**ECOLOGICAL RESTORATION OF BIOCRUSTS IN ALPINE TUNDRA BIOMES**

by

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# Abstract

Biocrusts are complex communities of bryophytes, algae, fungi, lichens, and cyanobacteria living at the uppermost surface of soils. They have a global distribution and commonly colonize early successional and newly disturbed habitats, where they play important functional roles by facilitating key ecosystem processes. While several studies have examined biocrust development and function in arctic and alpine environments, the potential to use biocrusts in the restoration of disturbed soils in alpine tundra biomes has rarely been examined. In a greenhouse trial, we evaluated the restoration of biocrust through artificial inoculation of soils with mature biocrust. Our results suggest that artificial inoculation with biocrusts increases soil surface nitrogen-fixation rates. In a field study, we characterized alpine biocrust communities from cool mesic and xeric environments and conducted an inoculation experiment to assess the recovery of biocrust structure and function. Together these studies offer a comprehensive description of the functions of biocrusts in alpine environments and provide key information regarding the efficacy of using biocrusts for ecological restoration.

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# 1. Introduction and literature review

## Soil surface disturbance ecology

Anthropogenic disturbances can have lasting effects on alpine plant communities, inducing decreases in plant and microbial diversity, native species abundance, soil organic carbon and nitrogen and mineralization rates [1]. The response of plant and soil communities to disturbance depends on the nature of the disturbance, the environmental conditions (abiotic factors) and the community composition (biotic factors).

Anthropogenic disturbances on alpine communities can be indirect, such as the impact of climate change [2], or direct, such as excavation for pipelines, roads, or mining [3]. Pipeline rights-of-way are a type of direct disturbance that can have profound and lasting impacts on alpine vegetation and soils [4, 5]. Environmental characteristics common to most alpine environments (i.e. short growing season, cold weather and nutrient poor soils) cause these biomes to have slow recovery rates following disturbance [1]. The response of alpine communities to linear disturbances, such as pipelines, will also depend on environmental factors such as climate, elevation, slope, aspect, topography, etc. [6]. Alpine plant and soil microbe communities can be disturbed by physical processes such as compression. Biocrusts are a key component of these alpine communities and those from arid and semi-arid regions are particularly sensitive to disturbances by pipelines [7], with factors such as compressional disturbances of biocrusts diminishing their resistance to erosion, thereby altering their ecosystem function by reducing their C and N inputs [8]. The community composition is also an important driver of its response to disturbance. The cyanobacterium *Microleus vaginatus* was highly susceptible to disturbance on sandy soils in cold-desert environments, whereas the lichen *Collema tenax* was less susceptible, but still exhibited decline in

nitrogenase activity after disturbance [9]. Nonetheless, alpine plant communities are resilient and have the potential to recover following disturbances [1, 10].

## Ecological restoration

Restoration can be defined as the process by which we aim to offset the impacts of contemporary anthropogenic disturbances on natural systems. The overarching goal of ecological restoration is to redirect a system towards its natural trajectory of successional processes, which can result in a resilient and self-sustaining system. Ecosystem restoration that allows for self-sustaining and resilient systems can be informed by naturally occurring diversity and its associated processes [11]. A function-based approach to ecological restoration seeks to facilitate the recovery of disturbed sites by prompting ecosystem processes that would naturally occur. The use of predisturbance community assembly as a guide relies on the premise that native local species are well adapted to local site conditions, however, this assumption is increasingly being challenged under changing climate conditions [12, 13]. For the most part, however, naturally occurring species remain those which are best adapted to restoring natural successional processes and therefore constitute an appropriate choice for restoration. This is echoed by the Society for Ecological Restoration guidelines which state that genetic integrity and regional biodiversity are fundamental in restoration projects [14, 15].

The use of non-native species is a common restoration approach used to facilitate the rapid establishment of ground cover. This is thought to reduce erosion and enhance soil physical properties so that native species can subsequently establish [16]. However, this approach has had limited success in montane environments [17]. As a result, native species are generally preferred for restoration protocols in ecologically sensitive alpine environments, where factors that limit growth and reproduction of native species including soil properties, topography and climate. Hagen

*et al.* [20] postulate that species from alpine environments are particularly sensitive to competition from non-native species and that the improvement of soil conditions may further increase their competitive advantage [18]. Although using native species can maintain the ecological integrity of a site, few studies have investigated the specific challenges associated with alpine restoration.

Various techniques have been used to restore native species in disturbed environments such as rights-of-way. An example is the soil transfer technique, which is commonly used in pipeline rights-of-way construction. It consists in translocating soil and the associated biota (fauna, flora and microbes) from a donor site to a disturbed site [5]. Another technique is hay transfer, the transfer from a donor site to a restoration site of mowed hay stalks with the desired seeds ripe and still attached, which can effectively restore pipeline rights-of-way located in grasslands [15]. In addition to the system's biodiversity, ecological restoration is also dependent on the environmental conditions. Short and cool growing seasons, minimal precipitation, and low soil nutrient levels are environmental conditions typical of alpine environments that pose specific challenges associated with the restoration of alpine sites.

## Spatial heterogeneity and site preparation

Alpine environments which are naturally highly variable provide a variety of niches for diverse plant communities. Topography is a major driver of variability in alpine environments and is an important element of successful ecological restoration which seeks to encompass the full range of a system's biodiversity. Site preparation techniques that create variation in topography and/or microtopography are important drivers of success in restoration. Spatial heterogeneity increases the number of niches in an ecosystem thereby enriching species diversity [19, 20].

Mounds have been widely used as a means to create spatial heterogeneity, hence promoting successful restoration [21]. Mounds create varying moisture conditions, such that the leeward side of the mound will be more consistently humid with less variation in moisture when compared with the windward side. Li et al. (2010) [22] found that biocrusts had higher biomass and cover in the mounds more humid hollows and on the leeward faces. At a smaller scale, microsites are associated with increased moisture and soil stability which are characteristics that foster successful restoration conditions [23]. The availability of microsites can be increased by augmenting the spatial variability of resources [19, 20]. Another technique that can create spatial heterogeneity is ripping which consists in mechanically disturbing the top few centimeters of soil [24]. This technique that loosens the topsoil (and in some instances the subsoil) with minimal soil mixing has been shown to promote establishment, increase water infiltration into the soil and reduce soil erosion [25].

## Biocrust communities

Biocrusts are communities of organisms forming a cohesive thin layer at the uppermost surface of soils. They are composed of algae, lichens, mosses, liverworts, cyanobacteria, and other primary successional species [26]. The accumulation of extracellular polysaccharide sheaths by cyanobacteria, a response to wetting-drying cycles at the soil surface, binds soil particles together, forming soil aggregates that stabilize soil surfaces and allow other species to colonize [27]. Biocrusts readily colonize disturbed soils, as well as, areas not occupied by vascular plant, where they can take advantage of maximum sunlight and precipitations. Although biocrusts are global in distribution, they are commonly found in alpine, boreal, and arctic ecosystems [28, 29].

## Abiotic factors influencing biocrust community assembly and function

Temperature and moisture are abiotic factors that can have a profound impact on biocrust function [30]. In a manipulative experiment, de Guevara et al. [31] demonstrated that soil warming caused a reduction in photosynthesis rates of biocrusts. Biocrusts require moisture to be metabolically active, hence moisture level has major influence on their recovery following disturbance. Biocrust growth can also be limited by nutrients, such as phosphorus, which is often not readily available since it is bound to unweathered minerals [32, 33].

The role of moisture availability in biocrust functioning is magnified by the poikilohydric nature of biocrusts. Nitrogen-fixation rates are strongly correlated with availability of water [34-38]. Several studies on boreal and arctic cyanolichens have found that limitations of moisture are a primary constraint on nitrogen-fixation across all temperatures [39]. In a large-scale study, Raggio et al. 2017 [40] demonstrated that metabolic activity of biocrusts could be predicted from macroclimatic data. The functions of biocrust are undoubtedly regulated by light and temperature although the interactions between these two factors and water at the soil surface are not well studied.

Not only do microclimatic conditions influence biocrust functions but they also play a role in regulating their community assembly. Differences in biocrust species composition are intrinsically linked to different environmental and microclimatic conditions. In a comparison between alpine and Antarctic biocrusts, Colesie et al. 2016 [41] found that compositional difference could be correlated to climatic conditions and the adaptations necessary to thrive in extreme environments. Accordingly, Li et al. 2010 [22]found that increasing soil moisture shifted species composition of biocrust. In addition, increased species richness has been linked to milder climates in North

American montane environments [42]. Together, these studies indicate that biocrust community composition and function are linked with environmental conditions.

Nutrient content is also a major driver of restoration success. Fertilization has been widely used in biocrust restoration efforts and has been found to influence both species composition and functions. Antoninka et al. 2015 [43] found that fertilization was correlated with an increase in nitrogen-fixation under greenhouse conditions, and Maestre et al. 2006a [44] observed a positive relationship between moderate fertilizer addition and nitrogenase activity under laboratory conditions. Elevated levels of nitrogen also have the potential to suppress the production of EPS and the nitrogen fixing activity of biocrusts [45, 46].

## Biocrust functions

Biocrusts are important in the development of ecosystems because they facilitate the shift to later successional seres by participating in several key ecosystem processes including nutrient cycling and soil pedogenesis. Cyanobacteria and cyanolichens in biocrusts influence the nitrogen dynamics of biomes through processes such as mineralization and fixation of organic nitrogen ($R\text{-}NH_2$) and atmospheric dinitrogen ($N_2$), respectively, into ammonium ($NH_4+$). The quantity of nitrogen fixed by biocrusts that is released depends on several factors such as season, light, soil moisture, and temperature and is estimated to be between 5 and 70% (e.g. Belnap et al. 2001 [47]). However, few studies focus on temperate mesic and xeric ecosystems (but see Veluci et al. [48]) where nitrogen inputs from atmospheric deposition and rates of nitrogen released may significantly differ from hot environments. Biocrusts also influence ecosystem hydrology through altering runoff and infiltration of water. The nature of their effect on water movement largely depends on biocrust

micromorphological features, as well as, associated soil characteristics [49]. The microtopography of biocrusts also provides a habitat for soil microbes which in turn affect soil respiration. Through these functions, biocrusts modify soils which affect vascular plants seed dispersal, germination, establishment, survival and nutritional status [26, 44, 50-53]. Given the key roles of biocrusts in ecosystem functioning, their restoration can promote the recovery of disturbed systems [54] by prompting fundamental processes such as carbon and nitrogen cycling.

## Extracellular polysaccharides

Extracellular polysaccharides (EPS) are compounds secreted by cyanobacteria and green algae in biocrusts that form an envelope surrounding the cells that bind soil particles and retain water [55]. Therefore, soils colonized by biocrusts have increased stability and are more resistant to erosion [56-58]. EPS attract and absorb water from their environment, which makes them particularly important in biocrust communities where bryophytes and other poikilohydrous organisms require externally provided water for growth and reproduction [30, 31]. Biocrusts are metabolically active only when hydrated, therefore limitation of moisture availability hinders the growth of these phototrophic communities [32, 59-61].

Biocrusts and the associated EPS can exert a strong influence on water movements. This influence is determined by several factors, such as, soil texture and moisture, rainfall intensity, and biocrust composition [62, 63]. EPS can modify the runoff-infiltration balance thereby increasing infiltration in some zones while reducing it in others [26, 64]. EPS are also known to increase the hydraulic conductivity of soils by increasing the amount of micropores present in biocrusts [62, 63].

EPS play an important role in carbon cycling in environments where soils are naturally carbon poor and EPS may provide the main input source of carbon. By stabilizing the soil, EPS reduce the carbon

loss due to soil erosion [65]. Carbon is also the energy source cyanobacteria use to fix nitrogen [66]. Therefore, carbon fixation is intimately linked to nitrogen fixation, which is another important ecosystem service provided by these organisms [62, 67].

## Nutrient cycling

Nutrient availability is lower in colder systems because low temperatures inhibit both chemical weathering and decomposition processes [68]. Therefore, alpine and arctic environments are generally nutrient poor and often have low soil nitrogen mineralization rates [69]. In addition, nitrogen typically limits primary production during early successional stages [32, 33]. Hence, the nitrogen-fixing organisms that compose biocrusts play a fundamental role in fixing nitrogen, thereby make it available to plants. Additionally, biocrusts may play a significant role in cycling of trace gases such as $CO_2$ [57, 61], as well as, retaining and concentrating nutrients such as sodium, potassium, magnesium, calcium, manganese, iron, nickel, copper and zinc [58, 62].

## Restoration techniques with biocrusts

It has been suggested that recovery of biocrust can be significantly accelerated by active restoration methods such as inoculation or cultivation [70, 71]. Inoculation of soils with biocrusts can speed up restoration processes [72] notably by increasing available nitrogen [51], a nutrient that is known to limit plant productivity in high montane and arctic environments. Inoculation approaches can range from placing discrete biocrust fragments onto the surface to spraying water enriched with microbial organisms. Several studies have investigated the assisted recovery of biocrusts in a plant community restoration context either through inoculation [73], pelletized cyanobacterial amendment or direct transplants. Stewart and Siciliano (2015) [74] found that

biocrust nitrogenase activity could recover on Yukon mine tailings in a growth chamber inoculation experiment. Buttars et al. (1998) [75] demonstrated that inoculation of soils by addition of pelletized cyanobacteria could increase recovery rates of BSC. According to Maestre et al. (2006) [44], inoculation in the form of slurry, as oppose to large discrete fragments, has the potential to foster recovery of BSC communities. Chiquoine et al. (2016) [76] found that inoculation with salvaged biocrusts dried for 2 years could hasten the biocrust recovery of disturbed drylands roads. Cultured cyanobacteria have been successfully applied to the field in Chinese deserts [77] and fully functional greenhouse-grown biocrust mosses and associated cyanobacteria have been produced by Antoninka et al. 2016 [78] but survival in the field has not yet been tested.

## Summary

Given the importance of biocrust establishment in restoration of soil surface properties (e.g. soil stabilization, nitrogen and carbon enrichment) following disturbance, the artificial enhancement of biocrust establishment on disturbed sites has been the subject of a number of studies, using techniques such as cultivation and/or inoculation [26, 70, 71, 79]. While previous studies have examined the efficacy of inoculation for establishing biocrust in grassland and desert sites [44, 73, 80], to our knowledge none have investigated the effects of assisted inoculation on alpine biocrust establishment and function.

Our main objectives were 1) to compare biocrust community composition and ecological function between inoculated and bare soil surfaces; 2) to evaluate the influence of microtopographic features (Flat, Microrills, Pit and mound) on the development of biocrusts in the inoculated soils. ; 3) to compare the composition and nitrogen fixation potential of natural biocrust associated with contrasting coastal and continental mountain ranges; 4) to assess the restoration potential of alpine biocrust communities; and to 5) to evaluate the influence of biocrust inoculation treatments

on nitrogen fixation, extracellular polysaccharide content, soil mineralizable nitrogen and dissolved organic carbon, as key indicators of soil ecological function.

# 2. Inoculation promotes microbial community reassembly and restoration of ecosystem function in alpine biocrusts – a microcosm experiment

## Introduction

Biocrusts are soil surface assemblages comprised of diverse organisms such as algae, lichens, mosses, liverworts, Cyanobacteria, as well as other primary successional bacterial, fungal [26, 81] and archaeal species [82]. Biocrusts are highly heterogeneous, capable of thriving in a variety of environments [83] and are commonly found in alpine ecosystems [29, 83] where they colonize exposed soil surfaces, taking advantage of the conditions of high insolation exposure and greater precipitation availability. Established biocrusts form a cohesive thin layer at the uppermost surface of soils, where production of extracellular polysaccharides (EPS) augments soil organic matter content, binds soil particles together [66], and generally exerts a strong influence on surface hydrological processes [84]. These soil surface communities can also influence soil nitrogen availability through their ability to fix atmospheric nitrogen [85]. Taken together these processes have a major influence on soil carbon and nitrogen cycling [54, 86, 87].

The harsh environmental conditions found in most alpine environments, including nutrient poor soils and short-cool growing seasons, can result in slow recovery rates after disturbance [68].

Anthropogenic disturbances, in particular, can have significant impacts on alpine vegetation and soils [3-5, 68], resulting in accelerated soil loss through erosion, decreased microbial diversity and abundance, and the alteration of soil carbon and nitrogen cycling, including mineralization [1, 75, 83].

Although biocrusts are sensitive to disturbances which can induce changes in their community composition and function [1, 80, 88, 89], they are also well adapted to harsh alpine environments [90]. The restoration of biocrust communities can therefore be a significant factor in the restoration of disturbed systems [54], promoting recovery of fundamental ecosystem processes such as nitrogen and carbon cycling [91-93]. It has been suggested that recovery of biocrusts can be accelerated by the adoption of active restoration methods such as inoculation or cultivation [26, 70, 71, 79]. The effects of inoculation on recovery of ecosystem function are poorly described, especially for alpine environments. Although previous studies have focused on separate aspects of assisted recovery [44, 73, 75], ecological function [29, 94], and community composition [83] of alpine biocrusts, few have investigated the combined effects of assisted inoculation on biocrust composition and function. Using a microcosm experiment we investigated the development of alpine biocrust communities on bare alpine soils over a 12-week period after inoculation with biocrust inoculant prepared from prior collections made in alpine environments. We also examined the influence of soil surface microtopography, which has previously been found to be an important factor in promoting heterogeneity and associated niche diversity of soil surface biocrusts (26). Our main objectives were 1) to compare biocrust community composition and ecological function between inoculated and bare soil surfaces; and 2) to evaluate the influence of microtopographic features (Flat, Microrills, Pit and mound) on the development of biocrusts in the inoculated soils.

Biocrust establishment after inoculation was assessed both directly, using soil surface cover measurements, and assessments of biocrust composition from bacterial 16S rRNA gene and fungal ITS2 amplicon sequencing, and indirectly, assessing ecological function from measurements of nitrogen fixation, EPS content, chlorophyll fluorescence, and *nif*H gene abundance. Contextual data or "controls" for the experiment were provided by identical assessments of ecological function in the initial inoculant, and in soils exposed to the same environmental conditions as treatments but with no biocrust inoculation, and soils that were collected then stored.

## Methods

### Site description

Samples were collected from two adjacent sites in the Coast Mountain Range of northern British Columbia, Canada; Trapper Mountain (N 54° 30.683', W 128° 27.317', 1187 m elevation) and Andesite peak (N 54° 13.868', W 128° 01.499', 1640 m elevation). Trapper Mountain occurs in the transition zone between subalpine forest and alpine tundra, with the vegetation community dominated by scattered clumps of *Abies lasiocarpa* (Hooker) Nuttall interspersed with *Cassiope mertensiana* heath. The site was characterized by Podzolic soils with acidic pH. Vegetation communities on Andesite peak were dominated by alpine tundra interspersed with rocky outcrops and late-melt snow beds in depressions. Vegetation on dry ridge top habitats on Andesite peak was dominated by *Cassiope mertensiana* and *Stereocaulon alpinum* heath, with *Saxifraga tolmiei* and *Ranunculus cooleyae* dominated vegetation communities common in wetter mid-slope habitats.

Andesite peak had Podzolic and Regosolic soils, with substrates of volcanic origin (meta-basalts and meta-andesites).

## Sample collection

Biocrust samples were collected on August 16 and 17, 2014. Collections were taken from sites with an intermediate soil moisture status, avoiding wet late-snowmelt depressions and xeric ridge-top habitats. Biocrusts were stored in ventilated bins held between 1 and 4°C during shipment (48 hours). Samples were then frozen at -20°C until the start of the experimental period on December 5, 2014 (17 weeks).

The impact of a prior period of frozen storage was assessed in a pilot trial using biocrusts from Trapper Mountain and Andesite Peak, where we compared acetylene reduction activity of freshly sampled biocrusts with that of previously frozen biocrusts. We found that after a six-day thawing period (moistened to field capacity daily and 16 hours photoperiod at 150 µmol/$m^2$/s during light hrs.; 20$^o$C daytime, 10$^o$C nighttime) rates of acetylene reduction were not significantly different between fresh and previously frozen biocrusts (Welch's t-test, t=0.921, p=0.4). Samples were thawed, under the same environmental conditions, for a six-day period immediately before the start of experiments.

Soils used in the experiments were collected from the Trapper Mountain site on August 16, 2014. Soil samples were collected at depths of 2 to 10 cm, after removing surface biocrusts and other vegetation.

## Microcosms

Biocrust development was examined under four soil surface microtopography treatments: i) Flat: soil with flat slightly compacted surface, ii) Microrills: soil with repeating microridges across the surface (gullies separated by ridges 1 cm in height and width- simulating raking), iii) Pit and mound: a single hummock-hollow complex 15 cm in height, iv) No top soil: coarse gravel without soil to emulate an absence of top soil application (Plate 3). Nine replicates of each treatment were established, each consisted of a shallow plastic tray (0.076 $m^2$ surface area) filled with 3 cm of crushed gravel topped with 2 cm of soil prior to establishing microtopography treatments. A control set of untreated replicates (referred to as "Uninoculated" in amplicon sequencing results) (n=9) did not receive any biocrust inoculant. In addition, excess soil used to make the microcosm treatments was stored (referred to as "Stored" in amplicon sequencing results) at -20 °C and used to determine the community composition of the soil prior to treatment.

Following soil surface treatments, each tray was wetted to field capacity and inoculated with biocrust at 10% surface area (i.e. 0.0076 $m^2$). The inoculant was obtained by homogenizing a combined mixture of mature biocrust fragments from our two collection sites through a 4mm sieve. After inoculation, each tray was watered with 300 mL DI water to ensure contact between the biocrust fragments and the underlying substrate. Trays were subsequently watered 3 times a week with 150 mL of DI water.

Microcosms were maintained under greenhouse conditions with a 16 hours photoperiod (150 µmol $m^{-2}$ $s^{-1}$ PAR and 20 °C daytime and 0 µmol $m^{-2}$ $s^{-1}$ PAR and 10 °C nighttime conditions). Illumination was provided by 168X-Pro Extreme LED Grow Lights (Hydrogrow, Sunrise, FL, USA). To maintain

relative humidity at approximately 70%, 4 large clear plastic tents were constructed, each overlying 11 or 12 trays. Under each tent, the trays were moved three times weekly to avoid location effect and ensure moisture and light levels were uniform between microcosms. Microcosms were maintained under experimental conditions for 12 weeks.

Two 14.5 $cm^2$ biocrust samples were removed from each tray at 6 and 12 weeks after the start of the experiment. The location of sampling within each tray was determined by overlaying a random numbers grid and randomly choosing four different grid numbers for sampling (the first two at week 6 and the others at week 12). For Pit and mound replicates, at each sampling period, three sets of two samples were taken across the microtopographical gradient from mound, mid-slope to pit. One soil sample was removed from each tray at week 12, after removing the top 0.5 cm including any biocrusts present. No soil samples were removed from the trays with the No top soil treatment.

## Bacterial 16S rRNA and fungal ITS2 sequencing

We randomly chose samples from each Soil surface treatment, the Uninoculated control and the Stored soil to be used for bacterial 16S rRNA gene (three replicates selected per Soil surface treatment and Uninoculated control but two replicates for Stored soil) and fungal ITS2 (four replicates selected per Soil surface treatment and Uninoculated control but three replicates for Stored soil) amplicon sequencing.

Primer sequences used for targeting the V4 region of the bacterial 16S gene were the 515F/806R sequences as described in [95]. 16S amplicons generated in this study were 450 bp in length (including adapter and index sequences). Primer sequences used for targeting the fungal ITS2 regions were the ITS86F/ITS4 sequences as described in [95] . The ITS86F/ITS4 primer pair (including adapter and index sequences) generated amplicons that were predominantly were 550 bp in length. Amplicons ranging in size from 500-600 bp were extracted from agarose gels for purification.

Prior to PCR amplification 10 ng of template DNA from each sample was pre-incubated with 0.5 µg BSA (New England Biolabs) for 10 minutes at 95 °C prior to the addition of master mix containing primers, 5 PRIME Hot Master mix (2.5 X stock) and nuclease free water (IDT). The final concentration of PCR components in a 25 µl reaction volume (including template and BSA) were 1X 5 PRIME Hot Master Mix and primers at 300 nM (bacterial 16S) or 200 nM (fungal ITS2).

The thermal cycle profile used for bacterial 16S amplification had an initial denaturation step at 94 °C for 3 minutes, followed by 30 cycles of 94 °C for 45 seconds (denaturation), 50 °C for 60 seconds (annealing) and of 72 °C for 90 seconds. A final extension at of 72 °C for 10 minutes ended the thermal cycle profile. The thermal cycle profile used for fungal ITS2 amplification had an initial denaturation step at 94 °C for 3 minutes, followed by 30 cycles of 94 °C for 45 seconds (denaturation), 55 °C for 45 seconds (annealing) and of 72 °C for 2 minutes. A final extension at of 72 °C for 7 minutes ended the thermal cycle profile. For each sample triplicate 25 µL PCR's were pooled and the amplicons were extracted from 1% agarose, 0.5X TBE gels after electrophoresis

using the GeneJET Gel Extraction and DNA Cleanup Micro Kit (ThermoFisher Scientific) and eluted in 20 µL of elution buffer.

DNA concentrations of the purified amplicons were determined with a Qubit Fluorometric assay (ThermoFisher Scientific) using the dsDNA BR assay kit. DNA concentrations were manually adjusted to 2 ng/ml and pooled and submitted for sequencing to the Génome Québec Innovation Centre's Massively Parallel Sequencing Services unit (McGill University, Montreal, QC, Canada). The amplicon pools were sequenced using a 250 nt paired-end run for bacterial 16S and a 300 nt paired-end run for fungal ITS2 on an Illumina MiSeq system.

## Biocrust establishment

Percent biocrust cover was visually estimated for each tray at weeks 6 and 12. All assessments were conducted by the same observer using a reference grid outlining 1% and 10% surface area increments.

## Maximum photochemical quantum efficiency

For every sample, taken at the height of day (between 10:00 and 14:00) at weeks 6 and 12, we determined the maximum photochemical quantum efficiency ($F_v/F_m$) by dark-adapted chlorophyll fluorescence using a pulse modulated fluorimeter ([96]; FMS2, Hansatech Instruments, Norfolk, UK). Samples were dark-adapted, using leaf clips, for a minimum of 10 minutes prior to fluorescence measurement.

## Nitrogenase activity

Nitrogenase activity was assessed using acetylene reduction assays (ARAs) [97] . Following chlorophyll fluorescence measurements, biocrust samples were placed in a 250 ml glass canning jar with a serum stopper lid. Samples were misted with 1 mL DI water prior to closure of jars and injection of 10% acetylene by volume. Jars were incubated 4 h at 150 µmol $m^{-2}$ $s^{-1}$ PAR and 20$^{o}$C. After incubations a 4-mL gas sample was removed from each incubation jar.

Each set of samples were processed along with two control samples: i) a biocrust sample not injected with acetylene, which served both as a temperature control and to ensure that no natural evolution of ethylene was occurring and ii) a jar with no biocrust that was injected with acetylene to ensure the absence of ethylene contamination in the acetylene.

Gas samples were injected into a gas chromatograph (SRI 8610A, Wennick Scientific Corporation, Ottawa, ON, Canada) fitted with a Porapak column (Alltech Canada, Guelph, ON, Canada) and a flame ionization detector for detection of ethylene. Hydrogen, used as the carrier gas, was held at a constant pressure of 32 psi while column temperature was held at 65$^{o}$C. The detection limits for acetylene reduction were equivalent to 3.7 µmol of ethylene $hr^{-1}$ $m^{-2}$.

After ARA assessments, biocrust samples were stored at -20$^{o}$C for subsequent analysis; samples from each tray were pooled and half was used for sequencing of bacterial 16S rRNA and fungal ITS2, and qPCR of *nif*H gene the other half to measure EPS content.

## nifH gene copy number

From each sample set aside for qPCR (one per replicate), we used a 0.25 g sub-sample to perform DNA extractions which were carried out using PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions. Extracted DNA was stored at -20°C. Primers used for quantification of *nif*H abundance were NifHF 5'-AAA GG**Y** GG**W** ATC GG**Y** AA**R** TCC ACC AC-3' and NifHR 5'-TTG TT**S** GC**S** GC**R** TAC AT**S** GCC ATC AT-3' from [98]. The standard used for the quantification of *nif*H was a synthetic double-stranded gene fragment generated at IDT Inc (Coralville, IA, USA) consisting of nucleotides 192-649 from the *Rhizobium meliloti nifH* gene sequence[1], *nif*H gene copies were quantified against a standard calibration curve obtained by serial dilution ranging from $10^2$ to $10^8$ *nif*H gene copies. Each 12.5 µL qPCR reaction was carried out in triplicate (samples, standards and no template control) and contained 6.25 µL Power Syber Green Mix (Applied Biosystems, Carlsbad, CA, USA), 0.25 µL *nif*HF primer (0.2µM) and 0.25µL *nif*HR primer (0.2 µM) (Life Technologies, Carlsbad, CA, USA) (sequences as per [98]), as well as 3.75µL of DI water and 2 µL of standards, nuclease free water (no template control) or DNA extract from samples (diluted 1 µL to 100 µL). Following the *nif*H qPCR method from [99], a 7300 Real Time PCR machine (Applied Biosystems, Germany) was used under the following thermal cycling conditions: hot start (95°C for 10 min.); amplification (95°C for 45s, 55°C for 45s, and 72°C for 45s) for 40 reps; dissociation (95°C for 15s, 60°C for 30s, and 95°C for 15s).

## Extracellular polysaccharide content

EPS content was determined using the phenol-sulfuric acid method [100, 101]. Each reaction was transferred to a well in a 96-well flat bottom microplate, with standards and template control

[1] NCBI Nucleotide database accession. version V01215.1.

carried out in triplicate. EPS content was measured as glucose concentration quantified at 480, 486 and 490 nm using a microplate reader (Varioskan, Thermo Fisher Scientific, Walthman, MA).

## Soil properties

Dissolved organic carbon and mineralizable nitrogen were assayed on soil samples collected at week 12 (n=36), and on soils sampled prior to biocrust inoculation (n=2).

Dissolved organic carbon was measured from a soil extract (1:10 soil to water ratio shaken for 1 h on a reciprocating shaker) filtered to 0.45 µm, with a total organic carbon analyzer (Formacs HT, Skalar Analytical B.V., Breda, Netherlands). pH at 12 weeks was measured by a 1:5 biocrust to DI water suspension with a hand-held pH meter (Oakton Instruments pH610, Vernon Hills, IL, USA).

Mineralizable nitrogen was measured by the anaerobic incubation method [102]. Soil samples were incubated under water-logged conditions for two weeks at 30°C. Ammonium produced was subsequently measured from a soil extract (1:10 soil to 1N KCl ratio shaken for 2 h on a reciprocating shaker) with a discrete analyzer using the phenate method for colorimetric determinations ([103]; SmartChem 200, Unity Scientific, Brookfield, CT).

## Data analysis

The amplicon sequencing data analyses used to generate inventories of 97% operational taxonomic units (OTU) was performed by the Bioinformatics unit at Génome Québec. For multivariate analysis of fungal community composition, we sub-sampled the fungal ITS2 OTU inventory to include only

clusters with greater than 250 sequences detected across all samples that were taxonomically classified to Class level. We also excluded four fungal OTUs classified as genus *Vrystaatia* that had conflicting sequence identifications when searched against the NCBI nr database using BLASTn. A total of 75 OTUs representing ~75% of the quality-filtered, clustered sequences were retained for community comparisons by Two-way Cluster Analysis using PC-ORD. For comparisons of bacterial community composition, we focused on exploring restoration treatments in re-establishing dominant biocrust- and soil-associated taxa by selecting 62 highly abundant OTUs from an initial inventory of 5,099 bacterial OTUs, included only if a minimum of 2,000 sequences total was detected within the specific sample groups (i.e. Inoculant, Stored soil, Uninoculated soil, Flat, Pit and mound, and Microrills treatments).

Two-way Cluster Analysis was performed in PC-ORD version 6 (MjM Software Design, Gleneden Beach, OR, USA) using a Sorensen distance and the flexible beta (-0.250) linkage method. The analysis with fungal and bacterial data was performed with an OTU table containing sequence counts averaged and relativized to sequence total (for only the OTUs included in analysis) per sample group.

Linear mixed models (LMM) were used to avoid pseudoreplication in analysis of the dataset created by having samples collected from the same trays at weeks 6 and 12. LMM included tray number as a random effect, week and measurement as fixed effects, and the interaction of week and measurement as fixed effects. To meet assumptions of normality and homoscedasticity, biocrust percent cover and acetylene reduction were log-transformed and EPS content Boxcox-

transformed[2]. Biocrust percent cover, acetylene reduction, and EPS content data were analysed using R package *lme4* [105] to generate LMM fitted by restricted maximum likelihood (REML) and the R package *lmerTest* to calculate degrees of freedom through the Satterthwait approximation [106]. As a post-hoc test, we used Tukey Contrasts from the R package *multcomp* [107].

Chlorophyll fluorescence data was analyzed using Kruskal-Wallis test and pairwise comparisons using Tukey and Kramer (Nemenyi) test with Tukey-Distance approximation for independent samples. *nif*H gene copy number and soil properties were Boxcox-transformed to meet assumptions of normality and homoscedasticity of the subsequent analysis using ANOVA and Tukey HSD post-hoc test. All statistical analyses were conducted in R (R Core Team, 2014).

## Results

### Bacterial 16S rRNA and fungal ITS2 amplicon sequencing

Two-way Cluster Analysis distinguished two main clusters (Fig. 1) of highly abundant bacterial OTUs (i.e. those with > 2,000 sequences detected in each sample group) where Cluster A consists of OTUs detected primarily in Inoculant, Flat, and Microrills sample groups. Cluster B consists of OTUs most consistently and abundantly detected in the soil surface treatments (Flat, Pit and mound, Microrills), Uninoculated soil, and the Stored soil sample groups (Fig. 1). Subcluster A1 contains OTUs that were abundant in Inoculant yet weakly established in the soil surface treatments. This subcluster included several Acidobacterial OTUs (in families Acidobacteriaceae and genus

[2] A power transformation such that $\times' = \frac{x^{\lambda} - 1}{\lambda}$, see 104. Box GE, Cox DR (1964) An analysis of transformations. Journal of the Royal Statistical Society Series B (Methodological): 211-252.

*Acidobacterium)* OTUs, one OTU assigned to the Chloroflexi family Ktednonbacteracae and one OTU assigned to the Alphaproteobacterial order Burkholderiales. OTUs in Subcluster A2 were most abundant in the Flat and Microrills treatments, least abundant in Uninoculated and Stored soils, and detectable with varying abundances in the Inoculant samples. Subcluster A2 is notable for the association of several Bacteroidetes-associated OTUs (numbers 36, 38, and 39, Fig. 1) that appear maximally established in the Microrills treatment. Subcluster B1 consists primarily of OTUs with highest abundances in Uninoculated and/or Stored soils (Fig. 1), which are also consistently detected in the soil surface treatments but not the Inoculant sample group. Within this subcluster are 22 Acidobacterial OTUs that exhibit strong soil associations but also three OTUs from candidate division WPS-2 that were maximally abundant in the Flat sample group. The Flat, Pit and mound, and Microrills treatments appear mostly comparable for relative abundance and consistent presence of subcluster B1 OTUs, which largely explained the clustering together of soil surface treatments in Fig. 1. The subcluster B2 contains OTUs with highest average relative abundance Inoculant sample group, and lower abundances in the Uninoculated, Stored, and soil surface treatments sample groups. This subcluster contains two Acidobacterial OTUs (numbers 11, and 12), two Chloroflexi-assigned OTUs (41 and 42), and three OTUs assigned to Gammaproteobacterial family Sinobacteraceae, all of which are exhibit the highest average relative abundance in the Inoculant sample group. The Flat and Microrills treatments exhibit subcluster B2 OTUs at greater abundance compared to the Pit and mound treatment. The subcluster B3 consists of two Alphaproteobacterial OTUs with maximal abundance of detected sequences in the Uninoculated soils sample group. Overall, Acidobacteria and Proteobacteria had the highest relative abundance across all treatments, the inoculant and the untreated control soil (Fig. 2). Although the relative abundance of Cyanobacteria was generally low across our microcosm samples, Cyanobacteria

relative abundance was highest in the inoculant (5%) and represented only 1% in the untreated control soils and 2-3% in the soil surface treatments at week 12 (Fig. 2).

Two-way Cluster Analysis distinguished three main fungal OTU clusters: Cluster A comprises isolates that are most abundant in the Inoculant, Cluster B most abundant in the soil surface treatments (Flat, Pit and mound, Microrills), and Cluster C most abundant in soils (Uninoculated and Stored) (Fig. 3). Subcluster A1 comprises OTUs that were abundant in Inoculant but that failed to establish in the soil surface treatments. This subcluster included several Zygomycota (*Mortierella)* OTUs, Ascomycota (OTUs with affinities to Pleosporales and Lecanoromycetes), Agaricomycetes (Polyporales, *Galerina*, *Psilocybe*, *Serendipita*) and the basidiomycetous yeast *Leucosporidium* (Fig. 3). Subclusters A2 and A3 were most abundant in the Inoculant but could also be found in treatments (A2) or soils (A3) (Fig.3). These clusters were dominated by *Serendipita* that were able to colonize the crust treatments but were not present in the soils, as well as several OTUs that were widely distributed in soil surface treatments samples, including yeast forms (Saccharomycetales, *Cryptococcus*), the chytrid *Pateramyces*, and several *Mortierella* (Fig. 3). Cluster B contained subclusters with OTUs that had a highest abundance in the Flat (B1), Pit and mound (B2) or the Microrills treatments (B3) (Fig.3). This cluster was dominated by *Serendipita* (18 of the 24 *Serendipita* OTUs occurred in Cluster B) the majority of which (16 OTUs) appeared to originate from the Inoculant and only 2 from soil. Other OTUS included *Mortierella* and *Eocronartium* (Fig.3). Cluster C showed highest OTU abundance in the Uninoculated soils (subcluster C1) or the Stored soil (subcluster C3), with subcluster C2 showing relatively high abundance in both Uninoculated and Stored soil (Fig. 3). This cluster was dominated by ectomycorrhizal (ECM) OTUs, including *Cortinarius*, *Inocybe*, *Sebacina*, *Laccaria*, and *Piloderma* (Fig.3). Overall, the inoculant also had the highest relative abundance of Lecanoromycetes in comparison to any other treatment (Fig. 4).

## Biocrust establishment

Except for the No top soil treatment, biocrust established on all soil surface treatments (Flat, Microrills, Pit and mound) where it was applied as an inoculant. Percent cover of biocrust was significantly higher in the Flat, Microrills, and Pit and mound treatments than in the Uninoculated soils and No top soil treatments at both week 6 and week 12 (Fig. 5a). Both treatment and duration of incubation (week) had a significant effect on percent cover ($F=366.25$, $p<0.01$ and $F=46.46$, $p<0.01$, respectively; Table 1). At week 6, there was no significant difference in biocrust cover between the soil surface treatments (Flat, Microrills, Pit and mound), however at week 12 the Flat and Microrills treatments had a significantly higher biocrust cover than the Pit and mound treatment (Tukey Contrasts, $p<0.01$). From week 6 to week 12, the Microrills and Flat biocrust cover increased from 37% ±1.7 to 52% ±1.6 and from 39% ± 3.2 to 52% ±4.3, respectively. Natural colonization of Uninoculated soils resulted in a 0.6% ±0.3 biocrust cover at week 12.

## Maximum photochemical quantum efficiency

The majority of soil surface treatment (Microrills, Flat, Pit and mound) $F_v/F_m$ values increased from undetectable (i.e. 0) at week 6 to above 0.8 at week 12. Most of $F_v/F_m$ values for the No top soil treatment and Uninoculated soils were undetectable at week 12 (both 89%). The $F_v/F_m$ values observed in soil surface treatments at week 12 were significantly higher than the No top soil and Uninoculated soils (Kruskal-Wallis, and pairwise comparisons using Tukey and Kramer (Nemenyi) post-hoc test (Chi-squared = 38.7, $p< 0.02$; Fig. 5b).

## Nitrogenase activity and nifH gene copy number

The highest rates of nitrogenase activity (NA) were found in the inoculant prior to application (17 ± 3.7 mean µmol of ethylene $hr^{-1}$ $m^{-2}$ ± SE) and the lowest rate in the Uninoculated soils (3.5±0.4). We observed recovery of NA where biocrust inoculant was applied with the exception of the No top soil treatment (Microrills 11.4±2.2, Pit and mound 10.8±2.33, Flat 9.1±1.9, No top soil 4.9±0.4 mean µmol of ethylene $hr^{-1}$ $m^{-2}$ ± SE) at week 12 (Fig. 5c). Although the linear mixed model fit by REML did not detect significant differences in NA due to duration of incubation (week), NA increased from week 6 to week 12 in soil surface treatments (Microrills, Flat, Pit and mound), whereas it declined in the No top soil and stayed the same in the Uninoculated soils (Table 1).

The general patterns of *nif*H gene copy number were reflective of trends in NA. The *nif*H gene copy number was highest in the inoculant ($7.5X10^6 \pm 2.3X10^6$copies/g of soil), lowest in Uninoculated soils ($6.5X10^5 \pm 1.0X10^5$copies/g of soil) and found at intermediate levels in the soil surface treatments (Flat $2.6\ X10^6 \pm 1.4X10^6$, Pit and mound $2.0\ X10^6 \pm 4.6X10^5$, Microrills $1.3\ X10^6 \pm 4.9X10^5$copies/g of soil) at week 12 (Fig. 6). The inoculant had a significantly higher *nif*H abundance than any of the soil surface treatments or Uninoculated soils (ANOVA, Tukey posthoc, $F=7.91$, all $p<0.04$; Fig. 6). There was no significant difference in *nif*H copy numbers between soil surface treatments (ANOVA, $F=0.912$, $p=0.45$).

## Extracellular polysaccharide content

Between week 6 and week 12 EPS content increased markedly in the Microrills soil surface treatment (16± 5.2 to 25±4.9 µg of glucose per g of biocrust) and Uninoculated soils (4.4±2.3 to 15±2.5 µg of glucose per g of biocrust) (Fig. 5d). A linear mixed model fit by REML showed that

week had a significant effect on EPS content, which was clearly driven by changes in the Microrills and Uninoculated soils ($F=9.98$, $p<0.01$; Table 1). Overall, we did not detect significant treatment effects on EPS content ($F=1.63$), however, EPS content in Microrills at week 12 (25 µg of glucose per g of biocrust) was higher than any other treatment.

### Soil Properties

Soil samples taken immediately below the surface biocrust were not significantly different across our microcosm treatments in both dissolved organic carbon and pH following 12 weeks of incubation (ANOVA, $F=0.817$, $p=0.5$ and $F=0.409$, $p=0.676$, respectively). Dissolved organic carbon ranged from an average of 118 ppm to 162 ppm and average pH ranged from 4.6± 0.04 to 4.8± 0.04 with both the highest DOC and pH detected in soils from the Microrills surface treatment. Mineralizable nitrogen was significantly higher in soils from the Pit and mound (12.1 ppm ±0.7) compared with both the Flat surface treatment (10.9 ppm ±0.5) and Uninoculated soils (10.8 ppm ±0.5; ANOVA, $F=4.36$, $p=0.01$; Table 2). Mineralizable nitrogen was also high in Microrills treatment (11.7ppm ±0.4) although it wasn't significantly different from any treatment.

## Discussion

### Fungal and bacterial communities of inoculated surfaces differ from uninoculated surfaces and from inoculant

The Inoculant was characterized by several OTUs that were annotated as fungi typically associated with bryophytes. The *Psilocybe* OTU was closest (99% identity) to *P. montana* according to a GenBank search; *P. montana* grows among mosses on alpine sites in North America. *Galerina* is also typically moss-associated [108]. These species were presumably associated with mosses of the

intact soil crusts and were not able to establish in the crust regeneration treatments. Two OTUs were annotated as *Eocronartium* and compared most closely to a GenBank isolate JX852332.1 that was isolated from bryophytes in Antarctica [109]. *Eocronatium* OTUs were common in the crust regeneration treatments and may have been associated with bryophytes within the developing crusts.

OTUs that were annotated as saprotrophic fungi occurred in all samples and treatments. Important saprotrophic OTUs included *Mortierella*, a fast-growing member of the Zygomycota that is often isolated from environmental samples, and basidiomycetous yeasts such as *Cryptococcus, and Leucosporidiella*, a member of the Leucosporidiales, an order of psychrophilic basidiomycetous yeasts commonly found in polar and alpine habitats [110]. The ability to produce mycosporines, UV-absorbing compounds, is found in a wide variety of basidiomyceteous yeasts [111]. The presence of these yeasts in biocrusts from alpine environments, which have high UV radiation exposure, is therefore not surprising. Their presence in the inoculant and the inoculated surfaces may indicate that inoculation promoted functional recovery of mycosporine-producing yeasts. Several abundant OTUs were annotated as Dothideomycetes with affinities to the Pleosporales. Several studies have noted Pleosporales to be dominant members of biological soil crusts [112-114] and Collins et al. [115] suggested that root endophytes in the Pleosporales may be involved in nutrient transfer between crusts and associated plants. Our OTUs likely represent these common crust inhabitants. Most saprotrophic OTUs showed little specificity for inoculant, treatments or soil.

Soils (Uninoculated and Stored) were dominated by typical soil-inhabiting ECM OTUs, particularly *Cortinarius*, *Inocybe*, *Laccaria*, and *Piloderma*. In nearly all cases these showed their highest

abundance in the Stored soils, with reduced abundance in the Uninoculated soils and very low abundance in the soil surface treatments, although *Laccaria* and *Piloderma* were detected in low frequency in other treatments. We expect that these fungi were associated with plant roots in the original soil and likely persisted on the dying root tissue in the soil used to construct the microcosms. There is little evidence that they were actively establishing in the soil surface treatments however, due to their declining abundance.

A novel finding of this study was the high proportion of *Serendipita* (Basidiomycota: Sebacinales) OTUs in the alpine crust and soil surface treatments. *Serendipita* belongs to the hyper-diverse and complex family Serendipitaceae, sister family to the equally diverse Sebacinaceae. While Sebacinaceae are predominantly ectomycorrhizal and early-diverging saprotrophic fungi, Serendipitaceae are mostly plant endophytes with derived ericoid, orchid and ectomycorrhizal lineages [116]. To more closely ascertain the phylogenetic affiliations of our *Serendipita* OTUs we generated a maximum-likelihood tree of our OTUs and representative *Serendipita* sequences chosen from the major clades (c-h) in Weiß et al. [116]. All of our OTUs were interspersed with members of subtrees c and g in their tree. Subtree c contains endophytic and ectomycorrhizal taxa and subtree g liverwort-associated taxa [116]. Although we cannot unambiguously determine the function of our *Serendipita* OTUs, the lack of ectomycorrhizal hosts in the Inoculant and restoration surface treatments suggests that they were likely associated with bryophytes or they were endophytes of vascular plants establishing in the soil crusts treatments.

Coleine et al. [83] reported that *Pezoloma ericae*, which forms root associations with plants in the Ericales and with liverworts, was a dominant member of alpine biological soil crusts in northern

Sweden and suggested that its activity as a root symbiont may support the “fungal loop” hypothesis of Collins et al. [115] which proposes that root-associated fungi, including endophytic species, may mediate nutrient exchange between soil crusts and vegetation in arid landscapes. It is not inconceivable that the root-associated *Serendipita* documented in this study could play such a role in alpine biological soil crusts. In particular, the potential of root-associated *Serendipita* to transfer nitrogen fixed by Cyanobacteria in the crusts to associated plants and liverworts deserves investigation.

Lecanoromycetes, the main class of lichenized fungi [117], declined in abundance in all treatments, compared to the initial inoculant, suggesting that this group might be particularly sensitive to treatment effects or to the modification of environmental conditions. The symbiosis in lichenized fungi is known to be quite sensitive to changes in environmental conditions [118]. Lichen thalli are intolerant of prolonged exposure to conditions of saturating water content, which causes the symbiosis to break down [119, 120]. The moist soils that were maintained under greenhouse conditions may therefore have favoured growth of free-living Cyanobacteria, instead of lichenized Cyanobacteria. Under field conditions, lichenized fungi may establish more readily due to naturally fluctuating environmental conditions. This highlights the importance of water content fluctuation in biocrust inoculation experiments in order to preserve this important functional group which is a well-documented component of biocrusts (e.g. [81]). However, lichens are a later successional species in biocrust [121], and the symbiosis may need a longer period to re-establish. Previous reports suggest that lichen reestablishment in biocrusts can take several decades after disturbance [88]. Lichen components are usually associated with thicker biocrusts and Wang et al. [122] found a time after disturbance to biocrust thickness relationship. The time scale of this experiment constrains the investigation of successional changes in biocrusts to early sere.

In contrast to our findings of marked differences in fungal taxa between treatments, we found less variation in bacterial communities between restoration treatments. The two-way cluster analysis did reveal that bacterial OTUs highly abundant in biocrust (Inoculant) were most effectively re-established by the Flat and Microrills treatments, while soil-associated bacteria were detectable to varying extents (abundances) in all of the soil surface treatments with biocrust after 12 weeks of incubation. The prevalent bacterial phylum in all treatments and the inoculant was the Acidobacteria, followed closely by Proteobacteria (data not shown). To our knowledge, no other studies on biocrusts have reported a bacterial assemblage dominated by Acidobacteria; although Kuske et al. [123] reported 51% of Acidobacteria-like sequences in arid soils and Nagy et al. [124] Acidobacteria as being a dominant (relative abundance of 11%) and diverse component of biocrusts of the Sonoran Desert, Arizona. The composition of the Acidobacteria clade is known to vary when associated with biocrusts [125]. Although Acidobacterial ecological function remains poorly defined, they are likely drivers of biocrust function, contributing to aggregate formation possibly through EPS production, and tending towards being abundant community inhabitants in low resource soils [126]. Our two-way cluster analysis also indicates that there might be distinguishable soil versus biocrust associations of Acidobacterial taxa, which may indirectly be related to different ecosystem services performed within this phylum along a vertical profile from biocrust to underlying material.

While some studies [80, 127] report a higher relative abundance of Proteobacteria, they also report higher Cyanobacterial abundance, in natural biocrusts when compared to below-crust soils. In contrast, a study on biocrust of temperate climate. Cyanobacteria are often seen as being the

precursor to microbial diversity in biocrusts [128] as their morphology and physiology provide a suitable environment for other microbes to proliferate [129]. Contrary to most studies reporting Cyanobacteria as the dominant biocrusts bacterial phyla [82, 130], reportedly up to 40% [130], representatives of this phylum were found in relatively low abundance in our samples (data not shown). Cyanobacteria are likely limited by acidic pH since they lack the ability to control their internal pH [131]. Although some found that Cyanobacteria growth was limited by pH in the range of 5.5 to 6 [132] or 4 to 5 [131],Dominic and Madhusoodanan [133] found Cyanobacteria in peaty bog lands at pH below 4. Nonetheless, they found decreasing Cyanobacteria abundance with increasingly acidic pH. This suggests that the low Cyanobacterial abundance in our samples may be a result of the acidic pH, 4.6 on average, of the experimental alpine soils. Also, the Cyanobacteria family Nostocaceae-classified OTUs found in our biocrust-containing samples are almost completely absent in the soils, suggesting that these taxa are specifically inoculant-associated, which is consistent with the presence of this phylum of bacteria in other well-studied biocrust systems [26, 81].

Differences in bacterial composition and Cyanobacteria abundance between our study of an alpine temperate environment and others of hot arid deserts may also be linked to differences in climate, geochemistry and primary producers [134]. Most of the current work [43, 89, 135] is focused towards Cyanobacteria, which are seen as the precursor to more diverse biocrusts mostly from arid lands and used to start ecological processes in disturbed lands. However, there is growing evidence that other bacterial species can also be important early colonizers depending on environmental conditions and soil geochemistry. Diazotrophs other than Cyanobacteria were likely abundant in our samples as evidenced by *nifH* detection using *Rhizobium*-specific primers, and due to the abundance of Proteobacterial sequences. Yeager et al. [65] report up to 10% of *nifH* sequences

potentially from Proteobacteria in a study of biocrusts of the Colorado Plateau. Nagy et al. [124] and Gundlapally and Garcia-Pichel [124, 136] also found Oxalobacteraceae (Betaproteobacteria) to be common members of the diazotrophic communities of Sonoran and Colorado Plateau respectively. Our results demonstrate that alpine biocrusts have a unique composition; with their diazotrophic community dominated by taxa different from Cyanobacteria possibly consisting of bacteria from the families Acetobacteraceae and Oxalobacteraceae (taxonomic affiliations for OTUs in our dataset for Inoculant samples, which were not included in the two-way cluster analysis). More research is needed to concretely identify members of and characterize the functioning of microbial diazotrophs in alpine biocrusts.

In addition to Proteobacteria, other "novel" prokaryote groups [124, 136] were found in our samples, including Chloroflexi, for which three Inoculant-associated OTUs were within our subsample of highly abundant taxa (Fig. 1) for hierarchical clustering analysis. The exact role Chloroflexi play in communities is unknown but they are potentially comprised of at least some photoautotroph clades [137]. It has been suggested that their distribution may be associated with Cyanobacteria [137]. Chloroflexi and Cyanobacteria have also been found in assemblages in hyper saline mats and hot springs [138].It has been hypothesized they utilize different light wavelengths to photosynthesise and the shorter wavelengths, with deeper penetrating power, could be utilized by Chloroflexi deeper in soils [137]. Others suggest that the potential for anaerobic photosynthesis from Chloroflexi is limited to the penetration depth (3mm) of incident light [127, 139]. While we cannot infer that inoculant-associated Chloroflexi are functioning as photobionts in the biocrusts we studied, the class-level taxonomy (Ktedonobacteria) of correlating OTUs is not associated with photosynthetic activity [140]. More research is needed to understand the role of Chloroflexi play in biocrusts and their rehabilitation.

Overall, bacterial and fungal communities of the inoculant were distinct from the Uninoculated soils used in this microcosm experiment. Analysis of bacterial 16S rRNA and fungal ITS2 sequences by hierarchical clustering analysis provided further confirmation that 12 weeks was insufficient to establish a microbial community composition similar to that of the inoculant. However, the observed changes in community composition following inoculation suggested that inoculation exerted a strong influence on community composition. Whether these shifts are reflective of natural restoration trajectories and whether they will lead to the similar late-seral soil crust communities is unknown. Furthermore, even if the induced community composition differs from that of natural communities, ecological function may remain similar. Further compositional characterization of early seral biocrust communities in alpine environments and an improved understanding of how changes in community composition drive ecological function are essential for examining soil development and the efficacy of biocrust for restoration.

## Inoculation and microtopography facilitate biocrust establishment

Soil surface treatment and inoculation facilitated biocrust establishment. Microtopography is generally regarded as a factor enhancing the rehabilitation of biocrusts on disturbed sites [141]. We found that biocrust cover was high in both the Flat and Microrills treatments. Nonetheless at a small scale microtopography was still an important variable within our treatments. In the Microrills treatment, for instance, we observed higher biocrust colonization in the gullies than in the ridges. These results demonstrate the influence of moisture on biocrust development. Li et al. [22] found that microtopography had a strong influence on water and material re-allocation and thus influenced biocrust community composition notably by increasing pH which was correlated to

increased cyanobacterial abundance. Similarly, Davidson et al. [72] found that microtopography had a strong influence on soil surface microclimate and nutrient availability, which, in-turn, was a major factor influencing the development of lichen communities within biocrusts. Since Microrills create an elevated moisture level in gullies, when combined with inoculation, they represent a practical restoration tool to facilitate biocrust establishment and function recovery.

### Inoculation and soil surface treatment promote function recovery

Overall, functional recovery, represented by nitrogen fixation potential, was highest in soil surface treatments and lowest in the Uninoculated soils. Our results suggest that artificial inoculation with biocrusts increased soil surface nitrogen-fixation rates. Similarly, Jeffries et al. [23] found that algae-moss biocrust inoculation increased NA on copper mine tailings, and Maestre et al. [44] that slurry increased NA in a microcosm experiment. Stewart and Siciliano 2015 [74] found that biocrust could successfully establish on Yukon mine tailings and mining impacted soils. Others (e.g. [73, 142]) found that artificial inoculation of soils with biocrust was an effective way of increasing diazotrophic community abundance.

Microrills were the most effective treatment in restoring NA, and this trend was also reflected in the *nif*H gene copy number. The non-inoculated surfaces (Uninoculated and Stored) had the lowest *nif*H abundance and NA rates, while the highest *nif*H quantity, NA values and 16S sequence counts were observed in the inoculant. The lack of a significant difference between soil surface treatments may be due to the high variability of *nif*H copy numbers. qPCR does not distinguish between viable and non-viable genes, which can obscure differences in viable gene copy numbers [143]. Furthermore, expression of a gene does not unequivocally imply that the enzyme is active [144,

145]. Despite these limitations, *nif*H has been shown to be largely consistent with 16S rRNA gene data and the study of *nif*H abundance can be used with nitrogen fixation data to understand linkages between community structure and function [65, 143]. The soil alone hosts a reduced population of nitrogen-fixers when compared to inoculated surfaces and therefore, the use of inoculant may effectively increase the diazotroph community of restored soils. Our results demonstrate that both *nif*H copy numbers and NA increased over time, suggesting that biocrust inoculation is effective at restoring nitrogen-fixation capability.

Inoculation of soil surface clearly facilitated the recovery of chlorophyll fluorescence. Maximum photochemical quantum efficiency of PSII ($F_v/F_m$) went from undetectable to higher than 0.8 in soil surface treatments, whereas it stayed mostly undetectable in Uninoculated and Stored soils. $F_v/F_m$ values higher than 0.8 correspond to the maximal efficiency for plants. The values associated with Cyanobacteria-dominated biocrusts are reportedly between 0.4 and 0.7 [146]. A limitation of $F_v/F_m$, cyanobacterial chlorophyll fluorescence is masked by concurrent values from plants, which are naturally higher [147]. Hence, it is uncertain if cyanobacterial chlorophyll fluorescence was present in the inoculated surfaces but chlorophyll fluorescence from plants was undoubtedly present. The chlorophyll fluorescence likely originated from the bryophytes colonizing the inoculated surfaces at week 12, which was accompanied by an increase in percent cover.

## Inoculation has limited effect on EPS and soil properties after 12 weeks

The increase in EPS content between weeks 6 and 12 was also significant in the Microrills treatment. Together with significantly higher nitrogen rates, these results are consistent with biocrust functional recovery following inoculation. EPS content is particularly relevant to measuring

early biocrust function since its compounds facilitate microbial life, notably by offering protection against desiccation [58, 66, 148]. EPS content can therefore be associated with increased functional recovery such as the higher NA we observed in the Microrills treatment. Similarly, in a study on induced biocrusts in China, Colica et al. [149] found that EPS content at an 8 year old site was significantly higher than at 3 and 5 year old sites and that the increase could be attributed to recruitment of EPS producing organisms since it was correlated to higher microbial abundance [150]. The short duration of our study could explain the apparent homogeneity between treatments for the EPS content, as well as, for the dissolved organic carbon and the mineralizable nitrogen levels. However, since we observed higher photosynthetic activity combined with higher nitrogen-fixation rates in the Microrills we believe that after a longer period these additional carbon and nitrogen inputs could be reflected in the EPS level and soil properties. The higher mineralizable nitrogen level detected in our soil surface treatments when compared to the Uninoculated soils may indicate that inoculation increases nitrogen availability in soils. This is consistent with other studies that found biocrust to be positively correlated with mineralizable nitrogen levels in soils [47, 85, 91, 151, 152].

The increase in EPS content over time may indicate an increase in EPS producing organisms on inoculated surfaces. However, it cannot be excluded that these changes may have been caused by changing rates of EPS production reflecting changing environmental conditions. The production of EPS by cultured Cyanobacteria is known to be influenced by factors such as C: N ratio, temperature, light intensity, pH, and nutrient availability, however the mechanisms by which these factors influence EPS production varies between Cyanobacterial strains and are largely unknown [153]. The moisture, temperature and illumination of our microcosms may have been more favorable to EPS production than field conditions which could explain the sharp increase in EPS from week 6 to week

12 in the Uninoculated soils. However, it may also be the result of an increase in EPS producing organisms present in the soil such as fungi or free-living bacteria.

## Conclusion

We demonstrated that inoculation with biocrust was an effective means of promoting the recovery of primary ecosystem function, notably by facilitating nitrogen fixation. Biocrusts can also support soil stabilization by EPS secretion, contributing to the establishment of vascular plant communities. Although bacterial communities were relatively unchanged after inoculation, relative contributions from the fungal communities to biocrust formation reflect compositional differences between Inoculant and soil samples. A novel finding was the high number of *Serendipita* OTUs associated with biocrusts; further research is needed to determine their role. Non-cyanobacterial diazotroph and other alpine pioneering species need to be the focus of research to develop a better understanding of soil development and effective management of disturbed alpine sites. Determining linkages between community changes and ecosystem functions at the soil surface is paramount in understanding the role of biocrust in ecosystem recovery and development.

# 3. Restoration of ecosystem function by soil surface inoculation of biocrust in mesic and xeric alpine ecosystems

## Introduction

Anthropogenic disturbances can have severe impacts on alpine vegetation and soils [3-5] causing decreases in plant and microbial diversity and the alteration of soil organic carbon and nitrogen cycling processes. [1, 75, 83]. The recovery of alpine environments after disturbances is commonly limited by nutrient poor-soils and short cool growing seasons [1]. One of the key components in the recovery of alpine soils after disturbance are biocrust, ubiquitous soil surface communities formed by a diversity of organisms such as algae, lichens, mosses, liverworts, Cyanobacteria, as well as other primary successional species [12-14]. Established biocrust communities play a key role in soil carbon and nitrogen cycling through processes such as the production of extracellular polysaccharides (EPS) and nitrogen fixation [54, 86, 87]. Although biocrust are sensitive to disturbances [80, 88, 89, 154] they are well-adapted to harsh alpine growing conditions [90] and can facilitate the recovery of ecosystem services such as nitrogen and carbon cycling [91-93].

Given that biocrust establishment can initiate restoration of soil surface properties (e.g. soil stabilization, nitrogen and carbon enrichment) following disturbance, the artificial enhancement of biocrust establishment on disturbed sites has been the subject of a number of studies, using techniques such as cultivation and/or inoculation [26, 70, 71, 79]. While previous studies have examined the efficacy of inoculation for establishing biocrust in grassland and desert sites [44, 73, 80], to our knowledge none have investigated the effects of assisted inoculation on alpine biocrust establishment and function.

We address this knowledge gap in a study of alpine biocrust in two contrasting sites: the mesic coastal mountain ranges of northwestern British Columbia (Terrace, British Columbia, Canada), and the xeric interior mountain ranges of the southern Yukon (Whitehorse, Yukon Territory, Canada). At each location, we examined the development of soil biocrust after disturbance in controlled experimental plots. Experimental treatments included plots with and without biocrust inoculation, and plots with and without fertilizer addition. This allowed us to investigate site-specific potential for biocrust restoration. Soil surface microclimate was monitored, and nitrogen fixation was measured at each site to provide a better understanding of the operating conditions under which physiological activity occurs in these contrasting biocrust communities. Our main objectives were 1) to compare the composition and nitrogen fixation potential of natural biocrust associated with contrasting coastal and continental mountain ranges; 2) to assess the restoration potential of alpine biocrust communities; and to 3) to evaluate the influence of biocrust inoculation treatments on nitrogen fixation, extracellular polysaccharide content, soil mineralizable nitrogen and dissolved organic carbon, as key indicators of soil ecological function.

## Methods

### Study areas

Coastal alpine plots (mesic) were situated on Andesite Peak (lat 54° 13.868'N, long 128° 01.499'W, 1640 m elevation) in the Coast Mountain range near Terrace, British Columbia. Mean annual precipitation in Terrace (at 706 m elevation) is 970 mm with roughly 40% falling as snowfall [155]. Predicted mean annual temperature and mean annual precipitation on Andesite Peak is -1 °C and 2019 mm respectively [156]. Vegetation communities on Andesite Peak were dominated by alpine tundra with a mixture of rocky outcrops and late-melt snow beds in depressions. Vegetation on dry

ridge top habitats is dominated by *Cassiope mertensiana* (Bong.) G. Don and *Stereocaulon alpinum* Laurer heath, while *Saxifraga tolmiei* Torr. & A. Gray and *Ranunculus cooleyae Vasey & Rose* dominated vegetation communities in wetter mid-slope habitats. This site is characterized by Podzolic and Regosolic soils [157], with substrates of volcanic origin (meta-basalts and meta-andesites) [158].

Continental alpine plots (xeric) were located on Mt McIntyre (lat 60° 38'07.0" N, long 135° 11'59.8" W, 1370 m elevation) on the Teslin Plateau near Whitehorse, Yukon, Canada. Mean annual precipitation in Whitehorse (at 706 m elevation) is 262 mm with roughly half as snowfall [159]. Vegetation at the Mt McIntyre site is dominated by low growing shrubs (e.g. *Salix* spp., *Vaccinium* spp., *Dryas* spp.), herbs (e.g. *Chamerion angustifolium* L., *Anemone* spp.) as well as bryophytes and lichens (e.g. *Stereocaulon* spp., *Cladina* spp., *Cladonia* spp.*)*. Located in the Mid-Cordilleran Alpine ecoclimatic region of Yukon, the area is characterized by sedimentary rocks (clastic/limestone) and Brunisolic soils [159].

## Microclimate monitoring of natural biocrust

At each site, a microclimate monitoring station was installed. At Andesite Peak, a CR1000 data logger (Campbell Scientific Inc., Logan, UT) was coupled to a AM416 multiplexer ( Campbell Scientific Inc., Logan, UT), fitted with a HPM45 temperature probe (Campbell Scientific Inc., Logan, UT), two EC-5 soil moisture/temperature probes (Decagon Devices Inc., Pullman, WA), two LiCor Photosynthetically Active Radiation (PAR) sensors (LiCor Inc., Lincoln, Nebraska), twelve Omega 0.001 mm copper/constantan (Cu/Cn) thermocouples (Omega Engineering, Stamford, Connecticut) inserted at the surface of mature biocrust (top 5 mm), as well as, twelve pairs of impedance clips [160] attached to mature biocrust. At Mt McIntyre, a CR23 data logger (Campbell Scientific Inc.,

Logan, UT) and a CR1000 data-logger (Campbell Scientific Inc., Logan, UT) coupled to an AM25T multiplexer (Campbell Scientific Inc., Logan, UT) were installed and fitted with the same probes and sensors as at Andesite Peak. The Andesite Peak microclimate monitoring station was located in an open alpine-tundra habitat, with no shrub cover. The Mt McIntyre microclimate monitoring station was located in a sparse shrub- alpine tundra complex. Consequently, soil surface probes were partially shaded from direct solar input at Mt McIntyre.

## Natural biocrust cover assessment

Natural biocrust cover was estimated by conducting a survey along transects (60m) at Andesite Peak and Mt McIntyre respectively. At 5m intervals, $1m^2$ plots were visually assessed for soil surface cover. The locations of transects were selected to represent the various slope/aspect combinations present at each site.

## Field experiment design

Four treatments were applied to artificially disturbed plots in a randomized blocked design with 9 and 10 blocks at Andesite Peak and Mt McIntyre, respectively. Each block was located in an area with uniform topography (slope / aspect) and where biocrust was present and vegetation was sparse. Each treatment was applied within a 1 $m^2$ area that was randomly assigned within each block with a buffer of 0.5m between treatment plots. The 4 treatments were biocrust with fertilizer (BF), biocrust only (B), fertilizer only (F), and a control (C) with no biocrust or fertilizer applied.

Biocrust used to prepare the inoculation mixture for restoration plots was collected from within the areas delineated for disturbance treatment plots. All biocrust within each treatment plot was removed to a depth of 2.5 cm, comprising ca. 0.5 to 2 cm of biocrust and 0.5 cm of underlying soil. Additionally, any vegetation present in the treatment plots was removed. Collected biocrust was sieved (to 4.5 mm) and homogenized. Once biocrust was removed from all the plots, the soil surface was disturbed by raking and cultivating to a depth of 3 cm. Homogenized biocrust was then applied within 1 $m^2$ plots for B and BF treatments (n=18 and n=20 for Andesite Peak and Mt McIntyre, respectively) at a rate of 10% surface area (i.e. 0.1 $m^2$ per plot). The F and C treatments (n=18 and n=20 for Andesite Peak and Mt McIntyre, respectively) did not receive biocrust applications. Subsequently, 30-30-30 fertilizer was added to the BF and F treatments at a rate of 110kg/ha. This application rate was selected because it represents industry standard in restoration work.

Soil surface samples were collected for analysis of biocrust recovery within experimental plots at weeks 0, 6 and 12 after the start of the experiment. A composite sample (64$cm^2$0.5-1cm depth) was collected from each treatment plot. Samples were packed in coolers for transport back to the laboratory, where they were subsequently stored in a dark refrigerator at 2°C prior to Acetylene Reduction Assays (ARAs). Stored biocrust was transferred to a growth chamber for pre-treatment (average summer conditions:16 hr photoperiod at 250 µmol $m^{-2}$ $s^{-1}$ during light hours; 20$^oC$ daytime, 10$^oC$ nighttime) for 24 hrs prior to ARAs.

## Natural biocrust sampling

Naturally occurring biocrust in undisturbed alpine tundra was sampled to provide an assessment of seasonal changes in mature biocrust nitrogenase activity. Mature biocrust was collected from alpine tundra adjacent to the experimental plots at the same time points as the experimental plots were sampled (i.e. 0, 6 and 12 weeks after the start of the experiment). At each site and at each sampling time point, 10 samples of undisturbed mature biocrust (0.5cm to 2cm depth) were collected from eight harvest locations. The samples were representative of biocrust composition at each site. The nitrogen fixation potential of each mature biocrust sample was assessed through ARAs following a pre-treatment of the biocrust for 24 hrs under optimal conditions (8hrs night at 15°C and 16hrs at 20°C and 350 µmol).

## Acetylene-reduction assays

The nitrogen fixation potential of soil surface samples (from mature biocrust and experimental restoration plots) was assessed using acetylene reduction assays (ARAs) [97]. Samples were placed in 250 ml glass canning jars with a serum stopper lid. Samples were misted with 1mL of deionized water prior to closure of jars and injection of 10% acetylene by volume. Jars were incubated 4 hr at 150 µmol $m^{-2}$ $s^{-1}$ PAR and 20°C. After incubation a 4-mL gas sample was removed from each incubation jar for analysis of ethylene concentration. Each set of samples were processed with two control samples: i) a biocrust sample not injected with acetylene, which served as a temperature control and ensured that no natural evolution of ethylene occurred and ii) a jar with no biocrust that was injected with acetylene to ensure the absence of ethylene contamination in the acetylene. Gas samples were injected into a gas chromatograph (SRI 8610A, Wennick Scientific Corporation, Ottawa, ON, Canada) fitted with a Porapak column (Alltech Canada, Guelph, ON, Canada) and a flame ionization detector for detection of ethylene. Hydrogen, used as the carrier gas, was held at a

constant pressure of 32psi while column temperature was held at 65$^{o}$C. After ARAs were completed, samples from week 0 and 12 collections were homogenized and kept frozen at -20°C, for subsequent EPS analysis.

## Extracellular polysaccharide content

Extracellular polysaccharide (EPS) content was measured on samples from experimental plots taken at week 0 and at week 12 (n=76 and 80 for Andesite Peak and Mt McIntyre respectively). EPS content was determined using the phenol-sulfuric acid method [100, 101]. Reactions were transferred to a 96-well flat bottom microplate, with standards and template control carried out in triplicate. EPS content was measured as glucose concentration quantified at 480, 486 and 490 nm using a microplate reader (Varioskan, Thermo Fisher Scientific, Walthman, MA).

## Soil properties

Dissolved organic carbon and mineralizable nitrogen were measured on soil samples collected at week 12 on composite samples obtained from each plot (n=36 and n=40 for Andesite Peak and Mt McIntyre, respectively). Soil was sampled immediately below surface cover to a depth of 3 cm.

Dissolved organic carbon was measured from a soil extract (1:10 soil to water ratio shaken for 1 hr on a reciprocating shaker) filtered to 0.45 µm, with a total organic carbon analyzer (Formacs HT, Skalar Analytical B.V., Breda, Netherlands).

Mineralizable nitrogen was measured by the anaerobic incubation method [102]. Soil samples were incubated under water-logged conditions for two weeks at 30°C. Ammonium produced was subsequently measured from a soil extract (1:10 soil to 1N KCl ratio shaken for 2 hr on a reciprocating shaker) with a discrete analyzer using the phenate method for colorimetric determinations ([103]; SmartChem 200, Unity Scientific, Brookfield, CT).

## Data analysis

Landscape level nitrogen fixation estimates were calculated for Andesite Peak data. These were derived by multiplying estimates of biocrust cover by modelled values for ARA rates per $m^2$ crust cover during the field experimental period. The modelled values for ARA were developed from incubations conducted under controlled conditions of light and temperature on mature biocrust harvested from Andesite Peak. Acetylene reduction was measured at a combination of two light levels (low-120 µmol and high- 450 µmol) and 3 temperatures (5, 15 and 20C°) for biocrust held at optimal water content. A linear regression model of ARA as a function of temperature was subsequently determined. ARA response to changes in crust water content was assessed under optimal temperature and light conditions (20°C and 350 µmol), where crusts were sequentially exposed to a desiccation series of moisture contents ranging from fully saturated to totally desiccated. A regression model for ARA as a function of moisture content was subsequently calculated. Field rates of ARA activity were estimated at hourly intervals by using microclimatic data and ARA models. Each hourly record was classified as being a low or high light exposure for crusts and the corresponding light/temperature regression equation was used to calculate potential ARA rates. These estimates of potential ARA activity were then corrected for actual crust water content, using the moisture content model. Application of the moisture content model assumed

that a similar moisture dependence of ARA activity would apply at other temperature/light combinations (see discussion below). Finally, landscape-level ARA estimates were calculated based on the summation of estimated ARA rates for each hourly period and landscape level biocrust cover measurements.

Linear mixed models (LMM) were used for the analysis of ARAs and EPS content. Since samples were collected from the same plots at multiple time points, pseudoreplication within plots was avoided by including plot number as a random effect. LMM included plot number as a random effect, week and measurement (i.e. ARAs and EPS content) as fixed effects, and the interaction of week and measurement as fixed effects. To meet assumptions of normality and homoscedasticity, all measurements except dissolved organic carbon content were log-transformed. Acetylene reduction, and EPS content data were analysed using R package *lme4* [105] to generate LMM fitted by restricted maximum likelihood (REML) and the R package *lmerTest* to calculate degrees of freedom through the Satterthwait approximation [106]. Post-hoc testing was carried out with Tukey Contrasts from R package *multcomp* [107]. Soil properties and biocrust percent cover were analyzed using ANOVA and Tukey HSD post-hoc tests. All statistical analyses were conducted in R (R Core Team, 2014).

## Results

## Characterization of alpine biocrust

### Community composition

On Andesite Peak, 40 lichen, 12 liverwort and 17 moss species were identified in mature biocrust. *Amygdalaria panaeola*, *Psoroma tenue var. boreale, Solorina crocea*, as well as *Stereocaulon alpinum*, *botryosum*, and *vesuvianum* formed the nitrogen fixing cyanolichen biocrust community. On Mt McIntyre, 57 lichen, 3 liverwort and 21 moss species were identified in mature biocrust. Nitrogen-fixing lichens were *Fuscopannaria praetermissa*, *Nephroma arcticum*, *Peltigera* spp., *Psoroma tenue var. boreale*, and *Stereocaulon alpinum* and *tomentosum*. Two nitrogen-fixing lichens were found at both sites (*Psoroma tenue var. boreale* and *Stereocaulon alpinum*), as well as 11 other lichens, 2 liverworts (*Anastrophyllum minutum var. minutum* and *Tritomaria quinquedentata*) and 3 mosses (*Dicranoweisia crispula, Pogonatum contortum, Polytrichum piliferum)* species (Table 3).

### Microclimate

At Andesite Peak, cumulative daily photosynthetically active radiation (PAR) showed a strong seasonal pattern, declining from over 55 mol $m^{-2}$ $day^{-1}$ in early summer to less than 25 mol $m^{-2}$ $day^{-1}$ by mid-September (Fig.7). Mt McIntyre has a similar pattern of decline, from high early summer PAR values (25 mol $m^{-2}$ $day^{-1}$) to low early fall (12 mol $m^{-2}$ $day^{-1}$) (Fig. 8). Overall lower solar input was observed at the Mt McIntyre site.

Mean daily biocrust temperatures were consistently higher than mean daily air temperatures both at Andesite Peak and Mt McIntyre (on average by 2.5 and 1.4°C respectively). At Andesite Peak, biocrust temperatures reached a maximum of 40.6°C on 6 July and a minimum of -1.6°C on 14

September. At Mt McIntyre, maximum biocrust temperature was 36.3°C on 25 June, and minimum biocrust temperature -4.0°C on 31 July. Biocrust temperatures below 0°C were recorded only once at Andesite Peak, on 14 September. In contrast, at Mt McIntyre, sub-zero temperatures were observed on 16 days over the summer. Overall, the ranges of biocrust temperatures were similar at both sites with Mt McIntyre biocrust reaching lower temperature minima and Andesite Peak higher temperature maxima despite a higher mean air and biocrust temperatures at Mt McIntyre.

Mean air temperature was higher at Mt McIntyre (9.1°C, 4 June – 24 August 2015) during the study period compared to at Andesite Peak (6.8°C, 10 June – 14 September 2015). Typical summer conditions at Andesite Peak included mean nighttime (21:00-8:00) air and biocrust temperature at 5 and 6°C respectively with mean daytime (9:00-20:00) temperatures increasing markedly in biocrust (14°C up to a maximum of 25°C) compared to air (8°C up to a maximum of 13°C) (Fig. 8). Fluctuating wet and dry periods were also typical of Andesite Peak microclimatic conditions (Fig.7).

The total duration of hydration episodes (defined as biocrust relative water content (RWC) >25%) for biocrust at Mt McIntyre (907 hrs) was considerably shorter than that of Andesite (1589 hrs), although the mean temperature of hydrated crusts was similar, at 9.4 and 12.1°C respectively. Overall biocrust and surface soils (i.e. 5 cm depth) were drier at Mt. McIntyre compared to Andesite Peak.

Mean volumetric soil water content was 26.6% at Andesite Peak and 21.0% at Mt McIntyre. RWC was on average 62.9% at Andesite Peak and 30.6% at Mt McIntyre. At Mt McIntyre, biocrust water

content was lowest in June (on average 22.0% RWC) with no drought events occurring over the monitoring period. At Andesite Peak, microclimatic data representing an extended period of desiccation was observed between 8- 11 July followed by rewetting (Fig.7). The drought period had daily high temperatures between 35 and 40°C and corresponding biocrust water content at or near 0%. Subsequently, daily high temperatures dropped to 15- 22°C and upon rewetting biocrust relative water content increased up to 100%.

## Nitrogen fixation

The response of acetylene reduction in biocrust from Andesite Peak was characterized against changes in temperature, light, and moisture availability. Acetylene reduction rates in biocrust were strongly correlated with gravimetric water content as shown by the regression model for acetylene reduction as a function of moisture ($R^2$=0.59) (Supp. Fig. 1). The highest acetylene reduction value (9.8 µmol ethylene $m^{-2}$ $hr^{-1}$) was measured at 450% moisture content while minimal acetylene reduction (<1 µmol ethylene $m^{-2}$ $hr^{-1}$) was detected at moisture contents below 100%.

Acetylene reduction was also strongly correlated with temperature, in both low and high light linear regression models ($R^2$=0.32 and 0.42, respectively) (Supp. Fig. 2). At all three temperatures, acetylene reduction rates were consistently higher under high light than under low light. Consequently, the maximum acetylene reduction rate (2.3 µmol ethylene $m^{-2}$ $hr^{-1}$) was obtained under high light at 20°C.

## Landscape nitrogen inputs

The landscape level cover of biocrust, estimated from the transect plots was 22.4±2.8% SE at Andesite Peak. The application of the acetylene reduction models to the biocrust microclimate data (PAR, biocrust moisture and temperature) yielded a modelled daily acetylene reduction rate of 2.1±0.1 X $10^6$ mol of ethylene/ha. Multiplication of this modelled rate by the landscape percent cover of biocrust yielded an estimated daily acetylene reduction rate of 4.8±0.3 X $10^5$ mol of ethylene/ha. A theoretical conversion ratio of three to one for ethylene reduced to nitrogen is commonly used. However, we chose to leave our data as mol of ethylene because of the wide range of conversion ratios reported for terrestrial species (0.1-6) along with a strong dependency on environmental conditions [161]. Although landscape nitrogen inputs were not modelled for Mt McIntyre because of time and budget constraints, the estimated landscape level cover of biocrust was 23.0±3.4%SE.

## Recovery of inoculated biocrust and associated ecosystem functions

### Biocrust establishment

Treatment plots receiving biocrust inoculation (B and BF) had significantly higher biocrust cover after 12 weeks (33% ±3 and 32% ±2 at Andesite Peak and 22% ±2 and 20% ±3 at Mt McIntyre; Table 4, Fig. 9). Addition of fertilizer with the biocrust inoculant did not significantly increase biocrust cover at either site. Similarly, fertilization alone did not significantly increase natural colonization. Fertilized plots (F) and control soils (C) had a 3% ±1 and 10% ±3 biocrust cover at Andesite Peak and 8% ±1 and 7% ±0.1 cover at Mt McIntyre. There was a significant interaction between site and treatment (Table 4). Inoculated plots (B and BF) had higher cover at Andesite Peak compared to Mt McIntyre, however biocrust cover of uninoculated plots (C and F) was not significantly different between sites (Fig.9).

## Site differences in acetylene reduction

Nitrogenase activity (NA) changed significantly over time at both sites; however, opposing trends were observed (Table 4, Fig.10). At Mt McIntyre, NA declined significantly from week 0 to 12, whereas at Andesite Peak, NA increased significantly from week 0 to 12 (Fig. 10). NA was significantly higher at Andesite Peak compared to Mt McIntyre.

## Effects of fertilization and inoculation on functional recovery

Extracellular polysaccharide (EPS) content was significantly higher in Mt McIntyre biocrust samples (Fig. 11, Table 4). There were no significant treatment or time difference at Andesite Peak; however, at Mt McIntyre, 12 weeks following inoculation, the fertilizer treatment (F) had a significantly lower EPS (Fig.11, 76±19 μg of glucose per g of biocrust) compared to all other treatments (Fig.11, B = 250±92, BF = 217±49, C = 170±42 μg of glucose per g of biocrust, respectively).

Twelve weeks after inoculation, soil mineralizable nitrogen (Min N) measured in the top three cm below crusts was significantly lower at Andesite Peak than Mt McIntyre, but not significantly different between treatments at either site with the exception of BF being significantly higher than B at Andesite Peak (Fig.12, Table 4). Conversely, dissolved organic carbon (DOC) was significantly higher at Andesite Peak than at Mt McIntyre (Fig.12, Table 4). At Andesite Peak, DOC was significantly higher in inoculated plots (B = 75±4.9 and BF = 122±14 ppm) than in uninoculated plots (C=9.2±1.3 and F=45±5.8 ppm). Higher DOC was observed in both inoculated (BF>B) and

uninoculated plots (F>C) that received the fertilizer treatment. Fertilization increased DOC in both the inoculated and uninoculated plots.

# Discussion

## Characterization of alpine biocrust

### Community composition

On Andesite Peak, biocrust covered 22% of the alpine tundra surface. Liverworts such as *Marsupella brevissima*, *Pleurocladula albescens*, and *Ptilidium ciliare* were abundant components of biocrust communities on Andesite Peak. Nitrogen fixing lichens were also common, especially *Solorina crocea*, *Stereocaulon alpinum, S. botryosum*, and *S. vesuvianum*. On Mt McIntyre biocrust accounted for 23% of soil surface cover and were dominated by lichens, including nitrogen-fixing species such as *Stereocaulon alpinum, S. tomentosum*, and *Peltigera* spp. Many of these species, such as the liverworts *Anastrophyllum minutum* and *Marsupella brevissima,* and *Tritomaria quinquedentata*, and lichen *Psoroma tenue* are found in both arctic and alpine soil crust communities [162-164]. Others, such as the liverwort *Cephaloziella divaricate*, moss *Dicranoweisia crispula* and lichen *Placynthiella icmalea* share affinities with grassland and desert soil crust communities [165-167].

## Microclimate

There was a clear contrast between the soil surface microclimate under the continental climate at Mt McIntyre and the coastal climate at Andesite Peak. In the Coast Ranges, regular periods of precipitation during the summer combined with abundant spring snowmelt resulted in prolonged

periods of biocrust hydration. In contrast, in the xeric continental site, seasonal moisture availability for biocrust was derived mainly from spring snowmelt, with biocrust becoming progressively drier in mid- to late-summer.

Contrary to the expectation that cumulative PAR exposure would be higher in biocrust communities on Mt McIntyre, due to its higher latitude and more cloud-free days, daily biocrust PAR exposure was consistently higher for the Andesite Peak crusts compared to Mt McIntyre. This discrepancy was likely due to the more abundant shrub cover at Mt McIntyre when compared with Andesite Peak. Although maximum recorded instantaneous PAR values were similar at Andesite and McIntyre, daily PAR values were considerably lower at Mt McIntyre, due to the obstruction of sky views at low sun angles by shrubs at this site. At Mt McIntyre biocrust were partially shaded by a widely-dispersed shrub community (e.g. *Betula glandulosa* and *Salix* spp.), ca. 90 cm in height. In contrast, biocrust on Andesite Peak were not shaded by taller shrubs, since the site was dominated by dwarf shrubs (e.g. *Cassiope mertensiana*) that are closely appressed to the alpine tundra surface. The spatial distribution of biocrust in relation to vascular plants within a given ecosystem can have a significant impact not only on the microclimatic conditions under which the biocrust operates, but also nitrogen and carbon cycling processes within the crust [168-170].

## Nitrogen fixation

We found that increased moisture availability was correlated to an increase in nitrogenase activity, which is in accordance with most studies of acetylene-reduction by biocrust from alpine and arctic environments [34-38]. Using Andesite Peak biocrust, our low light model (150 umol $m^{-2}$ $s^{-1}$) was correlated to a lower acetylene reduction rate than our high light (300 umol $m^{-2}$ $s^{-1}$) model. This is

contrary to Patova et al. (2016) [35] who found that at PAR above 100 umol $m^{-2}$ $s^{-1}$, there was no effect on acetylene-reduction of cyanobacteria-dominated biocrust. Similarly, Zielke (2002) [37] found that acetylene-reduction rates started to decrease only below 140umol $m^{-2}$ $s^{-1}$ for cyanobacteria-dominated and moss-cyanobacteria biocrust. These results suggest that the light saturation level of nitrogenase activity for our site might be higher than 150 umol $m^{-2}$ $s^{-1}$.

Zielke et al. (2005) found that only when moisture is not limiting does temperature become a driver of nitrogen fixation [36]. This may introduce a potential flaw in our nitrogen fixation model since the temperature and light responses were modelled under fully hydrated conditions, which may not be the case in the field. Otherwise, moisture remains the main factor affecting nitrogen fixation of the collective biocrust community. However, adaptations to water availability may be dependent on cyanobacteria species' ecology [36] and nitrogen-fixing difference between species may be correlated to hydrological regimes [38].

We observed a positive linear relationship between acetylene reduction and temperature between 10 to 20°C. Although studies from arctic regions report optimal nitrogenase activity temperature between 20 and 30°C (Zielke et al. [37] on Svlabard Island and Chapin et al. [34] on Devon Island NWT), some also report nitrogen fixation at low temperatures (30% of maxima at 4°C [37]).

Our study worked with biocrust samples containing a composite of lichens, mosses, and liverworts, as reported above. Nitrogen fixation in our biocrust was most likely primarily driven by cyanolichens such as *Stereocaulon*, *Solorina*, and *Peltigera* spp. Previous studies have found *Peltigera* spp. dominated biocrust in Sweden to fix up to 0.88 g N $m^{-2}$ yearly [171]. Nitrogen-fixing lichens such as *Peltigera* spp. can represent significant point sources for nitrogen enrichment of soil

surface horizons [172] however, the frequent association of mosses and liverworts with nitrogen-fixing cyanobacteria also provides a significant source of fixed nitrogen in biocrust communities [173]. Although association of cyanobacteria with liverworts are known to widely occur, many of these associations have yet to be characterized. Marchantia, Porella, Blasia and Clavicularia are four liverwort genera commonly known to host cyanobacterial associations [173]. None of these genera were observed at our sites, however, it likely that some liverwort cyanobacteria associations were present. *Pleurozium schreberi*, a moss species present at the Mt McIntyre site, commonly associates with the cyanobacterium *Nostoc* sp. and reported rates of nitrogen fixation range from of 1.5 – 2 kg N $ha^{-1}$ $yr^{-1}$ in boreal forests [174]. Cyanobacteria-moss associations supply most of the combined nitrogen in some Arctic and Boreal regions [36, 37]. Furthermore, many mosses and liverworts have adaptations that allow prolonged moisture retention, which creates hydric microenvironments allowing for cyanobacteria to readily colonize [40]. The more constant moisture availability at Andesite Peak, would have not only provided a more favourable operating environment for nitrogen-fixing species, but may have also provided the conditions for greater establishment and growth of nitrogen fixing components of the biocrust. Although we observed similar rates of biocrust establishment in our inoculated plots at the two sites, declining versus increasing rates of nitrogen fixation at Mt. McIntyre compared to Andesite Peak may indicate better establishment of nitrogen fixing species inoculated biocrust at Andesite Peak. Comparison of cyanobacterial colonization rates of recovering biocrust in contrasting environments would be highly informative and should be considered in future studies.

## Landscape-level nitrogen inputs

Several studies have assessed nitrogen fixation at a landscape level in low Arctic environments with nitrogen inputs varying widely between hydric and xeric environments (10.89 and 0.73 kg N $ha^{-1}$ $yr^{-1}$, respectively) [175]. In a study of biocrust disturbance Belnap et al. [50] estimated the annual input of nitrogen to 9 kg/ha and 1.4 kg/ha for biocrust of arid lands in Utah, US (dark cyanobacteria dominated and disturbed, respectively). Our estimate of nitrogen fixation of 4.8±0.3 X $10^5$ mol of ethylene/ha is in the lower range of values reported for both low Arctic and hot arid and semiarid lands. However, given the nitrogen-limitation often present in northern environments, including alpine, even smaller inputs of nitrogen can have significant effects. Although exact calculation of nitrogen inputs by alpine biocrust warrants a more detailed investigation, our study outlines the significance of nitrogen fixation by biocrust in alpine environments. We expect nitrogen fixation at a landscape level to be somewhat lower at Mt McIntyre than at Andesite Peak given the lower moisture level which would induce a decline in nitrogenase activity, given a similar percent cover of biocrust on both landscapes (23 and 22%, for Mt McIntyre and Andesite Peak, respectively). high

## Indicators of ecosystem function recovery in experimental plots

### Biocrust establishment

Mt McIntyre and Andesite Peak had similar biocrust cover 12 weeks after the start of the experiment, however we observed significantly faster recovery of biocrust function (i.e. nitrogen fixation and EPS) at Andesite Peak. The difference in cover observed between inoculated and uninoculated was not reflected as clearly in the measurements of functions which may indicate a shift in community composition at Mt McIntyre. Xiao and et al. (2008) [176] found that three years after disturbance a full cover of moss-dominated biocrust was established. This study however did

not measure nitrogen fixation nor EPS content. Most studies [1, 80, 88, 89] have observed slow nitrogenase activity recovery after disturbances. Although some have reported up to 60% biocrust cover in 4 years with algae-moss inoculation [71]. These studies are in hot arid or semi-arid environments which share the rugged character of alpine environments but have fundamentally different precipitation regimes. Our findings indicate that although a biocrust cover can be established within one growing season, recovery of key ecosystem functions, such as carbon and nitrogen fixation may require longer.

## Site differences in acetylene reduction pattern

In biocrust from experimental plots, opposing trends in acetylene-reduction were observed over time. At Andesite Peak, the acetylene reduction rate increased as the experiment progressed whereas at Mt McIntyre it decreased. The local climatic differences between both sites, notably the contrasting moisture regimes, may play in important role in the opposing trends.

In a study of cyanobacteria dominated biocrust of subpolar Ural Mountains, Patova et al. (2016) [35] reports hourly rates of acetylene reduction between 0.5 and 1.76 mg of ethylene/$m^2$ (18-62 µmol of ethylene/$m^2$)which is ten times higher than the rates measured in restored biocrust of Andesite Peak.

The difference in activity level could explain the lower rehabilitation potential of dry cold continental climate of Mt McIntyre when compared to moister and warmer coastal climate of Andesite Peak. Additionally, the declining pattern in acetylene-reduction over time at Mt McIntyre could be reflective of biocrust mortality linked to the uncharacteristically dry period at the beginning of our experiment; while the normal precipitation for June is 32 mm, June 2015 only

received 16mm [155]. This result suggests that a limitation in water availability can considerably slow restoration processes in montane environments. This is consistent with drylands where biocrust succession and functions are limited by water availability [177]. In more xeric and cold climates, different restoration protocols increasing surface moisture retention may be needed to successfully restore biocrust communities.

Differences in biocrust composition that are intrinsically linked to different environmental and microclimatic conditions between sites could also explain these opposing trends. In a comparison between alpine and Antarctic biocrust, Colesie et al. (2016) [41] found that compositional difference could be correlated to climatic conditions and the adaptation necessary to thrive in extreme environments. Accordingly, in a manipulative experiment, Li et al. (2010) [22] found that increasing soil moisture shifted the species composition to an increased proportion of mosses and lichens and a decreased proportion of cyanobacteria. Furthermore, Raggio et al. (2016) [178] found that lichens of dry and cold environments were less active than those from warmer moister climates. In addition, increased species richness has been linked to milder climate regimes in North American montane environments [42]. Collectively, these studies indicate that the community composition of BSCs and the ecological functioning (e.g. nitrogen fixation) of species assemblages is undoubtedly linked with environmental and operating conditions. Therefore, we expect that the mesic climate of Andesite Peak will result in a more active community than those located in xeric climate such as Mt McIntyre.

## Effects of fertilization and inoculation on functional recovery

There was no clear effect of fertilization on acetylene-reduction rates in the experimental plots of either site. Contrary to Maestre et al. (2006) who found a positive relationship between moderate fertilizer addition (in the form of a composted sludge) and nitrogenase activity under laboratory conditions [44]. In a recent study combining fertilizer addition and watering, Antoninka et al. (2015) [43] successfully produced fully functional moss-dominated biocrust. While the effect of fertilization on biocrust establishment remains unclear, these results suggest that hydration may be the key limiting factor in biocrust nitrogen fixation.

Experimental plots at Andesite Peak that received fertilization and/or inoculation (F, B, BF) had higher DOC in soils (3 cm depth). At Mt McIntyre, the DOC was extremely low in all soils and we did not observe any treatment differences. These extremely low levels of DOC are typical of northern latitudes, where primary productivity is limited and soil development slow [179]. However, it has been suggested that phototrophic microbial communities may play an important role in carbon uptake from the atmosphere in alpine environments where plant cover is limited [137]. Overall DOC was higher at Andesite Peak, which is likely caused by the coastal climate's association with higher primary productivity which creates better developed soils richer in carbon [180]. The difference in soil development level was also expected to be also reflected in the amount of mineralizable nitrogen present. Surprisingly, mineralizable nitrogen levels were found to be lower on Andesite peak than on Mt McIntyre. In Alaskan soils, nitrogen mineralization rates were found to be insensitive to temperature between 3 and 9°C but increased two folds or more between 9 and 15°C [181]. Consequently, higher average temperatures at Mt McIntyre when compared with

Andesite Peak could explain its higher mineralizable nitrogen concentrations. At Andesite Peak, DOC was approximately double in inoculated soils compared to uninoculated plots.

## Conclusion

Our study provides a comprehensive examination of biocrust communities in two contrasting alpine environments. Furthermore, our work on restoration of two sites with climatic and community compositional differences are a first step in understanding mechanisms driving successful restoration of biocrust in alpine environments. Although biocrust establishment was successful at both sites, only Andesite showed recovery of nitrogen fixation, demonstrating a need for better characterization of the early successional trajectories of nitrogen fixing communities in recovering biocrust. The decline of nitrogen fixation following inoculation at Mt McIntyre likely indicates a community shift, as well as, lower activity due to less optimal operating conditions. While restoration of key ecosystem functions is highly desirable, biocrust establishment alone could help mitigate physical impacts associated with soil surface disturbance, such as erosion. Furthermore, our study suggests that depending on restoration goals, protocols for dry and cold climates may need to include promoting soil surface moisture retention or providing additional water inputs for successful re-establish of biocrust communities.

# 4. Implications

Most restoration work with biocrusts has been conducted in desert and grassland ecosystems; by applying these restoration approaches in alpine tundra our study provides an important and unique contribution. Furthermore, few studies combine the concurrent examination of biocrust structure (species assemblages and functional groups), function (nitrogenase activity, eps production, carbon and nitrogen cycling) and field microclimate. We addressed this knowledge gap by the study of alpine biocrust in two contrasting sites: the mesic coastal mountain ranges of northwestern British Columbia (Terrace, British Columbia, Canada), and the xeric interior mountain ranges of the southern Yukon (Whitehorse, Yukon Territory, Canada). At each location, we examined the development of soil biocrust after disturbance in controlled experimental plots. Experimental treatments included plots with and without biocrust inoculation, and plots with and without fertilizer addition. This allowed us to investigate site-specific potential for biocrust restoration. Soil surface microclimate was monitored, and nitrogen fixation was measured at each site to provide a better understanding of the operating conditions under which physiological activity occurs in these contrasting biocrust communities.

We found that soil surface treatment and inoculation facilitated biocrust establishment. Since Microrills create an elevated moisture level in gullies, when combined with inoculation, they represent a practical restoration tool to facilitate biocrust establishment and function recovery. Our results demonstrate that both *nif*H copy numbers and NA increased over time, suggesting that biocrust inoculation is effective at restoring nitrogen-fixation capability.

We showed that inoculation with biocrust could facilitate the recovery of primary ecosystem function. Specifically, processes associated with nitrogen fixation as demonstrated by increased nifH copy numbers over time combined with increased nitrogenase activity. Although bacterial communities were relatively unchanged after inoculation in a greenhouse setting, relative contributions from the fungal communities to biocrust formation reflect compositional differences between Inoculant and soil samples. A novel finding was the high number of *Serendipita* OTUs associated with biocrusts; further research is needed to determine their role. Non-cyanobacterial diazotroph and other alpine pioneering species need to be the focus of research to develop a better understanding of soil development and inform the restoration practices for disturbed sites within alpine tundra biomes.

Our study examined nitrogen fixation by soil surface communities in natural alpine environments and is novel in that it quantifies the magnitude of nitrogen fixation at a landscape level. Our work on restoration of two sites with climatic and community compositional differences is a first step in understanding mechanisms driving successful restoration of biocrust in alpine environments. Although biocrust establishment was successful at both sites, only Andesite Peak showed recovery of nitrogen fixation, demonstrating a need for better characterization of the early successional trajectories of nitrogen fixing communities in recovering biocrust. The decline of nitrogen fixation level through time at Mt McIntyre likely indicates a community shift, as well as, lower activity due to less optimal operating conditions. However, biocrust establishment alone could help mitigate physical impacts associated with soil surface disturbance, such as erosion. Our study suggests that depending on restoration goals, protocols for dry and cold climate may need to include increased moisture retention or provide additional water inputs to successfully re-establish biocrust communities with pre-disturbance composition and function.

**Practical Implications for Restoration**

- In mesic climate conditions, inoculation of disturbed alpine environments with biocrusts may be an effective restoration technique
- In cold xeric climates, restoration protocols that increase surface moisture retention are likely needed for successfully restoration of biocrust communities. Timing might be particularly important in environments with high seasonal moisture variation.
- Restoration of biocrust cover may not be indicative of recovery of ecological function, such as soil surface nitrogen fixation. However, even if species assemblage has shifted some ecological functions such as erosion control might still be present.
- Alpine biocrusts appear to play a significant role in nitrogen and carbon input and restoration of these communities following disturbance may support ecosystem recovery.

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# Plates


Plate 1. A) Andesite Peak in the B.C. Coast Ranges is characterized by rolling topography, with small wet depressions surrounding by drier rock outcrops. B) Biocrusts on Andesite peak are comprised of a complex mix of lichens (orange thalli of *Solorina crocea* visible in image), mosses, liverworts, and free-living algae and cyanobacteria.

Plate 2. A) Mt McIntyre in Yukon is characterized by rolling topography covered by shrubs with biocrust in interstices. B) Biocrust on Mt McIntyre were lichen-dominated. C) A treatment block on Mt McIntyre.

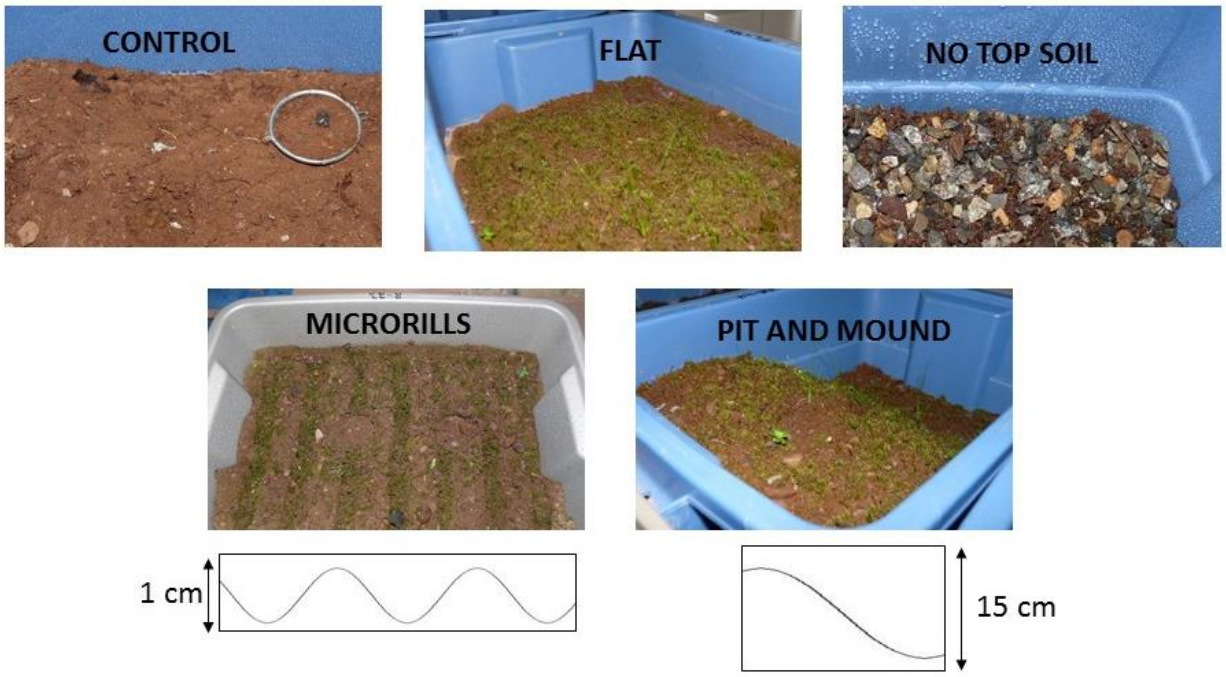



Plate 3. The control and four experimental treatments (flat, no top soil, microrills, and pit and mound) in the greenhouse inoculation experiment.

# Tables

Table 1. Analysis of variance (F values) for measurements taken at week 6 and 12. Fixed effects for the mixed ANOVA model included treatment, week, and a two-way interaction of treatment and week. Samples were taken from the same tray were treated as random variable to account for non-independence between samples within trays. * denotes a significant interaction

| **Analysis** | **Parameters** | **DF** | **F** | ***p*** |
|---|---|---|---|---|
| **Biocrust cover** | Treatment | 4, 40 | 366.25 | < 0.01* |
| | Week | 1, 40 | 46.46 | < 0.01* |
| | Treatment: Week | 4, 40 | 2.63 | < 0.05 * |
| **EPS content** | Treatment | 3, 78 | 1.63 | =0.19 |
| | Week | 1, 78 | 9.98 | < 0.01* |
| | Treatment: Week | 3, 78 | 1.13 | =0.34 |
| **ARAs** | Treatment | 4, 114 | 4.41 | <0.01* |
| | Week | 1, 114 | 1.91 | =0.17 |
| | Treatment: Week | 4, 114 | 0.23 | =0.92 |

Table 2.Properties of soils underlying experimental plots 12 weeks following inoculation. Dissolved organic carbon (DOC), potentially mineralizable nitrogen (Min N), and $pH_{H20}$ values (±SE). Min N was significantly higher in pit and mound than in the untreated and flat treatments (ANOVA with Tukey HSD, $p<0.02$).

| | **Untreated** | | **Flat** | | **Pit and Mound** | | **Microrills** | |
|---|---|---|---|---|---|---|---|---|
| **DOC (ppm)** | 154 | (12.1) | 119 | (21.1) | 133 | (12.2) | 163 | (21.3) |
| **Min N (ppm)** | $10.8^{A}$ | (0.5) | $10.9^{A}$ | (0.5) | $12.1^{B}$ | (0.7) | 11.7 | (0.4) |
| **pH** | 4.54 | (0.13) | 4.55 | (0.04) | 4.60 | (0.15) | 4.77 | (0.04) |

Table 3. Composition (bryophytes and lichens) of biocrust communities at Andesite and Mt McIntyre. Nitrogen-fixing lichen species are greyed. Bolded species name indicate that the species is present at both sites.

| Species on Andesite | Group |
|---|---|
| *Amygdalaria panaeola* | Lichen |
| ***Psoroma tenue var. boreale*** | Lichen |
| *Solorina crocea* | Lichen |
| ***Stereocaulon alpinum*** | Lichen |
| *Stereocaulon botryosum* | Lichen |
| *Stereocaulon vesuvianum* | Lichen |
| ***Anastrophyllum minutum var. minutum*** | Liverwort |
| *Andreaea rupestris* | Moss |
| *Anthelia juratzkana* | Moss |
| *Arthrorhaphis sp.* | Lichen |
| *Baeomyces carneus* | Lichen |
| *Blepharostoma trichophyllum* | Liverwort |
| *Bucklandiella microcarpa* | Moss |
| *Bucklandiella sudetica* | Moss |
| *Cephaloziella divaricata* | Liverwort |
| *Cetraria commixta* | Lichen |
| *Cetraria delisei* | Lichen |
| ***Cetraria islandica ssp. islandica*** | Lichen |
| ***Cladina arbuscula*** | Lichen |
| *Cladina rangiferina* | Lichen |
| *Cladonia bellidiflora* | Lichen |
| ***Cladonia borealis*** | Lichen |
| *Cladonia borealis* | Lichen |
| *Cladonia cervicornis* | Lichen |
| *Cladonia ecmocyna ssp. occidentalis* | Lichen |
| *Cladonia gracilis group* | Lichen |
| ***Cladonia macrophyllodes*** | Lichen |
| ***Cladonia pleurota*** | Lichen |
| *Cladonia singularis* | Lichen |
| ***Cladonia uncialis var. uncialis*** | Lichen |
| *Conostomum tetragonum* | Moss |
| *Cynodontium tenellum* | Moss |
| ***Dicranoweisia crispula*** | Moss |
| *Dicranum elongatum* | Moss |
| *Dicranum spadiceum* | Moss |
| *Diplophyllum taxifolium* | Liverwort |
| *Frutidella caesioatra* | Lichen |
| *Gymnomitrion concinnatum* | Liverwort |

| Species on Mt McIntyre | Group |
|---|---|
| *Fuscopannaria praetermissa* | Lichen |
| *Nephroma arcticum* | Lichen |
| *Peltigera cf. conspersa* | Lichen |
| *Peltigera didactyla* | Lichen |
| *Peltigera extenuata* | Lichen |
| *Peltigera leucophlebia* | Lichen |
| *Peltigera malacea* | Lichen |
| *Peltigera ponojensis* | Lichen |
| *Peltigera rufescens* | Lichen |
| *Peltigera venosa* | Lichen |
| ***Psoroma tenue var. boreale*** | Lichen |
| ***Stereocaulon alpinum*** | Lichen |
| *Stereocaulon tomentosum* | Lichen |
| *Alectoria ochroleuca* | Lichen |
| ***Anastrophyllum minutum var. minutum*** | Liverw |
| *Anzina carneonivea var. carneonivea* | Lichen |
| *Aulacomnium palustre* | Moss |
| *Barbilophozia hatcheri* | Liverw |
| *Brachythecium frigidum* | Moss |
| *Bryocaulon divergens* | Lichen |
| *Bryonora pruinosa* | Lichen |
| *Buellia papillata* | Lichen |
| *Caloplaca livida* | Lichen |
| *Caloplaca stilliciodorum* | Lichen |
| *Caloplaca tirolensis* | Lichen |
| *Catapyrenium cinereum* | Lichen |
| *Catapyrenium daedaleum* | Lichen |
| ***Cetraria islandica ssp. islandica*** | Lichen |
| *Cetraria odontella* | Lichen |
| ***Cladina arbuscula*** | Lichen |
| ***Cladonia borealis*** | Lichen |
| *Cladonia coccifera* | Lichen |
| *Cladonia macrophylla* | Lichen |
| ***Cladonia macrophyllodes*** | Lichen |
| *Cladonia phyllophora* | Lichen |
| ***Cladonia pleurota*** | Lichen |
| *Cladonia pocillum* | Lichen |
| *Cladonia pyxidata* | Lichen |

| | |
|---|---|
| *Kiaeria blyttii* | Moss |
| *Lecanora zosterae* | Lichen |
| *Lecidea hypnorum* | Lichen |
| ***Lecidoma demissum*** | Lichen |
| *Lepraria alpina* | Lichen |
| *Leprocaulon albicans* | Lichen |
| *Marsupella brevissima* | Liverwort |
| *Marsupella ustulata* | Liverwort |
| *Moerckia blyttii* | Liverwort |
| *Nardia geoscyphus* | Liverwort |
| ***Ochrolechia androgyna*** | Lichen |
| *Pertusaraia geminipara* | Lichen |
| *Pertusaria oculata* | Lichen |
| *Placidiopsis sp.* | Lichen |
| ***Placynthiella icmalea*** | Lichen |
| ***Placynthiella uliginosa*** | Lichen |
| *Plagiothecium laetum* | Moss |
| *Pleurocladula albescens* | Liverwort |
| ***Pogonatum contortum*** | Moss |
| *Pohlia sp.* | Moss |
| *Polyblastia gelatinosa* | Lichen |
| ***Polytrichum piliferum*** | Moss |
| *Porpidia contraponenda* | Lichen |
| *Ptilidium ciliare* | Liverwort |
| *Ptychostomum sp.* | Moss |
| *Racomitrium lanuginosum* | Moss |
| *Rhizocarpon expallescens* | Lichen |
| ***Thamnolia vermicularis*** | Lichen |
| *Thelenella muscorum var. muscorum* | Lichen |
| ***Tritomaria quinquedentata*** | Liverwort |
| *Umbilicaria hyperborea var. radicicola* | Lichen |

| | |
|---|---|
| *Cladonia sulphurina* | Lichen |
| ***Cladonia uncialis var. uncialis*** | Lichen |
| *Coelocaulon aculeatum s. lat.* | Lichen |
| *Dicranella heteromalla* | Moss |
| ***Dicranoweisia crispula*** | Moss |
| *Dicranum fuscescens* | Moss |
| *Distichium capillaceum* | Moss |
| *Ditrichum flexicaule* | Moss |
| *Flavocetraria cucullata* | Lichen |
| *Flavocetraria nivalis* | Lichen |
| *Hylocomium splendens* | Moss |
| *Hypnum bambergeri* | Moss |
| *Hypnum cupressiforme* | Moss |
| *Lecanora polytropa* | Lichen |
| *Lecidea alpestris* | Lichen |
| ***Lecidoma demissum*** | Lichen |
| *Masonhalea richardsonii* | Lichen |
| ***Ochrolechia androgyna*** | Lichen |
| *Pertusaria panyrga* | Lichen |
| ***Placynthiella icmalea*** | Lichen |
| *Placynthiella oligotropha* | Lichen |
| ***Placynthiella uliginosa*** | Lichen |
| *Platydictya jungermannioides* | Moss |
| *Pleurozium schreberi* | Moss |
| ***Pogonatum contortum*** | Moss |
| *Pohlia crudoides* | Moss |
| *Pohlia nutans* | Moss |
| *Polyblastia gothica* | Lichen |
| *Polytrichum juniperinum* | Moss |
| ***Polytrichum piliferum*** | Moss |
| *Protothelenella leucothelia* | Lichen |
| *Psoroma sp.* | Lichen |
| *Ptychostomum lonchocaulon* | Moss |
| *Ptychostomum pendulum* | Moss |
| *Ptychostomum pseudotriquetrum* | Moss |
| *Rinodina mniaraea var. mniaraea* | Lichen |
| *Rinodina mniaraea var. mniaraeiza* | Lichen |
| *Rinodina turfacea* | Lichen |
| *Sanionia uncinata* | Moss |
| ***Thamnolia vermicularis*** | Lichen |
| *Thelocarpon epibolum* | Lichen |
| *Trapeliopsis granulosa* | Lichen |
| ***Tritomaria quinquedentata*** | Liverw |

Table 4. ANOVA on mixed models of function parameters and linear models of soil properties and biocrust cover of experimental plots at Andesite and Mt. McIntyre (Site) sampled 0, 12 and weeks following inoculation (Time). TR = treatment (control, fertilizer only, biocrust only, and biocrust + fertilizer). Min N = mineralizable nitrogen and DOC= dissolved organic carbon. F-values are not available for the factor Time, for Cover, DOC and Min N that were only measured at 12 weeks.

| **Factor** | **Function Parameter** | | | **Soil Properties** | | | | |
|---|---|---|---|---|---|---|---|---|
| | df | NA | EPS | df | DOC | Min N | df | Cover |
| | | ----F-value ---- (p-value) | | | ----F-value ---- (p-value) | | | -F-value - (p-value) |
| TR | 3 | 0.96 (0.42) | 3.67 (0.02) | 3 | 40.4 (<0.01) | 2.03 (0.12) | 3 | 24.9 (<0.01) |
| Time | 2 | 16.21 (<0.01) | 0.70 (0.41) | 1 | - | - | - | - |
| Site | 1 | 40.30 (<0.01) | 76.08 (<0.01) | 1 | 157.1 (<0.01) | 34.8 (<0.01) | 1 | 1.64 (0.20) |
| TR * Time | 6 | 1.18 (0.32) | 0.50 (0.68) | 3 | - | - | - | - |
| TR * Site | 3 | 0.54 (0.66) | 2.86 (0.04) | 3 | 33.2 (<0.01) | 0.71 (0.55) | | 2.59 (0.06) |
| Time*Site | 2 | 81.64 (<0.01) | 1.71 (0.19) | 1 | - | - | - | - |
| TR*Time*Site | 6 | 0.94 (0.47) | 0.28 (0.84) | 3 | - | - | - | - |

# Figures

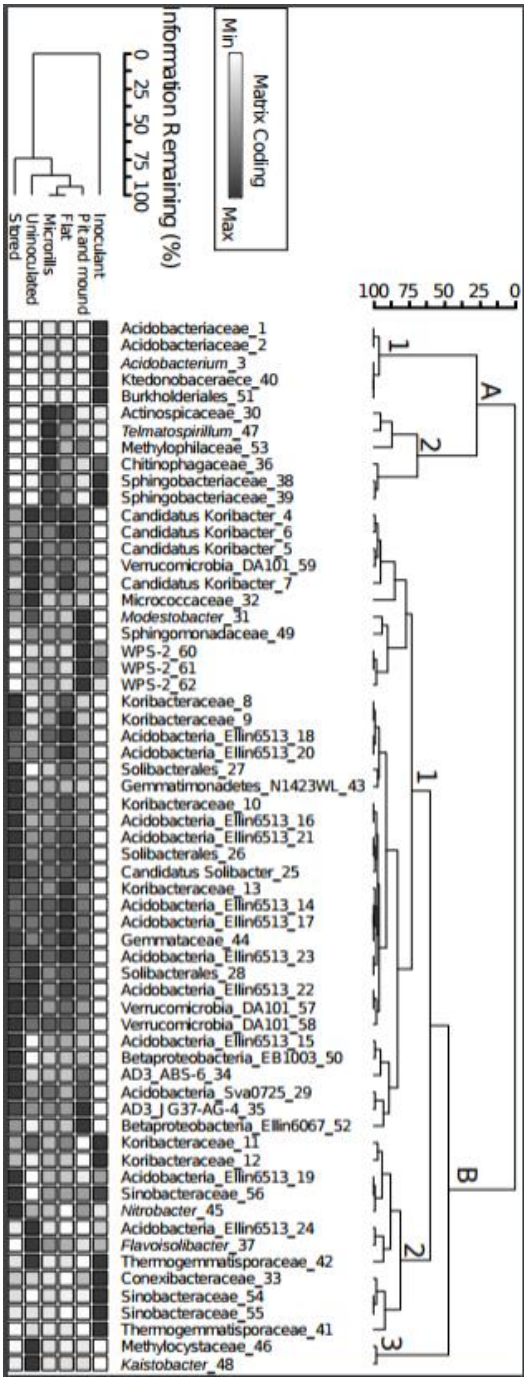



Figure 1. Two-way cluster dendrogram of 62 bacterial OTUs with high relative abundances within the six sample groups (i.e. minimum 2,000 sequences total across all replicates in group) defined by treatment or control category. Cluster A is comprised of OTUs detected mostly with high abundance in Inoculant, and nearly absent in Uninoculated and Stored soil control groups. Cluster B is dominated by OTUs most consistently detected in Uninoculated, Stored and soil surface treatment sample groups and least consistently detected in the inoculant group. Highest assigned taxonomic identifiers are shown, and genus-level assignments are indicated in italics.

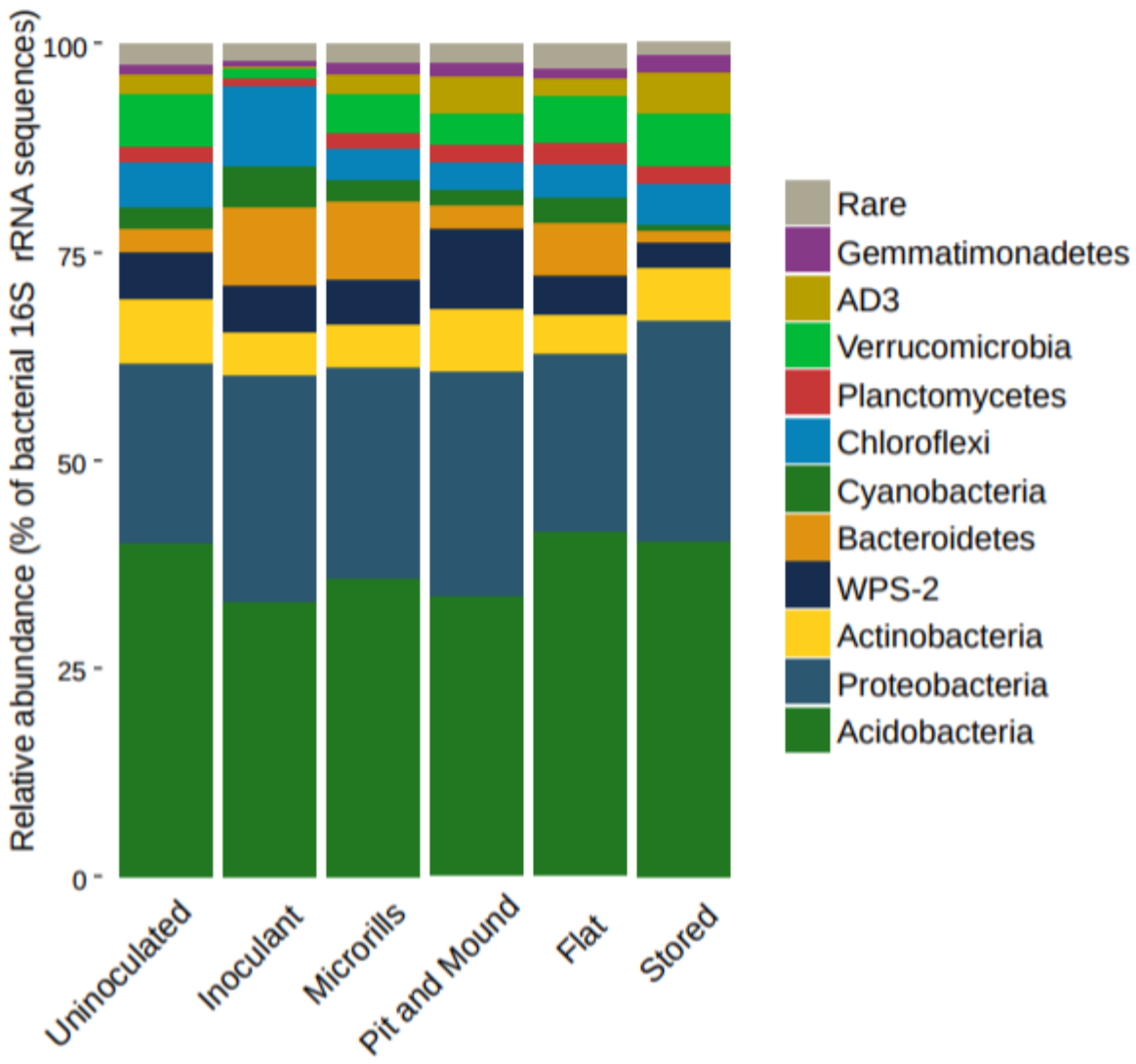



Figure 2. Relative abundance of bacterial 16S rRNA gene sequences. Soil bar represents unincubated soil that was sampled at the start of the experiment. Aciodobacteria were the most abundant across all treatments and most bacterial phylum were represented evenly across treatments. Chloroflexi were most abundant in the inoculant.

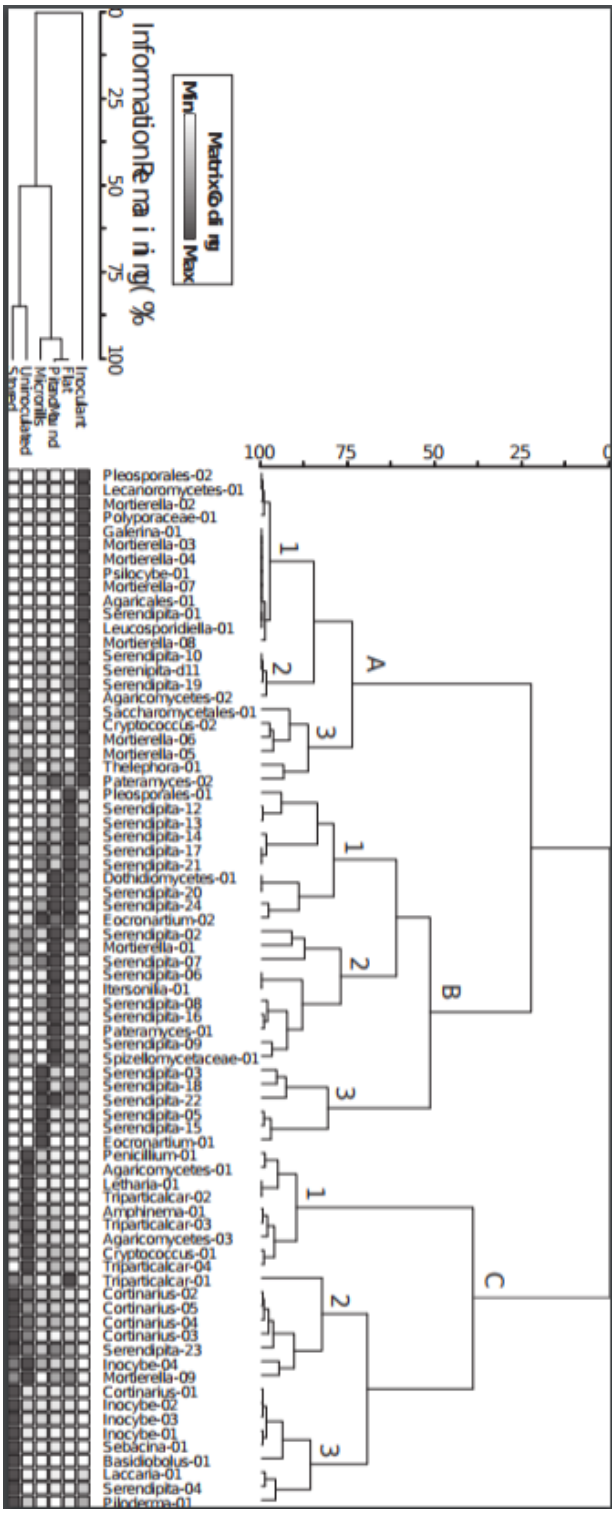



Figure 3. Two-way Cluster Analysis of 75 fungal OTUs averaged by sample units: Inoculant, biocrust surface treatments (Flat, Pit & Mound, Microrills), and soils (Uninoculated controls and Stored). Cluster A are OTUs that are most abundant in the Inoculant, Cluster B comprises OTUs that are most abundant in the soil surface treatments, and Cluster C comprises OTUs that are most abundant in the soil samples.

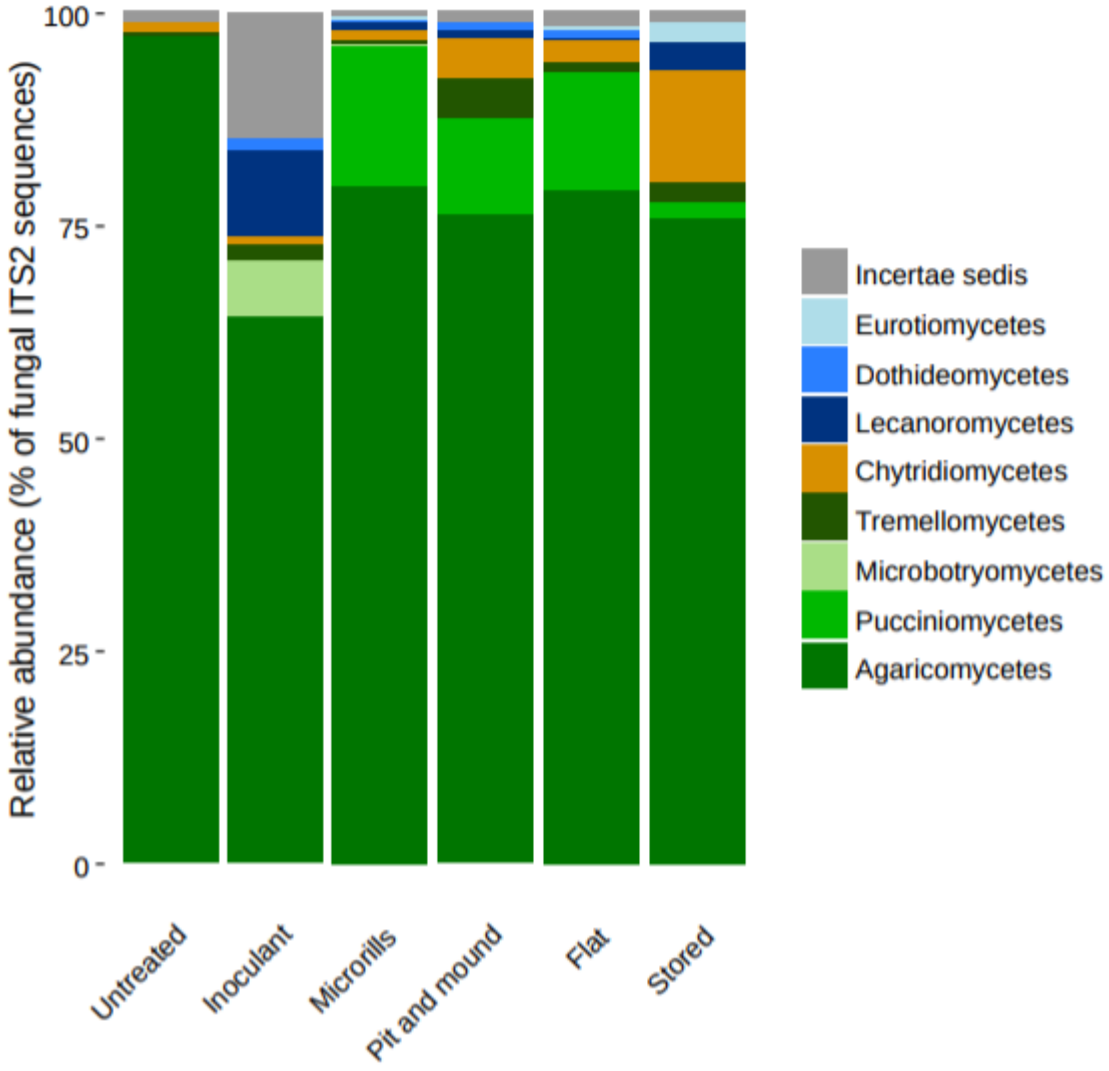



Figure 4. Relative abundance of fungal ITS2 region sequences. “Stored” bar represents unincubated soil that was sampled at the start of the experiment. Basidiomycota are shown in shades of green and Ascomycota in shades of blue.

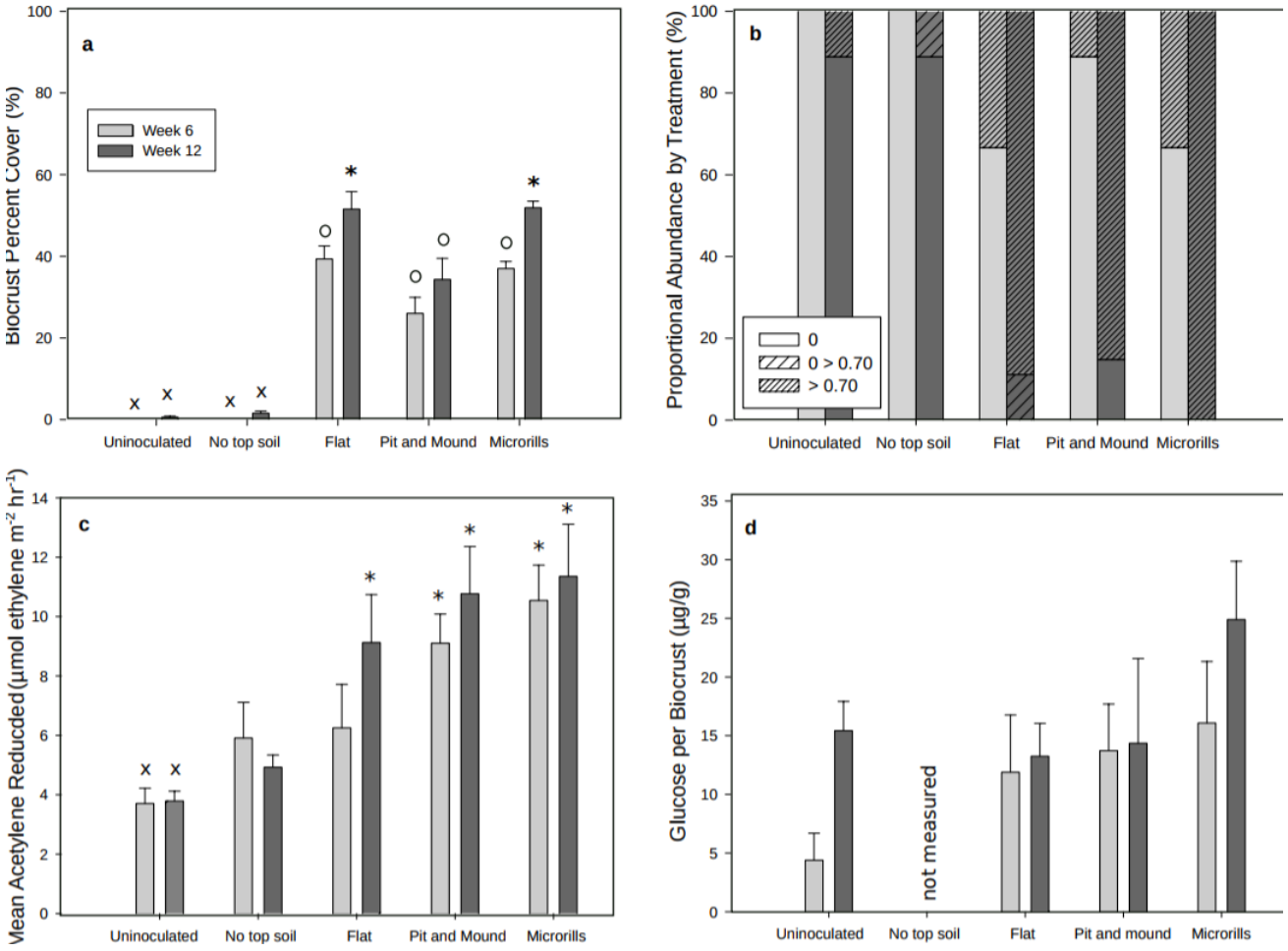



Figure 5. a. Mean (±SE) biocrust percent cover at 6 and 12 weeks (light and dark bar, respectively). Significant differences are indicated by differences in symbols. Percent cover was significantly higher in the flat, microrills, and pit and mound treatments than in the untreated and no top soil (ANOVA, Tukey HSD posthoc, $F=8.56$, $p<0.03$); b. Proportional abundance of Fv/Fm at 6 and 12 weeks (left and right bar, respectively) where no hash-line corresponds to no detected chlorophyll fluorescence (Fv/Fm =0), widely spaced hash-lines to Fv/Fm between 0 and 0.70, and closely spaced hash-lines to Fv/Fm larger than 0.70. Twelve weeks after inoculation, Fv/Fm was significantly higher in the flat, pit and mound, and microrills treatments than in the no top soil treatment and untreated (Kruskal-Wallis, and pairwise comparisons using Tukey and Kramer (Nemenyi) test with Tukey-Dist approximation for independent samples, chi-squared = 38.7, $p< 0.02$); c. Mean (±SE) acetylene reduced at 6 and 12 weeks (light and dark bar, respectively). Microrills treatment had significantly higher rates of nitrogen fixation than the untreated and no top soil treatments (ANOVA, TukeyHSD posthoc, $F= 9.01$, $p<0.01$). The detection limit for acetylene reduction was 0.2 µmol ethylene $m^{-2}hr^{-1}$; d. Mean (±SE) glucose content per biocrust at 6 and 12 weeks (light and dark bar, respectively). Extracellular polysaccharide content was significantly higher in the microrills treatment at 12 weeks than in the untreated at 6 weeks (ANOVA, Tukey posthoc, $F=9.23$, $p<0.03$).

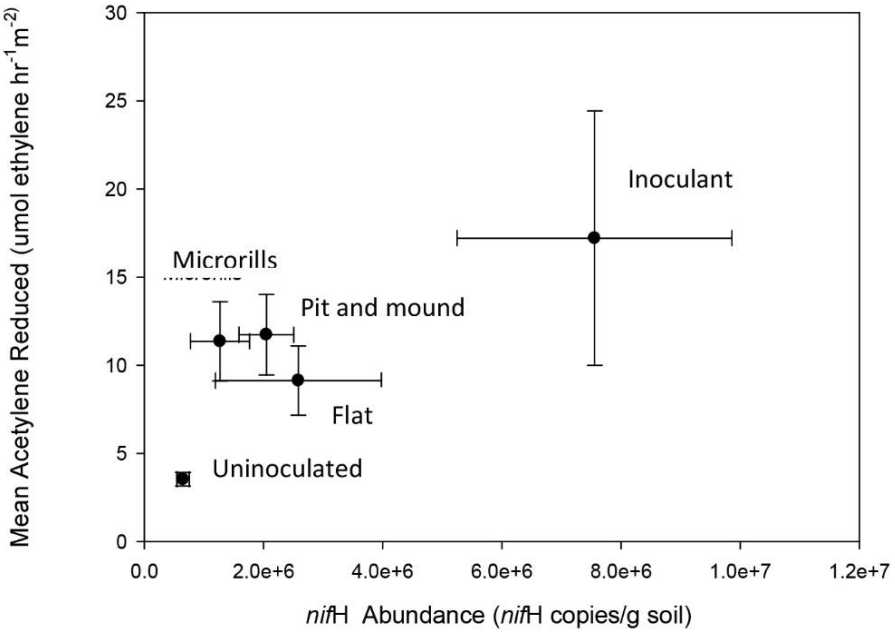



Figure 6. Mean acetylene reduced as a function of nifH abundance at 12 weeks. The inoculant had significantly more nifH copies per gram of soil than the untreated and all treatments (ANOVA, Tukey posthoc, $F=7.91$, all $p<0.04$). nifH gene copy number was not significantly different between surface microtopography treatments (ANOVA, $F=0.912$, $p=0.45$). The detection limit for acetylene reduction was 0.2 µmol ethylene $m^{-2}hr^{-1}$

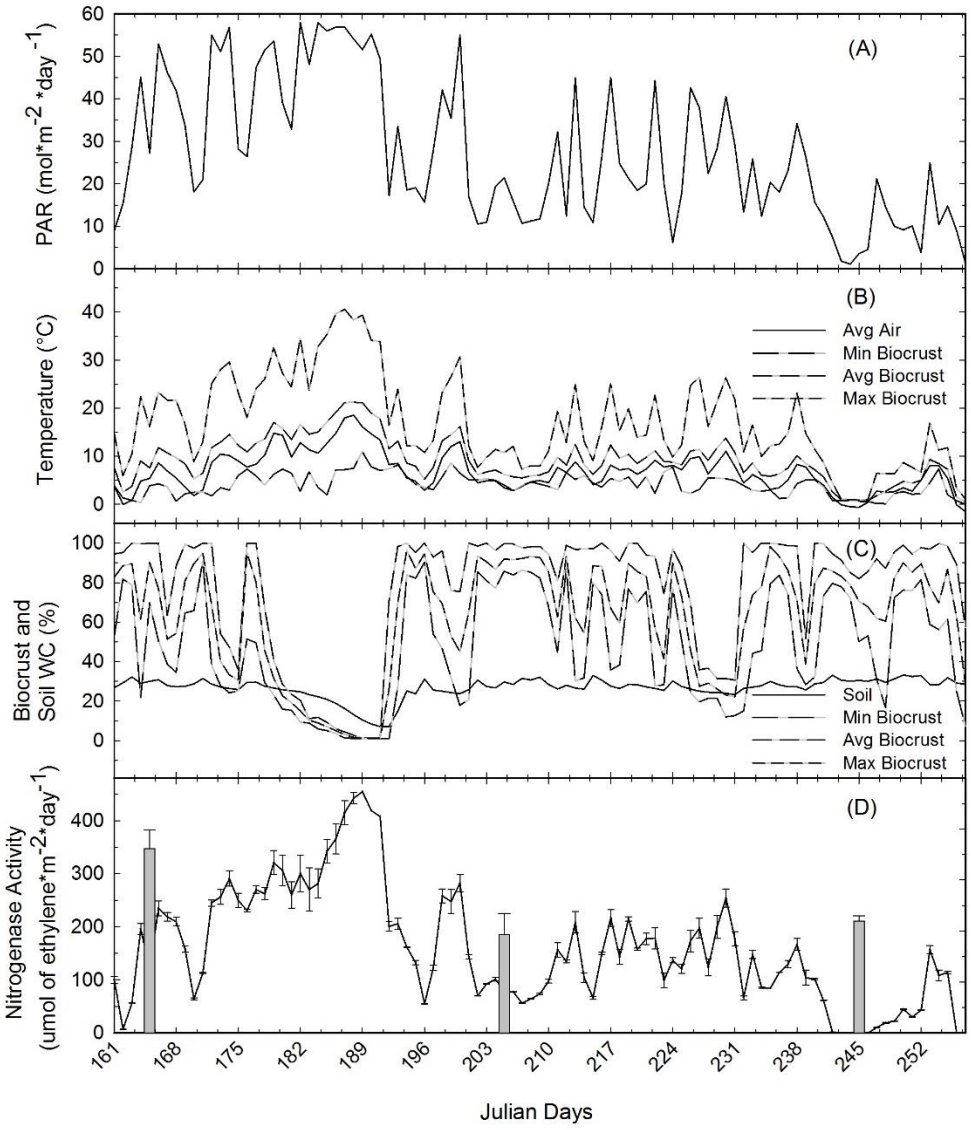



Figure 7. Microclimate data from mature biocrust monitoring station on Andesite Peak over the duration of the experiment, expressed in Julian days. Photosynthetically active radiation (PAR; mol $m^{-2}$ $day^{-1}$) (A). Minimum, average, and maximum biocrust temperatures (°C; long, medium, and short-dash respectively) (B). Average soil volumetric water content (%; solid line), as well as the minimum, average, and maximum biocrust relative water content (%, long, medium, and short dashed lines respectively) (C). Modelled estimation of nitrogenase activity (µmol of ethylene $m^{-2}$ $day^{-1}$) with error bars representing standard error for each daily record (D). The three bars represent measured nitrogenase activity under optimal conditions in mature biocrusts at three time points over the summer. ) The detection limit for acetylene reduction was 0.2 µmol ethylene $m^{-2}hr^{-1}$.

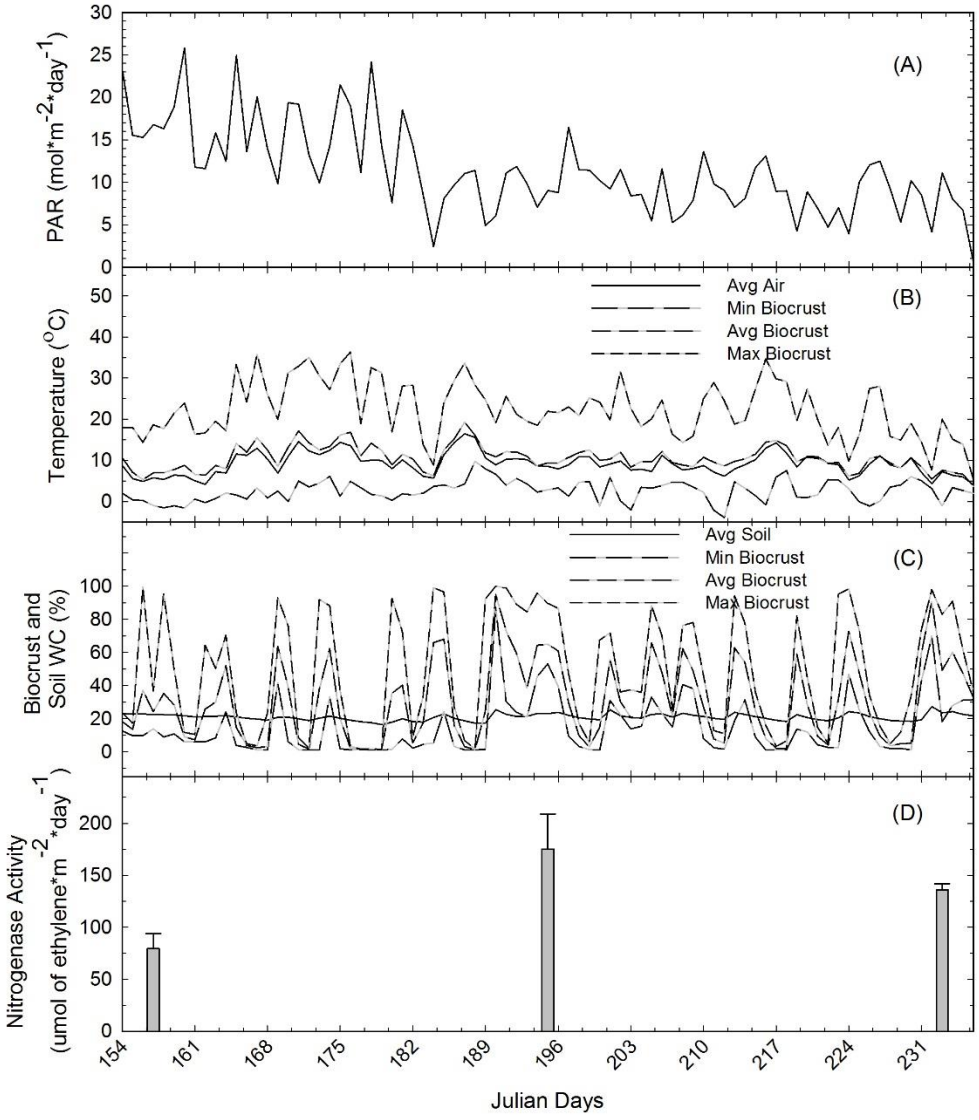



Figure 8. Microclimate data from mature biocrust monitoring station on Mt McIntyre over the duration of the experiment, expressed in Julian days. Photosynthetically active radiation (PAR, mol m-2 day-1) (A). Average air temperature (°C, solid line) and the minimum, average, and maximum biocrust temperatures (°C; long, medium and short-dashed lines respectively) (B). Average soil volumetric water content (%; solid line) as well as the minimum, average, and maximum biocrust relative water content (%; long, medium, and short-dashed lines respectively) (C). Nitrogenase activity under optimal conditions in mature biocrust at three time points over the summer (D). ) The detection limit for acetylene reduction was 0.2 µmol ethylene $m^{-2}hr^{-1}$.

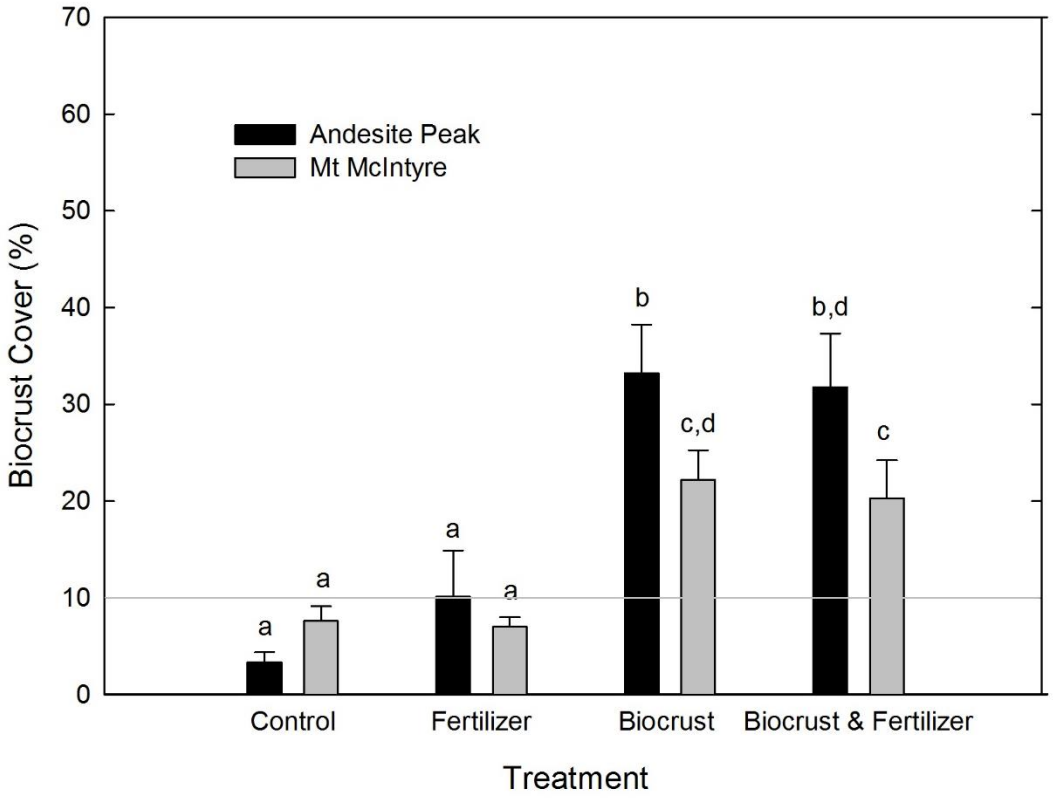



Figure 9. Biocrust cover 12 weeks following inoculation. The horizontal line represents the initial inoculation level of 10% biocrust (surface area based). At both Andesite (black bars) and Mt McIntyre (grey bars), plots inoculated with biocrust (Biocrust and Biocrust & Fertilizer) had a significantly higher biocrust cover than uninoculated plots (Control and Fertilizer). Different letters indicate significant differences. Bars represent mean with standard error.

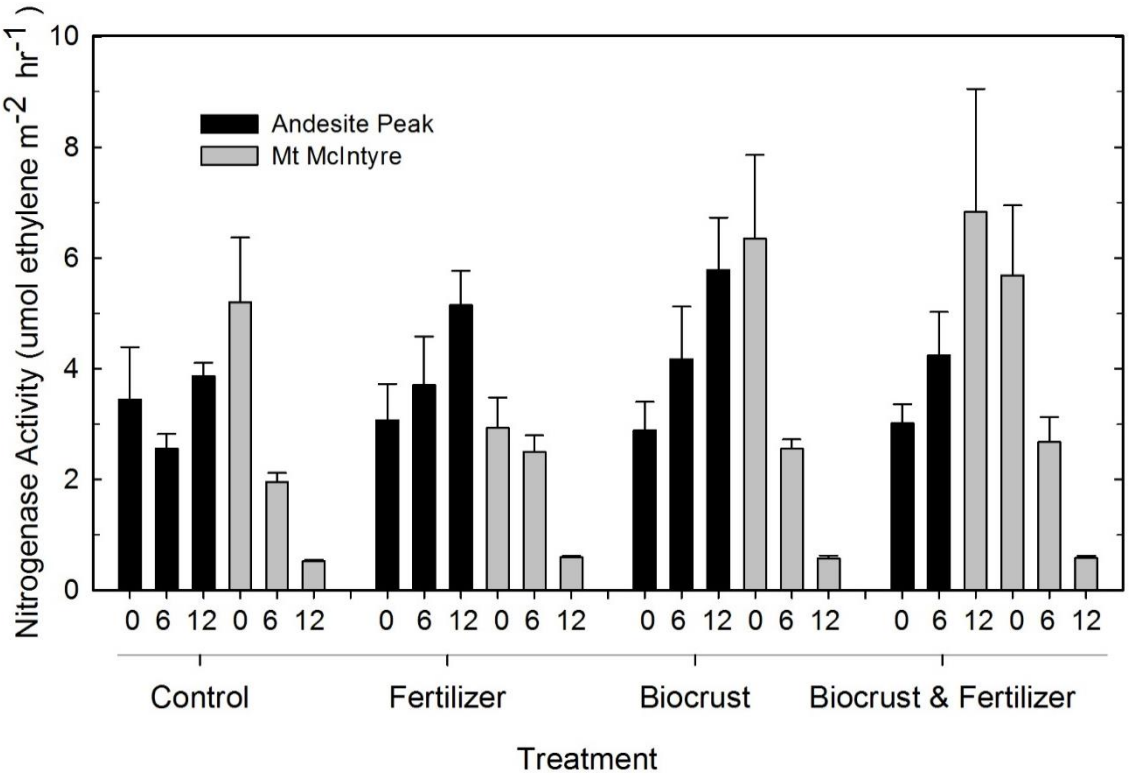



Figure 10. Acetylene reduced 0, 6, and 12 weeks following inoculation with biocrust at Andesite (black bars) and at Mt McIntyre (grey bars). Acetylene reduction as a measure of nitrogen fixation increased at Andesite and decreased at Mt McIntyre over the 12 weeks. Bars represent mean with standard error.) The detection limit for acetylene reduction was 0.2 µmol ethylene $m^{-2}hr^{-1}$.

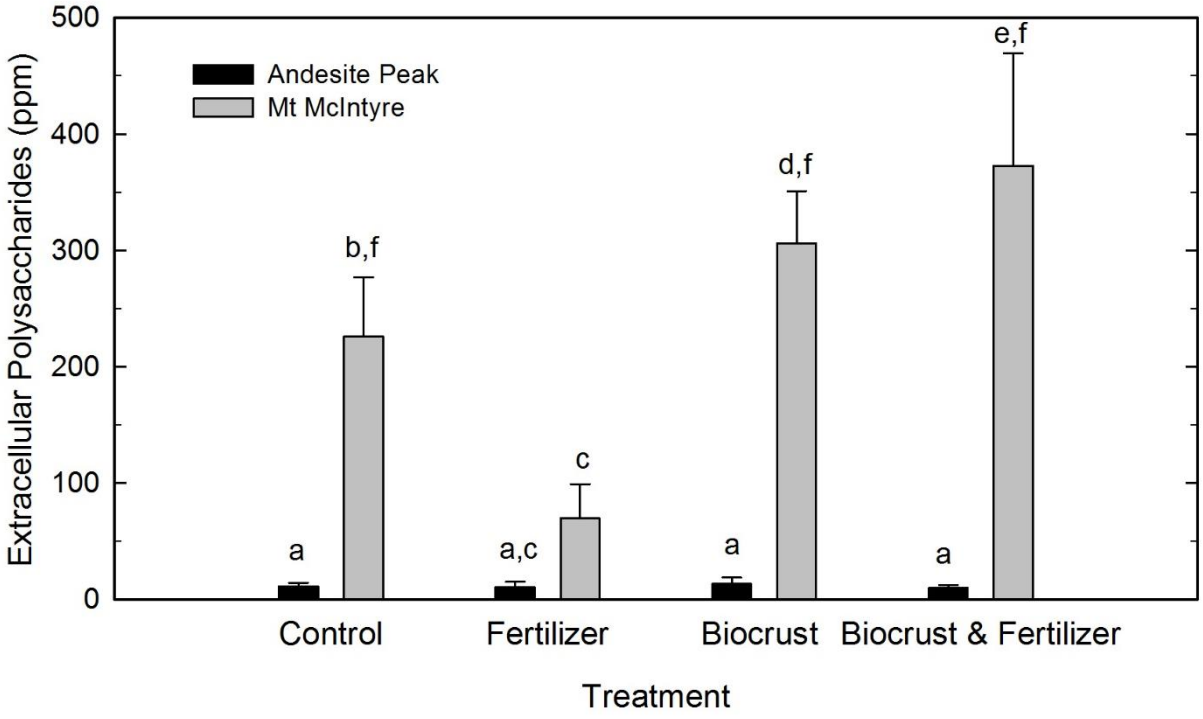



Figure 11. Extracellular polysaccharide content (EPS) of soil surface 12 weeks following inoculation at Andesite (black bars) and at Mt McIntyre (grey bars). Overall, EPS was significantly lower at Andesite than at Mt McIntyre. Different letters indicate significant differences. Bars represent means with standard error.

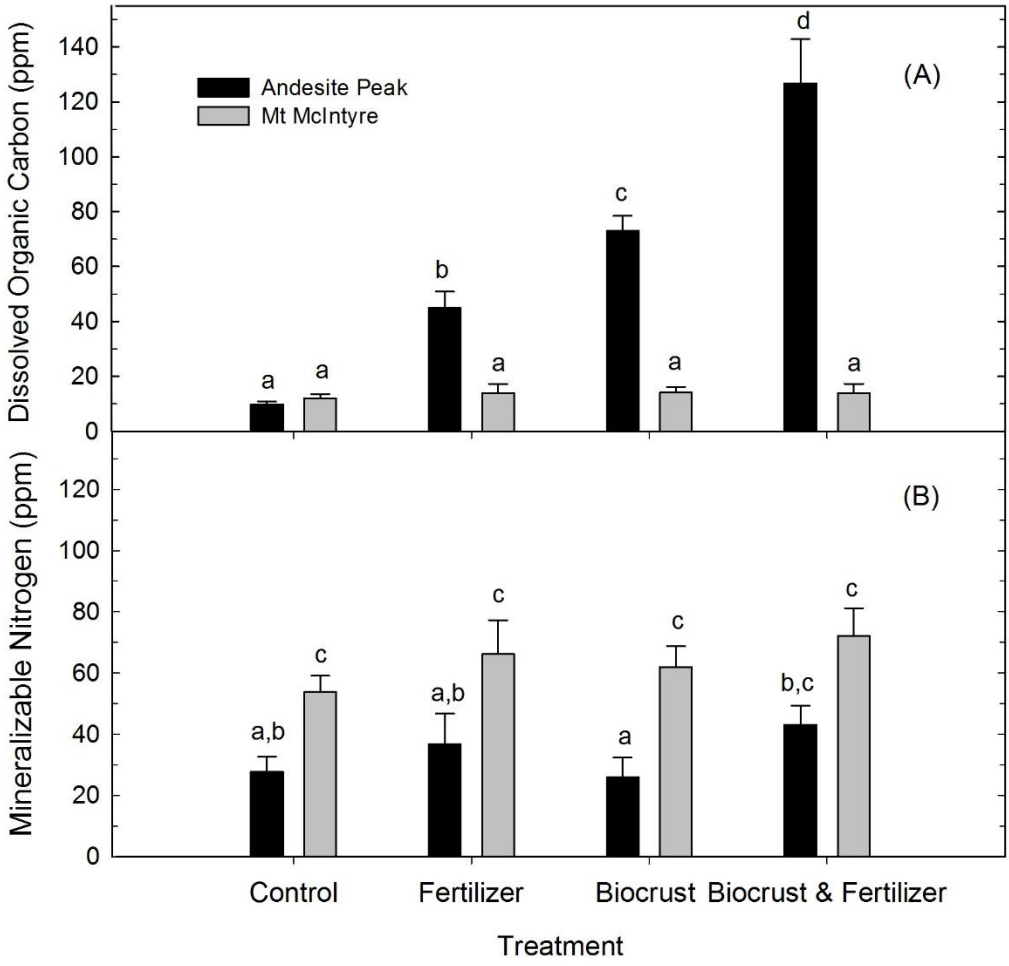
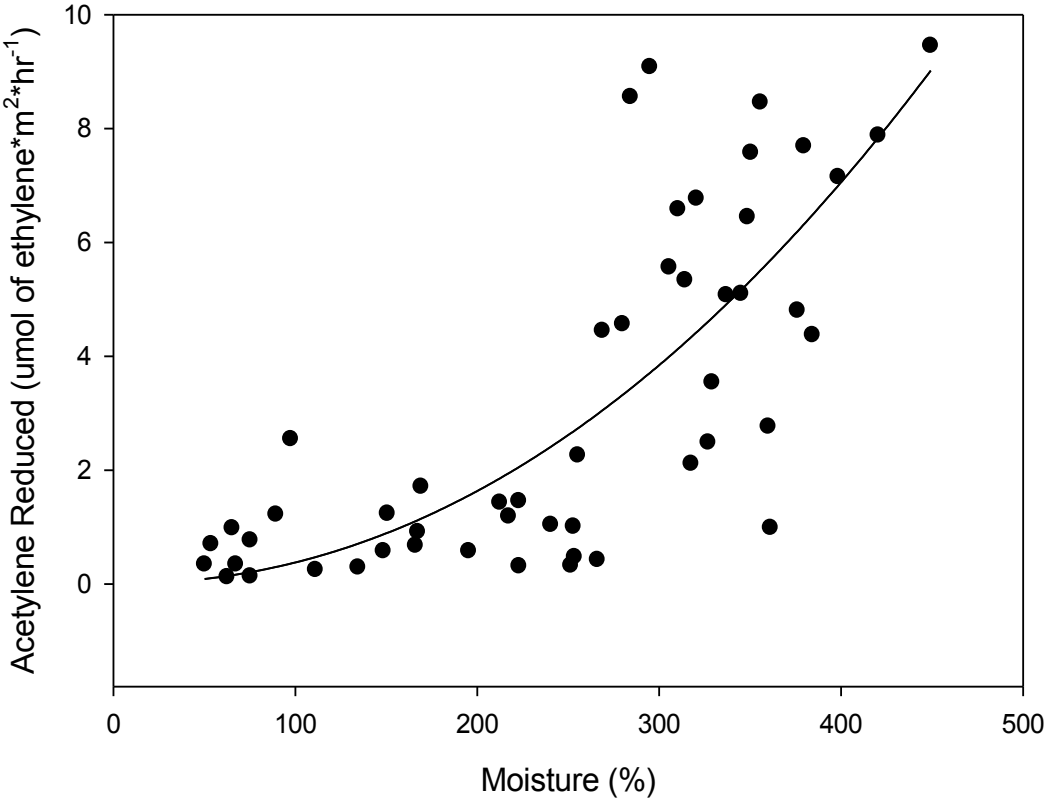
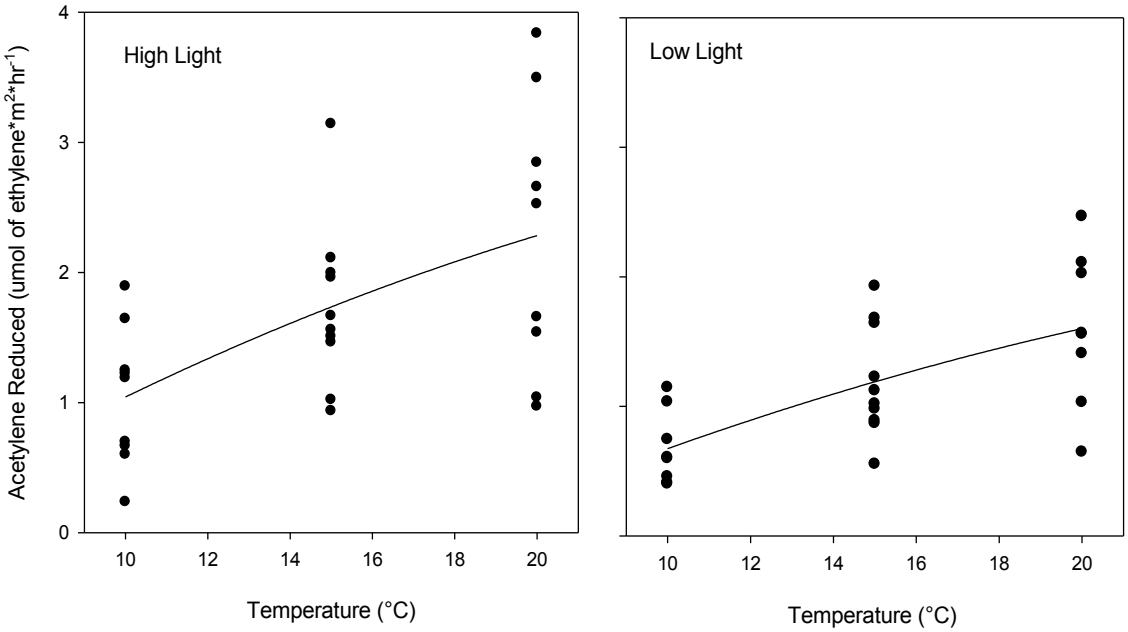



Figure 12. Soil properties 12 weeks following inoculation at Andesite (black bars) and at Mt McIntyre (grey bars). Dissolved organic carbon (A) was higher in inoculated plots (Biocrust and Biocrust & Fertilizer) at Andesite. Mineralizable nitrogen (B) was higher at Mt McIntyre than at Andesite but not significantly different between treatments. Different letters indicate significant differences. Bars represent means with standard error.




Supplementary Figure 1.

Acetylene reduced by mature biocrust as a function of gravimetric water content. The adjusted $R^2$ for the regression model is 0.59, $p<0.05$. ) The detection limit for acetylene reduction was 0.2 µmol ethylene $m^{-2}hr^{-1}$.




Supplementary Figure 2. Acetylene Reduction of mature biocrust under high light (left frame) and low light (right frame) as a function of incubation temperature. Adjusted $R^2$ are 0.42 and 0.39 for the high and low light regression models respectively, $p<0.05$). ) The detection limit for acetylene reduction was 0.2 µmol ethylene $m^{-2}hr^{-1}$.