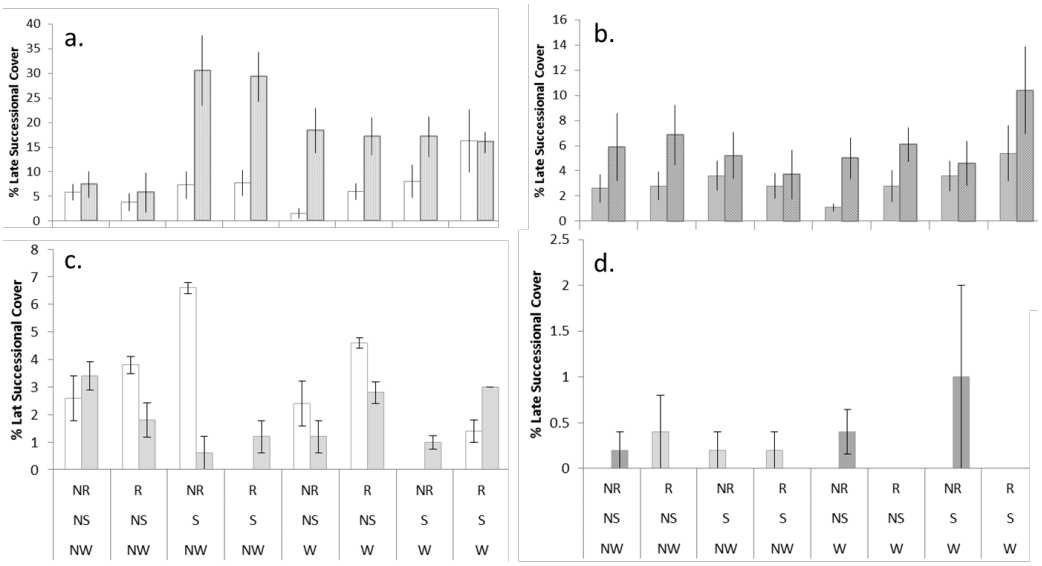


Fig. 19. Late succession cover (including dark cyanobacteria, lichens and mosses) from a) UTTR fine textured soil, b) UTTR coarse textured soil, c) JER fine textured soil, and d) JER coarse textured soil. Lighter bars (left side) are uninoculated, and darker or shaded bars (right side) are inoculated. Labels on the x-axis represent roughing (NR: control, R: roughened), shade (NS: control, S: shaded), and water (NW: control, W, watered).

Table 4. ANOVA results (F-value (p-value) for the habitat modification experiment at UTTR and JER. Statistically significant results are shown in bold. Only rows that had at least one significant effect are shown. Rows without any statistically significant effects are not shown.

Measurement	*Total biocrust cover*		*Late successional cover*		*Soil aggregate stability*	
Site	UTTR	JER	UTTR	JER	UTTR	JER
Soil type	**14.2 (0.0002)**	**54.0 (<0.0001)**	**52.2 (<0.0001)**	**211.4 (<0.0001)**	**2346.9 (<0.0001)**	88.7 (<**0.0001)**
Inoculum addition	**19.2 (<0.0001)**	0.03 (0.9)	**38.8 (<0.0001)**	0.3 (0.6)	**18.5 (<0.0001)**	0.0 (0.9)
Water	0.04 (0.8)	0.5 (0.5)	0.2 (0.64)	0.4 (0.5)	**6.9 (<0.0001)**	1.8 (0.2)
Shade	0.5 (0.5)	**7.8 (0.01)**	**17.2 (<0.0001)**	6.9 (0.01)	1.1 (0.3)	**11.8 (0.001)**
Surface roughening	1.0 (0.3)	0.2 (0.7)	0.8 (0.4)	0.4 (0.5)	**9.2 (0.003)**	0.3 (0.6)
Soil x inoc	0.2 (0.7)	0.5 (0.5)	**12.5 (0.0006)**	0.8 (0.4)	**14.5 (0.0002)**	0.0 (0.9)
Soil x water	0.3 (0.6)	0.0 (0.9)	0.02 (0.9)	0.7 (0.4)	**5.8 (0.02)**	0.6 (0.4)
Inoc x rough	**7.8 (0.01)**	4.9 (0.03)	0.5 (0.5)	0.0 (1.0)	0.9 (0.3)	3.1 (0.1)
Soil x water x shade	**6.4 (0.01)**	0.4 (0.5)	**8.2 (0.005)**	1.6 (0.2)	3.2 (0.1)	0.1 (0.8)
Inoc x water x shade	0.2 (0.6)	0.4 (0.5)	**10.5 (0.002)**	1.8 (0.2)	2.1 (0.2)	0.1 (0.7)
Soil x inoc x shade x rough	0.1 (0.8)	**3.8 (0.05)**	0.2 (0.6)	4**.5 (0.04)**	1.0 (0.3)	0.6 (0.4)
Soil x inoc x water x shade	**4.0 (0.05)**	2.0 (0.2)	0.9 (0.3)	1.3 (0.3)	0.8 (0.4)	0.0 (1.0)
Soil x inoc x water x shade x rough	0.8 (0.4)	0.9 (0.3)	1.2 (0.3)	**5.6 (0.02)**	0.6 (0.5)	0.0 (0.9)

Experiment 3 (Stability) Results: This experiment also yielded informative results. As in other experiments, soil type is the strongest driver of biocrust cover and soil aggregate stability at both sites (Table 5). At UTTR stability measures were important to biocrust cover, but varied by site and with and without inoculum, Polymer 2 (DirtGlue) and straw borders were strong performers on both soil types (Fig. 20, Table 5). Interestingly, inoculum addition was more important than stability measures in determining soil aggregate stability at Hill, but the shade+ roughening + polymer treatment had the highest stability values (Fig. 21, Table 5). At Jornada soil type and inoculation were important alone, and in conjunction with stability measures in determining biocrust cover.

Table 5. ANOVA results (F ratio (P-value)) from the soil stability experiment. Soil = silty and sandy, Inoculum = + inoculum, - inoculum, Stability Measure (6 levels) = PAM 1, PAM 2, PAM 3, Straw border, Straw border + PAM.

Measurement	*Total biocrust cover*		*Soil aggregate stability*	
Site	UTTR	JER	UTTR	JER
Soil type	**22.0 (<0.0001)**	**78.5 (<0.0001)**	**1198.0 (<0.0001)**	**207.5 (<0.0001)**
Inoculum addition	2.2 (0.1)	**8.1 (0.0004)**	**7.6 (0.007)**	1.7 (0.2)
Stability Measure	**8.9 (<0.0001)**	1.8 (0.1)	1.4 (0.2)	**7.6 (<0.0001)**
Soil x Inoc	0 (1.0)	**10.6 (<0.0001)**	**6.1 (0.01)**	0.2 (0.6)
Soil x Stability	0.9 (0.5)	**6.5 (<0.0001)**	1.3 (0.3)	2.0 (0.08)
Inoc x Stability	**4.8 (0.01)**	**3.4 (0.01)**	**3.1 (0.02)**	**2.8 (0.02)**

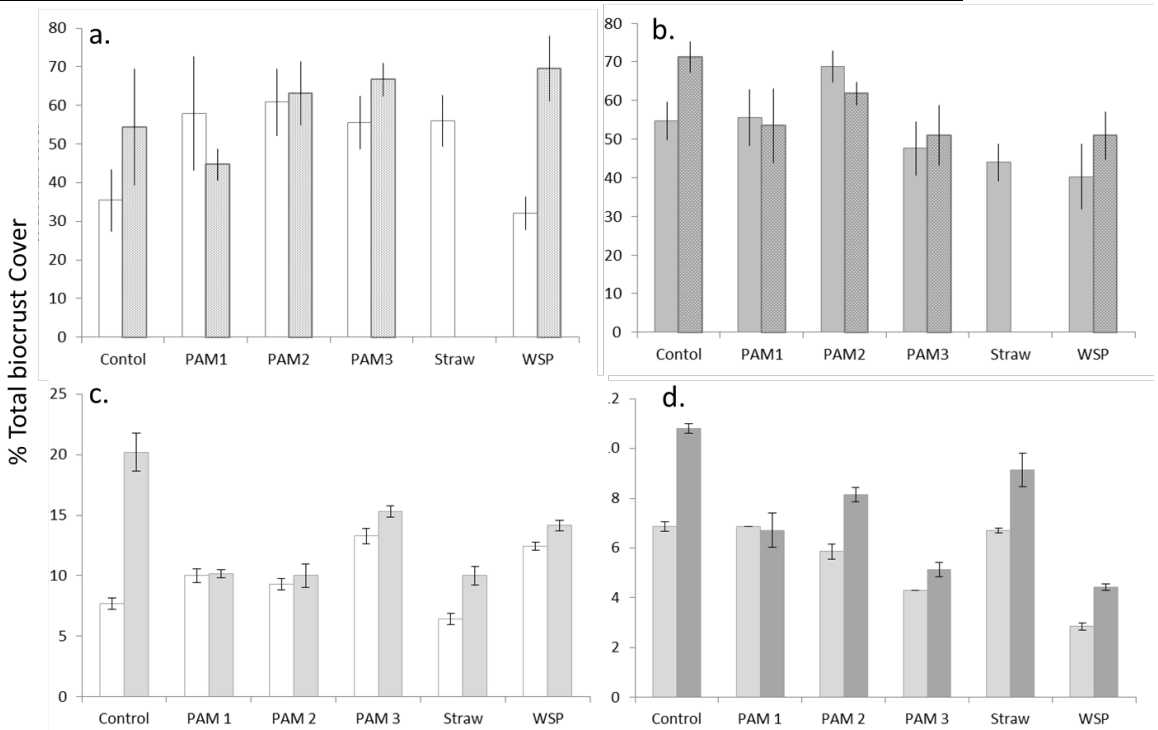


Figure 20. Percent biocrust cover mean values from a) UTTR fine textures soil, b) UTTR coarse textured soil, c) JER fine textured soil, and d) JER coarse textured soil. Lighter bars (left side) are uninoculated, and darker or shaded bars (right side) are inoculated. PAM 1-3 are three types of soil stabilizing polymers. Straw = straw

checkerboard stabilization. WSP = water + straw+ polymer.

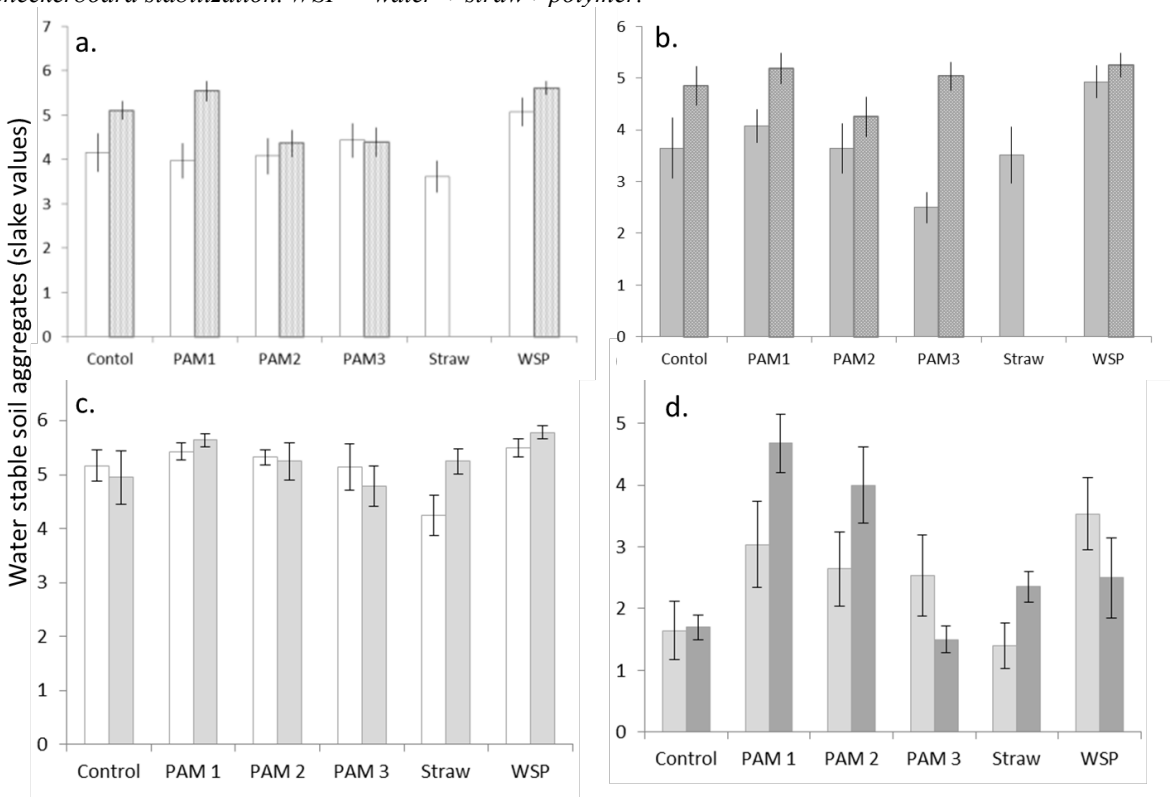


Figure 21. Water stable soil aggregate mean values from a) UTTR fine textures soil, b) UTTR coarse textured soil, c) JER fine textured soil, and d) JER coarse textured soil. Lighter bars (left side) are uninoculated, and darker or shaded bars (right side) are inoculated. PAM 1-3 are three types of soil stabilizing polymers. Straw = straw checkerboard stabilization. WSP = water + straw+ polymer.

Experiment 4 (Seed establishment): After 14 months, no seeds had germinated in this experiment. However, measurements after 24 months at UTTR show some grass seedlings.

Objective 3 —Evaluate plant and soil responses to biocrust restoration

In the multi-factorial field experiment, initial soil observation measurements in 2015 at the UTTR site showed the highest soil aggregate stability measurements in the control plots (CON-IN and CON-OUT) and in the polymer plots (PM) with no other strong treatment effect of stability (Fig. 22). One year after treatment the undisturbed controls which excludes the disturbed only (DIS-NA) maintained a high stability and in the silty site there was a general trend towards the polymer having a greater stability than the straw treatments, but not significantly so. After one year (2016), the UTTR inoculum treatments did not show any differences in soil stability. The hot desert site (JER) showed the same trends as the UTTR with the polymer demonstrating the highest stability, which was similar to the controls in the silty site but in 2016 the trends were less prominent. The JER sandy site had very low soil stability but was stabilized with the addition of polymers, which then declined after one year (Fig. 23). This decline in soil stability in the polymer treatments is not surprising since the polymers are designed to degrade over time (Seybold 1994). What wasn't as expected was the rapid stability recovery in the UTTR site regardless of treatment. While not reaching the undisturbed control levels, the recovery in the sandy site was commonly above a soil stability metric of four, which translates

out to a moderate to high level of soil stability. The JER sandy site unfortunately did not obtain any stability outside of the polymer treatments over time and considering the controls themselves had incredibly low stability this was consistent with what was initially expected (Fig. 23).

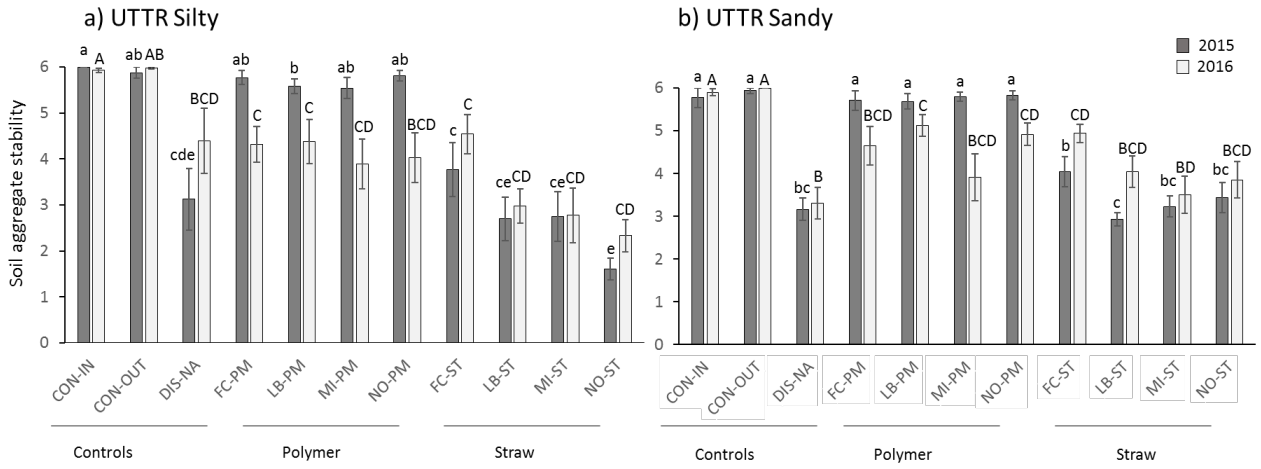


Figure 22. UTTR mean soil aggregate stability measurements in silty (a) and sandy (b) soils after implementation (2015) and one year after (2016). Lower case letters indicate significant differences between treatments in year 2015 and upper case letters for 2016. All error bars represent ±1 SE.

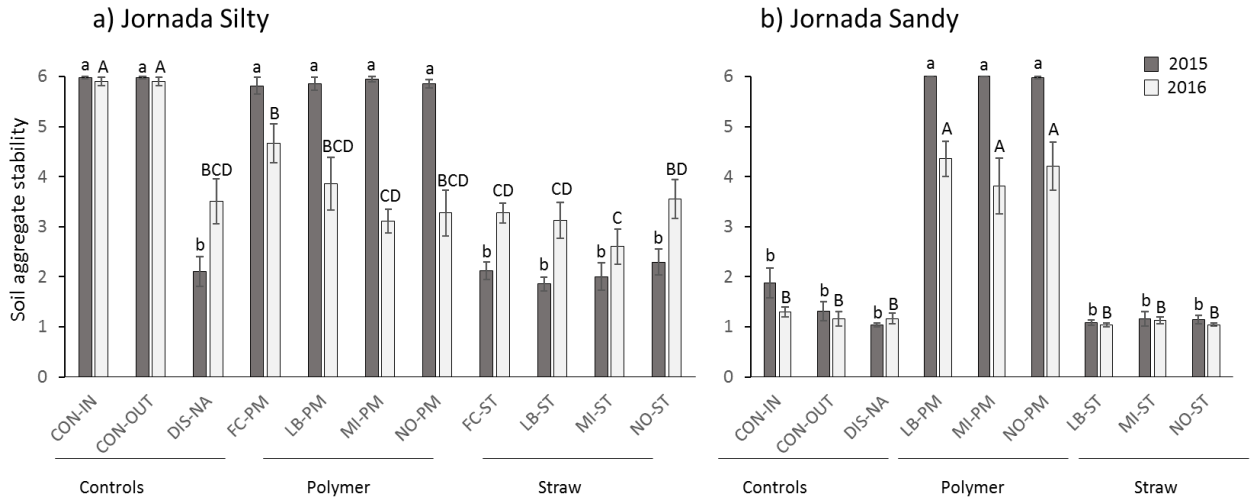


Figure 23. JER mean soil aggregate stability measurement in silty (a) and sandy (b) soils implementation (2015) and one year after (2016). Lower case letters indicate significant differences between treatments in year 2015 and upper case letters for 2016. All error bars represent ±1 SE.

Chl *a* in the undisturbed control plots were used as a general target level of chl *a* for the inoculum and soil stabilization treatments. At the UTTR sandy site the field collected with straw treatment (FC-ST) showed that initial levels were generally higher than most other treatments and this persisted into year one (Fig. 24). The UTTR field collected inoculum which was stabilized by both polymer and straw (FC-PM, FC-ST) again showed initial and continued trends of higher chl *a* levels. At the JER sandy site, chl *a* levels were extremely low (< 1 µg chl a/g soil) compared to the other sites (Fig. 25, upwards of 50 µg chl a/g of soil). The lack of chl *a* in the

JER sandy site suggests that further soil stability efforts and biocrust enhancement techniques may be required for this highly mobile site to achieve greater biocrust biomass. For the other three sites, there was a general recovery of chl *a* in some treatments but it's likely that we will see clearer treatments responses in years 2 and 3.

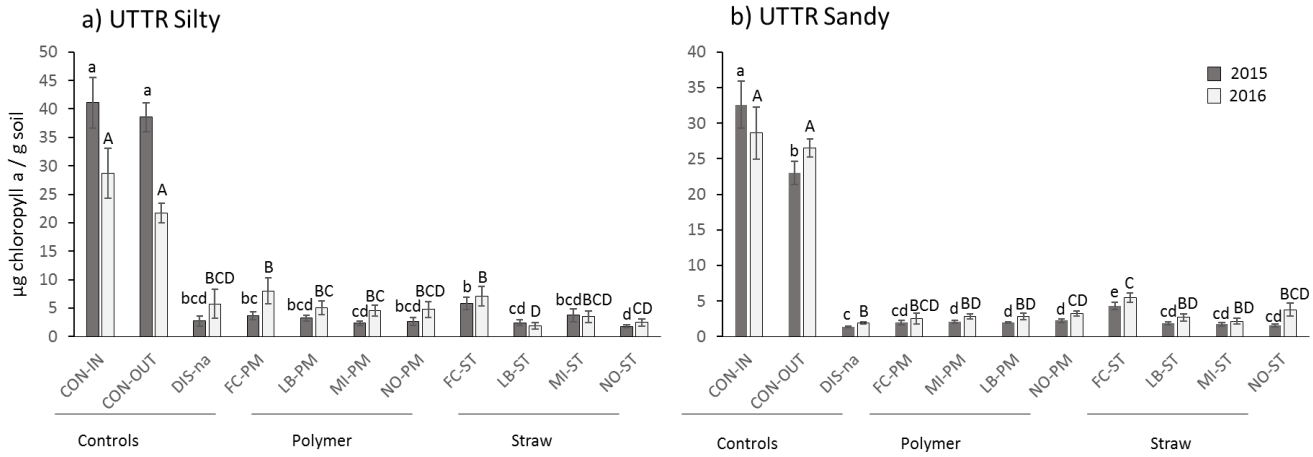


Figure 24. UTTR chl a measurements in the silty (a) and sandy (b) soils after implementation (2015) and one year after (2016). Lower case letters indicate significant differences between treatments in year 2015 and upper case letters for 2016. All error bars represent ±1 SE.

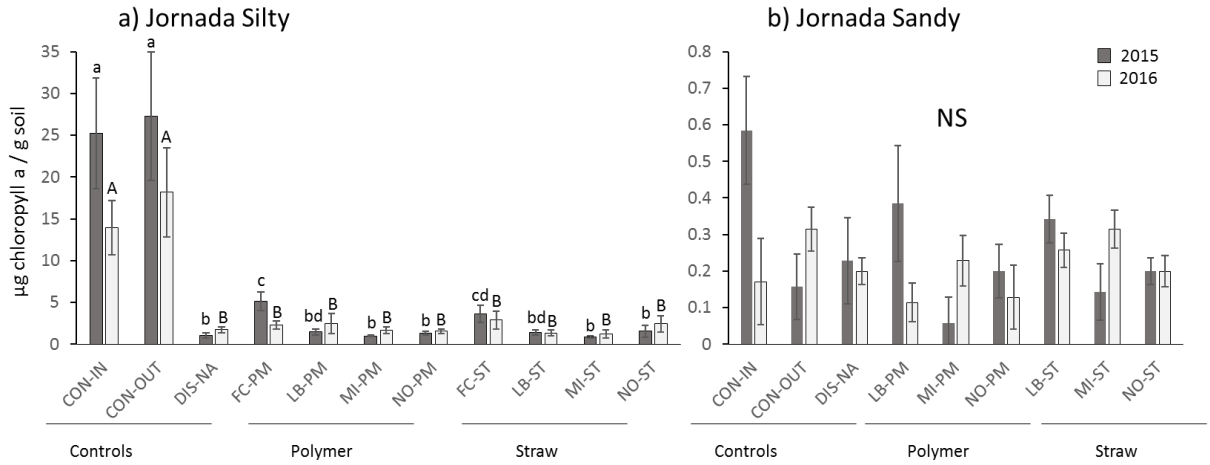


Fig. 25. JER chl a measurements in the silty (a) and sandy (b) soils after implementation (2015) and one year after (2016). Lower case letters indicate significant differences between treatments in year 2015 and upper case letters for 2016. All error bars represent ±1 SE.

The majority of torvane and penetrometer measurements did not have any significant differences or even trends across treatments at any of the sites with the exception of the UTTR sandy site measurements in 2015 (Fig. 26). Here the local biomass polymer (LB-PM) had a significantly higher sheer strength than the control within a disturbed area (CON-IN) and the local biomass and no inoculum straw (LB-ST, NO-ST) treatments. While biocrusts are known to increase the sheer strength of the soil, the CON-IN unexpectedly had one of the lowest values (Fig. 26). The high value observed in the LB-PM treatment in 2015 could be due to the presence of the polymer. Yet, the idea that the polymer could be impacting sheer strength wasn't supported in any of the other polymer treatments within this site, or any of the other sites. While

not significantly different across treatments the average sheer strength across all were highly variable. The lack of treatment differences within a site suggests that the signal may increase over time as trends further develop or, alternatively that differences may be at a fine enough scale the current instrumentation cannot detect it.

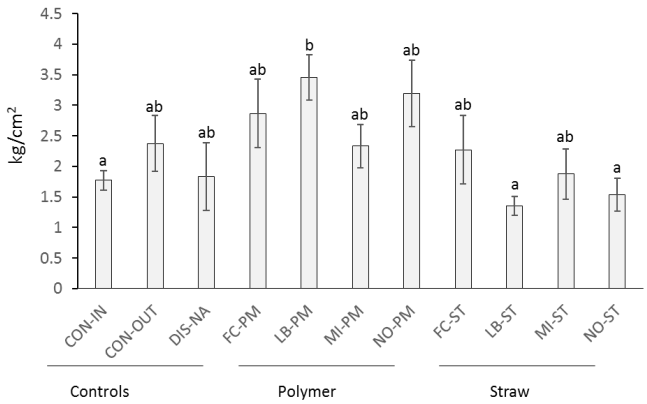


Fig 26. UTTR sheer strength measurements (kg/cm²)at the sandy sites. Letters indicate significant differences between treatments. All error bars represent ±1 SE.

Soil moisture were collected for the first time during the year one (2016) sampling campaign. The UTTR soil moisture readings at the different soil types were taken a few days apart with no rain event in between. While the mean soil moisture was lower in the sandy site than the silty in the UTTR, the strong observational difference across treatments was a significantly lower soil moisture in the undisturbed control plots as compared to all treatments (Fig. 27). Due to high variance across treatments at the JER there was no observable differences between soil moisture and the different treatments. Soil moisture fluctuates greatly across time and a single soil moisture measurement is often not sufficient to capture the variability at larger scales. However, it is interesting that at both the sandy and silty UTTR sites a strong decrease of soil moisture occurred in the undisturbed control plots. This could be due to the fact that there was no recent rain event replenishing the system and the biocrust created a more porous system than the highly compacted disturbed sites that experienced a physical crust that may hold in soil moisture. Hydrophobicity was collected at all plots and did not display any trends or significance levels for any of the desert types, soil types, or treatments.

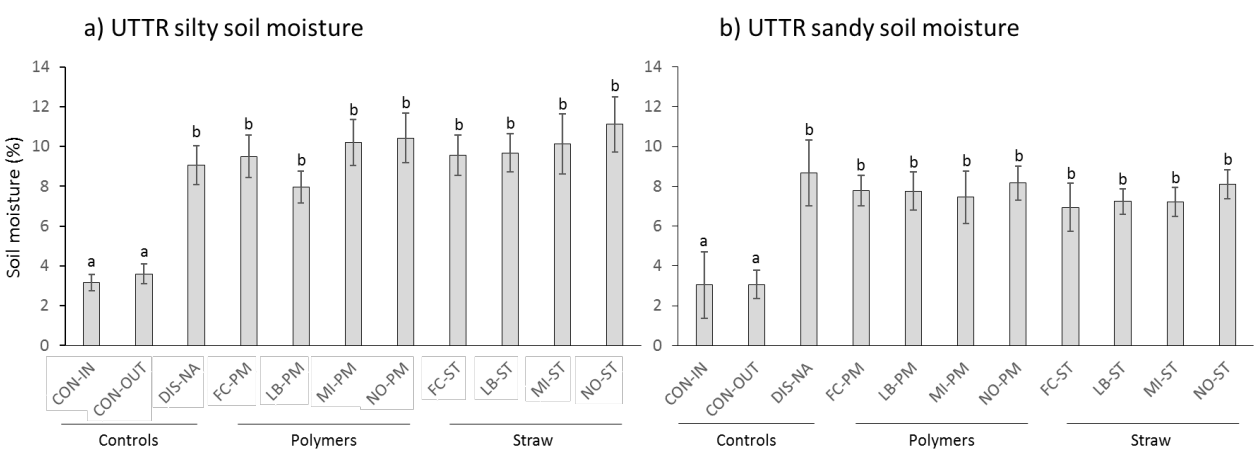


Fig. 27. Soil moisture at UTTR in 2016. No differences were observed at JER. Values are means ±1 SE.

Evaluate native plant restoration

UTTR site was monitored the May following treatment implementation and the JER site one year after in October 2016. Unfortunately, at both sites there was extremely low to no plant germination.

CONCLUSIONS AND IMPLICATIONS FOR FUTURE RESEARCH

Objective 1- Successful Establishment of Biocrust Nurseries

In years 1-3 we developed a two-pronged approach to growing biocrust inoculum. In growing LB inoculum, biomass across hot and cold desert sites was 4 to over 100-fold higher than field collected biocrusts with evidence that microbial community composition is stable and promotes early pioneer biocrust organisms. Our experiments clearly show that it is feasible to produce large amounts of biocrust biomass from low levels of natural inoculum within relatively short incubation times (several months). Key factors controlling biocrust growth are high watering frequency, light reduction and nutrient additions (N, P or N+P) which was specific to hot desert environments. Microbial community composition does not change at the bacterial phyla level but slightly at cyanobacterial level. Overall, we found that pioneer cyanobacterial organisms are easily cultivated in greenhouse facilities. Optimally nursed biocrusts attained or exceeded the biomass concentrations typical of field-collected mature communities. This was even in the presence of recurrent, full-scale cycles of desiccation and wetting designed to mimic the naturally pulsed nature of growth in biocrusts and to avoid allochthonous contamination by non-terrestrial forms in our open system. However, not all incubation conditions resulted in such positive outcomes, and several treatments resulted consistently in either poor growth or even in loss of inoculum biomass. Across different crusts types, incubations under enhanced watering regimes (equivalent to doubling the natural rainfall averages of origin) and decreased light stress consistently resulted in high growth rates. These results are in line with what could have been surmised from the literature: rainfall frequency and light intensity are among the most important factors contributing to the growth and activity of biocrusts. Exposing greenhouse grown biocrust to increasingly stressful conditions or "hardening" does not enhance growth biocrusts under field conditions. Thus, the extra step of hardening biocrust inoculum is unlikely to more successful establish biocrusts in the field. The exception to this was that mild hardening and lower-frequency watering led to the highest establishment of moss, lichen and dark cyanobacteria cover.

MI inoculum that is similar in biomass and community composition to field collected biocrusts may be created in a multi-step scaling up process from lab cultures in ~ 6 months. In a multi-step process, we designed protocols for the establishment of “microbial biocrust nurseries” to produce photosynthetic cyanobacterial inoculum for biocrust seeding at scale. We first report on the strategy for isolation, directly from the target site, of a large culture collection of cyanobacteria that included multiple representatives of the five most common biocrust taxa. After genetic pedigreeing of these isolates, we could select those that best matched filed populations genetically for scale-up cultivation. We then developed protocols for effective cyanobacterial scaling up to obtain sufficient inoculum. We have made significant advances in understanding the environmental conditions to promote the growth of the dominant early successional cyanobacteria, *Microcoleus. Microcoleus* spp. were shown to respond more positively to fog

than to liquid water and shows great promise for for scaling up of cyanobacterial biomass. This method offers a less labor intensive and time consuming technique, while achieving similar biomass yields in comparison to the method developed in years 1 and 2.

In cold desert environments, mosses are important functional component of these ecosystems. Experiments on limitations to moss growth showed that fertilizer and minimal watering enhance moss growth. Both moss species that are dominant in cool desert environments increased 6-fold in cover after 120 days of growth. For both moss species, the best weekly cultivation environment was two or three days hydration with biweekly fertilizer addition. We also showed that both biocrust mosses and one lichen (*Collema*) can be grown in the greenhouse over just a few months. This is desirable because later successional biocrust organisms offer additional ecosystem benefits.

All methods to develop biocrust inoculum were successful and future efforts will focus on growing biocrust inoculum for use in larger scale restoration and rehabilitation projects.

Objective 2 - *Identify* successful field application methods of biocrust inoculum

In our early trials, we showed that the level of biocrust inoculation did not strongly determine the long-term recovery of the biocrust community. This suggests that even small amounts of biocrusts inoculum added to degraded sites may enhance recovery. Shading of the soil surface has consistently show to be effective in enhancing the recovery of the biocrust community. Shading likely decreases water stress by increasing soil moisture through decrease surface evaporation and also directly by decreasing UV stress. In highly degraded sites where soils are actively eroding, the addition of synthetic soil stabilization agents and more specifically the polyacrylamide Dirtglue appeared to have no inhibitory effect on biocrust recovery. Thus, the use of soil stabilization products to increase soil surface stability before biocrust inoculation may work to prevent biocrust inoculum being buried by high mobile and eroding soils.

Objective 3 - Identify successful biocrust field applications

Addition of three inoculum types (field collected, greenhouse grown, lab developed) showed mixed results. Field collected biocrusts show modestly higher biocrust growth relative to greenhouse grown local biocrusts and lab grown mixed isolates. Again, addition of polyacrylamides to stabilize soils exhibited similar soil stability to intact biocrusts with no evidence of inhibiting biocrust recovery. What is clear is that significant barriers still exist to biocrust recovery under stressful field environments. This is likely due to resource limitation and more specifically to water and UV stress as demonstrated in our early field trials. Future work on successfully should focus on maintaining adequate water balance for biocrust recovery and the possibility of using natural shade structures in the field such as shrubs and other perennial plants. Inoculum placement on cooler, wetter north facing aspects of these natural shade structures may also further promote more rapid biocrust recovery.

Objective 4 - Share knowledge with land managers

We have drafted a biocrust restoration manual as a supporting document to share biocrust

restoration information with DoD and federal land managers. This document will continue to be revised as we increase our knowledge and understanding of biocrust restoration. Now that we have developed clear protocols for inoculum development and still face challenges in overcoming barriers to biocrust recovery under stressful field conditions. Our team will be submitting at least five and possibly more manuscripts to a special issue of Restoration Ecology in December 2017. Two of the post-doctoral research associates from our project will be guest editors of this special issue. Once this special issue has been published we will schedule meetings with DoD and federal land managers in the spring of 2019.

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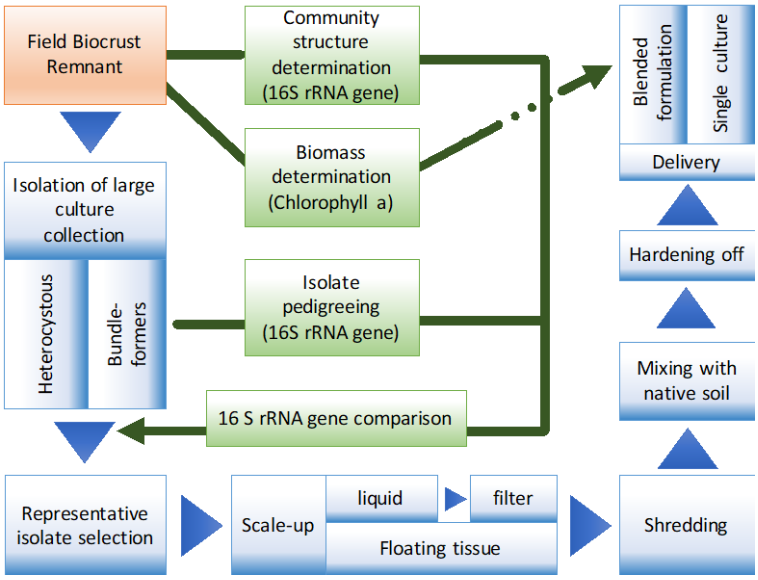
APPENDIX A Supporting Data

Appendix A.1. *Mixed isolate (MI) Scaling Up Protocol for Microcoleus spp. A) General protocol for the establishment of culture-based cyanobacterial biocrust nurseries. Blue arrows and boxes represent action flow. Green arrows and boxes represent information flow. B) Novel scale up process developed for the early successional biocrust pioneer species.*

A)



B)

SCALE UP TECHNIQUE TO PRODUCE LARGE QUANTITIES OF THE BIOCRUST PIONEERS *Microcoleus vaginatus* and *Microcoleus steenstrupii.*

Culture room conditions: 25 ± 2 C, 14:10 photoperiod (light:dark), 20 - 30 µmol m^{-2} s^{-1}

Keep a liquid cyanobacteria culture inoculum supply during the whole scale up process. To prepare the liquid inoculum supply, take a 1L flask Erlenmeyer and fill it with media up to 200mL (or the corresponded proportion when using different volumes). Inoculate at 2% with a well-homogenized cyanobacteria culture. Keep liquid inoculum supply in agitation (80 rpm), under culture room conditions.

Plate inoculation must be performed in the laminar hood (axenic conditions)

Filters (Kimwipes) need to be cut (according to plate size), and autoclaved previous to the scale up process.

- Take a plastic petri dish (bigger size found in the market: 14 cm ϕ). Add approximately 60mL of media to the bottom of the plate.

***Appendix A.2**. Hardening protocol by exposing biocrust mixed isolate (MI) inoculum to increasing environmental stress.*

HARDENING PROTOCOL

The hardening protocol is comprised by two main processes (acclimation to light and acclimation to dry-wet cycles). The two acclimation processes happen at the same time during the 'hardening period'. Autoclaved distilled water is recommended for the dry-wet cycles.

Total duration time: 14 days.

- Take the produced isolate mixed inoculum (cyanobacterial biomass mixed with native soil) and divided it in many flat containers as needed. Isolated mixed inoculum high per container should not be more than 1 cm. Containers must be transparent.

- First 2 days – culture room: place all the containers at the same culture growing conditions used during the scale up process. Wet the inoculum in the morning and let it to naturally dry over the day. Repeat the same wet-dry process twice during this time period. Wetting cycle should be gently enough to moisture the inoculum, but should no create pools.

- Green house (6 days): place all the containers under greenhouse conditions. Using a shade cloth, block 80% of the incoming sunlight for 48h. After 48h, remove the 80% shade cloth and replace it for a 40% shade cloth. After 48, remove the 40% cloth and let the inoculum expose to 100% of the incoming light from the next 48 h. Every morning, during the same time period (6 days), wet the inoculum and let it to naturally dry over the day. Wetting cycle should be gently enough to moisture the inoculum, but should no create pools.

- Total sunlight conditions (6 days): place all the containers under total sunlight conditions (open roof). Using a shade cloth, block 80% of the incoming sunlight for 48h. After 48h, remove the 80% shade cloth and replace it for a 40% shade cloth. After 48, remove the 40% cloth and let the inoculum expose to 100% of the incoming light from the next 48 h. Every morning, during the same period of time (6 days), wet the inoculum and let it to naturally dry over the day. Wetting cycle should be gently enough to moisture the inoculum, but should no create pools.

At the end of day 14 (make sure the inoculum is totally dry), sieve it (recommend: 0.5 cm), and mix it all together. Pay special attention at the homogenization process.

- Place the plate lid upside-down and using forceps, put an autoclaved filter inside it.

- Take 4mL of the culture from the liquid inoculum supply flask and with the help of a cell spreader ensure a homogenous distribution of the inoculum on the filter.

- Use forceps to transfer the filter from the plate lid to the plate bottom (containing the media). Avoid submerging the filter into the media.

- Close and label the plate.

- Place plates in the culture room.

- Cover plates with a white paper (Kimwipes can be used for this step as well) during the first 24 h.

- After 24 h, uncover the plates and let them grow for 8 to 10 days. Some strains may take longer time. It is important to keep track of the growing time. Plates will turn yellow from one day to another if this time is exceeded.

- After the growing period, remove the plates from the culture room and dry them inside the laminar hood. Open the plates when drying. Keep the lid of the plate inside the laminar hood as well. Drying period ranges are ~ 24 h.

- Store biomass at room temperature in dark conditions.

Appendix A.3. Protocol for straw checkerboard implementation for soil stabilization.

Straw implementation using the shovel method.

Equipment needed:
Straw
String and 4 nails to set up straight lines
Bucket to put straw in
Clippers to cut straw to correct size
Edger (or flat shovel)
Straw check sheet

Implementation:
Set a thin layer of straw along the soil surface (~10-12 inches long). Us the edger (or flat shovel) to push the straw into the slit so it folds upon itself and forms a vertical fence like barrier held in place by the soil.

Appendix A.4. Protocol for stabilizing soil surface with polymers.

Example Polymer delivery protocol
Dilution: 1:9 polymer to water ratio
Fill sprayer designated for polymer with 1 part polymer to 9 parts water to assure that we are getting 1 liter of water on the soil surface and the polymer is not too dilute. **Make sure the water used is filtered through the "pool filter" as this removes chemicals and minerals not wanted in the water**

Application rate:
Add 1 liter of polymer and solution per 1 m2 of soil surface.

Method of application:
Apply polymer solution in a grid like pattern for even distribution and coverage.

Time of application:
Polymer is ideally applied directly before adding the inoculum to also serve as the water treatment. Make sure when inoculating that the polymer has not dried and is still "sticky."

Equipment needed:
Polymer
Pool hose filter (to filter out pollutants and chlorine that may be harmful for biocrust species)
Containers to hold filtered water
Polymer sprayer (use only polymer sprayer as spray can get clogged)
Measuring cup to make polymer mixture.
Polymer addition checklist

Photo credit: A. Faist

Appendix B - List of Scientific/Technical Publications

Appendix B.1 Articles in peer-reviewed journals

1. Bowker, M.A., Antoninka, A.J. 2016. Rapid ex-situ culture of N-fixing soil lichens and biocrusts for the rehabilitation of drylands. *Plant & Soil* 408:415-428.
2. Antoninka, A.J., Bowker, M.A., Chuckran, P., Barger, N.N., Reed, S.C., Belnap, J. 2017. Maximizing establishment and survivorship of field-collected and greenhouse-cultivated biocrusts in a semi-cold desert. *Plant & Soil*.
3. Antoninka, A., M.A. Bowker, S. Reed, K. Doherty. 2016. Production of greenhouse grown biocrust *Restoration Ecology* 24: 324-335.doi:10.1111/rec/12311.
4. Doherty, K., A. Antoninka, M. Bowker S. Velasco, N.C. Johnson. 2015. *Restoration Ecology* 33: 13-16.
5. Velasco Ayuso, S., A. M. Giraldo Silva, C. J. Nelson, N. N. Barger & F. García-Pichel (2017). Microbial nursery production of high-quality biological soil crust biomass for restoration of degraded dryland soils. *Applied and Environmental Microbiology*, 83: e02179-16
6. Chock, T., A.J. Antoninka, A.M. Faist, M.A. Bowker, J. Belnap, N.N. Barger. In press. Soil exopolysaccharides (EPS) response to biological soil crust rehabilitation strategies. Journal of Arid Environments.

In preparation for the special issue on biocrust restoration in Restoration Ecology:

1. Antoninka, M.A. Bowker, J. Belnap, N. Barger. Addressing barriers to improve biocrust colonization and establishment in restoration.
2. Faist A.M., A.J. Antoninka[2], J. Belnap, M.A. Bowker, M. Duniway, F. Garcia-Pichel, A. Giraldo Silva, C. Nelson, S.C. Reed, S. Velasco Ayuso, and N.N. Barger. Using inoculum type and soil stability augmentation methods to return biological soil crust structure and function across desert types.
3. Fick S, N. Barger, M. Duniway, J. Tatarko. Resilience of restored biological soil crust to wind erosion.
4. Giraldo Silva, A., C.Nelson and F. Garcia Pichel. Clingy Cyanobacteria: solid surface availability enhances large scale biomass production of Microcoleus spp.
5. Velasco Ayuso, S., A. Giraldo Silva, Nichole N. Barger, Ferran Garcia-Pichel. Native soil is preferred over common substrate to produce high-quality biocrust biomass for restoration of degraded dryland soils.

Appendix B.2. Technical Reports

None

Appendix B.3. Conference symposium proceedings

None

Appendix B.4. Conference of symposium abstracts

1. Barger, N.N. Plenary talk. Society for Range Management. Restoration Challenges and Discoveries in Ecosystems of the Colorado Plateau. Society for Range Management. January 2017.
2. Barger N.N. Utah State University. Advances in Biological Soil Crust Restoration in North American Drylands. November 2016
3. Barger N.N. Northern Arizona University. Time Is On Our Side: Successes and Unintended Consequences of Ecological Restoration of Western U.S. Drylands. October 2016.
4. Barger N.N. Biocrust Restoration Symposium Introduction. Biocrust 3 International Conference on Biocrusts. Moab, Utah. September 2016. Bowker, M.A., Antoninka, A.J., Durham, R. 2016. Trait diversity and species interactions within biocrusts: Applications in ecological restoration. Ecological Society of America Annual Meeting, Ft. Lauderdale, Florida
5. Belnap. J. Ecological roles and restoration of biocrusts in arid lands. Departamento de Ecología, Facultad de Ciencias Biológicas Pontificia Universidad Católica de Chile. November 2016.
6. Bowker, M.A. 2016. Restoring the living skin of the Earth: greenhouse production of soil mosses to restore soil function in drylands. Restoring Ecosystem Functioning: Innovative Nature-Based Solutions Workshop supported by EDP Produção and Centre for Ecology, Evolution and Environmental Changes (cE3c), Porto, Portugal
7. Bowker, M.A. 2016. Restoring the living skin of the Earth: progress and hurdles in biocrust rehabilitation and prestoration. University of Nevada Las Vegas School of Life Sciences Dept. Seminar, Las Vegas, NV
8. Bowker, M.A. 2016. Vegetation management: what can soil organisms do for us? Southwest Vegetation Management Association, Twin Arrows, AZ
9. Bowker, M.A. 2016. Regrow the living skin of the Earth to save increasingly arid drylands. Ecological Society of America Annual Meeting, Ft. Lauderdale, Florida
10. Faist, A., Antoninka, A.J., Barger, N.N., Belnap, J., Bowker, M.A., Duniway, M.C., Giraldo Silva, A., Garcia Pichel, F., Nelson, C., Reed, S.C., Velasco Ayuso, S. 2016. Advances in biological soil crust rehabilitation in North American drylands. Ecological Society of America Annual Meeting, Ft. Lauderdale, FloridaX
García-Pichel, F., A. M. Giraldo Silva, S. Velasco Ayuso , C. J. Nelson & N. N. Barger (2016). Ecological dermatology: products to restore the soil skin of arid lands to its natural state and beauty . ESA 101st Annual Meeting, Fort Lauderdale, FL, USA, 7-12 August, oral communication
11. Nelson, C.J., A. M. Giraldo Silva, S. Velasco Ayuso, N. N. Barger, F. García-Pichel (2016). Creating the seeds of restoration: two approaches to producing compositionally explicit, location-specific biological soil crusts inoculum. 3rd International Workshop on Biological Soil Crusts, Moab, UT, USA, 26-30 September, oral communication
12. Barger, N.N., A. Faist, S.C. Reed, M. Duniway. Biocrust inoculation and soil stabilization to promote dryland soil restoration. SERDP-ESTCP Symposium. Washington D.C. November 2017
13. Barger, N.N., Faist, A., Antoninka A.J., Giraldo Silva, A., Velasco Ayuso, S., Bowker, M.A., Reed, S.C., Duniway, M., Garcia-Pichel, F., Belnap, J. Biocrust inoculum development and soil stabilization strategies to promote biocrust restoration. Drylands, Deserts, and Desertification Conference. Sde Boker, Israel. November 2017.
14. Bowker, M., Antoninka, A., K. Doherty, R. Durham, C. Tucker, L. Bailey, H. Grover, K. Young, S. Reed. 2018. Restoring biocrusts: a general strategy and an overview of emerging results. Society for Ecological Restoration - Europe. Reykjavik, Iceland.
15. Garcia-Pichel, F. 2018. Ecological dermatology: products to restore desert soil skin to its natural state and beauty. Society for Ecological Restoration - Europe. Reykjavik, Iceland.
16. Barger, N. N., A. Faist, A. Giraldo Silva, A. Antoninka, S. Velasco Ayuso, M. Bowker, S. Reed, M. Duniway, F. Garcia-Pichel, J. Belnap. Biocrust inoculum development and soil stabilization

strategies to promote biocrust restoration. Ecological dermatology: products to restore desert soil skin to its natural state and beauty. Society for Ecological Restoration - Europe. Reykjavik, Iceland.

Appendix B.5. Text Book or Book Chapters

1. Zhao, Y., Bowker, M.A., Zhang, Y., Zaady, E. 2016. Enhanced recovery of biological soil crusts after disturbance. Pages 499-523 In: Weber, B., Büdel, B., Belnap, J. (Eds.) Biological soil crusts: an organizing principle in drylands. Ecological Studies Series. Springer-Verlag, Berlin.