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Nitrogen Fixation by Biological Soil Crusts and Their Communities: Climatic and Grazing Controls

by

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A thesis

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To the Graduate Faculty:

The members of the committee appointed to examine the thesis of Stacy Gayle Schwabedissen find it satisfactory and recommend that it be accepted.

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NITROGEN FIXATION BY BIOLOGICAL SOIL CRUSTS AND THEIR COMMUNITIES: CLIMATIC AND GRAZING CONTROLS

Thesis Abstract-Idaho State University (2016)

Biological soil crusts (biocrusts) cover the soil surface of drylands worldwide. These communities provide essential nutrients of carbon and nitrogen through fixation, while also providing soil stability. Although there have been many studies examining climate change and disturbance factors on biocrusts, studies examining these factors on rolling biocrusts typically found in the more mesic cold deserts of the Intermountain West, USA is underrepresented and largely unknown. It is projected climatic changes will result in increasing temperatures and shifts in precipitation phases, yet it is unclear how rates of N_2 fixation and the bacterial communities that facilitate this process will respond, especially at elevations that are snow-dominated. Furthermore, it is unclear how N_2 fixation and N_2-fixing bacterial communities of rolling biocrusts will respond to disturbance (grazing). This study examined rates of N_2 fixation and N_2-fixing bacterial communities associated with rolling biocrusts along a climatic gradient in southwestern Idaho, USA, and tested how measured rates of N_2 fixation and these communities are affected by climate, grazing (exclosures), and shrub-canopy association. Climate affected rates of N_2 fixation and bacterial communities; elevation increased as N_2 fixation decreased, with the greatest rates occurring in the spring, when water is less limiting than in late summer/autumn. We detected dramatic shifts in bacterial communities with cyanobacteria dominating the warmer, drier climates and symbiotic N_2-fixers dominating the cooler, wetter climates. A unique dominate genus in cold desert sagebrush steppe ecosystems, *Oscillatoria* was the dominant cyanobacterium. Grazing did not affect N_2 fixation; however, grazing did slightly alter the N_2-fixing bacterial communities,

particularly decreasing cyanobacterial abundance at the warmer, drier climates, although it was statistically insignificant. The shrub-canopy had significantly higher rates of N_2 fixation than the interspace, but the N_2-fixing communities were not significantly different; however, there were greater symbiotic N_2-fixing bacteria in the shrub-canopy and cyanobacteria within the interspace. These findings indicate that controls on N_2 fixation vary seasonally and strongly with climate, and the bacterial community is more sensitive to climatic changes while grazing and the shrub-canopy can alter the N_2-fixing bacterial community.

General Introduction

There have been great advances in understanding the structure, function, and community composition of biological soil crusts (biocrusts), yet despite these advances in understanding of N_2 fixation by biocrusts, a systematic examination of climatic and disturbance controls on biocrusts communities and free-living N_2 fixation is lacking. The majority of biocrust studies have been on pinnacled crusts (Yeager et al. 2004; Belnap 2003; Belnap & Lange 2003), but less is known of how N_2 fixation by biocrusts vary in cold desert ecosystems, which have greater amounts of precipitation to support rolling crusts, and how climatic and disturbance controls will alter the N_2-fixing bacterial communities associated with them. In addition, no studies to my knowledge have examined the disturbance of rangeland practices (cattle grazing, road and foot compaction, dust, and recreation impact) via the use of long-term (>40 yr free of grazing) exclosures. Finally, studies generally examine only specific biocrusts such as dark and light rather than taking an ecosystem approach without looking for specific crusts.

This research examines the diversity of N_2-fixing bacteria community of biocrusts in the Reynolds Creek Critical Zone Observatory and utilizes a climatic gradient with grazed and long term exclosures to examine the ecological and disturbance controls of N_2 fixation of biocrusts. I ask the following questions: (1) How does N_2 fixation by biocrusts vary as a function of climate?; (2) How does disturbance in the form of rangeland management practices affect N_2 fixation?; (3) Are there differences between under-shrub canopy and interplant space free-living N_2 fixation rates?; and (4) How will the N_2-fixing bacterial community composition in the biocrust change with climate, disturbance, and shrub canopy?

Chapter 1 gives a brief review of the current knowledge on biocrusts regarding community composition, and the controls of temperature, moisture, carbon stores, and disturbance on N_2 fixation. There have been other more extensive reviews on the topics such as those by Reed et al. (2011) and Belnap & Lange (2003). Chapter 2 examines the controls of climate (temperature and moisture) and disturbance (cattle grazing) to see what effects they have on free-living N_2 fixation by biocrusts through a climosequence. The comparison of soil characteristics such as pH, electroconductivity, total C, total N, isotopes $\delta^{15}N$ and $\delta^{13}C$, and nutrient pools of available phosphorus, and nitrogen (nitrate + ammonium) on N_2 fixation will also be examined. Chapter 3 focuses on the N_2-fixing bacteria found in the biocrusts along the climatic gradient by 16S small subunit rRNA gene V3-V4 gene region paired-end sequencing. It addresses patterns in the identified genera within the phylum *Cyanobacteria* and other free-living N_2-fixing genera of *Azospirillum, Beijerinckia, Azotobacter, Clostridium,* and *Chlorobium.* To get a better idea of how N_2 fixation changes with elevation, we will also look at symbiotic N_2-fixing bacteria of the genera *Azorihizobium, Bradyrhizobium, Mesorhizobium, Rhizobium, Sinorhizobium,* and *Frankia.*

CHAPTER I: LITERATURE REVIEW

Nitrogen (N), next to water, is believed to be the main limiting resource for growth for the majority of organisms in many ecosystems (Yeager et al. 2004; Martinez-Espinosa et al. 2011; Strauss et al. 2012). Reduced N as ammonium is incorporated into amino acids, nucleic acids, and proteins, and therefore its availability can affect many processes on an ecosystem scale such as primary production and decomposition (Martinez-Espinosa et al. 2011; Fowler et al. 2014). In this review, I examine the distribution and environmental controls on biological soil crusts and their microbial communities as well as their role in the N cycle in dryland environments.

The N cycle (Fig. 1) is very complex in that it incorporates multitudes of organisms and various forms of N. The processes of dinitrogen (N_2) fixation and nitrate (NO_3^-) reduction both produce ammonium (NH_4^+) and are assimilatory processes, or anabolism (Martinez-Espinosa et al. 2011). Ammonium can be incorporated directly into carbon skeletons for growth, and attached to soil clay particles at cation exchange sites available for microorganisms and plants to use (Martinez-Espinosa et al. 2011). For plants, NH_4^+ is the main form of N that is assimilated other than NO_3^-, which is primarily produced through the processes of N mineralization and nitrification, respectively (Hart et al. 2011; Martinez-Espinosa et al. 2011). The dissimilatory, or catabolic, processes in the N cycle are nitrification and denitrification, in which ammonium is converted to NO_3^- (nitrification) and then to gaseous forms of N (N_2, NO, or N_2O; denitrification; Martinez-Espinosa et al. 2011). Seventy-eight percent of the atmosphere is N_2, which stays relatively constant because of the N_2 released to the atmosphere, via denitrification, is roughly balanced by the losses from the atmosphere by the process of N_2 fixation and

deposition by lighting and fertilizer production (Galloway et al. 2004; Hart et al. 2011; Martinez-Espinosa et al. 2011). Within an ecosystem, N is mainly stored in dead organic matter which is decomposed and converted into inorganic forms, and is then returned to the soil through animal wastes as NH_4^+ (Martinez-Espinosa et al. 2011).

Biological N_2 fixation is a process that fixes N_2 from the atmosphere into biologically-reactive N as ammonium (NH_4^+), and is limited to select bacteria and archaea (Galloway et al. 2004; Yeager et al. 2007; Reed et al. 2011). This large pool is otherwise inaccessible to many organisms (Galloway et al. 2004; Martinez-Espinosa et al. 2011). N_2 fixation is categorized into symbiotic and asymbiotic (also called free-living) fixation. Although the classification between the two forms is still debated, we will use the following definitions provided by Reed et al. (2011), for symbiotic and free-living N_2 fixation. Symbiotic N_2 fixation is performed as part of symbiotic relationships with plants, such as legumes and their *Rhizobium* symbionts. N_2 fixation rates from symbiotic fixation are usually higher than free-living fixation, with legume-associated rates reported to exceed 150 kg N ha^{-1} yr^{-1} in agricultural fields of alfalfa and soybeans. Other legumes have been shown to fix 30-50 kg N ha^{-1} yr^{-1} (Brady & Weil 2010; Reed et al. 2011). There are about 200 species of nonlegume plants that accommodate bacteria and archaea in developed nodules, which have lower rates of N_2 fixation than legume associated bacteria (Brady & Weil 2010). Free-living N_2 fixation encompasses all other forms of N_2 fixation, which can occur in many climates and surfaces including plants, leaves, fallen litter, decaying wood, and soil (Table 1) (Zahran 1999; Reed et al. 2011). Asymbiotic fixing heterotrophs such as those from *Alphaproteobacteria* and *Gammaproteobacteria* can have a significant influence in grasslands or natural forests

with rates of 5-20 kg N ha^{-1} yr^{-1} (Yeager et al. 2007; Brady & Weil 2010; Reed et al. 2011). Autotrophic bacteria are primarily composed of cyanobacteria, which are usually the most common, and often do not exceed 30 kg N ha^{-1} yr^{-1} in deserts (drylands and cold deserts), but commonly have rates around 4.0 kg N ha^{-1} yr^{-1} (Brady & Weil 2010; Reed et al. 2011).

Drylands encompass hyperarid, semiarid, arid, alpine, and polar region ecosystems and cover over a third of the terrestrial landmass (Kuske et al. 2012). Commonly observed in drylands are biological soil crusts, or biocrusts, which are soil surface community of mosses, lichens, liverworts, algae, and cyanobacteria, and are often the main influx of "new" N (Belnap 2002; Hawkes & Flechtner 2002; Yeager et al. 2007; Strauss et al. 2012). These biocrusts are also referred to as microbiotic, microfloral, organogenic soil crusts, cryptobiotic, microphytic, or cryptogamic crusts, and can reduce erosion of soil particles, decrease losses of carbon (C) and N via runoff, increase moisture retention, promote seedling germination, and decrease surface albedo through excreted polysaccharides and pigments (Eldridge et al. 1994; Belnap 2003; Belnap & Lange 2003, Barger et al. 2006; Kuske et al. 2012). Biocrusts are different than physical or chemical soil crusts, which are primarily formed by the loss of soil aggregates via compaction (raindrops, vehicles, or animal trampling), evaporation, and trapped gasses (Belnap & Lange 2003). Not only are physical or chemical crusts different in structure, their effects on the system are viewed as adverse because they inhibit vegetation seedling establishment, decrease water infiltration, increase runoff by sealing and smoothing the soil surface, and are difficult to remove (Belnap & Lange 2003).

There are four morphological types of biocrusts that vary with climatic conditions: (1) smooth; (2) rugose; (3) pinnacled; and (4) rolling (Belnap & Lange 2003; Yeager et al. 2004). Smooth or flat biocrusts are dominated by cyanobacteria and occur where soils do not freeze. These climates are classified as hyperarid or arid such as northwestern Australia, central Sahara Desert, the Arava Valley and Nizzana dunes in Iseael, and the Atacama Desert in Chile. Rugose biocrusts are dominated by cyanobacteria, and have limited lichens and mosses. Rugose biocrusts are found in arid and semiarid regions such as the Sonoran and Mojave Deserts and the Central Negev in Israel; however, smooth or rugose crusts can be found in disturbed regions (smooth), and successional (rugose) biocrusts in temperate regions. Pinnacled biocrusts also are dominated by cyanobacteria, but have lichens and mosses in soils that frost heave. These climates are located in mid-latitude, cool-desert regions such as those found on the Colorado Plateau and China. Pinnacled biocrusts are the most susceptible to disturbance because the raised structure can cause surface cyanobacteria to become buried and unable to photosynthesize (Belnap 2003). Rolling crusts have cyanobacteria and the highest amount of lichen and moss in soils that also undergo frost heaving. Rolling biocrusts dominate areas with higher precipitation and cooler regions such as those in the Great Basin, northern Mongolian steppes, and the Arctic (Belnap & Lange 2003; Yeager et al. 2004). Not only does morphology provide information about the biocrusts, color also is associated with inferring maturity of a biocrust, with lighter crusts mainly composed of early-successional cyanobacteria, and gray or almost black crusts are composed of later-successional cyanobacteria, mosses, and multi-colored lichens (including black) as the latest successional stage (Belnap 2002; Belnap & Lange 2003; Strauss et al. 2012).

The amount of N_2 fixed by biocrusts is thought to be determined by the ecological controls on these communities, which include but are not limited to temperature, soil moisture, C stores, community diversity, and disturbance (Belnap 2002; Vitousek et al. 2002; Barger et al. 2005; Yeager et al. 2007; Houlton et al. 2008; Delgado-Baguerizo et al. 2012). N_2 fixation is an enzymatic process; therefore, temperature plays a significant control in when fixation can occur. A study by Belnap (2002) showed active N_2 fixation between 1˚C and 26˚C, yet this range can change slightly depending on climate, such that biocrusts in the Artic have been shown to fix in temperatures as low as -7.6˚C and in Nigeria up to 30˚C (Belnap 2002). Studies examining warming temperatures on biocrusts show decreased cover, especially in those dominated by lichens and mosses (Escolar et al. 2012; Johnson et al. 2012; Ferrenberg et al. 2015; Steven et al. 2015).

Moisture (rainfall, snow melt, dew, or fog) is often considered a main control because cyanobacteria are only active when wet; the moisture threshold varies with species and habitat, ranging from 6% to complete immersion (Belnap 2002). Duration of the moisture affects N_2 fixation with prolonged periods of saturation decreasing rates and too little moisture can result in C starvation (Belnap 2002). Furthermore, C acquired by photosynthesis is vital to the hefty ATP requirements needed for N_2 fixation (Belnap 2002). A study by Johnson et al. (2012) and Ferrenberg et al. (2015) examined the effects of changing precipitation patterns associated with a temperature increase on biocrusts, and indicated that shifts in arid and semiarid ecosystems from snow dominated precipitation at higher elevations to smaller more frequent wetting altered biocrusts dominated by lichens and mosses to primarily cyanobacteria. Moreover, Reed et al. (2012) also found that this shift in precipitation could lead to a substantial decrease in

moss crust cover. This sensitivity to precipitation changes is believed to be because of the lack of vascular tissue or roots in mosses, which may determine particular climates that mosses can survive as precipitation and dew are the principal sources of water (Coe et al. 2012). The inability to tap into underground water may explain the decrease in cover, and therefore a decrease in N_2 fixation rates, found with precipitation shifts (Coe et al. 2012; Delgado-Baquerizo et al. 2012; Johnson et al. 2012; Reed et al. 2012). Furthermore, findings point toward a shift from an NH_4^+ dominated system to NO_3^-, which is easily lost in runoff and leaching and may contribute to greater N scarcity (Brady & Weil 2010; Johnson et al. 2012; Reed et al. 2012; Ferrenberg et al. 2015).

The communities associated with biocrusts are also considered to be strong determinants of the amount of N_2 fixed (Belnap 2002; Neff et al. 2005; Zhao et al. 2010), especially focusing on the major constituents of cyanobacteria, lichens, and mosses. Few of the factors that control the genera or species of cyanobacteria and lichens are often soil pH and chemistry (Belnap & Lange 2003). More alkaline, high salt, low precipitation soils tend to contain greater amounts of cyanobacteria, whereas lichens are found across various pH levels (Belnap & Lange 2003). An examination of the soil characteristics with community structure determined that well developed lichens favor higher sodium, and cover of biocrusts increased with higher pH and electrical conductivity (Ponzetti & McCune 2001). Studies have examined the community of cyanobacteria within biocrusts, and indicate that the cyanobacteria *Microcoleus vaginatus* is largely the first bacteria to initiate a biocrust formation (Belnap 2002; Belnap 2003). The biocrust formation is then followed by various stages of other cyanobacteria, lichens, and mosses to form mature crusts (Belnap 2002; Belnap 2003; Belnap & Lange 2003; Kuske et al. 2012; Rejeev et

al. 2013). Major cyanobacteria that have been found in association with biocrusts in North America biocrusts are those from the genera: *Nostoc; Schizothrix; Anabaena; Plectonema; Lyngbya; Porphyrosiphon; Oscillatoria; Scytonema; Phormidium; Leptolyngbya; Calothrix; Tolypothrix; Gleothece; Spirirestis; Chroococcus; Chroococcidiopsis;* and *Microcoleus* (Belnap & Lange 2003; Yeager et al. 2004; Yeager et al. 2007). Other N_2-fixing bacteria include representatives from the phyla *Proteobacteria, Actinobacteria, Chlorobi,* and *Firmicutes* and the genera of *Azotobacter, Azospirillum, Beijerinckia, Chlorobium* and *Clostridium* (Yeager et al. 2007). Those that have been associated with disturbance gradients are *Microcoleus, Nostoc, Syctonema, Phormidium, Porphyrosiphon,* and *Calothrix* (Jimenez Aguilar et al. 2009).

Lichens in biocrusts are often the association of late-successional cyanobacteria and fungi, and are found in mature crusts (Belnap & Lange 2003). Common genera that have been found in North American biocrusts are: *Catapyrenium; Collema; Acarospora; Heppia; Peltula; Desmazieria; Psora; Fulgensia; Toninia; Lecidea; Aspicillia; Candelariella; Placynthiella; Lepraria; Leptochidium; Leptogium; Massalongia; Ochrolechia; Physconia; Psorotichia; Peltigera; Acarospora; Pannaria; Trapeliopsis;* and *Texosporium* (Belnap & Lange 2003). It is hypothesized that fungal mycelia from biocrusts may link to other biocrusts and vegetation, therefore shuttling nutrient exchange between the systems (Bates et al. 2010).

Mosses that are established in biocrusts can dominate wetter (shrub or plant canopy) or drier (interspace between shrubs or plants) microclimates dependent on the species sensitivity to changes in precipitation and irradiances (Gómez et al. 2012; Coe et al. 2014). In North America common genera of mosses include: *Bryum; Crossidium;*

Totrula; Pterygoneurum; Syntrichia; Didymodon; Microbryum; Weissia; Pseudocrossidium; Ceratodon; and *Funaria* (Belnap & Lange 2003).

Although these are common genera in North America, certain species may be local or regional, and universal species such as the cyanobacteria *Calothrix parietina, Microcoleus vaginitus, Nostoc commune, Schizothrix calcicola, Scytonema hofmannii,* and *Tolypothrix tenuis* and found in nearly all studies (Belnap & Lange 2003). Ubiquitous species of lichen include *Collema tenax, Collema coccophorum, Catapyrnium squamulosum, Heppia lutosa, Psora decipiens,* and *Toninia sedifoia,* whereas mosses include *Bryum argenteum* and *Pterygoneurum ovatum* (Belnap & Lange 2003). Worldwide the common genera of (1) cyanobacteria, (2) lichen, and (3) mosses are (1) *Microcoleus; Nostoc; Oscillatoria; Lyngbya; Phormidium; Scytonema;* and *Tolypothris;* (2) *Fulgensia; Psora; Catapyrenium;* and *Toninia;* (3) *Bryum; Crossidium;* and *Tortula* (Belnap & Lange 2003).

In North America it has been documented that grazing has adverse effects on arid and semiarid ecosystems (Jones 2000; Chamizo et al. 2012; Concostina-Zubiri et al. 2014). The resulting compaction from grazing can lead to changes in soil physical structure and functional properties (Jones 2000). Grazing and human recreation disturbances can alter the community and physical structure of biocrusts, and therefore N-cycling within these drylands (Belnap 2002; Belnap 2003; Yeager et al. 2004; Gómez et al. 2012; Ferrenberg et al. 2015). Furthermore, grazing not only decreases biocrust cover, but also decreases the cover of grasses and shrubs (Jones 2000; Belnap & Lange 2003). This decrease in vascular plant cover can allow for increased biocrust cover upon the removal of grazing, because of quicker biocrust recovery in interspaces between

plants, but come at a cost with initially increasing erosion and decreasing water infiltration, C, and N (Belnap & Lange 2003). Moreover, the decrease in vascular plant cover also can lead to the invasion of certain plant species such as *Bromus tectorum*, which has been found to establish quicker on disturbed biocrusts (Belnap & Lange 2003). Biocrusts are very sensitive to disturbance when dry; therefore, timing of grazing can vary severity (Belnap & Lange 2003; Muscha & Hild 2006; Concostrina-Zubiri et al. 2014). It has been proposed and observed that grazing in early winter or when the soil is frozen will have little effect on biocrust cover (Belnap & Lange 2003; Muscha & Hild 2006). If grazing occurs in dry periods such as summer and late winter, biocrust cover and richness is greatly reduced (Kaltenecker et al. 1999; Belnap & Lange 2003; Muscha & Hild 2006). Furthermore, continuous grazing has greater implications than seasonal grazing (Concostrina-Zubiri et al. 2014). The intensity of grazing also can affect biocrust cover and richness, with light grazing showing no significant difference in cover and heavy grazing a large decline (Belnap & Lange 2003).

Not only does the cover of biocrusts and vegetation decrease with disturbance, but the infiltration of water can decrease depending on the severity of disturbance and maturity of the biocrust (Chamizo et al. 2012). Chamizo et al. (2012) observed that trampling, especially in previously non-grazed, areas had decreased the water infiltration. Further findings also indicate biocrusts dominated by mosses had the greatest water infiltration rate, which is alarming when biocrusts dominated by mosses are most sensitive to disturbance compared with lichens and cyanobacteria (Belnap & Lange 2003; Chamizo et al. 2012). Mosses are commonly found in shaded areas under shrubs, and studies observing their time to recover are conflicted (Muscha & Hild 2006; Gómez et al.

2012). Concostrina-Zubiri et al. (2014) observed mosses with rapid recovery, which they suggest is because of mosses ability to recolonize from fragments, and their use of low light availability. However, a study by Gómez et al. (2012) observed moss recovery to be slower than lichens, and mosses were only observed in the protection of the shrub canopy, suggesting full recovery has not taken place or other constraints. Differences observed are likely because of variations in climates, disturbance severity, and soil characteristics.

Disturbance of biocrusts can decrease N_2 fixation rates between 60 to 100% (Belnap 2002; Belnap & Lange 2003). This decrease in N_2 fixation occurs because mature crusts that are dominated by lichens and mosses cannot withstand the forces of trampling and compaction, and revert back to the cyanobacteria dominated biocrusts (Belnap & Lange 2003; Belnap 2003). Early biocrusts that are dominated by *Microcoleus* fix about 1 kg N ha^{-1} yr^{-1}, yet mature lichen dominated biocrusts can fix between 6 to 9 kg N ha^{-1} yr^{-1} (Neff et al. 2005). Ferrenberg et al. (2015) recorded similar outcomes of disturbance and climate change with mature lichen and moss dominated biocrusts shifting to early cyanobacterial biocrusts. Implications suggest decreased C and N_2 fixation rates, greater loss of C and N via runoff, and increased dust production, which can have strong influences of ecosystem processes (Ferrenberg et al. 2015).

Biocrusts within drylands are central to bringing in "new" N, and aiding in nutrient cycling, reducing erosion, and increase water infiltration (Belnap & Lange 2003). The process of N_2 fixation has many controls, but primarily is controlled by climate (temperature and moisture), C stores, community composition, and disturbance (Belnap

2002; Vitousek et al. 2002; Barger et al. 2005; Yeager et al. 2007; Houlton et al. 2008; Delgado-Baguerizo et al. 2012).

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CHAPTER II: LIMITS OF NITROGEN FIXATION BY ROLLING BIOLOGICAL SOIL CRUSTS IN A COLD DESERT SAGEBRUSH STEPPE

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ABSTRACT

In drylands worldwide, biological soil crusts (biocrusts) form a thin photosynthetic cover across landscapes, and provide vital benefits to these ecosystems in terms of stabilizing soil and fixing nitrogen (N) and carbon. Numerous studies have examined the effects of climate and disturbance (grazing) on biocrusts; however, few have assessed the rolling biocrusts typical of the Intermountain West, USA. With projected temperature increases and shifts in precipitation, it is unclear how biocrusts in this region will respond to climate change, and how their response could affect their capacity to perform key ecosystem functions such as providing 'new' N through biological N_2 fixation. To address this important knowledge gap, we examined nitrogenase activity associated with rolling biocrusts along a climatic gradient in southwestern Idaho, USA, and quantified how N_2 fixation rates change as a function of climate, grazing (exclosures), and shrub-canopy association. Our findings indicate that warmer, drier climates at lower elevations host greater biocrust surface cover and higher N_2 fixation rates compared with colder, wetter climates at higher elevations. The greatest N_2 fixation rates (0.5–29.3 μmol C_2H_4 m^{-2} h^{-1}) occurred during the spring, when water was less limiting than in late summer/autumn. Interestingly, patterns of N_2 fixation rates were strongly associated with soil pH and NH_4^+ concentrations, identifying these soil characteristics as strong controls on the process of biocrust N_2 fixation. We observed no significant differences in N_2

fixation rates with grazing, but biocrust N_2 fixation rates within the shrub-canopy were two to three times higher compared to biocrust N_2 fixation in the interspace between plants. Taken together, results indicate that the controls and rates of N_2 fixation by rolling biocrusts vary seasonally and strongly with climate in the Intermountain West, and that drier springs are likely to influence rates of N_2 fixation more than warmer summers.

INTRODUCTION

Nitrogen (N), after water, is commonly considered the resource most limiting to biota in drylands (Hooper & Johnson 1999; Yeager et al. 2007; Strauss et al. 2012), which span arid to dry sub-humid environments and make up ~40% of the terrestrial landmass (Belnap & Lange 2003; Reynolds et al. 2007; Kuske et al. 2012). Biological soil crusts (biocrusts) are commonly found in these environments within the interspace among vascular plats or beneath the canopies of grasses and shrubs. Biocrusts are a soil surface community primarily composed of cyanobacteria, mosses, and lichens. These communities provide vital functions to drylands, such as bringing 'new' N and carbon (C) into the ecosystem via the process of biological N_2 fixation and photosynthesis, respectively (Belnap & Lange 2003; Belnap 2003). Indeed, biocrusts may alleviate limitations to productivity via this supply of reactive N and the subsequent increase in nutrient availability (Belnap & Lange 2003; Belnap 2003; Hawkes 2003; Reed et al. 2007). Biocrusts can also indirectly affect nutrient availability to plants by reducing nutrient loss in runoff (Barger et al. 2006), reducing soil erosion from wind (Belnap 2003; Belnap & Lange 2003), enhancing establishment of vascular plants (Belnap 2003; Coe et al. 2014), lowering surface albedo by the excretion of UV protective compounds (Belnap & Lange 2003; Kuske et al. 2012), and regulating gaseous N losses (Barger et al.

2013; Weber et al. 2015). Despite their importance in drylands, the role of biocrusts bringing in new N into the ecosystem remains poorly characterized and understood across dryland conditions, especially for those biocrusts found in the more mesic and cooler semiarid regions of the Intermountain West, USA.

Most of our current knowledge of rates and controls on arid and semiarid biocrust N_2 fixation is limited to rugose and pinnacled crusts such as those on the Colorado Plateau, USA (Belnap 1996; Belnap 2002). Biocrusts, however, exhibit other morphologies, such as "rolling", a morphology more typically present in environments with higher amounts of precipitation and colder temperatures (Belnap & Lange 2003; Yeager et al. 2007). Each morphology can represent different abundances of lichen, moss, and cyanobacteria, and this community composition helps determine how much N_2 fixation can occur (Belnap 2002; Zhao et al. 2010; Barger et al. 2013). For example, mature lichen-dominated pinnacled biocrusts have been found to fix between 6 and 9 kg of N ha^{-1} yr^{-1}, whereas, early biocrusts with an increased abundance of *Microcoleus* fix about 1 kg of N ha^{-1} yr^{-1} (Neff et al. 2005). In addition to community, in pinnacled biocrusts N_2-fixing activity is thought to be strongly regulated by climate, with moisture and temperature controlling enzyme kinetics and the activity of soil organisms (Belnap 2002; Belnap 2003). Supply of other resources, such as C and phosphorus (P), are also known to help determine overall soil N_2 fixation rates (e.g., Reed et al. 2007). The process of N_2 fixation requires large amount of energy to break the triple bond in atmospheric N_2, and is completed by the enzyme nitrogenase (Reed et al. 2011). Carbon and P availability are thought to set energy constraints on the process of N_2 fixation and thus help determine biocrust N_2 fixation (Belnap 2002; Reed et al. 2011). Disturbance can

also play important roles in N_2 fixation rates (Belnap 2002; Belnap & Lange 2003; Yeager et al. 2004; Kuske et al. 2012). For example, studies have shown that disturbance from human recreation, vehicles, and livestock grazing can be detrimental to biocrusts, primarily when they are dry, by burying organisms that need the light to photosynthesize (Belnap 2003; Belnap & Lange 2003). Disturbance also can disrupt lichen and moss structure resulting in decreased biocrust cover (Belnap 2003; Belnap & Lange 2003). Indeed, studies document that disturbance of mature crust can result in a decrease in N_2 fixation by 60% or more (Belnap 2002; Belnap & Lange 2003), with important implications for biogeochemical cycling and primary production (Gómez et al. 2012). Recovery of biocrusts can take a few years to centuries, depending on the severity of disturbance, climate, and soil characteristics (Belnap and Lange 2003; Gómez et al. 2012). While our understanding of N_2 fixation in pinnacled biocrusts has made notable advances, nevertheless, we maintain a surprisingly poor understanding of the rolling biocrusts in the Intermountain West, although these biocrusts also perform critical ecosystem functions. To our knowledge, the few studies examining rolling biocrusts have focused on cover and identification of lichen and moss components with no recorded rates of N_2 fixation or assessment of its controls (Kaltenecker & Wicklow-Howard 1994; Kaltenecker et al. 1999).

Colder, more mesic drylands in the Intermountain West of the United States are undergoing directional climate changes with increases in temperature and shifts in precipitation (Nayak et al. 2010; Seyfried et al. 2011). Long-term studies across the Reynolds Creek Experimental Watershed, a semiarid rangeland watershed representative of the Intermountain West, documents significant trends of increasing temperatures,

especially minimum temperatures (Nayak et al. 2010). Maximum snow water equivalent and proportion of snow to rain have declined at all elevations with the largest and most significant declines at mid and lower elevations (Nayak et al. 2010). These changes in phase of precipitation and amounts as well as increasing temperatures will likely alter rates of biological N_2 fixation by biocrusts. Studies examining the responses of pinnacled biocrusts to experimental changes in precipitation and temperature show a strong decrease in cover of biocrusts (Reed et al. 2012; Steven et al. 2015; Ferrenberg et al. 2015), suggesting that N_2 fixation rates will also decline.

In this study, we examine how climate and disturbance factors influence N_2 fixation rates inrolling biocrusts by measuring nitrogenase activity (NA) along a climatic gradient in southwestern Idaho, USA. We posed the three following hypotheses: (1) N_2 fixation rates will change along the climatic gradient with the prediction that middle elevations will exhibit the highest rates of N_2 fixation due to optimal temperature and moisture conditions; (2) disturbance, in the form of grazing, will result in changes to N_2 fixation rates with the prediction of higher rates of N_2 fixation in experimental exclosures, because of the lack of grazing; (3) N_2 fixation rates will vary between the interspace between vascular plants and the shrub-canopy with the prediction of higher rates in the interspace, because of higher cover of lichen.

MATERIALS AND METHODS

Study Area & Experimental Design

We conducted this study at the Reynolds Creek Critical Zone Observatory (RC CZO) located in the Owyhee Range in southwestern Idaho near Murphy, ID, USA (Fig. 2). Reynolds Creek is a 239 km^2 watershed that has variable elevation and lithology with

several environments typical of the Intermountain West, US. The land is primary used for cattle grazing with little agriculture. Rain is the dominant form of precipitation at the lower elevations, whereas snow is the dominant form at higher elevations. In addition, at the lower elevations evaporation exceeds precipitation for half of the year, whereas at the higher elevations precipitation exceeds evaporation during most of the year (Fig. 3).

Four sites were established along an elevation gradient (climosequence) within the CZO (Fig. 2; Table 2). From low to high elevations, mean annual precipitation ranged from 235 to 803 mm, and mean annual temperature varied from 9.1 to 5.4˚C. Site selection was based on the location of the CZO core sites, which are areas with sufficient fetch and area for eddy covariance towers, and other instrumentation, and which is within the vicinity of historic climate stations, and grazing exclosures (>40 yr without grazing). Sagebrush (*Artemisia* spp.) was the dominant shrub species across the elevation gradient, but subspecies of sagebrush co-varied with elevation (Table 2). Variation in other soil forming state factors (Jenny et al. 1941), such as parent material, time, and topography, was minimized in selection of sites. For example, parent material at all sites is volcanic basalt, and topography at each site is relatively flat (<5% slope). The four sites from lowest to highest in elevation are Flats (F), Wyoming Big Sage (WBS), Low Sage (LS), and Mountain Big Sage (MBS).

Sampling Design

Five randomly, stratified plots had been established as part of the CZO at the four sites around the circumference of the CZO core location, which included an eddy covariance tower, soil moisture and temperature probes, sap flux data collection, and the exclusion of destructive sampling. At each site, biocrusts samples from outside the exclosures were

collected from paired interspace and shrub-canopy habitats from five randomly selected shrubs within each plot (for a total of 10 samples per site). Within the large exclosures, at each site a 50 m transect was laid and biocrust samples were collected from five randomly selected paired shrub and interspace locations. This resulted in 20 biocrust samples per site, for a total of 80 samples along the elevation gradient. Biocrusts were sampled by marking the collection area with a 10 x 10 cm metal square, and were excised with a spatula and soil knife to a depth of 2.5 cm. These samples were used for the acetylene reduction assay, total carbon and nitrogen, and isotopes of δ^{15}N air and δ^{13}C VPDB. These samples were placed in a previously ethanol-sterilized container. Soil cores were collected beneath the sampled biocrust an additional 5 cm in depth for analysis of pH, electrical conductivity (EC), inorganic N, and phosphorus. All samples were kept on ice during transportation and until processed.

Environmental conditions and soil characteristics

Environmental conditions were monitored at each site using existing infrastructure at the CZO. Precipitation and temperature were determined from established climate stations at the sites using rainfall gauges and air temperature sensors (Nayak et al. 2010; Seyfried et al. 2011). Soils directly below biocrusts were characterized for soil moisture, water holding capacity, pH, EC, nutrient availability including ammonium (NH_4^+-N), nitrate (NO_3^--N), and ortho-phosphate (ortho-P), soil total C and N, and isotopes δ^{15}N air and δ^{13}C VPDB. Upon returning to the laboratory, soils were sieved through a 2 mm sieve. Soil moisture was determined by percent gravimetric water content (GWC) by weighing out 10.0 ± 1 g of homogenized soil (owing to minimal sample sizes) in an aluminum tin

and drying at 105˚C for 24-48 h. The sample was then weighed again to determine amount of water loss.

Water holding capacity was determined on a soil sample from each site folling the method described by Romano et al. (2002). To a Whatman #1 filter (CAT:1001-090, Fisher Scientific, Pittsburg, PA), soil (10 ± 0.5 g) was added to vacuum filter funnel with an open bottom. Sufficient water (18.2 mΩ) was added to the soil to submerge the soil by 1 cm, and allowed to infiltrate and drain until just above the soil surface. Water was added a second time and allowed to infiltrate same as before. The third time, the soil was saturated and the funnel was covered with aerated plastic wrap for 48-72 h and then weighed.

Soil pH and electrical conductivity were determined using a 1:1 ratio of water to soil, except the highest site, due to high organics, which required a 2:1 ratio. Samples were stirred and allowed to sit for 1 h, and then pH and EC determined on a Dual Chanel pH/Ion/Conductivity Meter XL50 (Fisher Scientific, Pittsburg, PA). Nutrient availability was determined following standard methods. Inorganic forms of N were assessed following the methods outlined by Hart et al. (2011) using a 1:5 ratio of soil and 2 M potassium chloride (KCl, CAS: 7447-40-7, Fisher Scientific, Pittsburg, PA). Soil was weighed into a specimen cup, and 2 M KCl was added and then placed on a shaker for 1 h. Samples were filtered through Whatman #1 filters that were previously leached with 2 M KCl. The KCl extract was stored at 4 ˚C until analysis on a SmartChem 200 Discrete Analyzer (DA) Auto-Spectrophotometer (Westco Scientific Instruments, Inc, Brookfield, CT). A salicylate method (AMM-003-A) was used for NH_4^+, and a nitrate reduction method with a cadmium metal column (NO3-001-A) was used for NO_3^-. Soluble

phosphate (ortho-P) was extracted from 2.0 g soil with 40 mL of 0.5 M sodium bicarbonate ($NaHCO_3$, CAS: 144-55-8, Fisher Scientific, Pittsburg, PA). Samples were placed on a shaker for 1 h and then filtered through Whatman 40 filters (CAT:1440-110, Fisher Scientific, Pittsburg, PA) that were previously leached with 0.5 M $NaHCO_3$. The extract was stored at 4˚C until analysis on the DA with using the PHO-001-A method.

Total soil C, total N, and isotopes $\delta^{15}N$ and $\delta^{13}C$ were determined on an Elemental Combustion System 4010 (Costech Analytical Tech, Inc, USA) interfaced to a Delta V Advantage Mass Spectrometer (Thermo Scientific, Germany) at the Center for Archaeology, Materials, and Applied Spectroscopy at Idaho State University, Pocatello, ID. Soils were dried at 55˚C for 24-48 h prior to grinding soil and biocrust samples in a ball mill grinder until a fine powder was attained. Samples were then packed in 5 X 9 mm tin capsules. Standards of ISU Peptone, Costech Acetanilide, and DORM-3 are calibrated against international standards (IAEA-N-1, IAEA-N-2, USGS-25, USGS-40, USGS-24, IAEA-600), and were used to create a two-point calibration.

Acetylene Reduction Assay

To determine nitrogenase activity (NA), we used the acetylene reduction assay, which is a commonly used indirect assessment of fixation (Hardey et al. 1968). The nitrogenase enzyme can reduce acetylene to ethylene, which is reported at nitrogenase activity (NA; Belnap 1996; Belnap 2002; Belnap & Lange 2003), and to calculate accurate N_2 fixation rates calibration with ^{15}N is required. Therefore, values are reported in µmol C_2H_4 fixed m^{-2} h^{-1}. When calculating yearly rates, we used the common conversion factor of 3 moles of C_2H_2 reduced: 1mole of N_2 fixed (Eskey & Ting 1978; Zaady et al. 1998; Wu et al. 2009; Abed et al. 2010; Zhao et al. 2010) to obtain a conservative estimate since the

enzyme nitrogenase uses acetylene easier than N_2. Values were also adjusted using site-specific cover estimates taken in May 2015 described below. Pint, wide mouth, mason jars with hungate septa (CAT:50-121-5190, Fisher Scientific, Pittsburg, PA) sealed in the lids were used as chambers for the assay. Subsamples of the biocrusts were placed into the mason jars intact. "Actual" assays were assessed on samples as soon as possible, and these samples were incubated using the soil moisture concentrations at which they were collected. "Potential" assays were assessed on samples that were placed in the greenhouse for 48 h and kept at a constant 60% WHC by weight with 18.2 mΩ water. Because the samples were kept at more optimal moisture conditions, these samples were used to consider the potential for the biocrusts to fix N_2 under ideal environmental conditions. To make the acetylene, water was added to calcium carbide (CAS: 75-20-7, Alfa Aesar, Ward Hill, MA) until all bubbling ceased and the gas was collected in a bag with a port septum. Potential and actual assays were performed by adding acetylene to the sample jar headspace until a concentration of ~10% acetylene was attained. The samples were then incubated at room temperature under fluorescent lighting for 2 h. Gas samples of the headspace and were taken and placed in evacuated vials, which were sent to the Canyonlands Field Station USGS in Moab, UT for analysis a Shimadzu GC-14A gas chromatograph (Shimadzu Scientific Instruments, Kyoto, Japan) with a flame ionization detector equipped with an All Tech Haysep T 100/120 column (CAT:2814PC). Blanks assessing ethylene present in the acetylene gas, blanks assessing the soil samples capacity to emit ethylene, and calibrations with ethylene standards were all collected at the same time samples were incubated and analyzed.

Cover Estimates

To obtain a more accurate estimation of N_2 fixation rates from the acetylene reduction assay, biocrust cover estimates were determined at each site using a 10 x 10 grid point frame (28 x 28 cm) at the time of each sample collection in May 2015 (n=80 total point frame measurements, 20 per site). The four categories of moss, lichen, vascular plant vegetation, or bare ground were recorded from the intersecting points on the frame in grazed and ungrazed paired interspace and shrub-canopy plots. Total biocrust cover was determined by combining moss and lichen cover. Vegetation cover included various plant types, and bare ground included only that of visible ground. Total biocrust cover was used to adjust all NA ($\mu mol\ C_2H_4\ m^{2\ -1}\ h^{-1}$) and N_2 fixation annual rates ($kg\ N\ ha^{-1}\ yr^{-1}$).

Chlorophyll-a Extraction

A subsample for chlorophyll-a (Chl *a*) was collected from sampled biocrusts by coring the sample twice with a sterile 50 mL falcon tube. Tubes were then wrapped in foil to prevent light degradation and stored at -20˚C until analysis. Extractions for Chl *a* were achieved with a double-ethanol extraction (Lan et al. 2011) using the method described by MeKee & Mercer (2011) and Castle et al. (2011). Once thawed and air dried, samples were ground with mortar and pestle until homogeneous, and 3.0 g of sample was then added to a 15 mL sterile centrifuge tube with 6 mL of 95% ethanol (CAS: 64-17-5, Sigma-Aldrich, St. Louis, MO), which was previously neutralized with magnesium carbonate (CAS:546-93-0, Fisher Scientific, Pittsburg, PA) and filtered through a Whatman #1 filter. After the additional of ethanol samples were boiled for 5 min in a water bath, and placed on a shaker for 20 minutes. Samples were then spun in a centrifuge for 10 min at 4000 rpm. The supernatant was determined for Chl *a* on a

spectrophotometer (ThermoSpectronic Genesys 20 4001/4) by measuring at 665 nm and 750 nm, then at 665 nm and 750 nm following 90 sec after adding 50 µL 0.1M HCl (CAS:7647-01-0, Fisher Scientific, Pittsburg, PA). The entire extraction was conducted in minimal light.

Statistical Analyses

Permutational multivariate ANOVA (PERMANOVA) procedures (Anderson 2001) were used to test for differences among elevations with respect to soil characteristics. Environmental variables were graphically depicted in space by Principal Components Analysis using Euclidean distance (Ter Brakk 1986). We conducted a multiway ANOVA analyses to test for significance between elevations with respect to N_2 fixation rates, and Tukey-Kramer methods to determine significance among elevation treatments. Determinations of significant differences between potential and actual sample N_2 fixation rates was performed using a Wilcoxon signed ranked test. Multiway ANOVA analyses also were used for differences between the factors of grazing (grazing vs. no grazing in exclosure plots) and shrub-canopy (biocrusts living beneath shrub canopies vs. biocrusts living in the interspace between vascular plants) with respect to N_2 fixation rates at each elevation. We adopted a significance level of $\alpha = 0.05$ for all statistical tests. The statistical program R (version 3.2.0; R core team [https://www.R-project.org]) was used for all statistical tests, with heavy use of the packages “asbio” and “vegan” (Aho 2015; Oksanen et al. 2015).

RESULTS

Soil and Biocrust Characteristics

Soil characteristics were significantly different among sites of the elevational gradient (PERMANOVA; $F_{3,75} = 3.78$; $P = 0.001$). As elevation increased, pH decreased with a range of 8.2 ± 0.16 to 6.1 ± 0.08 ($\bar{x}$ ± SE) from the lowest site, Flats (F), to the highest site, Mountain Big Sage (MBS). Soil moisture (GWC) increased with elevation, ranging from 7.0 ± 0.76 to 30.3 ± 2.38 (Table 3) from the lowest site, F, to the highest site, MBS. Electrical conductivity (EC) showed no clear trend with elevation, but the second site along the elevation gradient, Wyoming Big Sage (WBS), showed the highest EC at 212.7 ± 51.52 µS cm^{-1} (Table 3). The greatest concentrations of extractable NH_4^+ and NO_3^- were at the highest elevation site, MBS (Table 3). The lowest elevation site, F, had the lowest levels of NH_4^+ (0.5 ± 0.04 g g dry $soil^{-1}$), and the middle elevation site, WBS, had the lowest levels of NO_3^- (1.2 ± 1.4 g g dry $soil^{-1}$). Ortho-P was highest at the middle elevation site, Low Sage (LS), and lowest at the lowest elevation site, F (Table 3). With respect to grazing and ungrazed areas, NH_4^+ had no clear pattern within an elevation. The concentrations of extractable NO_3^- was highest in the grazed locations at all elevation sites, yet varied little between the interspace and shrub-canopy. At the lower elevations, F and WBS, ortho-P was similar with grazing and ungrazed areas, but at the higher elevations, LS and MBS, ortho-P increased with grazing. Similar patterns for ortho-P between the interspace and shrub-canopy was also observed with increasing orhto-P within the shrub-canopy at the highest elevations. The other soil characteristics of pH, EC, and GWC did not have any consistent trends with grazing or between the interspace and shrub-canopy.

Biocrust characteristics of TN, TC, and chlorophyll-*a* (Chl *a*) increased with elevation, whereas $\delta^{15}N$ and $\delta^{13}C$ values decreased (Table 4). $\delta^{15}N$ values were higher in the interspace than in the shrub-canopy, and there was no clear pattern with grazing (Table 4). Variation in $\delta^{13}C$ values with grazing and shrub-canopy was small, but varied more with elevation. Biocrust cover was the highest at the lower elevations, and decreased at the highest sites (Table 4). Only the lowest elevation site, F, had greater biocrust cover within the ungrazed locations compared with the grazed locations. All other sites showed no effect of grazing exclosure on biocrust cover. All elevations showed higher biocrust cover, however, in the shrub-canopy than in the interspace. Vegetation cover increased two fold at the highest elevation sites, LS and MBS, compared with the lower elevations of F and WBS (Table 4). The two highest sites also had greater vegetation cover in the ungrazed plots relative to the grazed plots (Table 4).

N_2 fixation with Climate

Multiway ANOVA indicated significantly different actual N_2 fixation rates among the elevation gradient ($F_{3,3} = 12.99$; $P = 0.0317$). Potential NA also varied significantly with elevation. Post-hoc Tukey tests confirmed that actuals and potentials were significantly different at all elevation sites for October and May, except F and WBS were not significantly different from each other (Fig. 4). Measured actual nitrogenase activity (NA) was highest in the two lower sites, F and WBS, in October 2014 and May 2015 (Fig. 4). Actuals from October ranged from 0.3 to 16.4 µmol C_2H_4 m^{-2} h^{-1}, and May actuals were nearly double those in October at 0.5 to 29.3 µmol C_2H_4 m^{-2} h^{-1} (Fig. 4). Potentials from October ranged from 0.4 to 23.6 µmol C_2H_4 m^{-2} h^{-1}, and in May ranged from 0.5 to 29.2 µmol C_2H_4 m^{-2} h^{-1} (Fig. 4). October potential NA was significantly

higher than actual NA for all sites ($P < 0.01$), and the largest differences in actual vs. potential NA were in October at the lowest elevation sites, F and WBS (Fig. 4). May actual and potential NA were not significantly different except at MBS ($P = 0.014$; Fig. 4).

Rates of N_2 fixation were associated with soil and biocrust characteristics that varied along the elevation gradient as shown by the ordination projection (Fig. 5). In particular, there was a distinct separation between the lowest elevation site, F, and the highest elevation site, MBS (Fig. 5). Vector fitting of soil characteristics identified N_2 fixation rates ($P = 0.001$; $R^2 = 0.4944$), pH ($P = 0.001$; $R^2 = 0.6997$), EC ($P = 0.001$; $R^2 = 0.3017$), NO_3^- ($P = 0.001$; $R^2 = 0.6109$), NH_4^+ ($P = 0.001$; $R^2 = 0.6313$), ortho-P ($P = 0.002$; $R^2 = 0.002$), GWC ($P = 0.001$; $R^2 = 0.7761$), TN ($P = 0.001$; $R^2 = 0.7582$), TC ($P = 0.001$; $R^2 = 0.6769$), $\delta^{15}N$ ($P = 0.001$; $R^2 = 0.7582$), and $\delta^{13}C$ ($P = 0.001$; $R^2 = 0.6769$) significantly correlated to the ordination (Fig. 5). Correlated with the first dimension, higher N_2 fixation rates, pH, $\delta^{15}N$, and $\delta^{13}C$ were positively associated with the lower elevation sites while TN, TC, NH_4^+, and GWC were associated with the higher elevation sites (Fig. 5). Correlated with the second dimension was EC, ortho-P, and NO_3^- (Fig. 5). Chl *a* slightly increased with elevation; however, there was no association between Chl *a* ($P = 0.163$; $R^2 = 0.0452$) and the PCA projection (Fig. 5; Table 4). The lowest elevation site, F, was the only site with greater measured Chl *a* in ungrazed areas. The two lowest sites, F and WBS, had greater Chl *a* in the shrub-canopy while the two higher elevation sites, LS and MBS, had greater Chl *a* in the interspace (Table 4).

N_2 fixation with Grazing and Shrub-canopy

Actual NA was not significantly different between grazed and ungrazed areas when all elevations were assessed together. The lowest elevation site, F, was the only site that did show higher rates of NA in ungrazed areas (Fig. 6). All elevations had significantly higher actual NA within the shrub-canopy compared with the interspace ($P < 0.032$; Fig. 7). The two lowest sites, F and WBS, had significant interactions ($P < 0.001$) between grazing and shrub-canopy, while the higher sites, LS and MBS, did not. At the lowest elevations, F and WBS, NA was highest in the grazed shrub-canopy. The lowest NA at F was in the ungrazed interspace, while at WBS lowest NA was in the grazed interspace (Fig. 7). NA was highest in the ungrazed shrub-canopy, and lowest in the grazed interspace at the elevation site LS (Fig. 7). There was little difference in grazed and ungrazed NA at the highest elevation site MBS. Potentials showed nearly identical significance and interactions as the actuals.

DISCUSSION

N_2 fixation with Climate

To our knowledge, this study is the first to document rates of N_2 fixation associated with rolling biocrusts in the Intermountain West of the US and patterns associated with sagebrush steppe ecosystems along a climosequence. Results from our study only partially supported our expectations that N_2 fixation rates would change along the climatic gradient with the middle elevations exhibiting the highest rates of N_2 fixation because of optimal temperature and moisture conditions. Instead, we found that nitrogenase activity (NA) was consistently higher at warmer and drier climates (lower

elevation sites) compared to colder, wetter climates (higher elevation sites) across the growing season.

Our measured rates of N_2 fixation in biocrusts were comparable to those reported in biocrust studies from around the world (Table 5), suggesting that rolling biocrusts fix similarly important amounts of N_2. Like most of the studies we found, our study used the acetylene reduction assay to determine nitrogenase activity, and there are important limitations to this method (Belnap 1996; Belnap 2002). While annual estimates using this method (and any assessment of short-term point measurements scale to an annual rates) are problematic and should be viewed as extremely coarse, calculating coarse annual N_2 fixation estimates does allow us to contextualize the hourly acetylene reduction rates in useful ways. Using the common conversion factor of 3:1 (C_2H_4:N_2 ; Eskey & Ting 1978; Zaady et al. 1998; Wu et al. 2009; Abed et al. 2010; Zhao et al. 2010), average yearly rates for the rolling biocrusts at the warmest and driest elevations of this study were estimated at 12 kg N ha^{-1} yr^{-1}, while at the coolest and wettest elevations rates were estimated at 0.2 kg N ha^{-1} yr^{-1}. Less vegetation and greater open areas within the interspace within the warmer and drier climates allowed for greater biocrust cover, and therefore greater fixation since our NA were adjusted for cover. The significant differences between elevations and the shrub-canopy NA are attributed to cover, since unadjusted rates do not show the same trends.

The estimated rates of N_2 fixation at the warmer/drier elevations are 16 times higher than average annual rates of wet N deposition of 0.71 kg N ha^{-1} yr^{-1} reported from the National Atmospheric Deposition Program (NADP) site at the Reynolds Creek (1983-2014) indicating that biological N_2 fixation by biocrusts are a significant input of N into

the ecosystem, especially at the warmer/drier climates. At the highest elevation, MBS, *Lupinus* spp. cover some areas in addition to the low biocrust cover indicate that biocrusts are not a major input of N into the ecosystem at the highest site. Yearly rates from the warmer, drier climate sites are similar to those projected in pinnacled biocrusts of the Colorado Plateau (Belnap 2002; Strauss et al. 2012) and Mojave Desert, USA (Billings et al. 2003; Table 5). N_2 fixation rates from the highest elevation are most similar to early-successional biocrusts (Belnap 2002), and moss-dominated biocrusts (Zhao et al. 2010). Measured NA in our study appear to be a medium range compared to those reported in studies from around the world, and our rates are most similar to those reported by Belnap (2002) and Billings et al. (2003); however, even within individual desert types there are large ranges of measured NA (Table 5). These differences are likely due to variations in biocrust cover, biocrust communities, and climates (Belnap & Lange 2003; Schwabedissen et al. in prep).

It has been suggested that given sufficient water and light, N_2 fixation is primarily limited by temperature, with the range of -5–30˚C, and optimal temperatures between 15–30˚C (Belnap 2002; Belnap & Lange 2003; Wu et al. 2009). Studies show those periods of the year that receive adequate moisture and have temperatures > 15˚C display the greatest NA, and therefore N_2 fixation rates (Belnap 2002; Belnap & Lange 2003; Wu et al. 2009) Patterns along our climate gradient appear to support this. High rates of NA were observed at the lower elevation sites during the early summer/spring periods when water was not limiting and temperatures were > 15˚C (Fig. 3). Low rates of NA at the highest elevations are partly explained by the reduction in cover, as well as lower temperatures (< 20˚C), despite a surplus of water (Fig. 3).

Although biocrust photosynthesis and respiration quickly upon wetting (e.g., Reed et al. 2012, Coe et al. 2012), N_2 fixation has a lag time, which can vary by species and requires additional time to reach maximum potential (Belnap & Lange 2003). Our results showed strong priming effects of moisture on N_2 fixation patterns. Consistent with our expectations and with studies from pinnacled biocrusts (Belnap 2002; Belnap & Lange 2003), NA was higher in warmer, drier climates in the early summer/spring than late summer/autumn, likely owing to greater moisture during this time (Fig. 3). The early summer/spring (May) period followed a span of the year that had greater precipitation than evaporation (Fig. 3), resulting in consistently higher soil moisture contents than the late summer/autumn (October) where evaporation had exceeded precipitation for several months and is moisture limited (Fig. 3). During the early summer/spring, we found no differences in actual and potentials, except the highest site MBS, indicating a lack of water limitation; however, in late summer/autumn, there were significant differences between the actuals and potentials suggesting strong water limitations. Moreover, we know that winter can be an important time for under-snow biocrust activity (Darrouzet-Nardi et al. 2015) and lower resource stores from the long summer of desiccation could lead to decreased N_2 fixation potential in the late summer/autumn (Belnap 2002). In mid-summer, actual NA is expected to be minimal because of the greater moisture limitations and higher temperatures (Belnap 2002; Belnap & Lange 2003). Other studies on pinnacled biocrusts have shown that rates are typically highest in the spring and late autumn when there is adequate soil moisture and moderate temperatures (Belnap 2002; Belnap & Lange 2003). Collectively, the differences in the actual and potentials at the warmer and drier climates in October compared to May indicate that moisture is a

substantial seasonal control on NA and therefore N_2 fixation (Belnap 2002; Hartley & Schlesinger 2002).

Other controls on N_2 fixation that have received attention in the literature include nutrient availability and biocrust community composition (Belnap 2002; Belnap & Lange 2004; Yeager et al. 2004; Reed et al. 2011; Kuske et al. 2012; Wang et al. 2015), and our findings partially support these conclusions. The lowest levels of NH_4^+, NO_3^-, and TN were found with the highest NA, suggesting the possibility that, as has been seen in many ecosystems, increased N availability may lead to decreased N_2 fixation (Reed et al. 2011). Low amounts of N at the lower elevation sites indicate greater potential for N limitation and therefore a greater demand for N_2 fixation relative to the higher elevation site. At the highest elevation, since N_2 fixation is energy intensive, available N will likely be expended first (Reed et al. 2011), and additions of N have been found to alter bacterial communities of biocrusts (Wang et al. 2015), likely decreasing NA with high N. In addition, the lowest concentrations of extractable soil P were at the lower elevation sites, which could indicate that there is sufficient P to support higher NA and that N may be a stronger control over N_2 fixation than P within these ecosystems. Available sources of N for biocrust organisms are soil N pools, N_2 fixation, and N deposition, in which deposition is minimal (Evans & Belnap 1999). Both N_2 fixation and N deposition would be expected to result in lower $\delta^{15}N$ values, often ranging from -3 to +2‰ (Evans & Elheringer 1993; Ehleringer et al. 1998; Evans & Belnap 1999; Billings et al. 2003). The higher $\delta^{15}N$ values at the warmer, drier climates sites (~5 ‰; Table 4) where we have the greatest NA, the data suggest that N_2 fixation may be the main source of biocrust N (Evans & Belnap 1999); however, there is likely large amount of gaseous N loss via

volatilization or denitrification with N leaving the systems as ammonia, dinitrogen, nitrous acid, nitrous oxide, and NOx (Ehleringer et al. 1998; Barger et al. 2013; Weber et al. 2015), which would also increase $\delta^{15}N$ (Evans & Ehleringer 1993; Ehleringer et al. 1998; Evans & Belnap 1999). It has been estimated that 70% of fixed N_2 is lost through volatilization and denitrification (Evans & Belnap 1999), and the higher abundance of $\delta^{15}N$ at the warmer and drier climates compared with an increase in symbiotic N_2-fixers and lower $\delta^{15}N$ at the cooler, wetter climates (Schwabedissen et al. in prep) would indicate that biocrusts may exhibit greater gaseous N loss than symbiotic N_2-fixers.

Differences in biocrust community structure also appeared to drive differences in rates of NA at these sites. Values of $\delta^{13}C$ indicated a shift in biocrust community structure with $\delta^{13}C$ values of mainly lichens ($\leq$-23‰) at the lowest elevation (Evans & Belnap 1999; Billings et al. 2003). A greater abundance of cyanobacteria at the lower elevations (Schwabedissen et al. in prep) also may contribute slightly to lower $\delta^{13}C$ (Evans & Belnap 1999). As elevation increases, the $\delta^{13}C$ values are more indicative of mosses (<-26‰; Evans & Belnap 1999; Billings et al. 2003), which are supported by less lichen cover at the highest elevation (Table 4).

The soil characteristic pH has been highlighted as a strong correlate with biocrust community composition (Ponzetti & McCune 2001; Belnap & Lange 2003; Wang et al. 2015; Schwabedissen et al. in prep), and pH may represent an important control over N_2 fixation rates (Zhao et al. 2010). We observed alkaline soils in the warmer, drier climates along with higher NA and biocrust cover. Furthermore, the N_2-fixing bacterial community along the elevation gradient shifted from cyanobacteria dominated N_2 fixation at the warmer, drier climates to symbiotic dominated N_2 fixation at the cooler,

wetter climates (Schwabedissen et al. in prep), suggesting pH as a strong determinate of biocrust communities and functionality with respect to N_2 fixation. Interestingly, measured concentrations of Chl *a* did not vary greatly with elevation, but was slightly greater at the highest elevation, MBS, where we had the least abundance of cyanobacteria and biocrust cover (Schwabedissen et al. in prep; Table 4). We expected to see Chl *a* concentrations follow the patterns of cover and cyanobacteria N_2-fixers; however, the shift in bacterial N_2-fixing communities from cyanobacterial to symbiotic along the elevational gradient may explain why Chl *a* did not vary greatly with elevation. Furthermore, this may also indicate that Chl *a* within this system is not a good indication of cyanobacterial biomass, but rather indicates the photosynthetic capacity.

Similar to other studies, biocrust communities respond to changes in climate (Johnson et al. 2012; Ferrenberg et al. 2015; Steven et al. 2015; Schwabedissen et al. in prep), and climate-induced changes to biocrusts community composition have strong potential to affect N_2 fixation rates. There was a strong dissimilarity between the warmest, driest site F and the coldest, wettest site MBS with respect to NA (Fig. 4). With temperatures projected to increase, by the Intergovernmental Panel on Climate Change, and with shifts in precipitation already documented for the Intermountain West (Nayak et al. 2010; Seyfried et al. 2011), we would predict that N_2 fixation and biocrust cover will decrease at the warmer and drier elevations (Ferrenberg et al. 2015; Johnson et al. 2012; Reed et al. 2012). We further predict that the cooler and wetter climates at the higher elevations will become warmer and drier similar to the current conditions of the lower elevations sites, and that these areas would increase in biocrust cover and N_2 fixation rates.

N_2 fixation with Grazing and Shrub-canopy

It has been well documented that grazing disturbance decreases biocrust cover (Kaltenecker et al. 1999; Ponzetti & McCune 2001; Gómez et al. 2012; Concostrina-Zubiri et al. 2014), and therefore N_2 fixation rates. In contrast to our hypothesis and other studies, we found no significant difference in NA between grazed and ungrazed areas, except in the lowest elevation site, F, where NA was higher within the ungrazed areas (Fig. 6). This is the ecosystem where N_2 fixation rates were highest and thus significant differences in N inputs could be expected here, but the lack of a 40 year release on grazing effect on biocrusts was unexpected. Interestingly, there were some variations in the bacterial community associated with biocrusts in grazed and ungrazed areas at the warmest and driest climates, although it was statistically insignificant (Schwabedissen et al. in prep). This finding could indicate that grazing influences the biocrust community first, yet it did not alter the functional profile with respect to the capacity for N_2 fixation; however, other transformations within the N cycle and other nutrient cycles such as C might be altered. In particular, we found a 3-fold decrease in N_2-fixing cyanobacteria with grazing, which could mean greater implications at the ecosystem scale since cyanobacteria are primarily those bacteria that facilitate N_2 and C fixation within biocrusts (Schwabedissen et al. in prep). Studies have shown that the time of year that grazing occurs can affect the severity of the disturbance effect on biocrusts, with biocrusts being the most resilient during cool seasons (Kaltenecker et al. 1999; Belnap & Lange 2003; Concostrina-Zubiri et al. 2014). Our data suggests that the largest effect of the removal of grazing occurred at the warmest site, and other factors – such as the capacity for N_2 fixation – may play an important role in determining biocrust recovery

following disturbance. Furthermore, cattle stocking rates within a certain area also affects the original impacts of grazing. The lack of dramatic biocrust cover differences between grazed and ungrazed areas (Table 4) may in part be explained by the fact that grazing within our study area is seasonal, and the different elevations are grazed at different times of year (the lower elevations are grazed in the early spring and autumn and the higher elevations are grazed during the summer; Fig. 3).

Finally, it has been suggested that the soil beneath vascular plant canopies and the soil in the interspace between shrubs can host different biocrust communities (Elliott et al. 2014; Schwabedissen et al. in prep), and it is typically thought that the interspaces have higher cover and therefore higher rates of N_2 fixation. Results from our study did not support this hypothesis, and showed the opposite trend. Namely, there was significantly higher NA within the shrub-canopy than the interspace likely because of increased moss and overall biocrust cover within the shrub-canopy. Explanations as to why we observed higher biocrust cover within the shrub-canopy could have to do with more favorable microclimate, higher resource availability (Schlesinger & Pilmanis 1998), or increased protection from grazing and other physical disturbances (Belnap & Lange 2003; Gómez et al. 2012; Concostrina-Zubiri et al. 2014). It has been shown that mosses have a quicker recovery from disturbance than lichens because mosses can recolonize from fragments, and can inhabit the shrub-canopy as a result of their low light ability (Concostrina-Zubiri et al. 2014). Indeed, we observe higher moss cover in the shrub-canopy zones relative to the interspace. In this way, biocrust community composition (i.e., moss-dominated vs. lichen-dominated biocrusts) could affect fundamental ecosystem processes such as N_2 fixation. Thus, although grazing may not affect N_2

fixation rates at the ecosystem-scale (as assessed with the exclosures) or when comparing among the sites along the elevational gradient, gazing may influence biocrust properties at a much smaller scale.

CONCLUSION

Biocrusts maintain many vital roles within drylands, yet little is known about how the biocrusts typical of the Intermountain West are affected by climate and grazing disturbance with respect to their ability to fix N_2 from the atmosphere. To our knowledge, this is the first study to utilize an elevational gradient to examine climatic factors controls over biocrust N_2 fixation. Findings from this study indicate that climatic changes and the effect of shrub-canopy have the potential to alter N_2 fixation rates, while the effects of grazing on N_2 fixation may be relatively small (at least with the level of grazing experienced in this rangeland). As climates warm and precipitation patterns shift, we are likely to observe changes to biocrusts potential to fix N_2, suggesting reduced ecosystem N inputs and possibly greater N limitations as these biocrusts are the main source of N at the warmer/drier climates. Rates obtained from this study are similar to those found in other studies that host other biocrust morphologies. Further studies are needed to better understand the limitations and controls of N_2 fixation within the Intermountain West. This study lays a foundation for understanding the importance of rolling biocrusts in N cycling and for how rolling biocrusts may respond to climatic change and physical disturbance.

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CHAPTER III: DIVERSITY OF NITROGEN-FIXING BACTERIA IN ROLLING BIOLOGICAL SOIL CRUSTS OF THE INTERMOUNTAIN WEST: CLIMATE AND DISTURBANCE

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ABSTRACT

Biological soil crusts (biocrusts) colonize drylands worldwide, and are highly beneficial to those ecosystems in terms of soil stability and fertility. Numerous studies have examined bacterial communities in pinnacled biocrusts associated with hot semiarid drylands. None, however, have examined bacterial N_2-fixing communities in rolling biocrusts often associated with colder, more mesic drylands, especially in elevations that are snow-dominated. These ecosystems are likely to undergo warming and drying with climate change, and it remains unclear how their microbial communities will respond to both climate change and physical disturbance (e.g., grazing). We examined N_2-fixing bacterial communities associated with rolling biocrusts along a climatic gradient in southwest Idaho, USA, and quantified how these communities change as a function of local climate, grazing (exclosures), and shrub-canopy association. We detected dramatic shifts in bacterial communities from symbiotic N_2-fixing bacteria to cyanobacterial as temperatures increased and precipitation decreased with decreasing elevation. Surprisingly, the genus *Oscillatoria* dominated the cyanobacterial component of the population in all but the highest elevation biocrust communities; it has been found to typically be a minor portion of the cyanobacterial population in other biocrusts. *Microcoleus*, a cyanobacteria genus characteristically highest in early successional biocrust with grazing, decreased substantially in response to grazing. We also observed a

decrease in cyanobacterial abundance associated with grazing and shrub-canopies, whereas symbiotic N_2-fixing bacteria increased at lower (warmer and drier) elevations with grazing and shrub-canopy cover. Our findings illustrate that community composition of rolling biocrusts in the Intermountain West, USA, differs from other morphologies (e.g., rugose and pinnacled), and likely will shift from symbiotic N_2-fixing bacteria to cyanobacteria with warming and drying conditions.

INTRODUCTION

Drylands are characterized by low precipitation and limited vegetation (Belnap & Lange, 2003). These ecosystems cover over 35% of the terrestrial landmass (Kuske et al. 2012), and are commonly colonized by biological soil crusts, or biocrusts. Biocrusts, a soil-surface community of mosses, lichens, liverworts, algae, cyanobacteria, archaea, and other bacteria, vary dramatically in morphologies from flat to rolling to pinnacled structures depending on climatic conditions, particularly precipitation and frost heaving of soils (Belnap & Lange 2003; Belnap 2003; Yeager et al. 2004; Ferrenberg et al. 2015). Biocrust communities perform vital ecological functions including reduction of nutrient loss by runoff (Berger et al. 2006), decreasing erosion by wind (Belnap & Lange 2003), fixing carbon (C) and nitrogen (N_2) (Belnap & Lange 2003; Belnap 2003), facilitation of vascular plant establishment (Belnap 2003; Coe & Belnap 2014), and decreasing surface albedo (Belnap & Lange 2003; Kuske et al. 2012). Despite their importance in the structure and function in drylands, little is known about how these communities will respond to changing climatic conditions and disturbances in terms of composition, diversity, and function (Belnap 2002; Belnap & Lange 2003; Yeager et al. 2004; Kuske et al. 2012), especially the cyanobacteria and other N_2-fixing genera. Addressing these

knowledge gaps may be particularly important in environments whose winter precipitation is primarily snow, as these areas are likely to undergo directional climate changes with declines in amounts and shifts in precipitation phases (Nayak et al. 2010; Seyfried et al. 2011).

Nitrogen (N) often limits primary production in dryland ecosystems (Reed et al. 2011), wherein N_2 fixation by biocrusts can be the main influx of "new" N (Belnap 2002; Belnap & Lange 2003; Yeager et al. 2004; Yeager et al. 2007; Reed et al. 2011). The process of biological N_2 fixation is unique to prokaryotes in the domains Bacteria and Archaea. In biocrusts, cyanobacteria are the primary free-living N_2-fixers within the soil or associated with lichens and mosses (Belnap 2002). Numerous studies have examined the cyanobacterial diversity of flat, rugose, and pinnacled biocrusts from the Colorado Plateau (Yeager et al. 2004; Yeager et al. 2007; Kuske et al. 2012), and Chihuahuan Desert, USA (Yeager et al. 2004; Yeager et al. 2007), and in India (Kumar & Adhikary 2015), Israel (Hagemann et al. 2015), and Africa (Dojani et al. 2013). Across these biocrust types, cyanobacteria typically display greater abundance in well-drained, alkaline pH and saline soils (Belnap & Lange 2003). Generally, *Microcoleus* spp. are the first to colonize soil, whereas *Nostoc* and *Scytonema* spp. occur in greater abundance as lichens and mosses become established (Belnap 2002; Yeager et al. 2004; Kuske et al. 2012). Other genera that fix N_2 asymbiotically are from the groups *Alphaproteobacteria* and *Gammaproteobacteria*, but less is known about their distribution and function in biocrusts (Yeager et al. 2007). To our knowledge, examinations of the potentially complex diversity of cyanobacteria and other N_2-fixing genera in rolling biocrusts that often inhabit colder more mesic drylands remains lacking.

Colder, more mesic drylands such as those in the Intermountain West of the US are undergoing directional climate changes with increases in temperature and shifts in precipitation (Nayak et al. 2010; Seyfried et al. 2011). Long-term studies across the Reynolds Creek Experimental Watershed, a semiarid rangeland watershed representative of the Intermountain West, have documented significant trends of increasing temperatures, especially minimum temperatures (Nayak et al. 2010). At this watershed, maximum snow water equivalent and proportion of snow to rain have declined at all elevations with the largest and most significant declines at mid and lower elevations (Nayak et al. 2010). Changes in phase of precipitation and amounts as well as increasing temperatures will likely alter the abundance and community composition of biocrusts (Ferrenberg et al. 2015).

Altered disturbance regimes from anthropogenic activities such as recreation, grazing, fire, and agriculture are also likely to alter biocrust communities (Belnap & Lange 2003; Barger et al. 2006; Reed et al. 2011; Kuske et al. 2012; Coe et al. 2012). Biocrusts dominated by lichens and mosses, such as those in the Intermountain West, are considered extremely sensitive to disturbance (Belnap & Lange 2003; Belnap 2003), and once disturbed often are replaced by more tolerant cyanobacteria (Belnap 2003). After a disturbance, recovery can take over 1000 years, depending on soil characteristics, severity of disturbance, and climate (Belnap 2002). Thus, quantification of how rolling biocrust communities of N_2-fixing bacteria vary with disturbance and varying climates is vital to the understanding and management of these systems.

We used 16S rRNA V3-V4 gene sequencing to examine the abundance and diversity of N_2-fixing bacteria associated with rolling biocrusts along a climatic gradient in

Southwest Idaho, USA, to determine how their distribution changes with three factors: climate, grazing (versus exclosure), and shrub-canopy cover (versus interspaces). We considered three general hypotheses: (1) Biocrust communities of N_2-fixing bacteria and associated soil variables will change along the climatic gradient. We predicted that cyanobacteria will be observed in greater abundance within drier areas with alkaline pH and higher electrical conductivity, whereas other genera of N_2-fixing bacteria will be more abundant in wetter areas of acidic pH and lower electrical conductivity. In addition, we predicted that other soil variables such as the amount of ammonium (NH_4^+), nitrate (NO_3^-), and ortho-phosphate (ortho-P) will vary with elevation, and reflect changes with the N_2-fixing communities. (2) Disturbance, from grazing, will alter N_2-fixing bacterial communities. Specifically, we predicted that cyanobacteria associated with early-successional biocrusts, such as the genus *Microcoleus*, would be in greater abundance in grazed areas, whereas late-successional cyanobacteria, such as the genus *Nostoc*, would be in greater abundance in ungrazed areas. (3) N_2-fixing bacterial communities will differ between shrub-interspaces and shrub-canopy locations. We expected a higher abundance of cyanobacteria in interspaces.

MATERIALS AND METHODS

Study Area & Experimental Design

The study was conducted at the Reynolds Creek Experimental Watershed, now NSF Reynolds Creek Critical Zone Observatory (CZO), located in the Owyhee Range in Southwest Idaho, USA (Fig. 2). The Observatory is a 239 km^2 watershed with a broad elevational range (1170-2080 m). The vegetation of the CZO are typical of semiarid ecosystems in the Intermountain West and include sagebrush steppe (*Artemisia* spp.),

aspen forests (*Populus tremuloides*), and juniper woodlands (*Juniperus* spp.). The CZO is primarily used for grazing by cattle and, to a lesser extent, farming for hay and grain. At lower elevations rain is the predominant form of precipitation, whereas at higher elevations winter precipitation is primarily snow. Extensive spatial and temporal soil temperature and moisture data has been collected for ≥40 yr by the United States Department of Agriculture Agricultural Research Service Norwest Watershed Research Center in Boise, Idaho (Nayak et al. 2010; Seyfried et al. 2011).

Four sample sites were established along an elevational gradient (climosequence) within the CZO (Fig. 2; Table 2). From low to high elevations, mean annual precipitation ranged from 235 to 803 mm, and mean annual temperature varied from 9.1 to 5.4˚C. Site selection was based on the location of CZO core sites, areas with sufficient fetch and area for CZO eddy covariance towers, and other instrumentation and within vicinity of historic climate stations and grazing exclosures (>40 yr). Sagebrush (*Artimisia* spp.) was the dominate shrub species across this elevation gradient, but subspecies of sagebrush co-varied with elevation (Table 2).Variation in other state factors (Jenny et al. 1941; Brady & Weil 2010) such as parent material, time, and topography was minimized in selection of sites. For example, parent material at all sites is volcanic basalt in origin, and topography is relatively flat (<5% slope).

Sampling

Biocrust samples were collected in October 2014 from five randomly selected shrubs in grazed and ungrazed (≥40 years) areas at each site. Samples were collected from the interspace and shrub-canopy at each randomly selected shrub in both grazed and ungrazed (enclosures) areas. The sample area was defined with a 10 × 10 cm metal

frame, and biocrusts were excised by a spatula and soil knife to a depth of 2.5 cm. Samples were immediately placed in an ethanol-sterilized container. Soil cores were extracted from under the sampled biocrusts to an additional 5 cm in depth and sieved through a 2 mm sieve for determination of pH, electrical conductivity, and nutrient concentrations. All samples were kept on ice until processed.

Soil Chemistry

Determinations of soil pH and electrical conductivity were conducted at a 1:1 ratio of water to soil. Owing to the high organic matter at the Mountain Big Sage (MBS) site, a 2:1 ratio was required to obtain a similar ratio of water to soil achieved at the lower elevation sites. Following the addition of water to soil, samples were swirled for 1 min, and readings were recorded at 1 h by a Dual Channel pH/Ion/Conductivity Meter XL50 (Fisher Scientific, Pittsburg, PA). Soil was extracted for inorganic nutrients, ammonium (NH_4^+) and nitrate (NO_3^-), using a 1:5 ratio of soil to 2M potassium chloride (KCl, CAS: 7447-40-7, Fisher Scientific, Pittsburg, PA). Samples were placed on a shaker for 1 h and extracts were filtered through pre-KCL leached Whatman 1(CAT:1001-110, Fisher Scientific, Pittsburg, PA) filters. Samples were stored at 4˚C and then analyzed on a SmartChem 200 Discrete Analyzer Auto-Spectrophotometer (Westco Scientific Instruments, Inc, Brookfield, CT) for NH_4^+ with a salicylate method (AMM-003-A) and for NO_3^- with a nitrate reduction method with a cadmium metal (NO3-001-A). Ortho-phosphate (ortho-P) was extracted from a 1:20 ratio of soil and 0.5M sodium bicarbonate ($NaHCO_3$, CAS: 144-55-8, Fisher Scientific, Pittsburg, PA). Ortho-P samples were placed on a shaker for 1 h and then poured through pre-0.5M $NaHCO_3$ leached Whatman 40 (CAT:1440-110, Fisher Scientific, Pittsburg, PA) filters. The extract was stored at 4˚C

until analyzed colormetrically on the SmartChem 200 Discrete Analyzer Auto-Spectrophotometer using the PHO-001-A method.

Biocrust N_2-fixing bacterial community determination

Subsamples were gathered from biocrust samples for DNA extractions on the same day as field collection using a flame sterilized cork borer inserted to a depth of 1 cm into the biocrust until a volume of 1.75 mL was reached. The resulting materials were stored at -20˚C until extraction. DNA was extracted from all samples with the PowerSoil DNA Isolation Kit (CAT: 12888-100, MoBio Laboratories, Inc., Carlsbad, CA) following the manufacturer protocol (80 total samples). DNA was quantified by a NanoDrop ND-1000 Spectrophotometer at the Molecular Research Core Facility (MRCF) at Idaho State University (ISU), Pocatello, Idaho. DNA from the five field replicates were pooled into 16 samples representing grazing and vicinity to shrub treatments at each site (i.e., ungrazed and shrub-canopy, ungrazed and interspace, grazed and shrub-canopy, grazed and interspace), and then sequenced three times.

We conducted PCR reactions to provide amplicons for next-gen sequencing on the MiSeq Illumina platform (Illumina San Diego, CA). The 16S SSU rRNA V3-V4 gene region was targeted with the primer sequences of: 16SForward (5’ CCTACGGGNGG CWGCAG 3’); and 16SReverse (5’ GACTACHVGGGTATCTAATCC 3’) (Integrated DNA Technologies, Coralville, IA; Illumina). A 50 uL reaction volume was used that consisted of the primers, *Vent* polymerase (CAT: M0258S, New England Biolabs, Ipswich, MA), 1X ThermoPol Buffer (New England Biolabs, Ipswich, MA), 400 uM deoxynucleotide triphosphates (CAT: BP2564-1, Fisher BioReagents, Fair Lawn, NJ), nuclease free water, and 200 ng pooled template DNA. Individual mixes and negative

controls were used for each pooled sample. Amplifications were done with the following parameters: 95˚C for 10 min; 95˚C for 1 min; 41.5˚C for 2 min; 72˚C for 4 min for 34 cycle, and a final extension 72˚C for 10 min, followed by a hold at 4˚C until analyzed on a 1% agarose gel. Amplicons were submitted to the MRCF for paired-end sequencing. Raw data files obtained for the three sequencing runs from the MRCF were placed on the Galaxy server (Giardine et al. 2005; Goecks et al. 2010; Blankenberg et al. 2010) and were groomed, joined by paired ends, and converted to FASTA format. Files were then uploaded to the program Metagenomics Rapid Annotation using Subsystems Technology (MG-RAST) (Meyer et al. 2008; Wilke et al. 2015) for identification and analysis. Abundance values obtained from MG-RAST output from the sequencing runs were normalized to the highest abundance within that run, and then normalized again to the highest abundance in all three runs for comparisons. We note that the *nifH* functional gene parallels the 16S rRNA gene for bacterial identification (Reed et al. 2011), and that further classifications using *nifH* would ensure identified genera have the capability to fix N_2. However, our attempts to amplify the *nifH* gene in our samples were thwarted probably owing to high salts or other interference, despite repeated physiological assays indicating high potential rates of N_2 fixation (Schwabedissen et al. in prep). We utilized the 16S rRNA gene to reflect this previous usage in biocrust bacterial diversity studies (Gundlapally & Garcia-Pichel 2006; Kuske et al. 2012; Elliot et al. 2014; Hagemann et al. 2015), and to facilitate comparisons to this work. Furthermore, we utilized an abundance approach because this allowed us to examine how the three groups of cyanobacteria, symbiotic, and other N_2 fixers respond to climate, grazing, and the shrub-canopy.

Availability of sequence data

All sequenced data are publicly available at MG-RAST under the project "RCCZO Nfix run1", "RCCZO Nfix Run 2", and "RCCZO Nfix Run3". The identification numbers are 4637881.3–4637896.3, 4637897.3–4637912.3, and 4642664.3–4642679.3.

Statistics. We conducted permutational multivariate ANOVA (PERMANOVA) procedures (Anderson, 2001) to test for differences in the composition of the N_2-fixing microbial community with respect to climate, grazing, and shrub-canopy treatments. We graphically depicted multivariate relationships of sites in species community space using Nonmetric Multi-Dimensional Scaling (NMDS; Kruskal 1964). Bray-Curtis (Steinhaus) distance was used for all mulitivariate procedures requiring resemblance matrices (Bray & Curtis 1957), and a significance level of $\alpha = 0.05$ was used for statistical tests. We used the R (version 3.2.0; R core team [https://www.R-project.org]) for PERMANOVA and ordination statistical tests, with heavy reliance on the package "vegan" (Oksanen et al. 2015).

RESULTS

Climate and associated soil properties on N_2-fixing communities

Soil pH decreased with elevation, while ortho-P and NH_4^+ increased with elevation. Electrical conductivity and NO_3^- varied among elevations with no clear pattern (Table 6). Soil pH ranged from 6.2 ± 0.15 to 8.0 ± 0.19 ($\bar{x}$ ± SD). The highest elevation site, Mountain Big Sage (MBS), had the highest NH_4^+ levels (1.4 ± 0.10 µg g dry soil^{-1}), and the lowest site, Flats (F), had the highest NO_3^- (2.9 ± 0.89 µg g dry soil^{-1}). Ortho-P ranged from 8.0 ± 4.68 to 10.1 ± 10.57 µg g dry soil^{-1}, and electrical conductivity ranged from 54.1 ± 14.03 µS cm^{-1} to 212.7 ± 178.60 µS cm^{-1} (Table 6).

Biocrust N_2-fixing bacterial communities

We sequenced the 16S rRNA V3-V4 gene to identify bacteria within biocrusts samples. Identification of known N_2-fixers from this data was selected for further analyses. A total of 47 bacterial N_2-fixing genera were identified, and those with a genus abundance >15 after normalization were used for analyses. The 47 genera consisted of 38 cyanobacteria genera, six symbiotic genera, and three other genera.

PERMANOVA showed that biocrust N_2-fixing communities were significantly different between sites along the elevational gradient (R^2 = 0.68431, $F_{3,3}$= 3.1656; P = 0.0132). The lowest elevation site, F, had greater abundance of cyanobacteria (69% of N_2-fixers) than any other site, and MBS, the highest elevation site, exhibited the greatest abundance of symbiotic N_2-fixing bacteria with 90% of total N_2-fixing bacteria (Fig. 8A). The two middle-elevation sites, Wyoming Big Sage (WBS) and Low Sage (LS), contained communities intermediate to those at the lowest and highest elevations. The Shannon-Weiner diversity index was highest at the lowest elevation sites F and WBS, and decreased at the highest elevation site, MBS (Table 6).

The ordination projection highlighted PERMANOVA results of significant differences between sites along the elevational gradient, and explains 68% of the variability in the community distance matrix. Particularly evident was the distinctiveness of communities at the lowest elevation site, F, and the highest elevation site, MBS (Fig. 8A). With respect to N_2-fixing genera, vector fitting identified cyanobacteria (R^2 = 0.3791; P= 0.021) and symbiotic N_2-fixers (R^2 = 0.4819; P = 0.008) as significant correlates of the NMDS projection of communities while other N_2-fixing bacteria (R^2 = 0.0425; P = 0.745) were not (Fig. 8A). Higher elevations had less cyanobacteria, and

were more acidic (R^2 = 0.8897; P = 0.001; Fig. 8B). Conversely, NH_4^+ was positively correlated with symbiotic N_2-fixer abundance and elevation (R^2 = 0.8317; P = 0.001; Fig. 8). Levels of NO_3^- (R^2 = 0.4572; P = 0.017) were correlated to both the second altitude independent dimension and the abundance of other N_2-fixing bacteria genera (Fig. 8). No association was evident between ortho-P (R^2 = 0.0869; P = 0.557) and electrical conductivity (R^2 = 0.2216; P = 0.173) with the NMDS projection (Fig. 8B).

The four elevations were dominated by distinct genera N_2-fixing bacteria. The three most dominant genera at the lowest site, F, were *Oscillatoria* (48%), *Frankia* (26%), and *Nostoc* (11%). At WBS, the highest abundance genera were *Frankia* (45%), *Oscillatoria* (20%), and *Clostridium* (10%). The LS site was dominated by *Frankia* (61%), *Oscillatoria* (9%), and *Bradyrhizomium* (9%) genera. The highest elevation site, MBS, was primarily composed of the genera *Frankia* (69%), *Bradyrhizobium* (21%), and *Clostridium* (6%).

Grazing disturbance on biocrust N_2-fixing communities

Grazed and ungrazed treatments did not host significantly different biocrust N_2-fixing communities ($F_{1,3}$ = 1.3811; P = 0.2661), however, this may have been a byproduct of relatively subtle community changes that were undetected by our analyses (Fig. 9; Fig. 8A). Specifically, F displayed the greatest difference in the genera *Microcoleus* and *Nostoc* across all elevations, with three and 19 times greater abundance in ungrazed areas compared with grazed areas, respectively (Fig. 10). Symbiotic N_2-fixers were highest in the grazed locations, whereas cyanobacteria were highest in the ungrazed locations at the two lowest elevations, F and WBS (Fig. 9). The two highest elevations, LS and MBS, had similar abundance of cyanobacteria, symbiotic, and other N_2-fixing bacteria at grazed and

ungrazed treatments (Fig. 9). The lowest elevation site, F, was dominated by the genus *Oscillatoria* (52%) in ungrazed samples and the genus *Frankia* (46%) in the grazed samples. The other three elevations of WBS, LS, and MBS were dominated by the genus *Frankia* (37–72%) in both grazed and ungrazed samples. The highest elevation, MBS, contained the highest abundance of bacteria belonging to the genus *Frankia* (72%) compared with the other elevations.

Shrub-canopy and interspace biocrust N_2-fixing communities

Differences in shrub-canopy and interspace communities were not significant ($F_{1,6}$ = 1.5301, P = 0.2091), and no interactions were detected between grazing/ungrazed and shrub-canopy/interspace treatments ($F_{1,6}$ = 1.0612, P = 0.3875). Nonetheless, several observations are worth noting. First, the highest amount of cyanobacteria occurred in the interspace treatment at the lowest elevation, F (Fig. 11). Second, the abundance of symbiotic bacteria was highest in the grazed shrub-canopy throughout all sites (Fig 11); however, at the lowest elevation site, F, the ungrazed shrub-canopy and interspace was primarily cyanobacteria (Fig. 11). The abundance of the genus *Oscillatoria* was highest in the interspace locations compared with the shrub-canopy throughout all sites, and was the dominant bacteria for the lowest site, F, in the ungrazed interspace and shrub-canopy. The genus *Frankia* (63%) was highest in abundance within the grazed shrub-canopy at the lowest site, F, but those belonging to the cyanobacterial genus *Oscillatoria* were the highest within the ungrazed shrub-canopy. The two highest elevations, LS and MBS, were dominated in the interspace and shrub-canopy by the genus *Frankia*. The highest elevation site, MBS, contained the highest abundance of the genus *Frankia* compared with the other elevations.

DISCUSSION

Climate and associated soil properties on N_2-fixing communities

Our study area spanned a wide range of climatic conditions typical of the intermountain region of the western USA, that were driven by nearly 1000 m change in elevation. Along this gradient, examination of the 16S rRNA V3-V4 gene region showed that N_2-fixing bacterial communities vary with climate, grazing history, and shrub-canopy associations. Differences in communities were especially pronounced within groups of cyanobacteria and symbiotic N_2-fixers. In accordance with our first hypothesis, bacterial N_2-fixing communities differed substantially, with wetter and cooler (higher elevation) climatic conditions exhibiting more symbiotic N_2-fixers compared with dryer and warmer (lower elevation) climates which contained more cyanobacteria. Although symbiotic N_2-fixers did not change in diversity with climate (elevation), their abundance approximately doubled along the three-fold increase in annual precipitation and an almost a two-fold decrease in mean annual temperature from the lowest to highest elevation (Table 2). This shift from cyanobacteria to symbiotic N_2 fixation may indicate the structure of biocrust N_2-fixing communities may shift under changing climatic conditions. For Southeastern Utah, precipitation events are projected to become more frequent, but occur as smaller rain events, and soil temperatures are expected to increase 2–3˚C (Johnson et al. 2012; Steven et al. 2015; Ferrenberg et al. 2015). The effect of climatic changes on biocrusts in Southeastern Utah, USA, which is currently dominated by rain in late summer and early autumn with infrequent winter rain or snow (Johnson et al. 2012), has been previously examined (Ferrenberg et al. 2015; Steven et al. 2015). Ferrenberg et al. (2015) showed an increase in cyanobacteria and decrease moss and lichen cover with climate

manipulations. However, when increased temperatures and shifts in precipitation were combined, Steven et al. (2015), found that cyanobacterial populations decreased. In our study location, precipitation is higher and occurs more often as snow at the highest (coldest) elevation site, and biocrusts are dominated by symbiotic N_2-fixers. Conversely, precipitation at our lower (warmest) elevation sites is less frequent and occurs more often as rain, and biocrusts are dominated by cyanobacteria. Given regional precipitation and temperatures projections, we might expect a decline in lichens, mosses, and cyanobacteria, and an increase of cyanobacteria at higher elevations in the Intermountain West, USA.

The cyanobacteria genera *Oscillatoria* and *Nostoc* were dominant at the three lowest elevations sites. Studies conducted on biocrusts from SW United States and China, however, identified bacteria belonging to the genus *Microcoleus* as the dominant cyanobacteria, with the genera *Nostoc* and *Scytomena* occurring only in mature southwest USA biocrusts (Belnap & Lange, 2003; Yeager et al. 2004; Wang et al. 2006; Kuske et al. 2012 Wang et al. 2015). A study in India identified the genera *Tolypothrix*, *Scytonema,* and *Lyngbya* as dominant cyanobacteria (Tirkey & Adhikary, 2005; Kumar & Adhikary, 2015), whereas scientists in Israel identified cyanobacteria from the genera *Microcoleus*, *Leptolyngbya*, and *Trichocoleus* (Hagemann et al. 2015). All of those genera, except *Nostoc*, were abundant only at the three lower elevations, but composed minor (<1%) components of cyanobacteria. Furthermore, only one other study, conducted in Spain, identified cyanobacteria from the genus *Oscillatoria* as a major constituent in biocrusts (Pringault & Garcia-Pichel, 2003). The genus *Oscillatoria* is a non-heterocystous forming cyanobacteria, and motile in the soil, which allows migration to

the surface upon wetting, similar to bacteria found in the genus *Microcoleus* (Belnap, 2002; Belnap & Lange, 2003; Pringault & Garcia-Pichel, 2003). Species within *Oscillatoria* can conduct anoxygenic photosynthesis, which allows the production of ATP without producing oxygen and using water as the electron donor, such as the use of sulfide (Sybesma et al. 1986). The higher abundance of the cyanobacteria genus *Oscillatoria* at lower elevations in this study may indicate that soils higher in salts as indicated by higher conductivity, and likely higher sulfide, may select for *Oscillatoria* species (Pringault & Garcia-Pichel 2003).

Soil characteristics changed as a function of climate, along our elevation gradient. These changes were especially evident for soil pH and NH_4^+, which were strongly correlated with bacterial community composition (Fig. 8B). Similar to other studies (Belnap & Lange 2003; Wang et al. 2015), we found pH to be particularly strong correlate of biocrust bacterial communities. Symbiotic N_2-fixing bacteria abundance increased as soils became more acidic. In accordance with our prediction, both the abundance and number of cyanobacteria genera decreased as soils became more acidic (Fig. 8A). This pattern may indicate that pH is a weaker control for symbiotic and other N_2-fixers, but a stronger control for cyanobacteria. The observed pattern of correlation for NH_4^+ with N_2-fixing communities suggests that higher levels of NH_4^+ can decrease the abundance of particular cyanobacteria (Fig. 8B). Biocrust N additions have been shown to decrease abundance of the cyanobacteria genera *Lyptolyngbya* and *Microcoleus* (Wang et al. 2015). Higher N could possibly decrease the abundance of other cyanobacteria not identified within the high N addition study, but found within our study. Counter to our prediction, electrical conductivity was not correlated to the ordination projection, and was

highest at the middle elevation site WBS, which did not coincide with high abundance of cyanobacteria. This pattern may indicate that pH is a stronger control of N_2-fixing bacteria than conductivity. The higher conductivity (and concomitant salinity) at the WBS site may have selected for an increased abundance of cyanobacteria from the genus *Synechococcus*, compared with other sites. The lowest elevation site, F, had the highest levels of both NO_3^- and cyanobacteria. This may have occurred because of factors suggested earlier (Hagemann et al. 2015), but this requires further investigation. Phosphorus has been proposed as a limiting agent to biological N_2 fixation (Reed et al. 2011), which prompted our prediction that it would indirectly control community composition. The lack of correlation, however, with ortho-P in community space indicates that ortho-P is not a control on biocrust N_2-fixing communities in our system (Fig. 8B). In addition, concentrations of soluble reactive P as ortho-P were more than two times higher than N at all sites suggesting that this is not likely a limiting nutrient within our study.

Grazing disturbance on biocrust N_2-fixing communities

Although the differences were not significant, in accordance with our second hypothesis, grazing was correlated with decreasing cyanobacterial abundance, especially in drier and warmer (lower elevation) climates, thereby altering biocrust community composition. There were observed community differences occurred between grazed and ungrazed locations, albeit only at the two lowest sites, F and WBS (Fig. 9). At F, cyanobacteria abundance was greater in the ungrazed locations, and symbiotic and other N_2-fixing bacteria were greater in grazed locations indicating a shift in community composition with grazing. This outcome is consistent with a human disturbance study conducted in

southeast Utah, which also observed an increase in other phyla including symbiotic and other N_2-fixers, and a decrease in cyanobacteria with disturbance (Kuske et al. 2012), although our results were not as pronounced. Trampling by humans or cattle is likely to produce different effects on biocrusts, with severity controlling that effect. Shrub encroachment from grazing also may decrease cyanobacterial abundance (Elliott et al. 2014), and community differences between grazed and ungrazed areas are likely due to changes in abundance rather than presence or absence of genera, similar to patterns we observed (Ponzeti & McCune 2001).

Contrary to our expectations, the genus *Microcoleus* was less abundant at grazed locations, except for the LS site (Fig. 10). Other studies typically observe an increase of cyanobacteria from the genus *Microcoleus* with disturbance events (Belnap 2002; Yeager et al. 2004; Dojani et al. 2013), because early successional biocrusts predominantly have greater abundance of bacteria from the genus *Microcoleus*, with little or no presence of the genus *Nostoc* (Belnap 2003; Yeager et al. 2004; Dojani et al. 2013). In those studies, mostly conducted in southeast Utah, the genus *Microcoleus* is the dominant cyanobacteria. In our study, however, cyanobacteria from the genus *Oscillatoria* were dominant potentially explaining the discrepancy. The genus *Nostoc*, as we predicted, was higher in the ungrazed locations, following previously observed trends of greater abundance in undisturbed locations (Belnap 2003; Yeager et al. 2004; Dojani et al. 2013).

Shrub-canopy and interspace biocrust N_2-fixing communities

Regarding our third hypothesis, our results display altered bacterial N_2-fixing communities between the interspace and shrub-canopy in the two lowest sites, F and WBS (Fig. 11), although these differences were not significant. There were about three

times more cyanobacteria within the ungrazed shrub-canopy than in the grazed shrub-canopy at the lowest site (Fig.11) indicating that grazing may alter the biocrust community even at the microsite scale, particularly in warm, dry climates. With increasing elevation cyanobacteria became less abundant in the grazed and ungrazed interspace locations, while symbiotic N_2-fixers increased. We hypothesize that the decrease in cyanobacteria within interspaces may be a result of an increase in vegetation cover with higher precipitation. In this setting, cyanobacteria may be outcompeted by grasses and shrub bacterial communities within the smaller interspaces. Our results are similar with a previous study that found biocrust communities to be different between interspaces and the shrub-canopy understory locations (Elliott et al. 2014). In this prior study, cyanobacteria dominated the interspaces and were rare within the shrub and tree canopy biocrusts (Elliott et al. 2014). Our findings were similar, but cyanobacteria were abundant except at the highest elevation. Variations in cyanobacterial abundance are likely because of differences in climates and vegetation, and illustrate the sensitivity of bacterial biocrust communities to microclimate conditions.

CONCLUSION

Biocrusts are increasingly acknowledged as providing vital functions in drylands; however, the ecology of biocrusts associated with cool, more mesic drylands in the Intermountain West is unknown. Our study is the first to identify bacterial N_2-fixing community shifts corresponding to changes in climate, grazing history, and shrubs within a rolling biocrust community in southwest Idaho, and demonstrate that those factors are associated with shifts in N_2-fixing bacteria. Findings from this study indicate that climate change, grazing, and the loss of shrub interspaces could result in shifts of biocrust

communities with important ecosystem implications as these alterations affect both soil nutrients availability and stability. Further studies are needed to understand the distribution of observed biocrusts morphologies associated with our region. This research will aid in determining whether responses to environmental factors are similar, allowing predictions at the regional or greater scale. Taken together, our results indicate that rolling biocrust associated with colder more mesic semiarid dryland ecosystems share some similarities to pinnacled biocrusts that tend to dominate hot semiarid dryland ecosystems in terms that climate, grazing and shrub-canopy can alter the community composition. However, major constituents such as cyanobacteria from the genus *Oscillatoria* within our study suggest they are likely different in compositional aspects, and may change nutrient inputs and cycling within the system.

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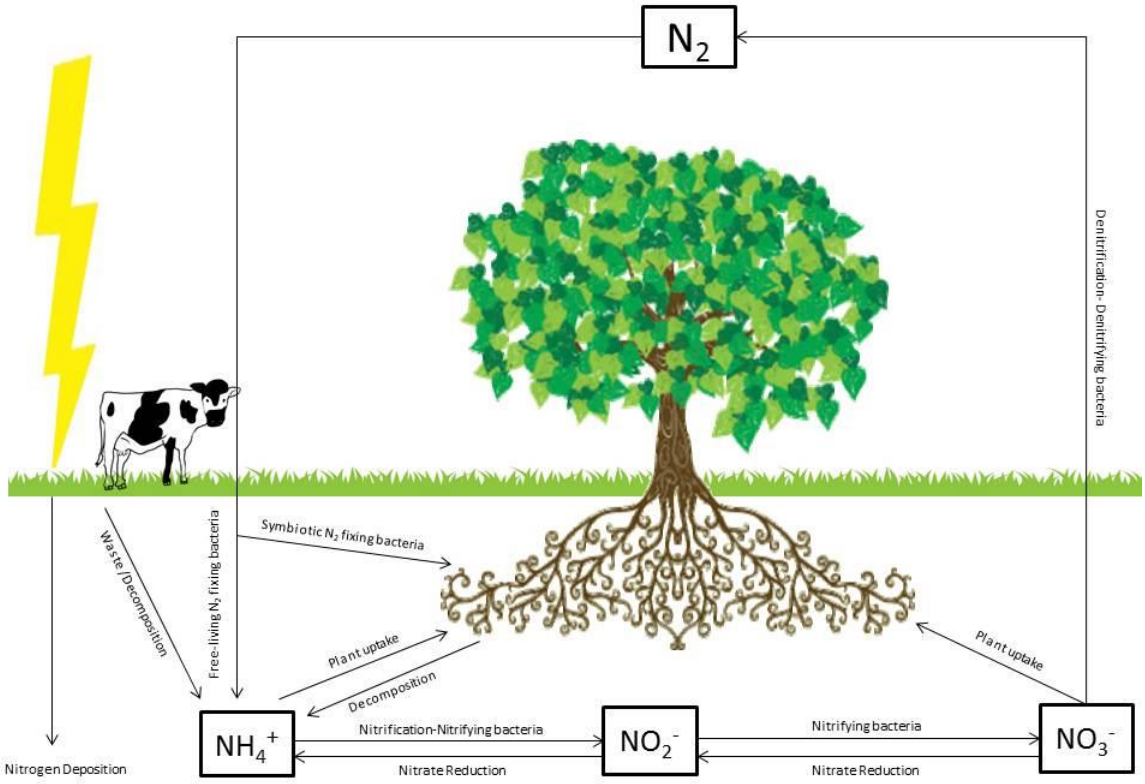

Figure 1: Nitrogen Cycle schematic portraying inputs, transformations, and losses from the system.

Table 1: Summary of studies done by Aranibar et al. (2003), Belnap (2002), Freiberg et al. (1998), Granhall et al. (1978), Malam Issa et al. (2001), Rosen et al. (2006) that show a comparison of free-living N_2 fixation rates found in many climates and ecosystems. Averages or ranges are shown if multiple locations and seasons were used.

Authors	***Location***	***Ecosystem***	***N_2-fixation rates ($Kg\ N\ ha^{-1}\ yr^{-1}$)***	***Substrate***
Aranibar et al. (2003)	Zambia and Botswana, Africa	Woodlands	8-44	biocrusts
Belnap (2002)	Cayonlands National Park, Utah, USA	Cold desert	1.4-9	biocrusts
Freiberg et al. (1998)	Provincia Alajucla, Costa Rica	Premontane rain forest	1.6 per leaf area index	phyllosphere
Granhall et al. (1978)	Stockholm, Sweden	Coniferous forest	0.3-38	phyllosphere, soil samples
Malam Issa et al. (2001)	Sahel, Niger	Arid	3.5	biocrusts
Rosen et al. (2006)	Sweden	Coniferous forest	1.5	biocrusts

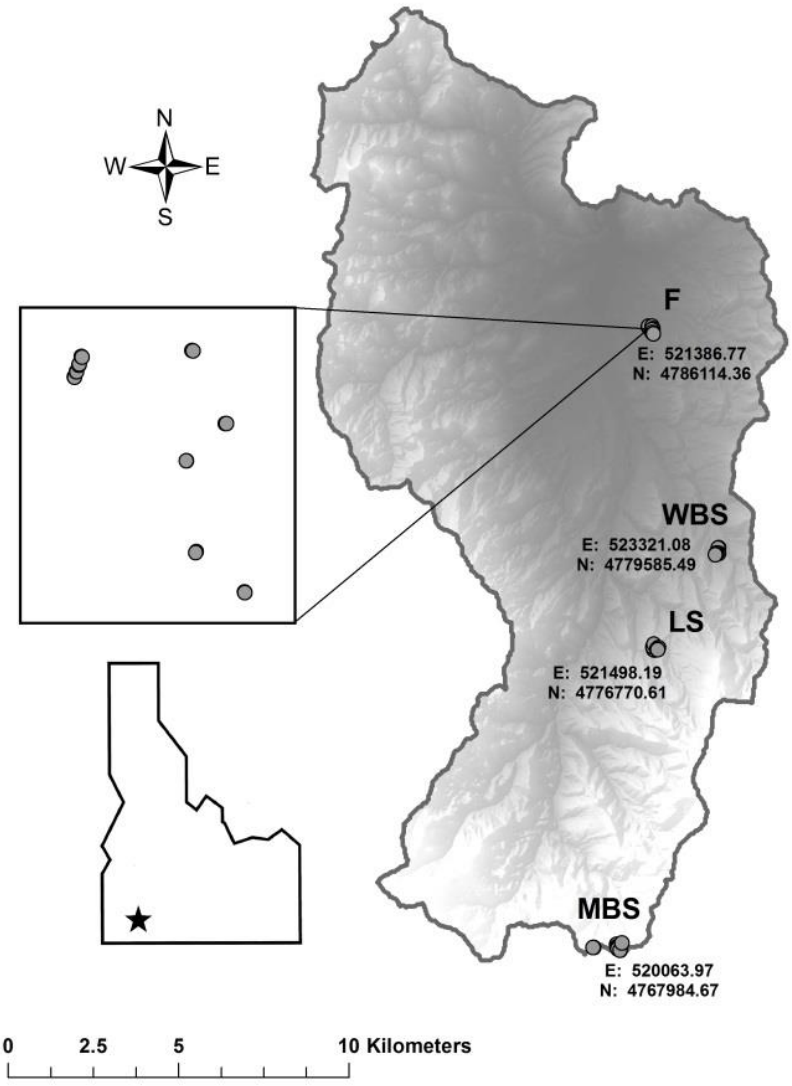

Figure 2: Location of sampling sites with black outline as boundary for Reynolds Creek Critical Zone Observatory watershed. Each cluster of points indicates a site. From north to south, sites are Flats (F), Wyoming Big Sage (WBS), Low Sage (LS), and Mountain Big Sage (MBS).

Table 2: Description of each site according to elevation (meters), GPS location (latitude and longitude), mean annual temperature (MAT) (˚C), mean annual precipitation (MAP) (mm yr^{-1}), and dominant vascular vegetation. Sites increase in elevation from Flats (F), Wyoming Big Sage (WBS), Low Sage (LS), to Mountain Big Sage (MBS).

Site	Elevation	GPS	MAT	MAP	Vegetation
F	1170	521386.77 E, 4786114.36 N	9.1	235	*Artemisia tridentate* ssp. *wyomingensis*
WBS	1400	523321.08 E, 4779585.49 N	9.2	298	*Artemisia tridentate* ssp. *wyomingensis*
LS	1600	521498.19 E, 4776770.61 N	8.5	345	*Artemisia arbuscula*
MBS	2080	520063.97 E, 4767984.67 N	5.4	803	*Artemisia tridentata* ssp. *vaseyana*

Table 3: Measured soil characteristics averaged from soil samples (2.5–7.5 cm depth) collected from October 2014 and May 2015 from beneath collected biocrust samples. Measured amounts of ammonium (NH_4^+), nitrate (NO_3^-), phosphorus (PO_4^{-3}), pH, electrical conductivity (EC), and gravimetric water content (GWC) were averaged from the two sampling time points and are given for each elevation. Means ± (SE) are reported. Sites increase in elevation from Flats (F), Wyoming Big Sage (WBS), Low Sage (LS), to Mountain Big Sage (MBS).

	F	WBS	LS	MBS
NH_4^+ (ug g dry soil^{-1})	0.5 (0.04)	0.6 (0.03)	0.7 (0.11)	1.5 (0.11)
NO_3^- (ug g dry soil^{-1})	1.6 (0.28)	1.2 (1.4)	2.1 (0.25)	5.4 (0.62)
Ortho-P (ug g dry soil^{-1})	6.8 (0.70)	9.5 (1.75)	10.9 (1.33)	10.2 (3.19)
pH	8.2 (0.16)	7.3 (0.08)	7.2 (0.09)	6.1 (0.08)
EC (µS cm^{-1})	189.6 (85.27)	212.7 (51.52)	54.1 (5.12)	103.5 (17.01)
GWC (%)	7 (0.76)	10.9 (0.57)	17.5 (0.96)	30.3 (2.38)

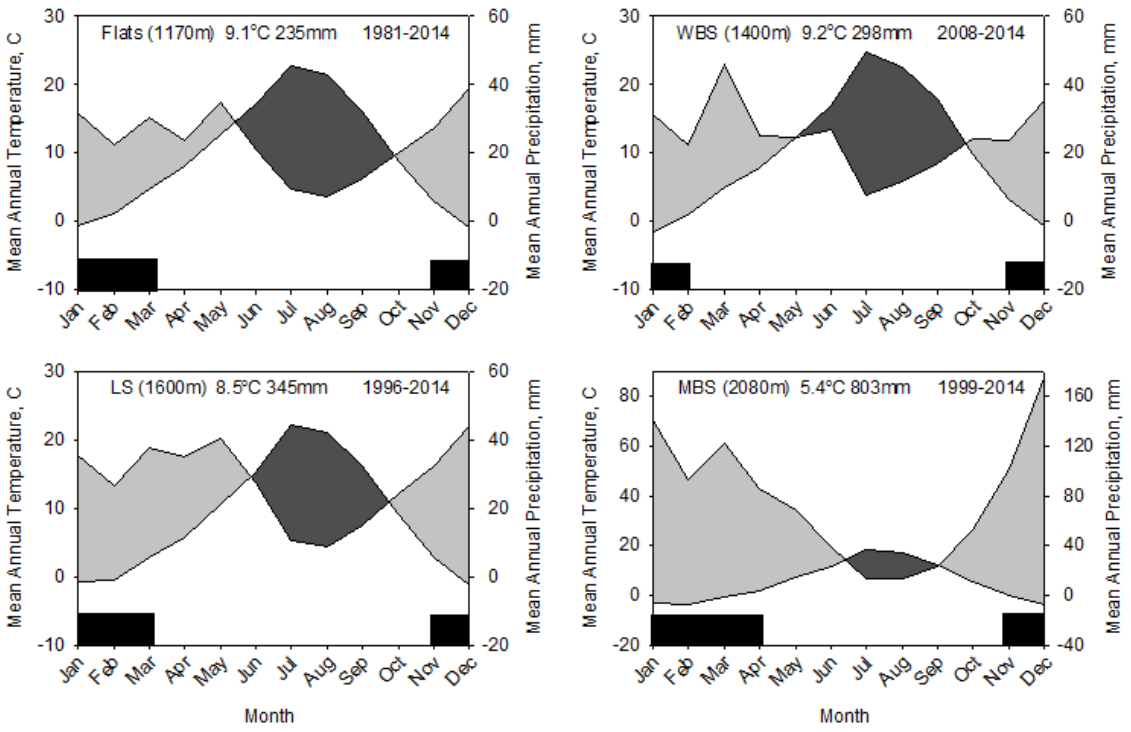

Figure 3: Walter-type diagram for each site including elevation, mean annual temperature, mean annual precipitation, and years monitored. Dark gray shaded area shows months with precipiation > evaoporation. Light gray shaded area shows months with precipitation < evaporation. Black area shows months with mean daily minimum temperatures were less than 0˚C. *Jan* January, *Feb* February, *Mar* March, *Apr* April, May, *Jun* June, *Jul* July, *Aug* August, *Sep* September, *Oct* October, *Nov* November, *Dec* December. Site in increasing elevation are Flats, Wyoming Big Sage (WBS), Low Sage (LS), and Mountain Big Sage (MBS).

Table 4: Measured biocrust characteristics. The means ± SE of total N (TN; %), total C (TC; %), δ15N (‰) and δ13C (‰), chlorophyll-a concentration (Chl *a*; ug g soil^{-1}), and percent cover of moss, lichen, total biocrust, and vascular plant vegetation at each elevation. Sites increasing in elevation are Flats (F), Wyoming Big Sage (WBS), Low Sage (LS), and Mountain Big Sage (MBS).

	TN	TC	$\delta^{15}N$	$\delta^{13}C$	Chl-*a*	Moss	Lichen	Crust	Vegetation
Flats									
Climate	0.2 ± 0.02	2.8 ± 0.39	5.5 ± 0.77	-22.9 ± 0.76	4.5 ± 0.73	44 ± 2.1	14 ± 3.6	58 ± 4.3	13 ± 1.3
Grazed	0.2 ± 0.04	2.6 ± 0.62	5.1 ± 0.99	-23.9 ± 0.73	4.0 ± 1.06	41 ± 1.1	5 ± 1.9	46 ± 2.4	14 ± 2.4
Ungrazed	0.2 ± 0.02	2.9 ± 0.54	6.0 ± 1.26	-21.9 ± 1.26	4.9 ± 1.07	46 ± 4.0	24 ± 3.6	70 ± 2.8	11 ± 0.7
Interspace	0.1 ± 0.00	1.3 ± 0.04	6.0 ± 0.21	-22.5 ± 0.75	3.3 ± 1.20	14 ± 2.2	28 ± 18.4	42 ± 20.6	8 ± 3.4
Shrub	0.3 ± 0.02	4.2 ± 0.37	5.0 ± 0.68	-23.3 ± 1.27	6.1 ± 0.78	74 ± 2.8	1 ± 0.0	75 ± 2.8	17 ± 0.0
WBS									
Climate	0.2 ± 0.02	2.7 ± 0.38	4.6 ± 0.23	-25.7 ± 0.11	5.3 ± 0.94	50 ± 3.4	10 ± 1.6	60 ± 2.6	13 ± 1.3
Grazed	0.2 ± 0.04	3.1 ± 0.66	4.8 ± 0.16	-25.9 ± 0.16	5.8 ± 1.59	55 ± 5.3	7 ± 1.3	62 ± 4.8	15 ± 1.8
Ungrazed	0.2 ± 0.03	2.3 ± 0.35	4.4 ± 0.43	-25.6 ± 0.13	4.9 ± 1.17	44 ± 2.9	13 ± 2.2	58 ± 2.1	11 ± 1.6
Interspace	0.1 ± 0.02	1.4 ± 0.26	5.7 ± 0.51	-25.3 ± 0.18	5.0 ± 1.62	22 ± 10.2	15 ± 2.6	37 ± 7.6	13 ± 3.2
Shrub	0.3 ± 0.06	3.9 ± 1.08	3.6 ± 0.10	-26.2 ± 0.17	5.7 ± 2.50	77 ± 0.4	5 ± 3.9	82 ± 3.5	13 ± 0.2
LS									
Climate	0.5 ± 0.07	7.4 ± 1.37	3.7 ± 0.33	-26.6 ± 0.19	6.1 ± 1.23	23 ± 4.6	12 ± 1.6	35 ± 6.0	37 ± 7.5
Grazed	0.5 ± 0.12	8.9 ± 2.42	3.4 ± 0.65	-26.3 ± 0.34	6.4 ± 1.71	30 ± 7.0	13 ± 2.9	43 ± 9.6	35 ± 10.8
Ungrazed	0.4 ± 0.06	5.9 ± 1.22	4.0 ± 0.15	-26.8 ± 0.06	5.8 ± 1.97	17 ± 4.9	11 ± 1.7	27 ± 6.2	38 ± 11.6
Interspace	0.4 ± 0.06	4.6 ± 0.80	4.4 ± 0.14	-26.0 ± 0.48	7.7 ± 0.79	8 ± 1.8	20 ± 0.7	28 ± 2.5	34 ± 4.7
Shrub	0.6 ± 0.07	10.2 ± 2.23	3.0 ± 0.70	-27.1 ± 0.09	4.5 ± 1.40	39 ± 11.7	4 ± 1.2	43 ± 12.9	40 ± 8.0
MBS									
Climate	0.7 ± 0.07	10.0 ± 1.29	3.7 ± 0.36	-26.8 ± 0.18	7.1 ± 1.05	1 ± 0.2	0 ± 0.0	1 ± 0.2	34 ± 5.4
Grazed	0.6 ± 0.07	8.5 ± 0.97	4.3 ± 0.31	-26.8 ± 0.27	8.4 ± 1.90	1 ± 0.5	0 ± 0.0	1 ± 0.5	30 ± 5.0
Ungrazed	0.7 ± 0.12	11.5 ± 2.32	3.1 ± 0.58	-26.8 ± 0.26	5.7 ± 0.66	1 ± 0.2	0 ± 0.0	1 ± 0.2	38 ± 9.8
Interspace	0.6 ± 0.04	7.7 ± 0.37	4.3 ± 0.17	-26.4 ± 0.20	11.0 ± 1.66	0 ± 0.0	0 ± 0.0	0 ± 0.0	38 ± 1.7
Shrub	0.8 ± 0.15	12.3 ± 3.37	3.0 ± 0.95	-27.2 ± 0.28	3.1 ± 1.05	2 ± 0.0	0 ± 0.0	2 ± 0.0	30 ± 9.8

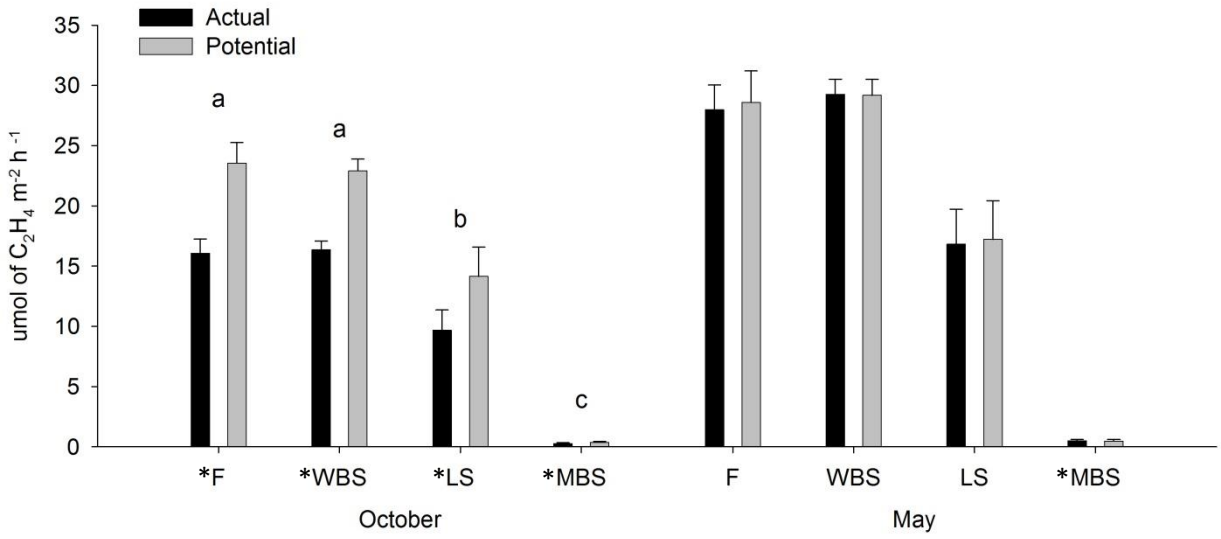

Figure 4: Nitrogenase activity for samples measured at field moisture (actual) and those where water was added (potential) from October 2014 and May 2015. Values are means ± SE. Different lowercase letters indicate significantly different nitrogenase activities among sites in October with same significance levels in May based on Tukey-Kramer *post-hoc* analyses. An * shown near the site name on the x-axis denotes significant differences between actual and potential activity based on Wilcoxon Sign ranked test. Sites increasing in elevation are Flats (F), Wyoming Big Sage (WBS), Low Sage (LS), and Mountain Big Sage (MBS).

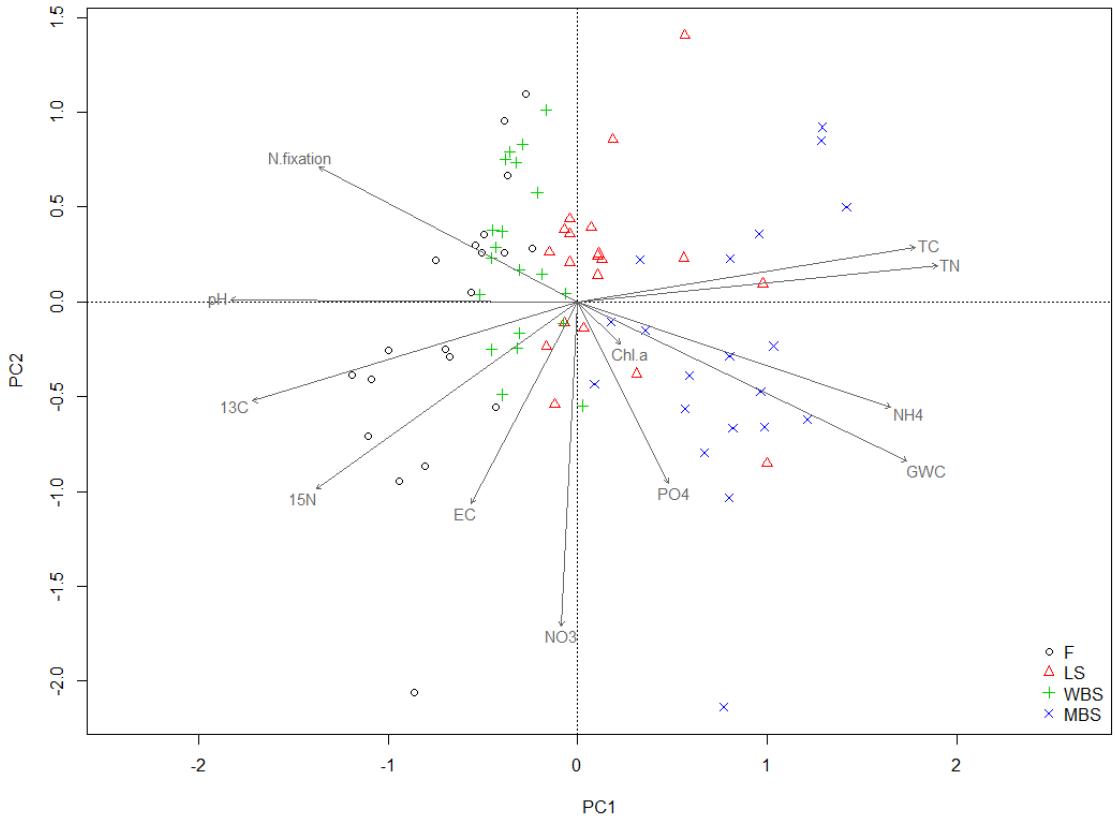

Figure 5: PCA ordination of measured environmental variables with vector fitting of soil characteristics identified N_2 fixation rates (P = 0.001; R^2 = 0.4944), pH (P = 0.001; R^2 = 0.6997), electrical conductivity (EC; P = 0.001; R^2 = 0.3017), NO_3^- (P = 0.001; R^2 = 0.6109), NH_4^+ (P = 0.001; R^2 = 0.6313), extractable PO_4^{-3} concentrations (ortho-P; P = 0.002; R^2 = 0.002), GWC (P = 0.001; R^2 = 0.7761), total N (TN; P = 0.001; R^2 = 0.7582), total C (TC; P = 0.001; R^2 = 0.6769), δ^{15}N (15N; P = 0.001; R^2 = 0.7582), δ^{13}C (13C; P = 0.001; R^2 = 0.6769), and chlorophyll-*a* concentrations (Chl a; P = 0.445; R^2 = 0.0208) with respect to elevation ($F_{3,75}$ = 3.78; P = 0.001).

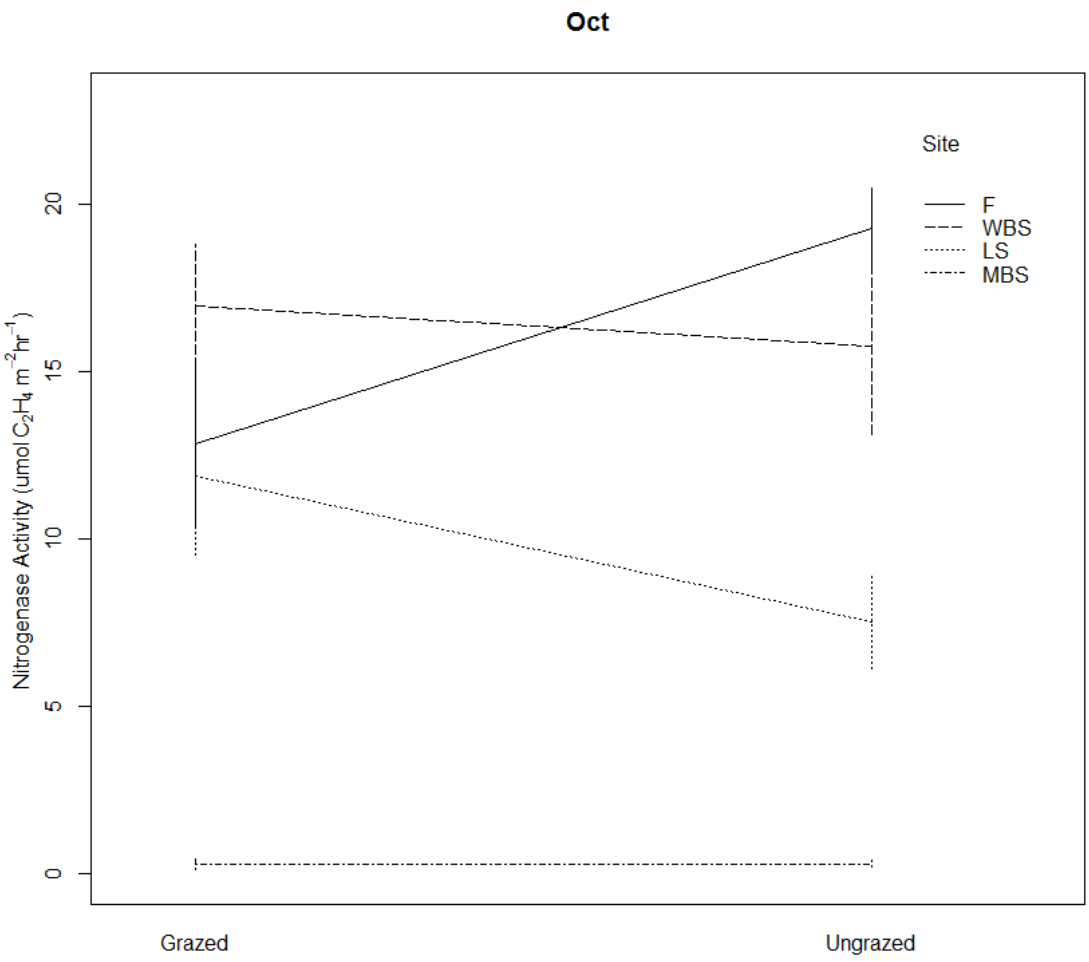

Figure 6: Interaction plot of nitrogenase activity from the October 2014 sampling for each site along the elevation gradient in samples that did not receive additional water (actual). May 2015 showed identical patterns, but May had higher nitrogenase activity. Values are means ± SE. Sites in order of increasing elevation are Flats (F), Wyoming Big Sage (WBS), Low Sage (LS), and Mountain Big Sage (MBS).

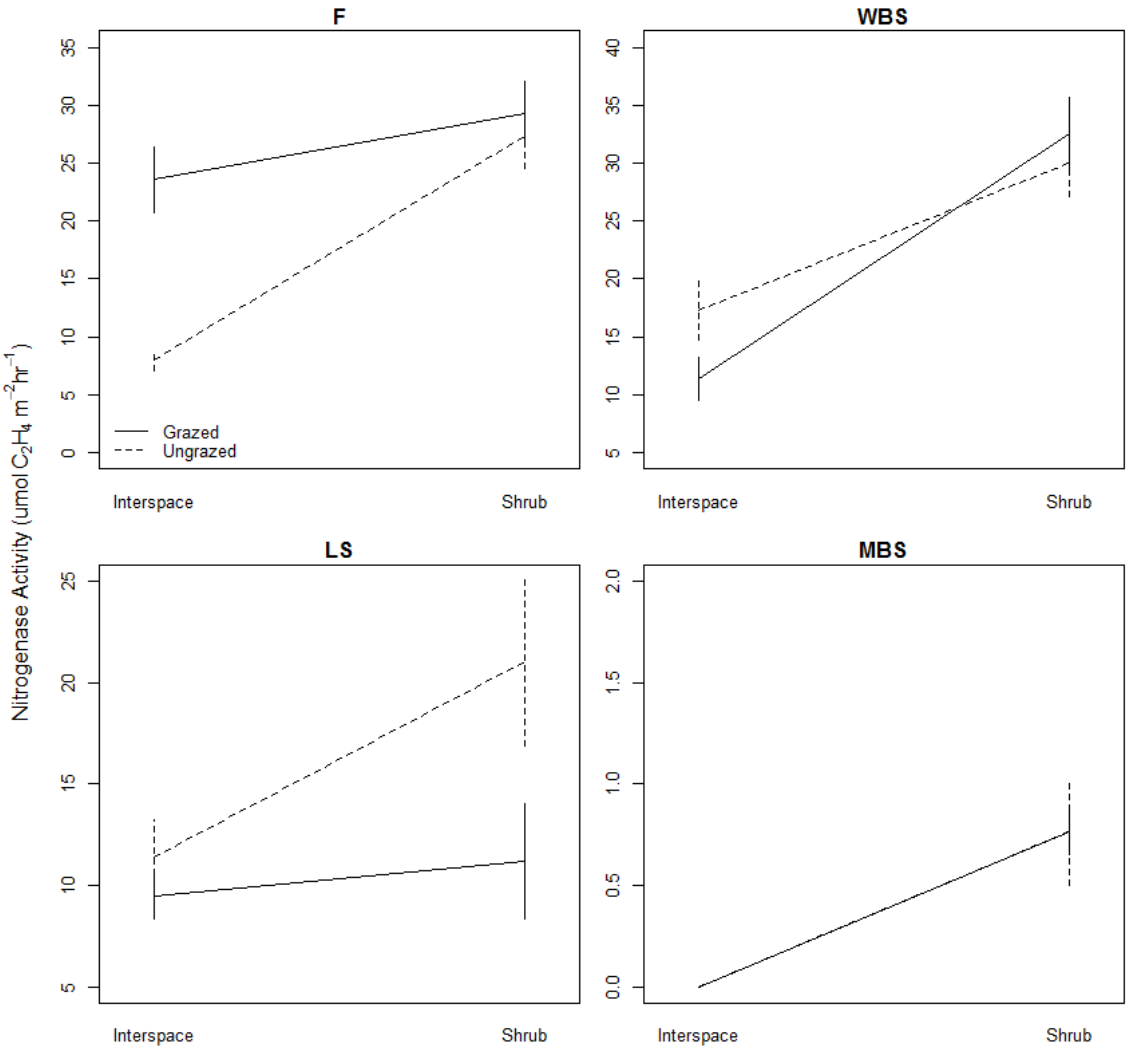

Figure 7: Interaction plots of nitrogenase activity within the grazed and ungrazed interspace and shrub-canopy for each site along the elevation gradient. Values are means ± SE. Sites in order of increasing elevation are Flats (F), Wyoming Big Sage (WBS), Low Sage (LS), and Mountain Big Sage (MBS).

Table 5: Literature review of biocrust nitrogenase activity in dryland ecosystems.

Southwest Idaho (USA)	
0.3–29.3 µmol m^{-2} h^{-1}	This study
Colorado Plateau (Southeast Utah, USA)	
25–50 µmol m^{-2} h^{-1}	Strauss et al. (2012)
1.5–120 µmol m^{-2} h^{-1}	Belnap (2002)
0.015–0.09 µmol m^{-2} h^{-1}	Belnap (1996)
0.01–0.05 µmol m^{-2} h^{-1}	Evans & Belnap (1999)
Chihuahuan Desert (New Mexico, USA)	
40–100 µmol m^{-2} h^{-1}	Strauss et al. (2012)
0.0004–0.013 µmol m^{-2} h^{-1}	Hartley & Schlesinger (2002)
Sonoran Desert (Arizona, USA)	
50–100 µmol m^{-2} h^{-1}	Strauss et al. (2012)
110 µmol m^{-2} h^{-1}	Eskew & Ting (1978)
Mojave Desert (Nevada, USA)	
25–200 µmol m^{-2} h^{-1}	Strauss et al. (2012)
0–33 µmol m^{-2} h^{-1}	Billings et al. (2003)
Loess Plateau (China)	
9–68 µmol m^{-2} h^{-1}	Zaho et al. (2010)
Muscat (Sultanate of Oman)	
39.4–58.5 µmol m^{-2} h^{-1}	Abed et al. (2010)
Sahel (Niger)	
34.7–42.04 µmol m^{-2} h^{-1}	Malam Issa et al. (2001)
Negev Desert (Israel)	
340–610 µmol m^{-2} h^{-1}	Zaady et al. (1998)
Zambia & Botswana	
0.117–0.535 µmol m^{-2} h^{-1}	Aranibar et al. (2003)

Table 6: Soil chemistry data from October 2014 soil core samples, and Shannon-Weiner diversity index from biocrust 16S rRNA V3-V4 gene region. Electrical conductivity (EC) is in µS cm^{-1}, nutrients (NH_4^+, NO_3^- , and ortho-P) are given in µg g dry $soil^{-1}$. Values for pH, EC, NH_4^+, NO_3^- , and ortho-P are means ± SD. Sites increasing in elevation are Flats (F), Wyoming Big Sage (WBS), Low Sage (LS), and Mountain Big Sage (MBS).

	pH	EC	NH_4^+	NO_3^-	Ortho-P	Shannon
F	8.0 ± 0.19	103.7 ± 29.21	0.5 ± 0.08	2.9 ± 0.89	8.0 ± 4.68	1.697
WBS	7.3 ± 0.13	212.7 ± 178.60	0.6 ± 0.14	1.5 ± 0.47	8.2 ± 5.43	1.790
LS	7.2 ± 0.28	54.1 ± 14.03	0.6 ± 0.04	2.2 ± 0.49	12.1 ± 4.18	1.461
MBS	6.2 ± 0.15	103.5 ± 18.41	1.4 ± 0.10	2.3 ± 1.27	10.1 ± 10.57	0.948

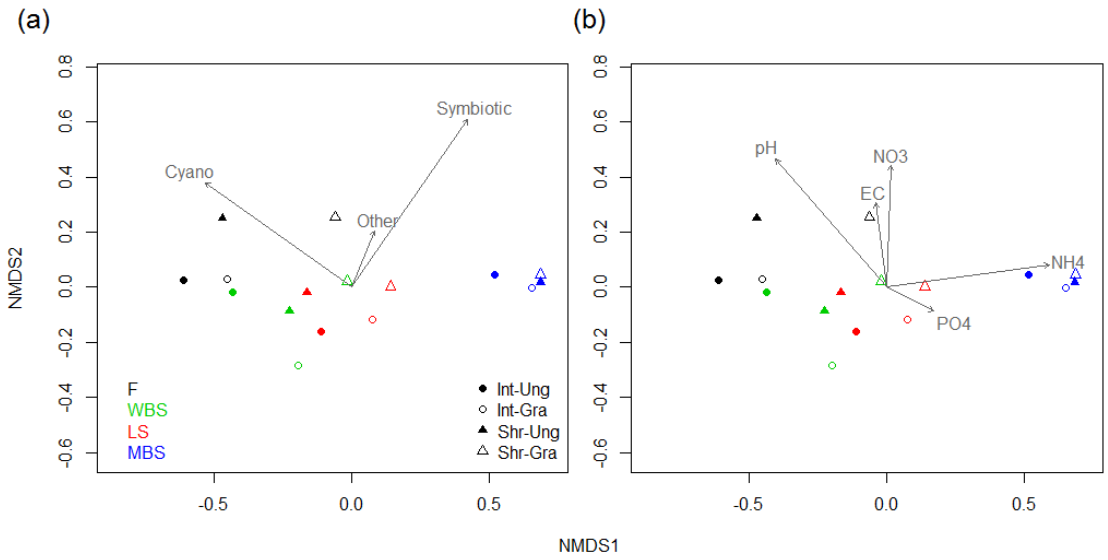

Figure 8: a) NMDS of N_2-fixing community genera with vector fitting for cyanobacteria (R^2 = 0.3791; P = 0.021), symbiotic (R^2 = 0.4819; P = 0.008), and other N_2-fixing bacteria (R^2 = 0.0425; P = 0.745). b) NMDS of N_2-fixing community genera with vector fitting of pH (R^2 = 0.8897; P = 0.001), electrical conductivity (R^2 = 0.2216; P = 0.173), NH_4^+ µg g dry soil^{-1} (R^2 = 0.8317; P = 0.001), NO_3^- µg g dry soil^{-1} (R^2 = 0.4572; P = 0.017), and ortho-P µg g dry soil^{-1} (R^2 = 0.0869; P = 0.557). Sites increasing in elevation are Flats (F), Wyoming Big Sage (WBS), Low Sage (LS), and Mountain Big Sage (MBS).

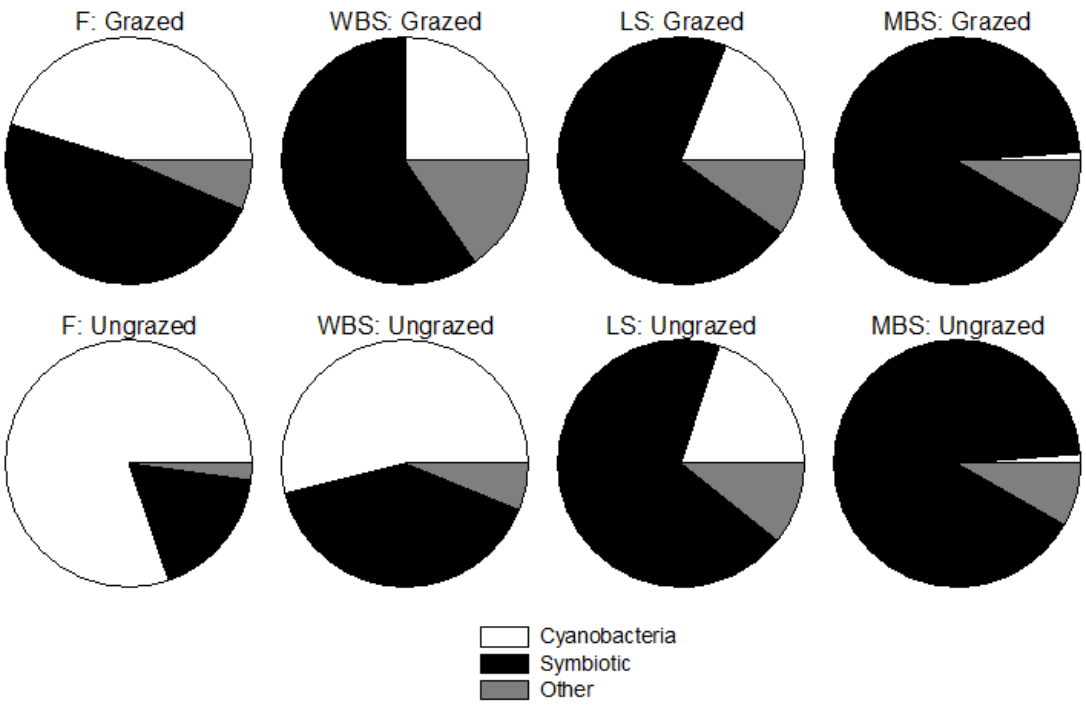

Figure 9: Proportions of the genera grouped into the categories of cyanobacteria, symbiotic, and other N_2-fixing bacteria with respect to grazing and ungrazed samples. Only one graph is shown for Low Sage (LS) and Mountain Big Sage (MBS) because grazed and ungrazed areas showed similar trends.

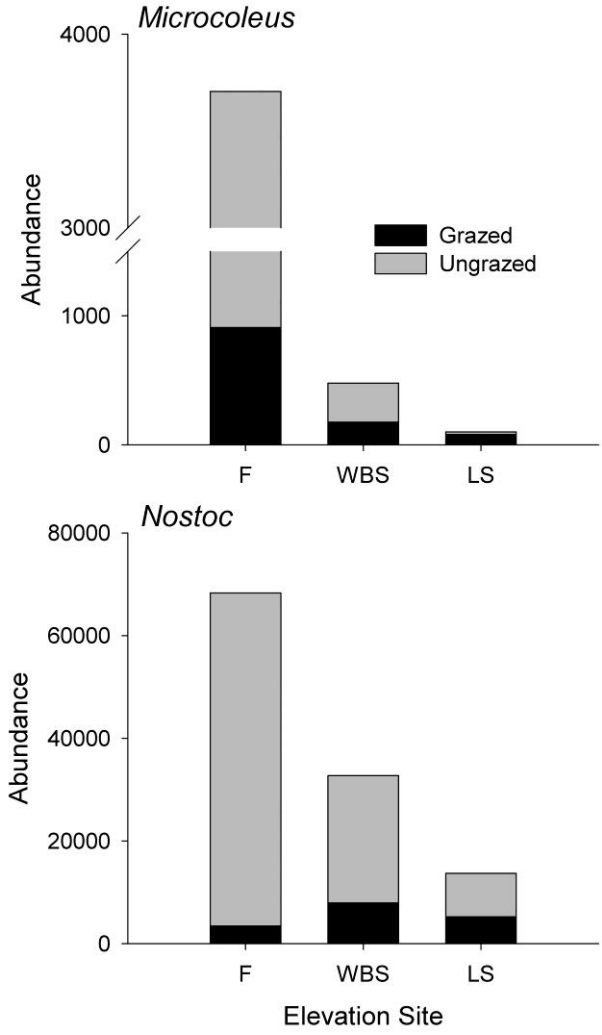

Figure 10: Comparisons of *Microcoleus* and *Nostoc* in grazed and ungrazed areas at the lower three elevations. The highest elevation site lacked the two genera in grazed and ungrazed areas. Sites increasing in elevation are Flats (F), Wyoming Big Sage (WBS), Low Sage (LS), and Mountain Big Sage (MBS).

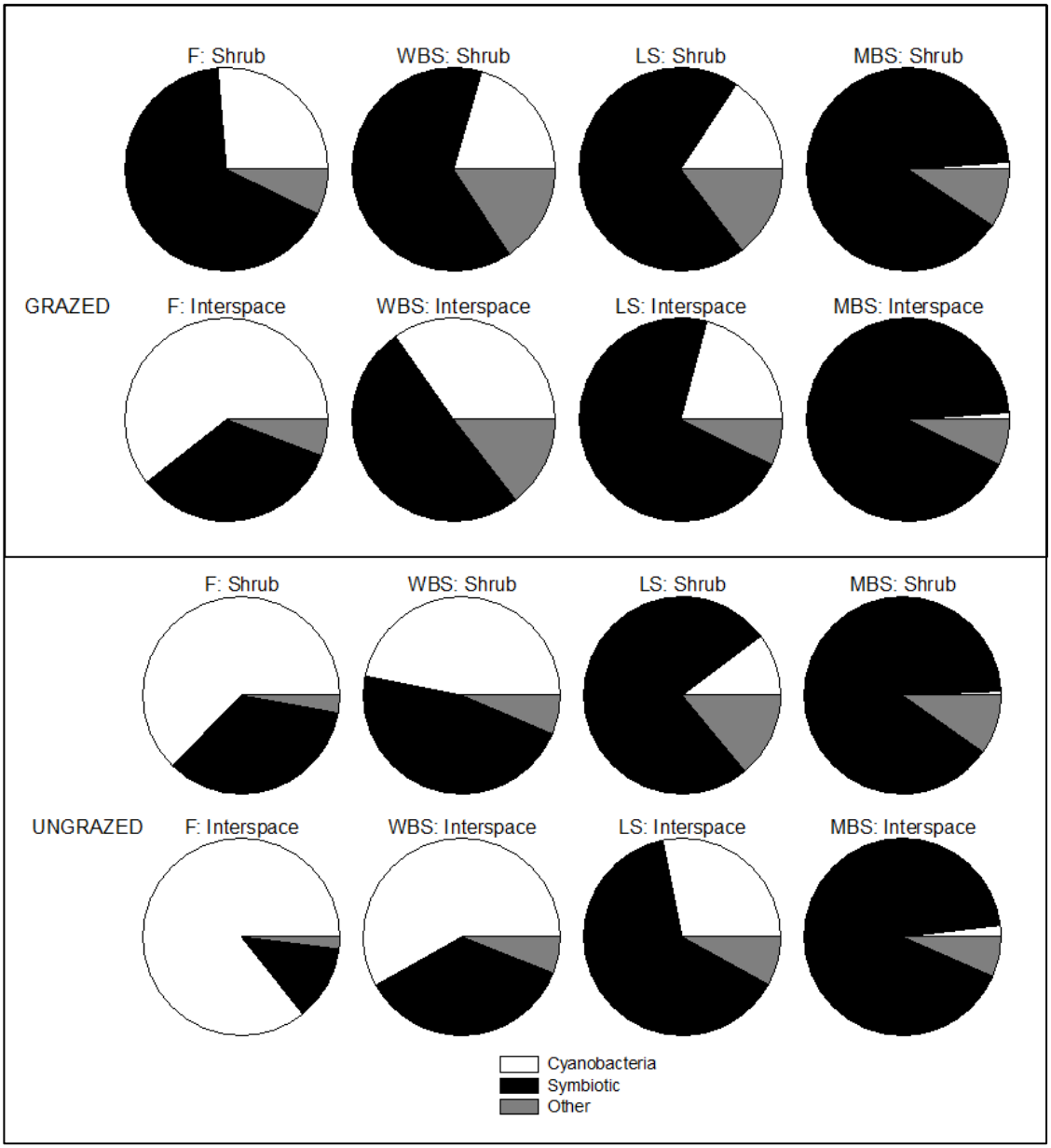

Figure 11: Grazed proportions of the genera grouped into the categories of cyanobacteria, symbiotic, and other N_2-fixing bacteria with respect to shrub-canopy and interspace at each elevation. Ungrazed proportions of the genera grouped into the categories of cyanobacteria, symbiotic, and other N_2-fixing bacteria with respect to the shrub-canopy and interspace at each elevation. Sites increasing in elevation are Flats (F), Wyoming Big Sage (WBS), Low Sage (LS), and Mountain Big Sage (MBS).