Abiotic and Biotic Drivers of Plant Range Shifts in the Alpine

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ABIOtic AND BIOtic DRIVERS OF PLANT RANGE SHIFTS
IN THE ALPINE

by

CLIFTON POWELL BUENO DE MESQUITA

B.A., Middlebury College, 2014

A thesis submitted to the
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This thesis entitled:
Abiotic and biotic drivers of plant range shifts in the alpine
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The final copy of this thesis has been examined by the signatories, and we
find that both the content and the form meet acceptable presentation standards
of scholarly work in the above-mentioned discipline.
ABSTRACT

Over the course of Earth’s biological history, organisms have many times had to shift their geographic distributions in order to track changing abiotic conditions suitable for their survival, growth, and reproduction. Now more than ever, in the new geologic epoch of the Anthropocene, organisms will have to rapidly shift their ranges to adapt to climate change and other anthropogenic disturbances. The rearrangement of organisms can be facilitated or inhibited by biotic interactions between organisms. Here I examine plant range shifts in an alpine ecosystem in response to climate warming and earlier snowmelt, as well as the role of biotic interactions between plants and microorganisms in facilitating or limiting upwards range shifts. First, I use remote sensing to show that plants are shifting their distributions concurrently with climate warming. Then I present work from bacterial and fungal surveys to demonstrate that diverse microbial communities, including key mutualistic taxa, co-occur with some of the highest vascular plant communities in Colorado, and suggest both of these groups of microorganisms could play an important role in mediating plant range shifts. Next, I present work from a manipulative snowpack and microbial inoculation experiment that suggests plant interactions with microbes are important for plant growth and survival as they colonize previously unvegetated soils as growing seasons lengthen. Lastly, I present work on plant litter-driven plant-soil feedbacks to demonstrate how differences in litter chemistry and litter microbiomes can lead to species-specific effects on unvegetated soil microbial communities, which can then feed back and affect plant growth.
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CHAPTER I

INTRODUCTION AND OVERVIEW

Introduction

Rapid, anthropogenic climate change is causing a mass reorganization of Earth’s flora and fauna (Walther et al. 2002, Parmesan and Yohe 2003, Parmesan 2006, Settele et al. 2014). Unfortunately, many species are not able to adapt to new climates or move to more suitable ones, and have become extinct in what is now considered to be the 6th great mass extinction on Earth (Brook et al. 2008). On the other hand, many taxa are capable of shifting their geographic distributions in order to “track” their suitable climate (Frei et al. 2010, Chen et al. 2011). However, there is considerable variation in the rate and success of species range shifts (Chen et al. 2011), and multiple interactions between organisms and the differential sensitivities and responses of organisms to abiotic change make it difficult to accurately predict species responses to climate change (Araújo and Luoto 2007, van der Putten 2012, Hille Ris Lambers et al. 2013).

Ecologists once modelled species distributions by studying the abiotic factors of where a species was present and absent, and then creating a “climate envelope” of suitable habitat for that species (Pearson and Dawson 2003). But while climate and abiotic conditions are indeed primary drivers of species distributions, biotic interactions are also critical for understanding species distributions. This was recognized as early as the 1950’s and 1960’s, with the ideas having a fundamental niche driven by abiotic factors which was then refined by biotic interactions to determine the realized niche (Hutchinson 1957). In a classic example of this idea, barnacles were shown to be excluded from otherwise suitable habitat because of competition with another barnacle (Connell 1961). Other examples of restricted ranges due to competition or other negative biotic interactions (predation, herbivory), now abound in a variety of systems and organisms (Leathwick and
Austin 2001, Meier et al. 2010, Pellissier et al. 2010, Giannini et al. 2013). More recently, positive biotic interactions (facilitation, mutualism) have been incorporated into this literature, showing that biotic interactions are also capable of expanding species ranges (Bruno et al. 2003, Afkhami et al. 2014).

Despite this recognition of the importance of biotic interactions in species distributions and distributional shifts, plant-microbe interactions are one important type of interaction that has been overlooked as a potential driver of species distributions and subsequently as a factor in distributional shifts (Pellissier et al. 2013a, Bueno de Mesquita et al. 2016). Plant-microbe interactions present a challenge for understanding plant distributional dynamics, because the diversity of taxa and number of potential interactions is so high, and the direction of the relationships can be either positive or negative (King et al. 2012, Tedersoo et al. 2014, Thompson et al. 2017). A growing body of work is demonstrating that the vast diversity of bacteria and fungi in soil can play important roles for plant fitness, whether it be via saprotrophic activity, decomposition, nutrient cycling, and soil fertility, or via direct interactions with plants as either pathogens or mutualists (Wardle et al. 2004, Smith and Read 2008, van der Heijden et al. 2008, Jackson 2009, Bardgett and van der Putten 2014, van der Putten et al. 2016).

As is the case with other biotic interactions in the context of climate change, in which both interacting organisms have their own environmental tolerances and sensitivities to climate (Hegland et al. 2009, Kudo and Ida 2013), microbes also have biogeographic patterns based on abiotic variables (Fierer and Jackson 2006, Fierer et al. 2007, 2009, Lauber et al. 2008, 2009, Tedersoo et al. 2014, Thompson et al. 2017, Delgado-Baquerizo et al. 2018). Due to differences in environmental tolerances and preferences, we cannot assume that plants and microbes will track climate lockstep. On the other hand, the combination of broader environmental tolerances and high rates of dispersal means that microbes can currently be present
where plants are not, presenting a new set of organisms that plants will encounter as they shift their ranges (Nemergut et al. 2007, Schmidt et al. 2008a, King et al. 2008, Sattin et al. 2010). Importantly, the potential patchy distribution of plant-associated microbial taxa beyond vascular plant ranges (Jumpponen 2003, King et al. 2010) could have important ramifications for future plant distributions. It remains unclear, however, how interactions between plants and these microorganisms and their spatial distributions will influence plant range shift dynamics.

**Thesis Overview**

To study the importance of climate and plant-microbe interactions in plant range shifts, I used a combination of remote sensing, landscape-level surveys of plants and microbes, and climate and microbial manipulative experiments in a high elevation alpine ecosystem at the upper range of vascular plant life. In Chapter II, I present landscape level vegetation change patterns of alpine tundra, shrubs, and subalpine forests, and identify plant colonization of unvegetated soils as a major type of land cover change. In Chapter III, I model plant distributions using different sets of predictor variables and show that adding soil bacterial abundances to plant species distribution models helps predict plant occurrence. In Chapter IV, I survey fungal root endophytes across a high elevation landscape and show that arbuscular mycorrhizal fungi and dark septate endophytes are prevalent and non-randomly distributed, and may play important roles for alpine plant nutrition in high alpine environments. In Chapter V, I manipulated growing season length and soil microbial community composition to study the role of these two factors in facilitating plant colonization of high elevation unvegetated soils, and find significant interactive effects between the two variables. Lastly, in Chapter VI, I examine the nature of plant-soil feedbacks as plant litter starts entering these
previously unvegetated and carbon-limited environments and discuss the role of these feedbacks in succession.
CHAPTER II

TOPOGRAPHIC HETEROGENEITY EXPLAINS PATTERS OF VEGETATION RESPONSE TO CLIMATE CHANGE (1972-2008) ACROSS A MOUNTAIN LANDSCAPE, NIWOT RIDGE, COLORADO

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Abstract

The distributions of biomes worldwide are predicted to shift as vegetation tracks climate change. Ecologists often use coarse-scale climate models to predict these shifts along broad elevational and latitudinal gradients, but these assessments could fail to capture important dynamics by ignoring fine-scale heterogeneity. We ask how the elevational ranges of vegetation types have changed in a mountainous landscape, and investigate the influence of fine-scale topographic, snowpack, and soil properties on vegetation change. We manually classified vegetation from high-resolution repeat aerial photographs from 1972 and 2008 at Niwot Ridge, Colorado, USA, and generally found that trees and shrubs colonized tundra, while tundra colonized barren soils. Only shrubs expanded their elevational range. Several fine-scale topographic, soil and snow characteristics, including elevation, slope, solar radiation, soil bulk density, and interannual snowpack variability, modulated where plant establishment occurred. Each vegetation type had a unique suite of variables best predicting its establishment in new areas. We suggest that fine-scale heterogeneity may strongly control how plants in mountainous regions respond to climate change, and different vegetation types may be sensitive to different aspects of this heterogeneity. An improved understanding of the factors controlling vegetation change gives us a broader understanding of ecosystem response to climate change, nitrogen deposition, and release from grazing.
Introduction

Rising temperatures have dramatically altered growing conditions for plant species in many regions of the globe (Walther et al. 2002, IPCC 2014). Global temperatures have increased by 0.6°C since 1951 and are expected to rise between 0.3°C and 4.8°C by the year 2100, depending on emissions scenarios (IPCC 2014). The effects of climate change are amplified in mountain ecosystems, where plants are generally more susceptible to habitat loss (Engler et al. 2011, Elsen and Tingley 2015). In addition to climate change, many sites are also experiencing higher levels of nitrogen (N) deposition, and in some cases, cessation from grazing (Dullinger et al. 2003, Galloway et al. 2008). Because mountain ecosystems have a high degree of topographic heterogeneity, which in turn leads to heterogeneity in microclimates, snowpack, and soil properties, plant responses to environmental change may not be uniform across the landscape (Ackerly et al. 2010, Geiger et al. 2012, Ford et al. 2013). For example, over the scale of tens of meters, there can be a shift from a dry, windswept knoll-top with little to no snowpack and shallow, rocky soil, to a leeward knoll-slope with a meters-deep snowpack, and deep, moist, organic soils (Bowman and Seastedt 2001, Bruun et al. 2006). Thus, to make accurate predictions about changes in species distributions, it is crucial to examine how these finer-scale factors mediate responses to environmental change (Luoto and Heikkinen 2008, Trivedi et al. 2008, Randin et al. 2009, Austin and Van Niel 2011, Barrows and Murphy-Mariscal 2012).

One of the primary impacts of climate change on mountain ecosystems has been shifts in the distribution of herbaceous plants, shrubs, and trees. Several studies have reported dramatic upward shifts in herbaceous alpine plant species (Pockley 2001, Parmesan and Yohe 2003, Peñuelas and Boada 2003, Lenoir et al. 2008, Parolo and Rossi 2008, Walther et al. 2009, Grabherr et al. 2010). Shrub encroachment into both arctic and alpine tundra has been widely reported as a
response to warming (Sturm et al. 2001, Tape et al. 2006, Hallinger et al. 2010, Myers-Smith et al. 2011, Elmendorf et al. 2012), and a global manipulative warming experiment provides strong evidence that warming is the driver of this response (Walker et al. 2006). In addition, tree range expansion toward higher elevations has been associated with increases in air temperature (Peñuelas and Boada 2003, Beckage et al. 2008, Lenoir et al. 2008).

Despite these results, shifts in the distribution of vegetation have not been uniform in the context of warming, indicating that other factors are at play. Importantly, fine-scale variables such as topography, snowpack, and soil properties could play a role in modulating vegetation responses to warming, especially in mountainous regions (Bourgeron et al. 2015). It is well established that all of these factors influence plant growth, phenology, and community composition in alpine tundra (Bowman and Seastedt 2001, Vonlanthen et al. 2008, Malanson et al. 2012, Rose and Malanson 2012, Körner 2013, Suding et al. 2015, Dearborn and Danby 2017, Theobald et al. 2017), subalpine meadows (Loneragan and del Moral 2006), treeline communities (Bader and Ruijten 2008, Holtmeier 2009, Grafius et al. 2012, Weiss et al. 2015), and montane forests (McKenzie et al. 2003). Thus, they could also play a role in influencing vegetation change over time, but this has been less well studied.

For example, some studies have found that topographic factors such as the slope and aspect of a site play a greater role than atmospheric temperature in governing the distributional responses of low-growing vegetation (Bennie et al. 2006, Scherrer and Körner 2011). Others have identified plants shifting to lower elevations as a result of climate-based changes in plant water balance (Crimmins et al. 2011). For shrubs, both habitat conditions and biotic interactions make expansion into tundra meadows spatially heterogeneous (Dullinger et al. 2006). Lastly, factors such as high wind speeds, which often occur in mountainous regions,
can negate the warming effect and prevent shifting of treeline (Holtmeier and Broll 2010). In other cases, upwards shifts in treelines have occurred, but only in certain aspects or slopes (Danby and Hik 2007, Treml and Chuman 2015). In a global review of treeline studies, which had an average length of 59 years, Harsch et al. (2009) found that treeline had only shifted to higher latitudes or elevations in half of the studies examined, and krummholz treelines, typical of elevational gradients, were less likely to have shifted with warming. The non-uniformity of elevational vegetation responses to warming in mountain regions warrants further investigation into the factors that can influence these responses.

Central to the discussion of this variation in responses to climate change is the difference between microclimate and macroclimate. Weather stations do not capture the fine-scale climatic variations at the landscape scale (Ashcroft et al. 2009, 2012, Scherrer and Körner 2010, De Frenne et al. 2013), which can be influenced by elevation, radiation, moisture, and exposure (Lookingbill and Urban 2003, Ashcroft et al. 2008). If plants are already confined to specific microclimates, then they will vacate microclimates at the trailing edge at the same rate as they would colonize new microclimates at the leading edge. However, the distribution of alpine microclimates and rates of warming are highly variable (Maclean et al. 2016), and therefore local vegetation responses to climate change differ significantly from predictions by macro-scale models.

In this study, we investigated the extent to which the distributions of high-elevation plant vegetation types (alpine tundra, subalpine forest, shrubs) have responded spatially to climate change over the past four decades. We utilized fine-scale topographic data, satellite-derived snow water equivalent estimates, and field-derived soil measurements to assess the influence of topography, snowpack, and edaphic properties on vegetation change. Because temperature and late melting snow are often thought to be limiting factors at the upper elevation ranges of all
these vegetation types, we expected (i) all vegetation types to show movement uphill in response to observed warming over the last several decades. Additionally, we expected (ii) a suite of fine-scale characteristics to also influence vegetation establishment, with greater establishment in microsites with greater exposure to warming (greater solar radiation; Maclean et al. 2016), shallower snowpacks that lengthen the growing season (Franklin et al. 1971), flatter slopes (that experience less disturbance from rock slides and receive more soil moisture that drains from hillslopes; Walker et al. 1996; Suding et al. 2015) and on deeper and less dense soil (reflecting greater soil development, water holding capacity and nutrient concentrations; Aina and Periaswamy 1985; Chaudhari et al. 2013). Thus, we expect that vegetation can move upwards in elevation, but only given certain conditions of other fine-scale variables.

Methods

Study Site

Our study was conducted at the Niwot Ridge Long Term Ecological Research (LTER) site and the adjacent valleys to the north (Brainard Lakes) and south (Green Lakes Valley), in the Front Range of the Rocky Mountains, Colorado, USA (Figure 2.1, 40° 3′ 20′ N, 105° 35′ 22′ W). The site borders the continental divide on its western end, and is about 25 km west of Boulder, CO (Figure 2.1). There is evidence of human activity at this site, including game-wall systems for hunting, that ranges in age from 7650 to 500 years before present (Bowman and Seastedt 2001). The site was also heavily grazed by sheep in the 1940's (Bowman and Seastedt 2001). Nitrogen (N) deposition has been increasing over the last few decades to current estimated rates of 6.2 kg N ha⁻¹ yr⁻¹ (Formica et al. 2014, Simkin et al. 2016). Average precipitation from 1952 to 2012 in the alpine at our site was 1090 ± 230 mm yr⁻¹, with a 60 mm yr⁻¹ increase over that time period, driven mostly
by increases in winter precipitation (Kittel et al. 2015). Summer temperature data from 1972 to 2008 at our site show a warming trend, especially since 1980 (Figure 2.2), and overall temperatures also increased between 1989 and 2008 (McGuire et al. 2012). This has led to increased positive degree days and earlier snow meltout times (Caine 2010, McGuire et al. 2012, Preston et al. 2016). Climate at our site is affected by larger scale oscillation patterns in the Pacific, and changes in climate in the 1980’s and 1990’s may be partly attributable to shifts in the Pacific Decadal Oscillation (Kittel et al. 2002). Treeline, or the upper limit of tree life including krummholz (stunted, windblown tree mats) (Tinner and Theurillat 2003), is mostly a gradual krummholz form, where the trees at their uppermost limit are stunted and windblown. Treeline species here are primarily *Picea engelmanni* (Parry ex Engelmann) (Engelmann Spruce), *Abies lasiocarpa* (Hook.) Nutt. (Subalpine Fir), and to a lesser extent, *Pinus flexilis* (James) (Limber Pine). Some *P. engelmanni* seedlings have been found 50 m above the main closed canopy timberline (Peet 1978, Daly and Shankman 1985). The most abundant shrub is the willow *Salix glauca* (Linnaeus). Alpine tundra on Niwot Ridge is representative of the region and includes wet, moist and dry meadow communities as well as fellfield and snowbed communities (Suding et al. 2015).
Figure 2.1. Study area and map of points where the three most common vegetation changes occurred, overlaid on the 2008 orthophoto, with hill shading and 100-m contours. Points where vegetation transitions did not occur are not shown. Bare to tundra n = 39, tundra to shrub n = 27, tundra to open forest n = 26. These points are a subset of 2000 points that we randomly generated across the landscape shown here.

**Aerial Images**

High resolution (0.6 m) orthographic photographs (orthophotos) taken in 1972 (color-infrared) and 2008 (true-color) included an approximately 38 km² region that encompasses subalpine forest, alpine tundra, subnival talus areas, and high peaks, with an elevation range of approximately 3100 - 4100 m. The photos were topographically corrected and are available on the Niwot Ridge LTER website (niwot.colorado.edu). We characterized ground cover at 2000 randomly generated points across the extent of the orthophotos. These points became our dependent variable in logistic regression models (0 for no vegetation change, 1 for vegetation change). The cover class categories were bare ground, permanent snowpack/glacier, rock, water, alpine tundra, shrub, open forest, and closed canopy forest. Both supervised and unsupervised maximum likelihood classification did a poor job in classifying these particular images into these desired classes, so we classified points manually. We defined open forest as points touching tree vegetation that existed in patches or islands with less than 75% canopy cover within a 25-meter radius of the
point. If the point landed on grassy vegetation within an open forest (i.e. a subalpine meadow), the point was classified as tundra vegetation, as these meadows still contain many alpine species. Of the 2000 points, 424 were water, snow, or rock in both years, leaving 1576 points with vegetation (Figure 2.1). Our analyses focused on 1532 of these points, which overlapped with our snow water equivalent (SWE) dataset (Jepsen et al. 2012, see below).

Figure 2.2. Mean summer (June, July, August) temperatures from the D1 Meteorological Station (3739 m.a.s.l.) on Niwot Ridge, CO, USA, showing a strong warming trend in the time between the orthophotos (1972, 2008). The linear regression line (red, p < 0.001, $R^2 = 0.31$) as well as a loess function (blue), with 95% confidence intervals (shaded) are shown.

Predictor Variables

Elevation, slope, and solar radiation were derived from a 2 m LiDAR-based digital elevation model and conferred to each point using QGIS (QGIS Development Team 2015). Solar radiation was calculated using the area solar radiation tool in Spatial Analyst in ArcGIS 10.2.2 (ESRI 2015). This tool calculates solar radiation based on slope, aspect, and shading from a DEM for a particular date and time. For consistency with our SWE data (see below), we calculated solar radiation at midday on June 1st, which is also representative of typical exposure to sun during the main part of the growing season in July. Solar radiation has a large impact on temperature and is commonly used to adjust temperature models of mountain
landscapes (Dubayah 1994, Daly et al. 2007, Fridley 2009). Because it is based in part on topography, we consider it as a topographic variable.

The SWE data is from a 12-year distributed SWE reconstruction model, which integrates hydrometeorological observations, a distributed snowpack energy balance model, and Landsat-derived snow cover from 1996 - 2007 (Jepsen et al. 2012). The model back-calculates SWE wherever the snow is deposited, but still suffers errors due to distribution by wind (Jepsen et al. 2012). In general, the dataset adequately identifies parts of the landscape receiving more snow than others. We acknowledge that errors in the model could lead to false negative or false positive results, which should be interpreted cautiously. Snow water equivalent is a measure of the amount of water contained in the snowpack, expressed as a linear depth for each 30 m grid cell. Since late spring snow determines the length of the growing season, which affects plant growth and reproduction (Kudo et al. 1999, Totland and Alatalo 2002, Kirdyanov et al. 2003), we focused on the 12-year mean, inter-annual variation, and 12-year trend (slope of linear regression model), of SWE on June 1st. Inter-annual variation was calculated as the coefficient of variation (CV, standard deviation/mean) for the 12 years. These variables were calculated for each pixel and then conferred to our classified points.

Lastly, because colonization is most likely to occur near sources of propagules, we calculated a percent cover (of trees, shrubs, and tundra) variable. Cover (proportion of points) and elevation had strong but nonlinear relationships. Thus, we made polynomial models of the relationship between elevation and the percent cover of each vegetation type and then used the coefficients from the model to calculate an approximate percent cover of each vegetation type at each of the 1532 points (Figure A2.1). This variable, however, can only be viewed as a rough approximation of the amount of viable seed produced by each vegetation type. Some herbaceous plant, shrub, and tree species show decreases in seed production at their
range edges (Jump and Woodward 2003, Myers-Smith et al. 2011, Vaupel and Matthies 2012, Hille Ris Lambers et al. 2013, Kroiss et al. 2015, Buechling et al. 2016). Two years of monitoring seed traps at our site captured no seed production at treeline (Robert Andrus, personal communication). Thus, it is likely that we overpredict the importance of cover on vegetation change. However, it is still important to include this variable as a basic representation of propagule sources.

In June-August 2015, we ground-truthed vegetation cover at a total of 187 points. Our overall classification accuracy of land cover identity was 92%, with respect to our 2008 classifications. Our sampling scheme involved surveying points in groups of three: a point at which the vegetation cover type had changed based on the orthophotos, a nearby (~20-200 m away) point where vegetation had not changed and was the same cover type as the first point in 1972, and a nearby point where vegetation had not changed and was the same cover type as the first point in 2008. In two instances, one point of the three was unable to be reached. At the locations we visited, we measured soil depth by hammering in rebar until it hit bedrock, and collected three soil cores to 10 cm depth to measure gravimetric water content, pH from a 1:2 soil to deionized water slurry, and bulk density. At points with shrubs and trees present, shrub and tree species were identified within a 2 m radius of the GPS point.

Analyses

We examined the minimum and maximum elevation values for each vegetation type in each year to determine if there were range expansions or contractions. Because this could be driven by outliers and not reflect any of the dynamics within the range, we also examined the 5th and 95th percentile elevation values for each vegetation type in each year (Zhu et al. 2012). Then we conducted both univariate and multiple logistic regressions to test our hypotheses about the influence of certain topographic, snowpack, and soil properties on vegetation...
change. In these models, a value of zero signified any vegetation class in 1972 other than the focal vegetation class that did not change to the focal vegetation class by 2008, while a value of 1 signified any vegetation class in 1972 other than the focal vegetation class that did change to the focal vegetation class by 2008. To be realistic, we limited the forest analysis to points < 3600 m, given that the treeline occurs at ~3550 m (Peet 1978; personal observation). The shrub analysis was similarly restricted to points lower than < 3760 m because the highest shrub identified in 2008 was at 3710 m. We limited the tundra analysis to points higher than the 3550 m treeline elevation. The tree and shrub ranges incorporate a 50 m elevation buffer where it is reasonably possible that a tree or shrub could disperse to and establish. This number is reasonable based on previous work on willow seed morphology, spruce and fir dispersal, and the winds at our site (Noble and Ronco Jr. 1978, Alexander and Edminster 1983, Uchytil 1992). Models including topography and SWE variables utilized the entire dataset of 1532 points, whereas models including soil factors only included data from the 187 ground-truthed points where soils were sampled.

To test our hypothesis about fine-scale topographic and SWE variables, we ran logistic regressions using an exhaustive, all subsets, AIC selection method (runs all combinations of predictor variables and selects models with the lowest AIC) to identify the best combination of predictors of a vegetation transition (R package ‘bestglm’; Mcleod and Xu 2017), which was then used to map predicted probabilities of change across the landscape. We excluded soil variables from this analysis, as we only sampled these variables at the ground-truthed points (12% of all the points). To make our analyses more robust and account for the spatial processes at play during vegetation change, as well as potentially complex distributional relationships and latent interactions, we also ran geographically weighted logistic regressions (GWLR, R package ‘GWmodel’; Gollini et al. 2015), which essentially
adds a spatial term to the logistic regression model, and random forest classification models (RFC, R package ‘randomForest’: Liaw and Wiener 2002), which is a nonparametric approach involving decision trees that can take into account interactions and nonlinearity. We ran these models to see if they gave the same results as the logistic regressions.

To specifically test for effects of soil variables on vegetation transitions, we conducted univariate logistic regressions for each predictor variable (soil depth, soil pH, soil bulk density). We interpreted terms with a p-value < 0.05 to be significant predictors, and determined the relationship between the predictor and response variables by the sign of the coefficient from the model.

Lastly, to help describe the data, rates of change per decade for each vegetation class were calculated using Equation 1, following Dial et al. (2007).

\[
\text{Rate of Change} = \frac{100 \times \ln(N_{2008} + N_{1972})}{3.6}
\]

where 3.6 represents the number of decades and \(N_{2008}\) and \(N_{1972}\) are the number of points of each vegetation class in that year. All analyses were performed with the statistical software R (version 3.4.0, R Core Team 2018).

**Results**

We found strong evidence of vegetation change across the landscape, especially encroachment by woody vegetation into areas previously characterized by other cover types (Table 2.1, Figure 2.1, Figure 2.3). The largest increase in cover was by shrubs, which increased by nearly 8% per decade. This expansion was driven by colonization of tundra and barren soil by *Salix glauca* at elevations between 3400-3500 m (Figure 2.3). For tree cover, open forest increased by 3.9% per decade while closed canopy forest cover increased by a mere 0.6% per decade. The increase in open forest was driven by infilling of tundra in the 3200-3400 m (subalpine tundra meadows) elevation range, mostly by *Picea engelmanni* and *Abies lasiocarpa*. 
Bare ground cover decreased by 4.9% per decade, constituting the largest decrease. Alpine tundra vegetation decreased overall by 0.5% per decade, but increased at higher elevations (Figure 2.1, Figure 2.3). Tundra vegetation colonized barren soils at higher elevations, with a 6.2% increase per decade at 3700-3800 m elevation (Figure 2.3). Plant species richness at ground-truthed plots that switched from barren soil to tundra vegetation (n = 13) ranged from 5 to 11. The forb *Geum rossii* (R. Br.) Ser. most frequently colonized barren soils. Individuals representing vegetation that had established since 1972 were typically smaller in size and isolated, suggesting that new seedlings established during the time period rather than in situ growth.

*Figure 2.3. Difference in the percentage of points in each land cover class between 2008 and 1972. At 3700-3800 m, tundra increased from 41.9% of the points to 52.4% of the points, which is a difference in percent of 10.5.*
Table 2.1. Land cover change matrix based on classification of orthophotos from 1972 and 2008. Numbers are the number of sample points out of 1576 within each category. Also shown are the rates of change per decade.

<table>
<thead>
<tr>
<th>Land Cover Classification</th>
<th>2008</th>
<th>Total (1972)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bare</td>
<td>Tundra</td>
</tr>
<tr>
<td>1972 Bare</td>
<td>291</td>
<td>39</td>
</tr>
<tr>
<td>Tundra</td>
<td>0</td>
<td>578</td>
</tr>
<tr>
<td>Shrub</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Open Forest</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Closed Canopy</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total (2008)</td>
<td>291</td>
<td>620</td>
</tr>
<tr>
<td>Rate of change (% per decade)</td>
<td>-4.9</td>
<td>-0.5</td>
</tr>
</tbody>
</table>

Elevation Expansion or Contraction

No vegetation type experienced a range contraction at the lower end of their elevational range. Tundra vegetation and open forests did not experience range expansion at the upper end of their elevation range, while shrubs moved uphill 14 meters. The distribution of points within the ranges tended to shift upwards in elevation, with all vegetation types showing increases in the 95th percentile (Table 2.2).

Table 2.2. Minimum, 5th percentile, 95th percentile, and maximum elevation (meters above sea level) values for each vegetation type in each year.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Closed Canopy</td>
<td>3121</td>
<td>3121</td>
<td>3147</td>
<td>3147</td>
<td>3365</td>
<td>3367</td>
<td>3412</td>
<td>3412</td>
</tr>
<tr>
<td>Open Forest</td>
<td>3124</td>
<td>3124</td>
<td>3171</td>
<td>3175</td>
<td>3420</td>
<td>3421</td>
<td>3482</td>
<td>3482</td>
</tr>
<tr>
<td>Shrub</td>
<td>3198</td>
<td>3198</td>
<td>3246</td>
<td>3253</td>
<td>3536</td>
<td>3593</td>
<td>3697</td>
<td>3711</td>
</tr>
<tr>
<td>Tundra</td>
<td>3141</td>
<td>3141</td>
<td>3255</td>
<td>3256</td>
<td>3801</td>
<td>3813</td>
<td>3972</td>
<td>3972</td>
</tr>
</tbody>
</table>

Topography, SWE, and Soil

There were no consistent effects of topographic, SWE, or soil variables on vegetation expansion across vegetation types. Solar radiation was the only predictor variable common to all three of the vegetation types, but the direction of the relationship ranged from positive (tree and shrub) to negative (tundra). Each vegetation transition had its own suite of important predictor variables (Table 2.3).
Table 2.3. Predictor variables included in the best-fit logistic regression model for expansion of each vegetation type. Variables bolded and italicized were also present in the best geographically weighted logistic regression model and random forest classification model. Italicized variables were only included in one of the other modeling techniques. The AIC was calculated as AICnull model – AICbest model where the null model is an intercept only model. Lower AIC’s indicate better model fits. A decrease in AIC of more than 2 typically means significant model improvement. The R2 value is Nagelkerke’s pseudo R2 value for logistic regression. AUC is the area under the receiver operating characteristic curve and is a measure of model accuracy that ranges from 0 to 1 with 1 being a perfectly accurate model (no false positives or negatives). SWE = snow water equivalent. SWE trend is the slope of a linear regression model of SWE over 12 years (1996-2007); SWE CV is the coefficient of variation (standard deviation/mean) of SWE.

<table>
<thead>
<tr>
<th>Response Variable</th>
<th>Predictor Variables (direction of relationship)</th>
<th>AIC</th>
<th>ΔAIC</th>
<th>R²</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Open Forest Expansion</td>
<td>Tree Cover(-), Elevation(-), Solar Radiation(+)</td>
<td>318.88</td>
<td>-40.61</td>
<td>0.17</td>
<td>0.81</td>
</tr>
<tr>
<td>Shrub Expansion</td>
<td>Shrub Cover(+), Elevation(+), Solar Radiation(+), SWE trend (+)</td>
<td>352.63</td>
<td>-30.98</td>
<td>0.13</td>
<td>0.77</td>
</tr>
<tr>
<td>Tundra Expansion</td>
<td>SWE CV(+), Solar Radiation(-), Slope(-)</td>
<td>138.42</td>
<td>-9.84</td>
<td>0.14</td>
<td>0.73</td>
</tr>
</tbody>
</table>

For open forest expansion, tree cover (range = 0 – 93%, $b = -0.26$, $p = 2.70e^{-05}$), elevation (range = 3121 – 3600m, $b = -0.08$, $p = 5.71e^{-05}$), and solar radiation (range = 11 – 28 WH m$^{-2}$, $b = 0.27$, $p = 0.00842$) was the best combination of predictor variables for both logistic regression and GWLR (n = 1004). Open forest expansion was more likely in lower elevation areas with less tree cover and higher solar radiation (Figure 2.4c). Random forest classification models suggest that mean SWE is also an important variable driving open forest expansion (Table A2.1). Soil characteristics did not appear to influence open forest expansion (n = 42, $p > 0.10$). However, when just analyzing Engelmann spruce (Logistic regression, n = 42), there was a significant effect of soil bulk density (range = 0.11-0.87g cm$^{-3}$, $b = 297.32$, $p = 0.03$), with spruce colonizing areas with higher bulk density.

Shrub expansion was best predicted in logistic regression by a combination of shrub cover (range = 0-12%, $b = 0.48$, $p = 3.62e^{-05}$), elevation (range = 3121-3760m, $b = 0.02$, $p = 7.13e^{-05}$), solar radiation (range = 8-28 WH m$^{-2}$, $b = 0.14$, $p = 0.0614$), and SWE trend (range = -0.13-0.02 m yr$^{-1}$, $b = 12.51$, $p = 0.1578$) predictor variables (Logistic Regression, n = 1305, Table 2.3). Shrub expansion was more likely in higher elevation areas with greater shrub cover, greater solar radiation, and increasing snowpack (Figure 2.4b). In GWLR and RFC models, mean SWE replaced solar radiation as an important predictor variable and slope was also an important
variable in GWLR (Table A2.1). There were no significant effects of any of the soil variables (Logistic regression, n = 48, p > 0.05).

For tundra expansion (n=215), the best-fit model included SWE CV (range = 0.25–2.59, $b = 1.36$, $p = 0.00142$), solar radiation (range = 8 – 28 WH m$^{-2}$, $b = -0.22$, $p = 0.01003$), and slope (range = 1 – 65˚, $b = -0.07$, $p = 0.01648$) predictor variables for both logistic regression and GWLR (Table 2.3). Tundra expansion was more likely in areas with greater interannual variation in snowpack, lower solar radiation, and flatter slopes (Figure 2.4a). Tundra cover and elevation replaced solar radiation and slope as important predictors in the RFC model (Table A2.1). There were no significant effects of any of the soil variables (Logistic regression, n = 36, p > 0.05).

![Figure 2.4. Predicted probabilities of transitions to tundra (>3550m), shrub (<3760m), and open forest (<3600m) from 1972 to 2008. A 0.1 means there is a 10% chance of that point changing to the vegetation type of the map. Note the different ranges of probabilities. Values are calculated using the intercept and slope coefficients from the best-fit logistic regression model for each (Table 2.2). Blue areas are lakes.](image-url)
Discussion

Over the past four decades, we observed changes in the distributions of several plant communities in conjunction with a directional increase in summer warming. Unlike early climate change predictions for the alpine tundra and treeline (IPCC 1990), we did not observe directional shifts in vegetation moving upslope, with the exception of shrubs. Consistent with other studies, our data suggest an upward expansion of shrubs into tundra vegetation. While tundra vegetation and open forests did not increase their maximum elevations, their distributions within the elevation range shifted uphill, as evidenced by increases in the 95th percentile of their elevation range. We observed vegetation-specific responses to suites of topographic, snowpack, and edaphic factors. These results suggest that in mountain ecosystems it is crucial to examine fine-scale factors to make accurate predictions about changes in species distributions.

Elevation Expansion or Contraction

Given that other studies have found uphill responses of vegetation to warming, and that plants are expected to track changing climates, we hypothesized that all vegetation types would show directional uphill shifts. Our results partially support this hypothesis, with all vegetation types increasing at the 95th percentile. Interestingly, shrubs were the only one of the three vegetation types to experience a true range expansion (uphill shift in its maximum elevation). While tundra plants and trees are establishing in areas in which they were formerly not present (Table 2.1), this is occurring at the upper end of, but not beyond, their 1972 elevation range, and can thus be considered infilling.

The lack of tree colonization into higher elevation tundra could be due to low winter temperatures, which have not increased over time (McGuire et al. 2012), high wind speeds, ice abrasion, or low soil moisture (Norton and Schonenberger 1984, Hadley and Smith 1986, Harsch et al. 2009, Holtmeier and Broll 2010, Moyes
et al. 2013). In addition to these abiotic factors, biotic factors such as herbivory, 
granivory, and lack of seed production and dispersal can also inhibit treeline shifts 
from treeline at our site showed no seed rain into the tundra from treeline (Robert 
Andrus, personal communication). Our results are consistent with the 41 of 50 other 
alpine krummholz treelines that have not shifted uphill over time (Harsch et al. 
2009).

The lack of tundra colonization into higher elevation barren soil could be due 
to the late melting of snow, poor soil development, nutrient limitation, or lack of 
microbial mutualists, and testing the effects of these factors on plant growth beyond 
their range is an important avenue for future research (Chapin et al. 1994, Darcy et 
al. 2018b). The barren soils we collected had high bulk density and low water 
content, suggesting poor development and water holding capacity. The effects of 
nutrient limitation on alpine plant range expansion depend on site characteristics 
such as climate and age. Recent studies in the high alpine of Perú and Alaska 
suggest that plant colonization is limited by phosphorus, likely due to low 
weathering rates (Darcy et al. 2018b). Sites with greater weathering rates and 
phosphorus availability could be limited by nitrogen (Raffl et al. 2006), while other 
sites with developed soils may not be limited by nutrients. Since phosphorus levels 
and microbial enzyme activity in unvegetated and sparsely vegetated soils at our 
site are similar to those reported in Perú and Alaska, plants at our site could also be 
limited by phosphorus in some areas (King et al. 2008, Bueno de Mesquita et al. 
2017). We have fertilization experiments in place to test this hypothesis.

*Topography, SWE, and Soil*

We hypothesized that all vegetation types would benefit from the same 
advantageous topographic, soil, and snowpack conditions. On the contrary, trees, 
shrubs, and tundra plants each had their own suite of predictor variables that
predicted their establishment in new areas. Solar radiation appears to be an important determinant of the distributions of all three vegetation types. There was a positive relationship between radiation and both tree and shrub expansion, which is consistent with greater tree establishment and shrub growth on more sun exposed slopes (Stueve et al. 2011, Liu et al. 2015). This suggests that higher energy inputs from the sun and the resulting warmer microclimate are important factors for woody plant establishment and growth in the tundra. Solar radiation was also included in the best fit model for tundra expansion, but the direction of the relationship was negative. One potential explanation of this trend is that plant colonization in higher elevation areas beyond intact tundra meadows is more limited by soil moisture, and the higher soil water evaporation on sun exposed slopes (Isard 1986) is thus detrimental to plant establishment. This is consistent with findings on a glacial chronosequence in Austria, where early vegetation developed faster in shaded areas (Raffl et al. 2006).

For trees, negative relationships with both tree cover and elevation suggest that establishment was likely to occur in the lower part of their elevational range in this study, but where open meadows (lower tree cover) were present. This trend of infilling over a period of time with warming summers and increases in precipitation is consistent with trends in Yellowstone National Park (Jakubos and Romme 1993). The increases in winter precipitation at our site could be particularly important, as winter snowpacks are important for insulating tree seedlings over winter (Holtmeier 2009). We expected soil moisture to be beneficial for tree establishment, as low soil moisture has been shown to increase seedling mortality in subalpine fir (Cui and Smith 1991), Engelmann spruce (Hessl and Baker 1997), and limber pine (Moyes et al. 2015), in both lower and upper subalpine forests (Moyes et al. 2013). The lack of a relationship is likely due to limitations of our single time point measurement, which does not reflect the water stress that may become more
apparent later in the growing season after our sampling. No relationship with SWE variables is in contrast to the suggestion by Franklin et al. (1971) that the snow free period affects tree establishment in subalpine meadows, but is in line with the recent findings of Bader et al. (2018), who found that early snowmelt by two weeks had minimum effects on tree seedling establishment. No effect of soil depth is consistent with other studies (Butler et al. 2004) and suggests that these trees are able to colonize areas with shallow soil. We found that Engelmann spruce was more likely to colonize areas with higher soil bulk density. This is surprising, given that lower soil bulk density typically is associated with less soil moisture and organic matter and more sand, but may be explained by less competition with tundra plants. Other studies have reported conifer growth on thin, rocky soils as opposed to deeper, finer soils where a thick mat of herbs can inhibit tree establishment (Peet 1988, Malanson and Butler 2013). Another explanation is that the coarser soils facilitated seed trapping and moisture retention around seeds, which facilitated the establishment of subalpine fir in a glacial chronosequence (Jumpponen et al. 1999). Interestingly, we found no relationship between bulk density and subalpine fir establishment, but our results combined with those of Jumpponen et al. (1999) suggest that coarse soils can be beneficial for both of these treeline species.

For shrubs, positive relationships with both shrub cover and elevation highlight their establishment at higher elevations and where there were shrubs to provide propagules. Importantly, we also report a positive relationship between shrub expansion and the trend in SWE. While other studies have reported shrub expansion, to our knowledge this is the first study to connect this expansion to a detailed snowpack dataset at the landscape scale. Areas with increasing snowpack over time appear to be beneficial for shrub establishment, likely because snowpack can insulate soil (Brooks et al. 1996, Myers-Smith and Hik 2013), and protect shoots from winter damage (Tape et al. 2006, Myers-Smith et al. 2011), which has been
shown to increase shrub survival (Formica et al. 2014). This result indicates that shrub expansion should be most rapid in areas undergoing simultaneous increases in temperature and snowfall, both of which are occurring at our site (Formica et al. 2014). Furthermore, the rates of N deposition at our site also likely promote shrub growth (Formica et al. 2014), and shrubs also likely started to increase following the cessation of grazing in 1949 (Bowman and Seastedt 2001). The lack of a significant relationship with slope suggests that shrubs are colonizing areas of tundra with both steep and flat slopes, as has been seen in other studies (Tremblay et al. 2012). Slope was not correlated with SWE in our dataset.

Tundra was more likely to expand on flatter slopes and in areas with greater interannual variation in snowpack. Many of the higher elevation sites in our study landscape are steeper than the main stretch of intact tundra on Niwot Ridge, which creates an unstable landscape with frequent rockslides and snow avalanches that can inhibit establishment (Walker et al. 1996). Our results show that there are areas of shallower slopes at these elevations that are more conducive to plant establishment. The relationship with greater variability in snowpack suggests that plant establishment over time benefits from a combination of years with deep snowpack and years with shallow snowpack. Germinating seeds, for example, may benefit from higher growing season moisture content (Sayers and Ward 1966) provided by later melting snow, while seedlings with established root networks may benefit from earlier melting snow and longer growing seasons by increasing their cover and reproductive output (Kudo 1991, Galen and Stanton 1993, Totland 1997). While we did not measure or include this in our analyses, the presence of larger rocks (>20 cm diameter) can also create microhabitats suitable for plant establishment in these environments (Jumpponen et al. 1999).

Conclusions
We find that changes in vegetation distribution with time are affected by fine-scale variation, which suggests that fine-scale factors are important in mediating vegetation change in mountainous areas. In particular, the increase in cover and range expansion of shrubs was substantial and should be expected in areas experiencing both increases in temperature and precipitation. Although no one suite of variables was beneficial for all three vegetation types, the factors important for each specific vegetation type are likely to be important in other mountainous regions, but this warrants further study. The lack of a unified response across vegetation types demonstrates how environmental change can lead to a broader reorganization of vegetation. Contrary to our original predictions of similar advantageous microclimates for alpine plants, shrubs, and trees, our data show that each of these vegetation types establishes in different microhabitats as climate changes. One interesting contrast among the vegetation types is that woody species (both trees and shrubs) appear to perform better in warmer, higher energy microclimates (high solar radiation), while flat and cool microclimates are important for herbaceous species moving into unvegetated areas. This result may reflect differences among vegetation types in limitations by energy versus moisture. A key conclusion from our results is some form of heterogeneity matters for all vegetation types, but the importance of topography, versus soil, versus snow depends on type of vegetation. While we only presented findings from one site, our work supports the idea that some plant species may only need to migrate tens of meters to track climate instead of hundreds of meters uphill or hundreds of kilometers poleward (Ford et al. 2013). It is important to note, though, that our results pertain only to a limited number of plant species that we surveyed (i.e. 3 tree species, 1 shrub species, and alpine plant communities). There are certainly many other plant species in our landscape that have not expanded their range and could be experiencing range contractions (Pauli et al. 2007). Local models
incorporating topographic heterogeneity lead to drastically different extinction predictions than continent-scale models (Randin et al. 2009). Important future research about the role of fine-scale heterogeneity in distribution modeling should address whether habitat loss has been overpredicted (Luoto and Heikkinen 2008, Randin et al. 2009, Austin and Van Niel 2011, Barrows and Murphy-Mariscal 2012) or underpredicted (Trivedi et al. 2008). In any case, incorporating finer scale data will be crucial in shaping local biodiversity conservation planning and management in the face of climate change.
CHAPTER III
INCORPORATING BIOTIC FACTORS IN SPECIES DISTRIBUTION MODELING: ARE INTERACTIONS WITH SOIL MICROBES IMPORTANT?

**Abstract**

It is increasingly recognized that species distributions are driven by both abiotic factors and biotic interactions. Despite much recent work incorporating competition, predation, and mutualism into species distribution models (SDMs), the focus has been confined to aboveground macroscopic interactions. Biotic interactions between plants and soil microbial communities are understudied as potentially important drivers of plant distributions. Some soil bacteria promote plant growth by cycling nutrients, while others are pathogenic; thus, they have a high potential for influencing plant occurrence. We investigated the influence of soil bacterial clades on the distributions of bryophytes and 12 vascular plant species in a high elevation talus-field ecosystem in the Rocky Mountain Front Range, Colorado, USA. We used an information-theoretic criterion (AICc) modeling approach to compare SDMs with the following different sets of predictors: abiotic variables, abiotic variables and other plant abundances, abiotic variables and soil bacteria clade relative abundances, and a full model with abiotic factors, plant abundances, and bacteria relative abundances. We predicted that bacteria would influence plant distributions both positively and negatively, and that these interactions would improve prediction of plant species distributions. We found that inclusion of either plant or bacteria biotic predictors generally improved the fit, deviance explained, and predictive power of the SDMs, and for the majority of the species, adding information on both other plants and bacteria yielded the best model. Interactions between the modeled
species and biotic predictors were both positive and negative, suggesting the presence of competition, parasitism, and facilitation. While our results indicate that plant-plant co-occurrences are a stronger driver of plant distributions than plant-bacteria co-occurrences, they also show that bacteria can explain parts of plant distributions that remain unexplained by abiotic and plant predictors. Our results provide further support for including biotic factors in SDMs, and suggest that belowground factors be considered as well.

**Introduction**

Understanding the drivers of species distributions is a central topic in ecology, and species distribution modeling today is more important than ever for predicting the impacts of climate change on plant and animal populations (Hutchinson 1957, Araújo and Luoto 2007, Hille Ris Lambers et al. 2013). Recent work has demonstrated that biotic interactions may be essential to incorporate in species distribution modeling, in essence modeling a species’ realized niche rather than fundamental niche (Hutchinson 1957, MacArthur 1972, Pulliam 2000, Bruno et al. 2003). A growing number of studies show that adding biotic variables, such as the abundance of competitors or host plants, improves the fit and predictive power of topo-climatic models for both animals (Davis et al. 1998, Araújo and Luoto 2007, Heikkinen et al. 2007, Preston et al. 2008, Hof et al. 2012) and plants (Leathwick et al. 1996, Meier et al. 2010, Pellissier et al. 2010, Meineri et al. 2012, Hille Ris Lambers et al. 2013, Giannini et al. 2013). However, these studies have all focused on aboveground interactions among macroorganisms. Given that plant interactions with soil microbes are ubiquitous and often critical for plant survivorship (Wardle et al. 2004, van der Heijden et al. 2008), vegetation models should incorporate interactions with belowground organisms.
Soil microbes are important drivers of aboveground patterns of vegetation productivity, diversity, community assembly, and community structure (Klironomos 2002, Wardle et al. 2004, van der Heijden et al. 2008). Thousands of plant species are dependent on microbial symbionts for growth and survival (van der Heijden et al. 2008), and many plants are also harmed by soil borne microbial pathogens (Jackson 2009). Furthermore, these organisms are not everywhere – fungi and bacteria show biogeographic patterns based on environmental and biotic variables (Fierer et al. 2009, Lauber et al. 2009, Tedersoo et al. 2014). Thus, in addition to abiotic factors, the distributions of microorganisms could be important drivers of plant distributions. In the only example to date, Pellissier et al. (2013) found that including fungal diversity as a predictor variable improved species distribution models (SDMs) of alpine plants.

Soil bacteria may play a particularly important role in mediating plant species distributions in high elevation alpine habitats, where plants are at the limits of their physical tolerances (Sheng et al. 2011, King et al. 2012). In these habitats, soil is shallow, undeveloped, and low in nitrogen and phosphorus (Aide and Cwick 1998, Seastedt and Vaccaro 2001). Bacterial taxa that perform nitrogen fixation, mineralization, immobilization, and nitrification are important in governing plant nutrient availability and may be important facilitators of plant growth (Schmidt et al. 2008a). Furthermore, several genera of bacteria can solubilize phosphorus (Rodriguez and Fraga 1999), which may also limit plant growth in these landscapes (Darcy et al. 2018b). Conversely, plants may be especially susceptible to negative interactions when at their distributional limits. Many groups of bacteria are pathogens, parasites, or root herbivores that could hinder plant establishment (Wardle et al. 2004, Sinclair and Lyon 2005, Jackson 2009). Bacteria may also compete with plants for resources, which can be detrimental to plants in nutrient limited systems (van der Heijden et al. 2008).
Overall, bacteria, through these positive and negative relationships with plants, could play a role in determining a plant’s realized niche.

Here we incorporate species co-occurrences into species distribution modeling by adding abundances of co-occurring plants and relative abundances of co-occurring bacteria to abiotic distribution models of 12 vascular alpine plants and bryophytes. To our knowledge, no other study has included bacterial clade relative abundances in plant distribution modeling. Our work takes advantage of a rich dataset across an elevation gradient, which previously showed that pH, plant abundance, and snowpack are the primary drivers of the soil bacteria community (King et al. 2010), and demonstrated the presence of species-specific plant-bacteria associations (King et al. 2012). To examine the potential importance of soil bacteria in plant species distribution modeling, we asked two main questions: (1) Are bacteria-plant co-occurrences more important than plant-plant co-occurrences in predicting plant species distributions? (2) Does including a combination of three predictor sets – abiotic, plant, and bacteria factors – yield the best distribution model? To address these questions, we used an information-theoretic approach to compare models with various sets of predictor variables.

**Methods**

*Study Area and Data collection*

The study site was the upper Green Lakes Valley (GLV), located in the Colorado Front Range on the south side of the Niwot Ridge Long Term Ecological Research site. The site is a matrix of block slope, late-melting snowbanks overlaying unvegetated gravel soils, fellfields, and small patches of vegetation (King et al. 2010). Soil texture is high in sand content and soil depth is shallow. Precipitation averages 930 mm/year, 80% of which falls as snow (Nemergut et al. 2005). Sampling was done in September 2007 and August-September 2008, at locations spanning an
elevation gradient of 3635 – 3935 m. Locations were spaced every 50 m across the landscape (50 plots) except in three targeted 30 m x 30 m areas where they were spaced every 5 m (16 plots) (see King et al. (2010) for more detailed sampling information).

The abiotic factors measured were altitude, pH, soil moisture, snowpack, total phosphorus, inorganic phosphorus, total dissolved nitrogen (TDN), dissolved organic carbon (DOC), and percent sand. Soil samples were collected on September 4-8, 2007, by homogenizing in situ ~ 100 g of soil at that location to a depth of 5 cm and then scooping 50 g of soil into a new sealable plastic bag. Soils were stored at 4°C for a maximum of 1 week while TDN and DOC measurements were taken, and then stored at -20°C. Altitude measurements are from a Garmin eTrex Vista GPS (Garmin International). Soil pH was measured from a slurry of 2 ml water and 2 g soil, shaken for 1 h. Soil moisture was measured gravimetrically. Snow depth values at each point are from kriging interpolations of snow surveys in the GLV from 1997 to 2003 (NWT LTER, http://culter.colorado.edu/exec/Database/gis_layer_query.cgi). Soil texture analysis was done for samples collected in September 2008 in the South Dakota Soil Laboratory (South Dakota State University, Brookings, SD, USA). Plants were identified based on Webber and Whittmann (2001) and abundances were based on exhaustive stem counts of 1 m radius plots established in August 2008 around the location where soil was sampled (King et al. 2012). Although the plant and soil texture surveys were done a year later than the original bacterial sampling, we expect little year-to-year variation in these measures. To focus on locations recently colonized by plants and to limit circularity in our models by avoiding plots where the microbial community is more strongly influenced by plants, only plots with <100 stems were included in the analysis (n=66; Figure A3.1), a subset of previously studied sites (King et al. 2012, n=76). The majority of
plots had fewer than 100 stems, while several plots from our previous work (King et al. 2012) that had well over 100 stems were removed.

For the bacterial community assessment, DNA was extracted from 1 g of soil using a MO BIO PowerSoil DNA Isolation Kit (MO BIO Laboratories) and PCR was used to amplify the V1-V2 hypervariable region of the bacterial 16S SSU ribosome gene using 27F and 338R primers following the methods of Fierer et al. (2008). Sequencing was performed on the Roche 454 platform using FLX chemistry. Raw sequence data were processed using the methods of Hamady et al. (2010), resulting in 6151 representative Operational Taxonomic Units (OTUs) at 3% similarity with 10,000 total reads and an average of 200 (ranging from 50 to 1073) reads per sample (King et al. 2012). Data were not rarefied, and the Good's coverage was 0.65. While this coverage is sparse, we focused on the relative abundance of family to phylum level clades (Table A3.1), and did not do any analyses of diversity or rare OTUs. Clades were defined by selecting all nodes on the full community tree that aggregated at least 100 sequences, which resulted in 22 clades. To further minimize the inclusion of bacteria that are influenced by plant abundance in the SDMs, we removed the seven clades that were significantly correlated (Kendall's tau, p<0.05) with total stem count, leaving 15 clades to be included in the modeling analyses. Relative abundances (Table A3.1) were calculated by dividing the number of each OTU’s sequence reads by the total number of sequences in a sample. More details about the bacterial community analysis can be found in King et al. (2010, 2012).

Modeling

Models of plant presence/absence were constructed in the generalized linear model (GLM) framework with a logit link and binomial distribution. We modeled presence/absence to focus on plant colonization and establishment in the subnival talus. The plants selected for the modeling analysis (n=13) were present in at least 9 of the 66 plots we analyzed; those that were found in fewer than 9 plots were not
modeled but were included as predictors for the modeled species. An SDM built on
data from less than 9 sites likely would not accurately reflect a plant’s niche, and a
cutoff greater than 9 would have limited the number of modeled species. Previous
work used 10 occurrences as a cutoff for their modeled species (Pellissier et al.
2013a). On average, our modeled species were present in 21 of 66 sites. Abiotic
factors were included as the baseline model for all models and biotic factors were
added in addition to abiotic factors. For each species, four models were constructed:
one with abiotic factors (ABIOT), one with abiotic and plant factors (ABIOT +
PLANT), one with abiotic and bacteria factors (ABIOT + BACT), and one with all
three predictor sets (FULL; Figure 3.1). The order of variable input for the FULL
model was abiotic factors, then plants, then bacteria. The direction of the
association (positive or negative) with a factor was inferred by the sign of the
coefficient in the model.

For each species, the best combination of abiotic factors was selected using an
exhaustive all subsets method (package bestglm, Mcleod and Xu 2017) with the
Akaike Information Criterion (AIC; Akaike 1974) as the selection criterion. We then
calculated the corrected Akaike Information Criterion (AICc, Sugiura 1978;
Burnham and Anderson 2002) and performed AICc selection on the AIC selected
variables (bestglm does not include AICc as an option). We used the AICc because of
the small sample size and relatively large number of parameters ($n/K < 40$; Burnham and Anderson 2002). For the ABIOT + PLANT model, the abundances of neighbor plant species (rare or modeled species) were added as predictors using the forward/backward stepwise method, using AICc as the criterion. The same was done for bacteria clades to yield the best fit ABIOT + BACT model. For the FULL model, bacteria clades were added to the ABIOT+PLANT model using the forward/backward stepwise method and AICc selection.

We evaluated our models with several different metrics. To assess the amount of variance explained by each model, we calculated the adjusted $D^2$ value (package ModEvA, Barbosa et al. 2014). In likelihood methods, the $D^2$ value is the amount of variance explained by the model, and the adjusted $D^2$ value takes into account the number of predictor variables in the model and allows for direct comparison between models (Guisan and Zimmermann 2000, Meier et al. 2010). Nagelkerke’s pseudo $R^2$ value was also calculated, but will not be presented as it yielded the same results as the adjusted $D^2$ (Nagelkerke 1991, Field et al. 2012). To examine the redundancy of the biotic predictor sets (Meier et al. 2010), the joint $D^2$, or the amount of overlap in the deviance explained by plants and bacteria (Figure 3.1), was calculated by subtracting the $D^2$ value of the ABIOT model from that of the ABIOT + BACT model, subtracting the $D^2$ value from the ABIOT + PLANT model from the FULL model, and taking the difference of these two values ($\left( D^2_{\text{ABIOT+BACT}} - D^2_{\text{ABIOT}} \right) - \left( D^2_{\text{FULL}} - D^2_{\text{ABIOT+PLANT}} \right)$). To determine if bacteria improved models for certain types of plants more than others, we compared the $D^2$ attributed to bacteria ($D^2_{\text{ABIOT+BACT}} - D^2_{\text{ABIOT}}$) by plant elevation average and functional group and whether the plant was a talus specialist or tundra generalist (Table A3.2). We considered *Carex nardina*, *Carex phaeocephala*, *Cirsium scopulorum*, *Oxyria digyna*, and *Senecio fremontii* to be talus specialists, whereas the other species are all commonly found in intact tundra locations at Niwot Ridge.
(Spasojevic and Suding 2012) or are known to have broad ranges in many habitats (Harberd 1961, Berg et al. 1997). We evaluated model accuracy internally by 10-fold cross-validation. There was no external data set for an external validation of the models. For some of the rarer species, some (no more than 4) of the runs resulted in errors because of no presence. Model predictive power was assessed with the area under the Receiver Operating Characteristic curve (area under the curve, AUC). The AUC value is a description of the predictive power of the logistic regression model in that it is a measure of the rate of false positives and negatives and rate of true positives and negatives, while the adjusted $D^2$ is a measure of the amount of variance the model explains. Lastly, for each modeled plant, the four models were compared with Akaike weights, which are the probabilities that each model was the best predictor of the data. The highest Akaike weight is the most probable best model (Burnham and Anderson 2002).

The models were tested for independent errors using the Durbin-Watson test and for multicollinearity using VIF statistics (Table A3.3, Field et al. 2012). All statistical analyses were performed with the software R (version 3.1.2, R Core Team 2018).

**Results**

According to all four of our criteria for assessing alternative models, incorporating biotic interactions was important to understand plant species occurrences; biotic interactions increased model fit (AICc, Akaike weights), deviance explained (adjusted $D^2$), and predictive power (AUC). For all 13 plants, the best fit model included abiotic predictors plus plant predictors, bacterial predictors, or both (AICc, Akaike weights, Table 3.1). The best fit model substantially increased both the explained deviance (mean increase in adj. $D^2 = 0.19$, Figure 3.2), and predictive
power (mean increase in AUC = 0.09) compared to the ABIOT model, demonstrating the increased explanatory power and accuracy of models including biotic factors.

Table 3.1. Comparison of model fit (AICc, Akaike weights), deviance explained ($D^2$, adjusted for the number of predictor variables), and predictive power (AUC) for the four models. Bolded values are the lowest AICc, and highest Akaike weight (multiplied by 100), $D^2$, and AUC values.

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Including abiotic, plant, and bacteria variables (the FULL model) outperformed the other models for the majority of plants (Table 3.1). The FULL model had the highest predictive power (AUC) for all plants we analyzed, and it generally explained the most variance in the dataset (adjusted D², 12 of 13 plants) and was the best fit (AICc) and most likely the best prediction of the data (Akaike weights, 9 of 13 plants). Inclusion of bacteria abundances did not improve our ability to describe the occurrences of two plants (Carex nardina and Festuca rubra): the ABIOT+PLANT model was the best fit model. For two other plants, (Kobresia myosuroides and Senecio fremontii), inclusion of plant variables was not important: the ABIOT+BACT model was the best fit model. Considering abiotic factors alone (the ABIOT model) was never superior to the inclusion of biotic factors.
Some of the focal plant species more strongly associated with other plant species while other focal plant species more strongly associated with bacteria (ABIOT+PLANT versus ABIOT+BACT model comparisons, Table 3.1). Overall, plant–plant co-occurrences appeared to be somewhat more important than plant–bacteria co-occurrences: for over 60% of the plant species, the ABIOT+PLANT models were better fit (9 of 13 plants) and explained more deviance (8 of 13 plants) than the ABIOT + BACT model. However, there were several cases where metrics of model performance gave differing indications of the superior model (Carex phaeocephala, Trisetum spicatum and bryophytes), indicating that there were only subtle differences between the importance of the plant and bacteria variables. Growth form did not help clarify the patterns across species; The ABIOT+PLANT model had a better fit in 5 of 7 graminoids and 3 of 5 forbs.

Plant species exhibited strong associations with abiotic variables, indicating filtering along elevation, growing season, moisture, and nutrient gradients (Table A3.4). For many of the plant species we analyzed, variation in snowpack and elevation were important factors. A distinct group of species was more likely to occur at high elevation (Carex nardina, Festuca rubra, Silene acaulis, bryophytes), while others were more likely to occur at lower elevations (Kobresia myosuroides, Cirsium scopulorum). Similarly, some plants occurred more frequently in high snowpack areas (C. nardina, Oxyria digyna, bryophytes), while others preferred less snowpack (Elymus scriberneri, Trisetum spicatum, C. scopulorum, S. acaulis). The distribution of some species was also influenced by soil moisture, DOC, total phosphorus and inorganic phosphorus, pH, total dissolved nitrogen, and percent sand. For instance, some plant species tended to occur in areas with high DOC (C. nardina, Geum rossii, S. acaulis) or high pH (Deschampsia cespitosa, O. digyna, Senecio fremontii), two variables that had consistently positive relationships with the modeled plants (Table A3.4).
Neighboring plant species both positively and negatively co-occurred with the focal plant species. Of the 31 co-occurrences we identified, a little over half (58%) were positive (Table A3.5). Several focal species (sedges Carex nardina and Carex phaeocephala, forb Geum rossii) were more likely to occur with other plant species. On the other hand, two of the talus specialists (Oxyria digyna, Senecio fremontii), as well as moss, were less likely to occur in association with other plant species. A group of forb species (Angelica grayi, Geum rossii, Hymenoxis grandiflora) most frequently had positive co-occurrence patterns with the modeled plants. In contrast, several graminoid species (Carex phaeocephala, Festuca rubra, Kobresia myosuroides) had the most negative associations with the modeled plants, although each of these species was also positively associated with some focal plant species as well.

Bacteria also both positively and negatively co-occurred with the focal plant species. Of the 28 co-occurrences we identified, slightly over half (53%) were positive (Table 3.2). Five bacterial clades had positive relationships with some plant species and negative relationships with other plant species, indicating diverse and species-specific co-occurrence patterns. However, several clades (the three Acidobacteria groups and Deltaproteobacteria) frequently and consistently exhibited positive associations with plants. These clades include some of the more abundant clades (Deltaproteobacteria) as well as some of the rarer clades (Acidobacteria Gp3) that were analyzed (Table A3.1). We also identified several clades (Burkholderiales, Oxalobacteraceae, Rhodospirillales) that most frequently had negative associations with plants.
Figure 3.3. Correlations between the relative abundances of bacteria clades and the plants’ predicted probability of occurrence from the ABIOT+BACT model. The clades shown are the clades that most improved the AICc value of the model for each plant. Correlation coefficients and significance levels are from Kendall’s tau rank correlation test.

The majority of the clades tested in the analysis (13 of 15), including both the most abundant and most rare clades improved the SDM for at least one plant; only Clostridiales and Rubrobacteraceae did not improve any of the SDMs. Clostridiales and Rubrobacteraceae were two of the rarer clades included in the analyses. TM7, however, was the rarest clade included in the analysis, and improved the SDMs of two plants. The relative abundances of the most important clade for each plant (clade that most improved AICc, see Figure 3.3) were significantly correlated with the predicted probability of occurrence of the plant for 8 of 12 vascular plants (Kendall’s tau, p<0.05, Figure 3.3).
The amount of deviance explained shared by plants and bacteria in the FULL model was only 0.017. This corresponds to only a 20% overlap between plant and bacteria contributions to the variance explained. There was no significant relationship between the deviance explained attributed to bacteria and the elevation average of the plant (Spearman's rho = -0.09, p>0.05). There were marginally significant trends of higher deviance explained attributed to bacteria in forbs (mean = 0.12) than in graminoids (mean = 0.06, T-test, t=2.30, p=0.07) and in talus specialists (mean = 0.10) than in tundra generalists (mean = 0.06, Wilcoxon signed rank test, W=26, p=0.09).

Table 3.2. Bacteria clades that improved plant distribution models, with the number and type of associations with the modeled plants, and information on plant symbioses and metabolism. Note that information is for some taxa in the clade, but does not reflect the entire diversity of functions that could be present in a clade. carpha = Carex pheaophala, decses = Deschampsia cespitosa, elyser = Elymus scrberneri, silaca = Silene acaulis, carnar = Carex nardina, fesrub = Festuca rubra, kobmyo = Kobresia myosuroides, oxydig = Oxyria digyna, senfre = Senecio fremontii, geuros = Geum rossii, circo = Cirsium scopulorum, trispi = Trisetum spicatum.

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<th>Clade</th>
<th>Plant</th>
<th>Symbiosis</th>
<th>Metabolism</th>
<th>References</th>
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<td>Acidobacteria Gp3</td>
<td>carpha, deces, elyser</td>
<td>1 silaca</td>
<td>Unknown</td>
<td>Unknown Lee et al. (2006)</td>
</tr>
<tr>
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<td>carmar, fesrub, kobmyo</td>
<td>0</td>
<td>Unknown</td>
<td>Unknown Lee et al. (2006)</td>
</tr>
<tr>
<td>Acidobacteria Gp7</td>
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<td>Unknown</td>
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<td>silaca</td>
<td>0</td>
<td>N-fixation, endophytic, roots</td>
<td>N-fixation, heterotrophy Benson and Sylvester (1993)</td>
</tr>
<tr>
<td>Burkholderiales</td>
<td>senfre, silaca</td>
<td>2</td>
<td>N-fixation, P-mobilization, pathogenic</td>
<td>Heterotrophy, endosymbiont Rodriguez-Diaz et al. (2008), Compton et al. (2008)</td>
</tr>
<tr>
<td>Deinfluorobacteria</td>
<td>circo, geuros, oxydig</td>
<td>1</td>
<td>elyser</td>
<td>Heterotrophy, S-reduction, Fe reduction Herrier et al. (2005)</td>
</tr>
<tr>
<td>Klodinobaceteraceae</td>
<td>senfre</td>
<td>1</td>
<td>Unknown</td>
<td>CO-oxidation Webber and King (2010)</td>
</tr>
<tr>
<td>Oxalobacteraceae</td>
<td>fesrub, bryophytes</td>
<td>2</td>
<td>Potential pathogen</td>
<td>Heterotrophy Green et al. (2007), Monteiro et al. (2008), Olick et al. (2012)</td>
</tr>
<tr>
<td>Pseudomonadaceae</td>
<td>trispi</td>
<td>1</td>
<td>oxydig</td>
<td>Heterotrophy, S-oxidation, N-reduction Rechert et al. (1998), Chen et al. (2009)</td>
</tr>
<tr>
<td>Rhodospirillales</td>
<td>carmar, bryophytes</td>
<td>2</td>
<td>N-fixation, P-mobilization</td>
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</tr>
<tr>
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<td>1</td>
<td>Indole-3-acetic acid</td>
<td>Heterotrophy, phototrophy Kosako et al. (2000), Kumar et al. (2012), Tsvetkova et al. (2012)</td>
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<tr>
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<td>1</td>
<td>circo</td>
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</table>
Discussion

Consistent with many recent studies across a range of systems and interaction types, we find strong evidence for the importance of biotic factors in species distribution modeling. Including neighbor plants or bacteria as predictors improved the models for all focal plant species. Furthermore, incorporating both plant-plant and plant-bacteria associations (the FULL model), generally had the best fit, most deviance explained, and greatest predictive power of all the models.

Comparing models including plant-plant associations versus models including plant-microbe associations, we found that neighbor plant species were more predictive of plant species distributions than bacterial associations; however, this was not the case for all species, and for several species the inclusion of bacterial associations was as predictive as neighbor plant species. The superior performance of the model with both plant and bacteria variables shows that even after accounting for abiotic factors and other plants, bacteria still explained a substantial part of the remaining variance and improved the model’s ability to accurately predict presence or absence. Furthermore, the overlap in the plant and bacteria contributions to the variance explained was small (20%), suggesting that bacteria explain a substantial and distinct part of plant distributions that neighboring plants do not.

Thirteen different bacteria clades improved distribution models for at least one plant. The almost equal prevalence of both positive and negative associations illustrates the various ways in which bacteria can interact with plants (Table 3.2), and confirms previous work showing the presence of positive plant-microbe interactions in alpine and subnival systems (Sheng et al. 2011, King et al. 2012). Positive associations may be due to either mutualistic relationships, microbes facilitating plants, plants facilitating microbes, or shared responses to an unmeasured environmental variable (Pellissier et al. 2013a). Because our focus was
on the influence of microbes on plant distributions, we removed bacterial clades significantly correlated with total plant abundance and did not include occurrences for plots with high plant density. Negative associations may represent antagonistic interactions initiated by either the plant or the microbe, or different responses to an unmeasured environmental gradient (Pellissier et al. 2013a).

Information on bacteria function can guide the interpretation of why bacteria improved vegetation SDMs, although for many clades function remains unknown. Groups from the Acidobacteria were some of the most common bacteria that improved plant SDMs. Acidobacteria are an abundant and functionally diverse group of bacteria (Bryant et al. 2007), so it is not surprising that there were positive and negative associations with plants, but it remains unknown exactly how these bacteria interact with plants. Previous work in high elevation unvegetated soils has described an active and diverse microbial community with clades capable of N-fixation and P-mobilization (Nemergut et al. 2007, Schmidt et al. 2008a, King et al. 2008). There were several such clades present at our site, but surprisingly, some of these clades had negative relationships with plants (Table 3.2). This could be due to plants negatively affecting bacteria that are adapted to plant-free landscapes (Knelman et al. 2012) or possible pathogenic functions in the clade. For example, Rhodospirallales, the most abundant clade at our study site, are negatively correlated with plant abundance likely due to light competition (King et al. 2010). Given some previous work on cyanobacteria-plant symbioses (Meeks 1998, Adams et al. 2013), it is surprising that the cyanobacteria only improved the SDMs for one species - *Geum rossii* - and the effect was negative. Depending on how much nitrogen the cyanobacteria fix, the negative association with *Geum* could be due to the negative impact of higher soil nitrogen levels on *Geum*. Previous work near our site showed that *Geum* declined with nitrogen additions with and without the presence of its co-dominant *Deschampsia cespitosa* (Suding et al. 2008, Farrer et al.
Actinomycetales were positively associated with *Silene acaulis*, likely because some taxa in the Actinomycetales (notably *Frankia*) are N-fixers that can colonize plant roots and form mutualisms with a wide range of host plants (actinorhizal plants), including tundra plants (Benson and Sylvester 1993).

If bacterial taxa abundance influences plant species distributions, it would be useful to determine if they influence certain types of plants more than others. Pellissier et al. (2013) showed that the deviance explained by the fungal diversity in alpine plant SDMs increased as the elevation average (average elevation of where a plant is found at a site) of the plants increased, suggesting that soil microbes may be more important for plant establishment and survivorship in harsher locations. In our analyses, the amount of deviance explained by bacteria (adj. $D^2_{\text{ABIOT+BACT}} - \text{adj. } D^2_{\text{ABIOT}}$) was not correlated with the average elevation of the plant species (Spearman’s rho = -0.09, p>0.05). One potential reason for this lack of correlation is that our plants’ elevation averages did not vary much within this dataset—there was only a 95 m difference between the averages in the highest and lowest elevation species. However, many of the plant species in this analysis are abundant at lower elevation sites not included in this survey. The strength of plant-bacterial associations might indeed dissipate if lower elevations were surveyed.

We largely found species-specific, rather than functional group-specific, interactions with bacteria clades (Table 3.2, Bezemer et al. 2006; King et al. 2012). A trend towards more dependence on bacteria in forbs compared to graminoids may suggest that forbs may be more strongly by microbial symbionts or pathogens than are graminoids. In a tallgrass prairie, mycorrhizal colonization in forbs is greater than in cold season C$_3$ grasses (Wilson and Hartnett 1998) and this pattern may extend to bacterial root symbionts as well. Lastly, talus plant specialists appeared to be more dependent on bacteria than tundra generalists, consistent with our
expectation that soil microbes may play a particularly important role in governing where colonizers establish in the largely unvegetated talus (King et al. 2012).

Plant-plant co-occurrences are added to distribution models to account for competitive or facilitative interactions or shared environmental tolerances that affect species occurrence (Leathwick et al. 1996, Meier et al. 2010, Pellissier et al. 2010). We found many positive (18) and some negative (13) relationships among plants in our SDMs. These relationships may be indicative of competitive and facilitative interactions, or they may represent unmeasured abiotic gradients to which the focal species is responding. In the harsh, windswept, sparse habitats of the subnival zone, plant establishment is difficult, so it is likely that some of the positive associations are due to facilitation (Brooker and Callaghan 1998, Dormann and Brooker 2002). For example, *Silene acaulis* and moss positively affected two plant species distributions, and cushion plants and moss are generally known to facilitate establishment in the tundra (Arroyo et al. 2003, Freestone 2006, Cavieres et al. 2007). Cushion plants can ameliorate extreme soil temperatures and improve soil moisture (Arroyo et al. 2003, Cavieres et al. 2007) and moss can improve seed retention and moisture levels, stabilize sediment, and protect seeds from consumers (Freestone 2006). *Angelica grayi* positively affected three plant distributions, which could be due to a nursing effect. *A. grayi* is a large alpine plant, and could facilitate other plants’ establishment by providing shelter or contributing to soil development and fertility (Callaway 1995). Some negative associations are probably due to different responses of species to unmeasured abiotic gradients, since competitive exclusion in this habitat is unlikely. Although soil texture and moisture were measured, other factors such as soil depth were not. Furthermore, plants can also represent an integration of abiotic factors throughout the year, while our soil moisture measurement only represents one time point in the year. For example, *Deschampsia cespitosa* prefers moist, wet environments (Bowman and Seastedt
2001) while *Senecio fremontii* specializes in rocky, shallow soils (Mooney and Billings 1961), thus a negative correlation between them is likely due to differing environmental preferences that were not well explained by our environmental measures. Other studies incorporating plant-plant interactions for many modeled species found more negative relationships than positive ones (Meier et al. 2010, Pellissier et al. 2010). If our models reflect species interactions, the higher prevalence of positive associations would be consistent with other research that has shown that facilitation is more common than competition in harsh environments (Brooker and Callaghan 1998, Choler et al. 2001, Callaway et al. 2002, King et al. 2012). A next step would be manipulative experiments to determine the nature of positive associations and the role of species interactions.

*Limitations*

A key limitation to our work is that our approach was correlative, and we cannot conclude that co-occurrences are interactions. Experimental manipulations are necessary to understand if species interactions are the mechanism by which SDMs are improved by including abundances of co-occurring species. A combination of field and greenhouse experiments that involve growing plants with different microbial inoculations would be useful to examine the effects of certain microbes on plant growth and survivorship. Additionally, growing plants with different microbes in growth chambers would allow researchers to test specific hypotheses about the role of microbes in enhancing plant tolerance to stressors such as freezing, drought, and low nutrients. Co-occurrence models such as those we have presented here provide strong data for choosing specific plants and bacteria on which to do experiments to test such hypotheses.

One limitation to incorporating soil microbes into plant SDMs is the lack of knowledge of the function of soil bacteria. For some clades we simply lack knowledge of function altogether (Table 3.2), while for others we are limited by
taxonomic resolution (Pellissier et al. 2013a). For some clades we infer function based on example taxa in the clade (Table 3.2), but because of the broadness of the resolution (family to order level) we cannot conclude for certain the function of each of the clades and how their relative abundances influence plant distributions.

A last key caveat in our approach and in other studies where there are positive biotic interactions shaping distributions is the problem of circularity. While the bacteria at our site are primarily structured by soil pH, plant abundances are also an important factor structuring the microbial community (King et al. 2010). Thus, plant-microbe interactions can work in both directions, even for clades not significantly correlated with total stem count. Previous work near our site and elsewhere has shown that the microbial community is active and diverse at the highest elevations above the limit of plant life (Schmidt et al. 2008a, King et al. 2008), which suggests that some of the clades included in our modeling were present before plant colonization of our site. Microbes that are present in unvegetated locations may be important for facilitating plant colonization (Schmidt et al. 2008a). More work is currently being done at our site to tease apart the temporal relationship between plant and bacteria establishment in new locations, which will help inform us of the direction of plant-microbe interactions in shaping new distribution patterns. Our findings support those of Pellissier et al. (2013) in showing that soil organism data enhance the power of plant SDMs, further highlighting the need for manipulative field experiments to inform the causality behind such models of plant interactions with soil microbes.

**Conclusions**

Our work provides strong evidence that incorporating biotic factors into SDMs can improve the models. Biotic factors can improve SDMs by explaining unmeasured abiotic gradients or through interactions that actually influence the range of the modeled species. Building on the work of Pellissier et al. (2013), our
results suggest that soil microbes can enhance distribution models of vegetation by improving model fit, deviance explained, and predictive power. As sequencing costs continue to decline, more microbial datasets are becoming available that can be of use for plant ecologists. While biotic interactions can be important even at macro scales (Araújo and Luoto 2007), the incorporation of soil microbes into vegetation distribution modeling will be most feasible, and likely most important, at small scales. Our work can be expanded into other systems to discover more about which bacteria have important associations with plants, and which types of plants are more affected by soil microbes than others.
CHAPTER IV

PATTERNS OF ROOT COLONIZATION BY ARBUSCULAR MYCORRHIZAL FUNGI AND DARK SEPTATE ENDOPHYTES ACROSS A MOSTLY-UNVEGETATED, HIGH-ELEVATION LANDSCAPE


**Abstract**

Arbuscular mycorrhizal fungi (AMF) and dark septate endophytes (DSE) are two fungal groups that colonize plant roots and can benefit plant growth, but little is known about their landscape distributions. We performed sequencing and microscopy on a variety of plants across a high-elevation landscape featuring plant density, snowpack, and nutrient gradients. Percent colonization by both AMF and DSE varied significantly among plant species, and DSE colonized forbs and grasses more than sedges. AMF were more abundant in roots at lower elevation areas with lower snowpack and lower phosphorus and nitrogen levels, suggesting increased hyphal recruitment by plants to aid in nutrient uptake. DSE colonization was highest in areas with less snowpack and higher inorganic nitrogen levels, suggesting an important role for these fungi in mineralizing organic nitrogen. Both of these groups of fungi are likely to be important for plant fitness and establishment in areas limited by phosphorus and nitrogen.

**Introduction**

Arbuscular mycorrhizal fungi (AMF) and dark septate endophytes (DSE) are two fungal groups that can directly influence plant success in a given environment, but little is known about how their abundances vary over local environmental and...
plant density gradients. AMF are obligate symbionts of living plant roots (Smith and Read 2008) that form a monophyletic group in the subphylum Glomeromycotina (Spatafora et al. 2016) (formerly classified as the phylum Glomeromycota; Schüßler et al., 2001) and are characterized by the formation of arbuscules for nutrient exchange (Smith and Read 2008). AMF have been shown to protect plants from pathogens (Sikes et al. 2009), help plants cope with drought stress (Augé 2001), and aid plants in the uptake of phosphorus (P) and nitrogen (N) (Johnson et al. 2010).

DSE are facultative fungal symbionts that can live on organic debris and in biological soil crusts in addition to plant roots (Menkis et al. 2004, Green et al. 2008, Day and Currah 2011), and several studies have reported enzymatic activities by DSE capable of degrading organic matter (Mandyam and Jumpponen 2005, Mandyam et al. 2010, Knapp and Kovács 2016). They are a polyphyletic group with members typically found in the phylum Ascomycota and are characterized by their dark, melanized, septate hyphae (Jumpponen and Trappe 1998). DSE are relatively less studied than AMF, but have also been shown to have important beneficial impacts on plant growth (Mandyam and Jumpponen 2005, Newsham 2011), perhaps due to N mineralization (Newsham 2011), protection from pathogens (Mandyam and Jumpponen 2005), or uptake of N during snowmelt (Mullen et al. 1998).

Cold environments at high latitudes and high elevations that are experiencing rapid climate change provide an interesting context in which to study these two fungal groups. Recent work has argued that biotic factors need to be considered to accurately predict the effects of climate change on species distributions (van der Putten et al. 2010). For example, plant-pollinator interactions, plant-herbivore interactions, and plant-plant interactions have all been suggested to mediate distributional responses to climate change (Leathwick et al. 1996, van der Putten et al. 2010, Hille Ris Lambers et al. 2013). While less
studied, plant-microbe interactions are a very important type of interaction that can affect plant distributions as well (Pellissier et al. 2013a, Bueno de Mesquita et al. 2016). AMF and DSE both appear to play important functions in plant growth in cold environments, as they have both been found in abundance in arctic and alpine systems (e.g. Schmidt et al., 2008; Väre et al., 1992). In particular, the high melanin concentrations in DSE have been cited as an adaptation to cold temperatures, and AMF are capable of producing cold-active enzymes (Robinson, 2001). Nutrient cycling is typically slow in cold environments, so plants may rely on fungi to acquire adequate nitrogen and phosphorus. Studying the landscape distribution of fungi in cold environments, including over plant density and snowpack gradients which are changing with climate warming, helps us understand the potential effects of climate change on plant-fungal interactions. Interestingly, Kytöviita and Ruotsalainen (2007) found that the benefits of an arbuscular mycorrhizal fungus increased at warmer temperatures, suggesting that some arctic or alpine fungi have good potential to respond to warming.

While both AMF and DSE can be important determinants of a plant’s ability to colonize and persist in new environments, little is known about how these plant-microbial interactions vary across the landscape (Ranelli et al. 2015). Interestingly, there have been global studies about the distributions of AMF (Davison et al., 2015), but we lack information on their distributions in extreme environments. For DSE, there is substantial information on their occurrence in extreme environments, but no global studies. Here we focus on high-elevation alpine environments because alpine plants are vulnerable to climate change due to an often greater magnitude of change (Pepin and Lundquist 2008) and susceptibility to habitat loss (Engler et al. 2011, Elsen and Tingley 2015) compared to lower-elevation plants. Previous work suggests that AMF colonization increases at lower elevations (Kotilínek et al. 2017) as well as lower P, N, (Johnson et al. 2015) and moisture levels (Augé 2004, Smith
and Read 2008, Camenzind et al. 2014). Mechanisms discussed in this literature to explain these trends include both the temperature and moisture optima of the fungus, as well as levels of fungal propagules, and the amount of photosynthate the plant devotes to the fungus. On the other hand, studies have found higher levels of DSE colonization at higher elevations, high nitrogen, and low moisture levels, and no relationship with phosphorus levels (Newsham 2011, Kivlin et al. 2013, Ranelli et al. 2015). In addition to these landscape level patterns for each fungal group, AMF and DSE may interact with each other. In one of the few studies to assess such interactions, Ranelli et al. (2015) hypothesized that AMF and DSE would be negatively correlated due to competition for similar host tissue, but instead found positive correlations between AMF and DSE.

In this study, we build on this prior work by examining colonization across a wide range of different alpine plant species (forbs, grasses, sedges, rushes, N-fixers), including some not previously studied for fungal infection, and by adding snowpack and plant density into models that typically include elevation, soil moisture, and soil nutrients. In many alpine environments, snowpack governs both growing season length and soil moisture (Williams et al. 2009) and provides insulation during winter; we know less about how these effects may translate to plant-fungal interactions. Alpine environments can also vary drastically in plant density. At our field site in the Colorado Rocky Mountains, which is located at the upper edge of the elevational range of vascular plants, plant density varies from intact tundra meadows to sparsely-vegetated talus slopes. These gradients may influence fungal colonization of plants because intact meadows should have higher levels of fungal inoculum in the soil (Cázares et al. 2005). We also tested for effects of plant functional group and plant phylogenetic relatedness on root colonization levels. These variables take into account the broader plant functional traits and evolutionary histories, which can play a role in determining plant-associated
microbial communities (Scheublin et al. 2004). These variables have been studied in the context of broader microbial community composition in soils (e.g. Leff et al. 2018), but are rarely included in models of root colonization (but see Ranelli et al. 2015).

Here, we asked: (1) How does colonization by AMF and DSE vary among plant host species, functional group, and phylogenetic distance? (2) Do plant host and environment jointly predict fungal colonization? (3) What is the relationship between AMF and DSE colonization levels? and (4) Do plant hosts and environment influence the community composition of AMF and DSE taxa? We hypothesized that (H1) both AMF and DSE would show patterns of differential colonization among plant hosts and functional groups, with higher AMF colonization in forbs due to their thicker root architecture, and higher DSE colonization in graminoids, based on the literature (Ranelli et al., 2015) (H2) AMF and DSE colonization levels are influenced by both host plant and the environment, but host plant plays a more important role in AMF colonization due to their obligate status (Ranelli et al., 2015); both fungi would show higher colonization levels as environmental harshness increases (higher elevation, more snow, fewer plants and less nutrients), except at the highest elevations with low plant densities, where AMF will decline (Kotilínek et al., 2017), while DSE will remain abundant (Schmidt et al., 2008; Figure 4.1) (H3) AMF and DSE colonization would be negatively correlated due to different responses to the environmental and plant gradients, and potential competition for host plant tissue (Figure 4.1) and (H4) AMF and DSE community composition will vary among plant hosts and change over environmental gradients, in a similar manner to which the percent colonization does (i.e. different taxa at the harsh end of the environmental gradient).
Figure 4.1. Conceptual diagram of how arbuscular mycorrhizal fungi (AMF) and dark septate endophyte (DSE) root colonization levels are predicted to change over environmental and plant gradients. A) We predict that AMF colonization will increase as environmental harshness increases, but then decline as plant density declines. We predict that DSE colonization will increase steadily with environmental harshness. The bolded line for AMF and DSE shows average colonization levels over all host plants across the environmental gradients; the two thinner lines around the bold line represent the variation in colonization among different host plant species. The spacing of the small lines reflects the expectation of a greater influence of the host plant on AMF colonization levels. B) Environmental harshness increases moving up in elevation as nitrogen (N) and phosphorus (P) become more limiting, snowpack increases, and plant density decreases. We expect AMF to be driven primarily by phosphorus and plant density, and DSE by nitrogen. We also expect the community composition of AMF and DSE taxa to shift across the gradient.

Materials and Methods

Study Site

Our study was conducted along a 2-km portion of a south facing slope at the Niwot Ridge Long Term Ecological Research (LTER) site, in the Front Range of the Rocky Mountains, Colorado, USA (40° 3’ 20’ N, 105° 35’ 22’ W, Figure 4.2). Average precipitation from 1952 to 2012 in the alpine at our site was 1090 ± 230 mm yr⁻¹, with a 60 mm yr⁻¹ increase over that time period, driven mostly by increases in winter precipitation (Kittel et al. 2015). Recent (2011-2014) mean annual temperatures at the nearby D1 Meteorological station range from -4°C to -7°C, while mean summer (July-August) temperatures range from 4°C to 10°C (Losleben 2017). Overall temperatures have been increasing over the past several decades (McGuire et al. 2012). This has led to increased positive degree days and earlier snow meltout times (Caine, 2010; McGuire et al., 2012; Preston et al. 2016). Our plot locations range from continuous tundra meadows to sparsely vegetated talus (plant density gradient of 243 to 3 stems m⁻²) near the continental divide, across an elevation
gradient of 3636 to 3933 m.a.s.l. The landscape is a matrix of block slope, late-melting snowbanks overlaying unvegetated gravel soils, fellfields, and small patches of vegetation (King et al. 2010). The most abundant plant species in this landscape are *Festuca brachyphylla* (Poaceae), *Trisetum spicatum* (Poaceae), *Carex pyrenaica* (Cyperaceae), *Geum rossii* (Rosaceae), *Oxyria digyna* (Polygonaceae), *Senecio fremontii* (Asteraceae), and *Deschampsia cespitosa* (Poaceae). Soils are shallow and show limited development, with mean sand contents of 71% (King et al. 2010). Circular plots (n=160) with a radius of 1 m were established in 2007 in a spatially explicit sampling grid (plots were spaced 50 m apart, with 3 focal clusters with plots spaces 5 m apart) (King et al. 2010). In this study, we sampled plants and measured botanical and environmental variables (see below) from 74 of these plots (Figure 4.2).

**Variables**

Elevation for each plot was obtained from a 2 m resolution LIDAR-based digital elevation model. Mean May snowpack depth was calculated for each plot based on krigged snow depth data for the study site from 1997 to 2015. Kriging was done each year on point depth data from annual snow surveys, in which snow depth was measured manually at an average of 483 random locations ~50 m apart across the study site during approximate peak snowpack in May.

In August and September of 2015, we conducted vegetation surveys and collected soils for nitrogen analysis. We identified all plants at the species level and conducted exhaustive stem counts of each species at each plot. At each plot, plant density was calculated as the number of stems per square meter. We collected three soil cores of 3 cm diameter and 4 cm depth, composited them into a plastic bag, gently homogenized them, and transported them on ice to the lab by the end of the day. Soil total inorganic nitrogen (TIN) was measured via soil extractions with 0.5 M K₂SO₄ and analyzed on a Lachat QuikChem 85000 Flow Injection Analyzer.
Lachat Instruments, Loveland, CO, USA). Soil total dissolved inorganic phosphorus (DIP) data comes from a 2007 survey of these same plots (King et al. 2008) and could have changed some over time. However, the relative levels of DIP across the landscape (i.e. high DIP versus low DIP areas) is likely the same. DIP was determined by extracting the Olsen phosphorus with 0.5 M NaHCO$_3$ (Olsen 1954) at pH 8.5 (pH was adjusted using 1 M NaOH). In 2016, we counted the number of arbuscular mycorrhizal fungal spores in 2.5g of the 2015 soils (stored at -70°C for ~11 months). We acknowledge that this is a small amount of soil, less than what is usually used (25 g, Sieverding et al., 1991), but still provides an estimate of the relative numbers of spores across our landscape. We followed the differential water/sucrose centrifugation method to extract spores from the soil (Allen et al., 1979; Ianson and Allen, 1986; Sieverding et al., 1991) and then placed the suspended spores in a petri dish to view under an inverted microscope at 100x.

From August 15-25, 2016, we harvested 177 individual plants from 74 plots from a total of 35 different species for microscopy and sequencing. We sampled one individual from the three most abundant species in each plot, but only if there were > 5 individuals of that species, so as not to destroy locally rare populations. This resulted in sometimes sampling only the one or two most abundant plant species in a plot. Bulk soil was shaken off in the field, and plants and rhizosphere soil were placed in Ziploc bags and transported to the lab on ice, where samples were flash frozen in liquid nitrogen and then placed in a -70°C freezer due to planned molecular work. Within 1 month, roots were rinsed with DI water, surface sterilized with ethanol and bleach, and then a subset of roots were placed in FAA (63% ethanol, 30.15% water, 5% glacial acetic acid, 1.85% formaldehyde) and stored at 4°C until staining and microscopy within 1 month. Another subset of roots was sequenced to identify the DSE and AMF taxa using the internal transcribed spacer (ITS) part of the genome (Schoch et al. 2012).
Figure 4.2. Map of the 74 1m radius plot locations (blue circles) across the upper Green Lakes Valley in the Front Range of the Rocky Mountains, Colorado, USA. At each of these locations we measured soil nitrogen and phosphorus, counted the number of plant stems in a 1 m radius circle, conferred snow depth from annual snow surveys, and sampled 1-3 individual plants for fungal analyses (sequencing and microscopy).

Staining and microscopy were performed following the procedures of Koske & Gemma (1989), Schmidt et al. (2008) and McGonigle et al. (1990). Roots were rinsed 3 times with DI water to remove FAA and then cleared with 10% KOH for 1 hr in a 90°C water bath. In some instances, if roots were still pigmented after this step, they were cleared with alkaline hydrogen peroxide for 10-45 minutes. Roots were rinsed 3 times with DI water to remove KOH and then soaked in 0.5% HCl at room temperature for 20 minutes. After another triple rinse with DI water, roots were soaked overnight in acidic glycerol with 0.05% trypan blue. In the morning, roots were destained with acidic glycerol and stored in acidic glycerol at 4°C until microscopy was performed within 1 week. Several fine root segments and their branches, adding up to 20-30 cm of root were placed horizontally across slides, covered with a cover slip, and viewed at 200x magnification under a microscope with a crosshair on the ocular. Passes were made up and down the slide at random intervals and the presence of AMF or DSE structures at each of 100 intersections with the crosshair was recorded. Fine endophytes, now classified as in the
Mucoromycotina (Orchard et al., 2017), were present and were included in counts for AMF. Percent colonization for each fungal group is the number of times out of the 100 intersections that a fungal structure was present. If no fungal structures were observed in the first 100 intersections, the entire slide was scanned for structures. Any fungi discovered in this case were given a score of 0.5%, to note their presence at a very low percentage (Schmidt et al., 2008). Doing this provides a more accurate method for determining the mycorrhizal status of the plants. It is more accurate to say that a low abundance fungus is 0.5% than to give it a zero, because a zero would reflect that it was not present in the root at all.

For DNA sequencing to identify AMF and DSE taxa, 0.1 g of wet roots from each of the 177 individual plant samples were frozen in liquid nitrogen and ground into a fine powder with a sterile mortar and pestle. Each individual plant was processed as a separate sample, although this included multiple roots from each individual. DNA was extracted from this powder using the DNeasy Plant Extraction Kit (Qiagen; Hilden, Germany) and PCR was used to amplify the ITS1 region using the ITS1F forward primer and ITS2 reverse primer (White et al., 1990) following the methods of the Earth Microbiome Project (Amaral-Zettler et al. 2009, Caporaso et al. 2012, Smith and Peay 2014). Amplified samples were purified and normalized with the SequalPrep Normalization Kit (Invitrogen Inc., CA), combined into a single pool of an ITS amplicon library and sequenced on one lane of an Illumina MiSeq2000 (2 x 300 bp paired-end) at the University of Colorado BioFrontiers Institute (Boulder, CO). Data were processed using a combination of UPARSE (Edgar 2013) and QIIME (Caporaso et al. 2010) pipelines to demultiplex and merge sequences, remove singletons, and then cluster sequences (including chimera filtering) into OTUs at 97% sequence identity and assign taxonomy using the UNITE database (Abarenkov et al. 2010). Sequences were rarefied at 5238 sequences per sample. AMF genera were identified as any genus in the subphylum
Glomeromycotina (Spatafora et al., 2016). DSE genera were selected from reports of known DSE taxa (Jumpponen and Trappe 1998, Jumpponen 2001, Mandyam and Jumpponen 2005, Newsham 2011). Sequences for all of the OTUs are available on GenBank, accessible via the accession numbers SUB3901920: MH238510-MH240826.

Statistical Analysis

To test for differences in colonization levels among plant species (only for species with at least 3 samples) and functional groups, we used the Kruskal-Wallis test (kruskal.test function in the R Package ‘stats’) followed by Nemenyi post-hoc tests (posthoc.kruskal.nemenyi.test function in the R Package ‘PMCMR’, Pohlert, 2014), because the data were not normally distributed (Shapiro-Wilk Test, p < 0.05) and the variance was not homogenous among groups (Levene Test, p < 0.05). We defined functional groups as forbs, grasses, and sedges. Due to low sample sizes, N-fixers (1 species, 4 samples) and rushes (1 species, 2 samples) were removed from the functional group analysis. Because species differences and differences in species replication could mask differences among functional groups, analyses for functional groups were performed on species’ means colonization percentages. We also tested for a phylogenetic signal in species’ mean DSE and AMF colonization using the multiPhylosignal function (R Package ‘picante’, Kembel et al., 2010). The phylogenetic supertree of the plant species (identified to species and sampled at least 3 times) for this analysis was created using the software Phylomatic (Webb and Donoghue 2005). Since we did not have a molecular phylogeny of our species, we subset the supertree down to our species. To test for spatial autocorrelation in DSE and AMF colonization, as well as in the predictor variables, we calculated Moran’s I (Moran.I function in the R package ‘ape’, Paradis et al., 2004). To test for relationships between spore counts and environmental variables and plant density, we ran univariate linear regressions (lm function in the R package ‘stats’, R Core
To find the best combination of variables that predicted AMF and DSE percent colonization, we used an exhaustive all subsets method to select models based on the Akaike Information Criterion (AIC) (Akaike, 1974; function bestglm in the R package 'bestglm', Mcleod & Xu, 2017). Instead of using forward or backward selection to select variables, this function tests all possible combinations of predictor variables. Predictor variables were plant species, elevation, snowpack, plant density, TIN, and DIP. We did not include plant functional group in the analysis, because plant species had a much larger effect. These models did not take into account how plant species abundances vary over environmental gradients (i.e. species are not distributed evenly across the landscape; some species may be only present at low elevation areas with higher plant density). To improve the normality of the response variables and some predictor variables, colonization levels were logit transformed, and plant density, TIN, and DIP were log transformed. To test for associations between AMF and DSE colonization levels and richness of genera, we used the Spearman rank correlation. Due to a possible exponential decay relationship after data visualization, we also evaluated an exponential decay model, which we fit using the nls function (R package ‘stats’). We ran this analysis on the full data set as well as on species’ mean DSE and AMF levels, and on samples that contained both DSE and AMF. Lastly, to test for differences in AMF and DSE community composition among plant host species and functional groups, we used permutational multivariate analysis of variance (PERMANOVA, Anderson, 2001) on Bray-Curtis dissimilarities at the genus level, implemented with the adonis function in the R package ‘vegan’ (Oksanen et al., 2018). To test for environmental drivers of community composition we used the envfit function in ‘vegan’. We visualized compositional data using Principle coordinates analysis, calculated with the cmdscale function in ‘vegan’. All statistical analyses were performed using the statistical software R (version 3.4.0, R Core Team, 2017).
Results

The majority of the 177 plant individuals sampled, 86%, were colonized by either AMF or DSE, or both. A total of 43 individuals were colonized by AMF only, a total of 36 individuals were colonized by DSE only, and 71 individuals contained both AMF and DSE. Percent of plant root length colonized ranged from 0·72% by AMF and 0·64% by DSE. We report colonization (or lack thereof) by AMF and DSE for 9 plant species not previously characterized in the current literature as well as colonization of 7 species that had previously been described as non-mycorrhizal (Table 4.1).

Neither of the fungal groups’ colonization levels were spatially autocorrelated (Moran’s $I = 0.01$, $p = 0.16$). The density of AMF spores increased significantly with plant density ($R^2 = 0.32$, $p < 0.001$), and were not significantly correlated with the other variables.
Table 4.1. Plant taxonomy, sample size, ranges of arbuscular mycorrhizal fungi (AMF) and dark septate endophyte (DSE) colonization, mycorrhizal status, ranges of number of genera in the root (from ITS sequences), and references where plants have been previously studied for AMF or DSE colonization. Abbreviations in the Other Reference column (AM = arbuscular mycorrhizae, DS = dark septate endophytes, EM = ectomycorrhizae, NM = non-mycorrhizal) note which fungi, if any, had been found in previous samples of the species. Obligate, facultative, or non-mycorrhizal status was assigned based on if a species always, sometimes, or never has DSE or AMF based on our findings and the literature. Codes marked with a cross (†) were previously thought to be non-mycorrhizal until our study.

<table>
<thead>
<tr>
<th>Family</th>
<th>Species</th>
<th>Code</th>
<th>n</th>
<th>AMF % Status &amp; Gen.</th>
<th>DSE % Status &amp; Gen.</th>
<th>Other References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apioideae</td>
<td>Angelica grayana</td>
<td>AnaGr</td>
<td>3</td>
<td>0-52 Facultative 0-7</td>
<td>0-4 Facultative 0-4</td>
<td>First report</td>
</tr>
<tr>
<td></td>
<td>Oregnalis alpina</td>
<td>OraAlp</td>
<td>2</td>
<td>0-4 Facultative 0-5</td>
<td>0-4 Facultative 2-3</td>
<td>First report</td>
</tr>
<tr>
<td>Asteraceae</td>
<td>Antennaria media</td>
<td>AntMed</td>
<td>4</td>
<td>5-45 Obligate 0</td>
<td>0-1 Facultative 0-1</td>
<td>First report</td>
</tr>
<tr>
<td></td>
<td>Cirsium scopulorum</td>
<td>CirSco</td>
<td>4</td>
<td>0-70 Facultative 1-8</td>
<td>0 Non-DS 1-2</td>
<td>First report</td>
</tr>
<tr>
<td></td>
<td>Eriogonum simplex</td>
<td>EriSim</td>
<td>3</td>
<td>6-15 Obligate 6</td>
<td>0-27 Facultative 2</td>
<td>AM/DS1,2</td>
</tr>
<tr>
<td></td>
<td>Senecio freynii</td>
<td>SenFre</td>
<td>9</td>
<td>0-32 Facultative 0-11</td>
<td>0-3 Facultative 1-4</td>
<td>NM/AM/EM1,3</td>
</tr>
<tr>
<td>Boraginaceae</td>
<td>Mentzelia lanceolata</td>
<td>MerLan</td>
<td>1</td>
<td>2 Obligate NA</td>
<td>0 Non-DS NA</td>
<td>NM/AM4</td>
</tr>
<tr>
<td>Caryophyllaceae</td>
<td>Cerastium arvense</td>
<td>CerArv</td>
<td>1</td>
<td>0.5 Obligate 1</td>
<td>0 Non-DS 0</td>
<td>NM/AM/DS4,5,6</td>
</tr>
<tr>
<td></td>
<td>Minuartia obtsiobra</td>
<td>MinObt</td>
<td>3</td>
<td>0-5 Facultative 0-2</td>
<td>0-1 Facultative 2-3</td>
<td>NM1</td>
</tr>
<tr>
<td></td>
<td>Stenea acaulis</td>
<td>StAca</td>
<td>6</td>
<td>0-28 Facultative 2-12</td>
<td>0-64 Facultative 1-2</td>
<td>NM/AM1,7,9,10,11,12,13,14</td>
</tr>
<tr>
<td></td>
<td>Stellaria umbellata</td>
<td>StUmb</td>
<td>5</td>
<td>0-5 Facultative 0-1</td>
<td>0-1 Facultative 2-5</td>
<td>First report</td>
</tr>
<tr>
<td>Cyperaceae</td>
<td>Carex eleocharis</td>
<td>CarElc</td>
<td>5</td>
<td>3-20 Obligate 0-9</td>
<td>0-2 Facultative 1-3</td>
<td>NM1</td>
</tr>
<tr>
<td></td>
<td>Carex heteroneura</td>
<td>CarHet</td>
<td>2</td>
<td>3-4 Obligate 0</td>
<td>0-1 Facultative 1</td>
<td>First report</td>
</tr>
<tr>
<td></td>
<td>Carex perglabosa</td>
<td>CarPgl</td>
<td>1</td>
<td>5-45 Obligate 1</td>
<td>0 Non-DS 4</td>
<td>First report</td>
</tr>
<tr>
<td></td>
<td>Carex phaeochelina</td>
<td>CarPhe</td>
<td>3</td>
<td>12-40 Obligate 2-8</td>
<td>0-1 Facultative 1-4</td>
<td>NM18</td>
</tr>
<tr>
<td></td>
<td>Carex pyrenaica</td>
<td>CarPyr</td>
<td>11</td>
<td>0-12 Facultative 0-3</td>
<td>0-1 Facultative 1-3</td>
<td>NM1</td>
</tr>
<tr>
<td></td>
<td>Carex rupistris</td>
<td>CarRup</td>
<td>1</td>
<td>0-3 Non-AM 7</td>
<td>0 Non-DS 3</td>
<td>NM4,15</td>
</tr>
<tr>
<td></td>
<td>Carex scopolorum</td>
<td>CarSco</td>
<td>2</td>
<td>0-1 Facultative 5</td>
<td>3-8 Obligate 1</td>
<td>NM/DS1</td>
</tr>
<tr>
<td></td>
<td>Carex spicata</td>
<td>CarSpi</td>
<td>2</td>
<td>0-3 Non-AM 4</td>
<td>0 Non-DS 3</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Kobresia myosoroides</td>
<td>KobMyo</td>
<td>5</td>
<td>0-3 Facultative 0-6</td>
<td>0-4 Facultative 1-3</td>
<td>EM/DS10,17</td>
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<tr>
<td>Fabaceae</td>
<td>Trifolium dasyphyllum</td>
<td>TriDas</td>
<td>1</td>
<td>0-3 Non-AM 0-6</td>
<td>0-13 Facultative 0-3</td>
<td>NM14</td>
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<tr>
<td>Junaceae</td>
<td>Lotus spicata</td>
<td>LuzSpi</td>
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<td>0-1 Facultative 2-4</td>
<td>NM16,17</td>
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<tr>
<td>Liliaceae</td>
<td>Liliaa serotina</td>
<td>LioSer</td>
<td>1</td>
<td>10 Obligate NA</td>
<td>12 Obligate NA</td>
<td>AM/DS2</td>
</tr>
<tr>
<td>Poaceae</td>
<td>Agrisotis variabilis</td>
<td>AgrVar</td>
<td>1</td>
<td>0 Non-AM 1</td>
<td>4 Obligate 2</td>
<td>NM1</td>
</tr>
<tr>
<td></td>
<td>Deschampsia cepitosas</td>
<td>DesCes</td>
<td>8</td>
<td>3-21 Obligate 0-17</td>
<td>0-5 Facultative 1-5</td>
<td>AM/DS1,19</td>
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<tr>
<td></td>
<td>Elymus scribneri</td>
<td>ElyScr</td>
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<td>0-20 Facultative 0-3</td>
<td>AM/DS20</td>
</tr>
<tr>
<td></td>
<td>Festuca brachyphylla</td>
<td>FestBr</td>
<td>35</td>
<td>0-12 Facultative 0-5</td>
<td>0-40 Facultative 0-5</td>
<td>AM/DS1,20,21</td>
</tr>
<tr>
<td></td>
<td>Poa species</td>
<td>PoaSpe</td>
<td>3</td>
<td>1-2 Obligate 0-3</td>
<td>0-4 Facultative 0-1</td>
<td>AM/DS1,14,20,22,*</td>
</tr>
<tr>
<td></td>
<td>Trisetum spicatum</td>
<td>TriSpi</td>
<td>19</td>
<td>0-14 Facultative 0-7</td>
<td>0-55 Facultative 0-4</td>
<td>NM/AM/DS30,23,24</td>
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<tr>
<td>Polygonaceae</td>
<td>Oriaia digyna</td>
<td>OryDig</td>
<td>9</td>
<td>0-28 Facultative 0-1</td>
<td>0-9 Facultative 0-4</td>
<td>NM/AM3,11,12,14</td>
</tr>
<tr>
<td>Ranunculaceae</td>
<td>Aquilegia caerulea</td>
<td>AquCer</td>
<td>1</td>
<td>15 Obligate 3</td>
<td>2 Obligate 1</td>
<td>First report</td>
</tr>
<tr>
<td>Rosaceae</td>
<td>Geum rosseum</td>
<td>GeuRos</td>
<td>10</td>
<td>0-30 Facultative 0-6</td>
<td>0-34 Facultative 0-3</td>
<td>AM1,2,25</td>
</tr>
<tr>
<td></td>
<td>Silphium parvifolium</td>
<td>SilPar</td>
<td>10</td>
<td>0-15 Obligate NA</td>
<td>7 Obligate NA</td>
<td>NM/AM1,13,17,26</td>
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<tr>
<td>Scrophulariaceae</td>
<td>Bessisia arctica</td>
<td>BesAlp</td>
<td>4</td>
<td>29-72 Obligate 0-6</td>
<td>0-5-3 Obligate 0-4</td>
<td>First report</td>
</tr>
</tbody>
</table>

*1Cripps & Eddington 2005  
2Schmidt et al. 2008  
3Cázares et al 2005  
4Kovacic et al. 2004  
5Pawlowska et al. 1996  
6Casonova-Kanyo et al. 2011  
7Nepiak 1953  
8Stutz 1972  
9Haselwandt & Read 1980  
10Rend & Haselwandt 1981  
11Teu et al. 1996  
12Vare et al. 1992  
13Vare et al. 1997  
14Vare et al. 1998  
15Bledsoe et al. 1990  
16Radulé & Aiken 1998  
17Kohn et al. 1990  
18Katinen 1965  
19Lago et al. 2012  
20Lisent & Aestibus 1986  
21Zubek et al. 2008  
22Fu et al. Patpina, P. arctica, P. glauca  
23NM/AM/EM1,3
(Kruskal Wallis, $X^2 = 54.65$, df = 20, $p < 0.001$, Figure 4.3b). The highest levels of AMF and DSE colonization were found in *Besseya alpina* and *Silene acaulis*, respectively. While AMF colonization did not differ among functional groups (Figure 4.4a), there were significant differences in mean DSE colonization among plant functional groups (Kruskal Wallis, $X^2 = 6.48$, df = 2, $p = 0.039$, Figure 4.4b). Grass species had greater DSE colonization than sedge species (Nemenyi post hoc test, $p < 0.05$). There was no phylogenetic signal in mean AMF colonization ($K = 0.75$, $p = 0.42$) or DSE colonization ($K = 1.71$, $p = 0.16$).

**Figure 4.3.** Percent of root length colonized by a) AMF (arbuscular mycorrhizal fungi) and b) DSE (dark septate endophytes) across the 20 different plant species that were sampled at least 3 times. Plant host species had a greater influence on AMF than DSE. Plants are ordered phylogenetically. For plant family, genus, and species names, refer to Table 4.1. Boxplots show the median, 25-75th quantiles, 95% confidence intervals, and outliers.

**Figure 4.4.** Percent root colonization by a) AMF (arbuscular mycorrhizal fungi) and b) DSE (dark septate endophytes) across forb (n = 18 species means), grass (n=6), and sedge (n=9) functional groups. Different letters represent significant differences in colonization based on the Nemenyi post hoc test ($p < 0.05$). Boxplots show the median, 25-75th quantiles, 95% confidence intervals, and outliers of species' means.
Plant and Environmental Predictors of Colonization (H2)

For AMF, plant species was the most important variable driving colonization levels and was a significant predictor in all of the top 5 models (Table 4.2). Plant species explained more variation in the data than environmental variables (Table 4.2). After plant species, several other variables were also important. The best model for AMF colonization included TIN, elevation, and snowpack, with percent colonization increasing at lower elevation areas with less snowpack and less nitrogen (Table 4.2). A slightly less parsimonious model that had similar support (AIC difference <1) also included DIP, with which there was a negative relationship (Table 4.2, Figure 4.5a). For DSE colonization, the best model included snowpack and TIN, with the highest levels of colonization in areas with less snowpack (Figure 4.5b) and higher soil nitrogen (Table 4.2). A less parsimonious model with similar support also demonstrated a positive relationship with plant density (Table 4.2).

Figure 4.5. a) Kriging interpolation and scatterplot of total dissolved inorganic phosphorus (DIP) and the percent of arbuscular mycorrhizal fungi (AMF) root colonization b) Kriging interpolation and scatterplot of mean snowpack and the percent of dark septate endophyte (DSE) root colonization. DIP and snowpack were two of the significant predictors of AMF and DSE colonization, respectively.
Table 4.2. Results of all subset modeling of arbuscular mycorrhizal fungi (AMF) and dark septate endophyte (DSE) colonization. Shown are the included variables and their coefficients of the top 5 models for each fungal group, the AIC scores, and partial $R^2$ scores for plant and environmental variables. All models except for the 5th DSE colonization model were significant. The spatial distributions of a significant continuous predictor variable (DIP for AMF, Snowpack for DSE) are shown in Fig. 5. TIN = Total inorganic nitrogen. DIP = Total dissolved inorganic phosphorus.

<table>
<thead>
<tr>
<th>Response Variable</th>
<th>Best Predictor Variables (coefficient sign)</th>
<th>AIC</th>
<th>Plant $R^2$</th>
<th>Env $R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMF Colonization</td>
<td>Species, TIN(-), Elevation(-), Snowpack(-),</td>
<td>357.23</td>
<td>56.06</td>
<td>8.57</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Species, TIN(-), Elevation(-), Snowpack(-), TDI</td>
<td>357.53</td>
<td>56.30</td>
<td>9.71</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Species, TIN(-), Elevation(-)</td>
<td>357.64</td>
<td>56.60</td>
<td>4.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Species, TIN(-), Elevation(-)</td>
<td>357.76</td>
<td>56.93</td>
<td>5.58</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Species, TIN(-)</td>
<td>358.03</td>
<td>56.22</td>
<td>1.96</td>
</tr>
<tr>
<td>DSE Colonization</td>
<td>Snowpack(+), TIN(+)</td>
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<td>NA</td>
<td>5.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Snowpack(+), TIN(+), Density(+)</td>
<td>382.87</td>
<td>NA</td>
<td>5.32</td>
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<td></td>
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<td></td>
<td>Snowpack(+), Density(+)</td>
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<td>5.38</td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>Snowpack(+)</td>
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<td>NA</td>
<td>4.90</td>
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</tr>
<tr>
<td></td>
<td>Snowpack(+), Elevation(+)</td>
<td>384.89</td>
<td>NA</td>
<td>3.98</td>
</tr>
</tbody>
</table>

**AMF and DSE Co-Occurrence (H3)**

There was no significant correlation between AMF and DSE colonization across the whole dataset ($S = 883300$, Rho = 0.04, p = 0.56) or across species means ($S = 6742.1$, Rho = 0.06, p = 0.75). However, when looking only at roots that contained both DSE and AMF, the percent colonization of the fungal groups was significantly negatively correlated ($S = 75608$, Rho = -0.27, p = 0.02, Figure 4.6). On the other hand, the genus richness of AMF and DSE was significantly positively correlated ($S = 20222$, Rho = 0.34, p < 0.01) for roots containing both fungal groups. There was no significant exponential decay of AMF colonization across DSE colonization levels for the whole dataset ($\lambda = 19.97$, p > 0.05), within species’ means ($\lambda = 41.25$, p > 0.05), or across samples containing both fungal groups ($\lambda = 19.90$, p > 0.05, Figure 4.6).

![Figure 4.6. Arbuscular mycorrhizal fungi (AMF) versus dark septate endophyte (DSE) root colonization for individuals that contained both fungi (n = 71). Root colonization levels of the two groups were significantly negatively correlated ($S = 75608$, Rho = -0.27, p = 0.02). An exponential decay function was not significant ($\lambda = 19.90$, p > 0.05).](image-url)
**AMF and DSE Community Composition (H4)**

The roots sampled contained a total of 29 genera of AMF and 14 genera of DSE. The most widespread AMF genera were *Acaulospora* and *Entrophospora*, found in 35 samples each. The genera *Archaeospora*, *Claroideoglomus*, and *Glomus* were also widespread, found in over 25 samples each. The most widespread DSE genus was *Phialophora* found in 125 samples, followed by *Capronia*, found in 43 samples. The genera *Leptosphaeria*, *Exophiala*, and *Cryptosporiopsis* were also widespread, each found in over 25 samples. AMF community composition did not differ significantly among plant species or functional group (PERMANOVA, p > 0.05). AMF community composition was driven by elevation, nitrogen, and plant density gradients (Figure 4.7A). DSE community composition differed significantly among plant species and functional groups (PERMANOVA, p < 0.05), and was also driven by snowpack and nitrogen gradients (Figure 4.7B).

![Figure 4.7](image)

**Figure 4.7. Principle coordinates analysis of arbuscular mycorrhizal fungi (A) and dark septate endophyte (B) community composition at the genus level based on Bray-Curtis dissimilarity. Vectors show significant variables that drive community dissimilarity, as determined by the envfit function in the R package “vegan”. Also shown are vectors for percent colonization, to represent on the graphs areas where there was higher percent root colonization by each fungal group.**

**Discussion**

The majority of plant individuals and species sampled contained AMF, DSE, or both, highlighting the importance of these fungi in alpine ecosystems (Haselwandter and Read 1980). Our work provides insights into several questions
that we posed based on previous work (Ranelli et al. 2015), including decreases in colonization with higher elevations, increased AMF colonization at both lower nitrogen and phosphorus levels, increased DSE with higher nitrogen levels, and a negative relationship between AMF and DSE fungi.

**Plant Species and Functional Group (H1)**

The high amount of variation in colonization among different plant species is not surprising and has been reported elsewhere (Ruotsalainen et al. 2004, Ranelli et al. 2015). However, a lack of differences among species has also been reported, possibly due to a low number of species sampled (Casanova-Katny et al. 2011). Our study confirms differences in colonization among plant hosts across a wide variety of species (n = 35).

Our hypothesis that forbs would show greater levels of colonization than other groups was not supported by our results. While forbs may generally have thicker roots than grasses, a trait which can be positively correlated with AMF infection (Maherali 2014), we did not measure this trait and the lack of difference in colonization levels between the two groups could have been masked by high inter- and intra-specific variation in root architecture within the groups. Furthermore, variability in other traits, such as cool- or warm-season grasses, photosynthetic pathway, or clonal mobility, all of which can impact mycorrhizal infection levels (Hetrick et al., 1991; Wilson and Hartnett, 1998; Lugo et al., 2012; Onipchenko and Zobel, 2000), may not have been captured by our functional groups. For DSE, forbs (all dicots except one individual) had similar levels of DSE colonization to graminoids and sedges (all monocots), contrary to previous results showing greater colonization in monocots (Weishampel and Bedford, 2006, Newsham, 2011). The low level of DSE colonization in the sedges we studied was surprising, as it was much lower than in other studies (Read and Haselwandter 1981), and DSE have been shown to benefit sedge growth (Haselwandter and Read 1982). However, many
sedges can take up organic nitrogen without the help of mycorrhizae (Raab et al. 1999), and increasing rates of atmospheric deposition of inorganic nitrogen at our site (Burns 2003) may negate the beneficial effects of DSE for some plant species.

**Plant and Environmental Predictors of Colonization (H2)**

Plant species was included in all of the top 5 multivariate models for AMF percent root colonization, but none for DSE. Plant species also explained more of the variation in AMF colonization than environmental variables. This result supports the hypothesis that AMF are obligate symbionts and are expected to depend on plant hosts more than the environment, while DSE are facultative symbionts that may be structured more by environmental variables (Ranelli et al. 2015), though this remains to be tested in other systems. There were several important environmental predictors of AMF and DSE colonization in the multivariate models, including elevation, snowpack, plant density, phosphorus, and nitrogen.

AMF were more abundant at lower elevations, while DSE showed no significant relationship with elevation. Decreases in AMF root colonization with increasing elevation have been reported in most other mountain ranges (Haselwandter and Read, 1980; Lugo et al., 2012; Ruotsalainen et al., 2004; Schmidt et al., 2008; Väre et al., 1997, Shi et al., 2014; Kotilínek et al., 2017). Samples from even lower elevation, intact tundra meadows on Niwot Ridge (3500 m elevation) have shown over 80% colonization in some samples, which further supports this trend (Schmidt et al. 2008). This could be due to a lack of inoculum at high elevations (but see Marín et al., 2017 for increases with elevation), which could be a function of low plant density or a negative effect of cold temperature on AMF (Lugo et al., 2008; Yang et al., 2016). Since DSE are common in arctic and alpine environments, can tolerate cold temperatures (Mullen et al. 1998), and have been found in abundance at over 5250 m.a.s.l. in the Andes (Schmidt et al. 2008), and at 6000 m in the Himalayas (Kotilínek et al., 2017), our finding of no relationship with
elevation makes sense and is consistent with other studies (Ruotsalainen et al. 2004, Schmidt et al. 2008b, Zubek et al. 2009).

Both AMF and DSE showed higher colonization levels in areas with shallower snowpacks, which may be explained by more developed soils and plant communities, and subsequently more fungal inoculum, in these areas. Another explanation for this relationship is that both AMF (Augé 2001) and DSE (Barrow 2003) can help plants cope with low soil moisture conditions where snow melts earlier (Williams et al. 2009). Snowpack is a variable expected to decline as climate warms. While our site shows increasing winter precipitation trends (Kittel et al. 2015), there are declining trends in snow meltout date and lake ice-off dates over the past few decades due to warming and less spring snow (Preston et al. 2016). Our results suggest that fungal inoculum could increase in these conditions and plants could become more colonized by fungi to cope with moisture limitations, but this remains to be tested.

Despite a positive relationship between plant density and AMF spores, there was no relationship between AMF colonization and plant density. Interestingly, percent root colonization and spore density are not necessarily correlated (e.g. Aguilera et al., 2017). Since AMF are obligate symbionts, they are expected to be more abundant where there are more individual plants that associate with them (regardless of plant species richness). We originally thought DSE would be most important for plants in sparsely vegetated areas because we did not expect them to decline with plant density; however, DSE colonization did decline with plant density, even though they can survive without a plant host. Thus, AMF may be just as important as DSE for plant survival in sparsely vegetated and newly vegetated areas. As climate warms at our site, vascular plant density is increasing in the subnival zone (Bueno de Mesquita et al. 2017). Most of the stem counts from our 2015 survey were higher than those in a 2008 survey of the same plots (King et al.
This increase in plant density may lead to both an increase in AMF inoculum levels and DSE colonization levels, which may in turn be important for alpine plants to cope with warmer maximum temperatures and lower soil moistures (Kivlin et al. 2013).

The negative relationship between phosphorus and AMF colonization supports our hypothesis and suggests that AMF help plants acquire phosphorus in high-alpine systems, as has been shown in other systems (e.g. Lingfei et al., 2005, Camenzind et al., 2014). Phosphorus is a limiting nutrient for plant growth at our site in both tundra meadows (Seastedt and Vaccaro 2001) and in the subnival zone (King et al. 2008), as well as in the high alpine of Perú and Alaska (Darcy et al., in review). P limitation at our site could be due to phosphorus-poor granite-gneiss parent material, or insufficient weathering (King et al. 2008; Vitousek et al. 2010; Bowman and Seastedt, 2001; Porder and Ramachandran, 2012). Consequently, plants may devote more photosynthate to AMF in low P areas to increase P uptake via the fungus (Johnson et al. 2010). Furthermore, previous work at our site demonstrated correlations between plant tissue P and the amount of arbuscules in the roots (Mullen and Schmidt 1993), which is strong evidence of AMF-mediated P uptake in the field.

There were higher levels of AMF colonization at low soil inorganic nitrogen levels, suggesting that AMF are also helping plants acquire N at our site, consistent with previous studies (Augé 2004, Smith and Read 2008, Camenzind et al. 2014). On the other hand, there was a positive relationship between DSE colonization and TIN, also consistent with our hypothesis and other studies (Newsham 2011). This result, especially given the context of our alpine study site, where decomposition rates are slow and N can be stored in organic form for long periods of time (Bowman and Seastedt 2001), supports the idea presented by Newsham (2011) that DSE break down organic N in the rhizosphere into more plant-available forms.
Together, these results are consistent with the idea that DSE help make inorganic N available, and AMF help plants take up the inorganic N, especially when it is more limiting (Smith and Read 2008, Newsham 2011).

**DSE and AMF Co-occurrence (H3)**

Our results contradict those of Ranelli et al. (2015), who found a positive relationship between AMF and DSE colonization. In our data, there appeared to be a trend of an exponential decay relationship, though this was likely confounded by many instances of low colonization of both DSE and AMF, and thus was not significant. In the 71 individuals colonized by both AMF and DSE, AM and DSE colonization levels were negatively correlated. This could be due to different responses to environmental and plant gradients or competition for host plant root tissue. Interestingly, there was no negative correlation in the number of DSE and AMF genera in the roots, suggesting that multiple taxa can share the same root, even if some taxa are at low abundances. While many studies have examined both AMF and DSE colonization, few of them have explicitly analyzed correlations between the two (Ranelli et al., 2015; Kotilínek et al., 2017). Competition studies between AMF and DSE are necessary to tease apart these dynamics.

**DSE and AMF Community Composition (H4)**

The most abundant AMF genera identified in the roots show a cosmopolitan distribution, as was found in a global distribution study on the Glomeromycotina (Davison et al., 2015). *Acaulospora* has been reported in a wide variety of ecosystems and countries (Zhang et al., 2004 Oehl et al., 2004, Andrade et al., 2009, Schenk and Smith, 1982, Oehl et al., 2006), and has been shown to increase plant foliar P concentrations (Klironomos, 2000). *Entrophospora* is similarly widespread (Palenzuela et al. 2010), but to date, no targeted inoculation studies have been conducted to determine its function. While the percent root colonization by AMF
varied among plant species, community composition did not. These results show that different plant species may be more dependent on AMF than others, but they do not necessarily rely on only certain taxa. In other words, the plants in our study associate with a variety of AMF taxa, and AMF taxa colonize a wide variety of plant species. This is in contrast to other studies that have found differences in community composition among plant hosts (Vandenkoornhuyse et al. 2003; Hausmann and Hawkes, 2009; Martínez-García et al., 2015), which may be explained by the number of plant species we sampled and the environmental gradients we encompassed compared to other studies. Interestingly, AMF composition is driven by the harshness gradient, with certain taxa more abundant in high-elevation, sparsely-vegetated areas, and others more abundant at high plant densities and N concentrations.

The abundant DSE genus *Phialophora* has been previously found in alpine forbs (Schadt et al. 2001) and conifer roots (Wang et al. 1985), as well as moss in Antarctica suggesting a circumpolar distribution for the taxon (Yu et al. 2014). *Capronia* has been found in lichens in Bolivia (Etayo et al. 2013), Turkey and Spain (Halici et al., 2010), in oak forests (Friebes 2011), and even in intertidal environments (Au et al. 1999). While *Capronia pilosella* is cited as being a dark septate fungus (Jumpponen and Trappe 1998), most sequences in our study aligned more closely to *Capronia peltigerae*, which is a lichenicolous fungus (Untereiner et al. 2011). While the *Exophiala* genus also contains non-endophytic soil fungi, our sequences closely aligned with an endophyte isolated from fine pine roots in the mountains of Montenegro (Lazarević and Menkis 2017). Unfortunately, there are no studies of how targeted inoculations with these taxa affect plant fitness, which is an avenue for future research. DSE community composition varied among plant species and functional groups, just as percent root colonization did. Similarly, nitrogen and snowpack gradients, the key drivers of percent root colonization, also
drove community composition, with certain taxa associated with high N, low snow areas, and others in late-melting snowbed areas.

Conclusions

Our work builds on other surveys of AMF and DSE colonization and contributes to a growing body of knowledge on these two important fungal groups. Our data show that colonization levels of both fungal groups vary significantly among different plant species. As plants shift their ranges in response to climate change and changes in snowpack, interactions with these two fungal groups will be important for some plant species and not others. Our work provides new insights on how colonization varies across the landscape, and highlights the important role of AMF in plant acquisition of both nitrogen and phosphorus, and of DSE in nitrogen cycling in alpine ecosystems. Interactions with these two fungal groups will likely prove crucial for alpine plants responding to global change (Kivlin et al. 2013), which is an important avenue for future research. For example, plants moving uphill to track warming may have to colonize unvegetated areas, which could be limited by phosphorus or nitrogen, and fungi could help facilitate movement into these habitats.
CHAPTER V
THE IMPORTANCE OF PLANT-MICROBIAL INTERACTIONS INCREASES AS PLANTS COLONIZE AREAS ON THE EDGE OF THEIR ABIOTIC RANGE

**Abstract**

As organisms shift their geographic distributions in response to climate change, biotic interactions have emerged as an important factor driving the rate and success of range expansions. Plant-microbe interactions are an understudied but potentially important factor governing plant range shifts. We studied the distribution and function of microbes present in high elevation unvegetated soils, areas that plants are colonizing as climate warms, snow melts earlier and the summer growing season lengthens. Using a manipulative snowpack and microbial inoculation transplant experiment, we tested the hypothesis that growing season length and microbial community composition interact to control plant elevational range shifts. We predicted that a lengthening growing season combined with arriving in patches of soils with more mutualistic microbes and fewer pathogenic microbes would facilitate plant survival and growth in previously unvegetated areas. We identified negative effects on plant survival in both short and long growing seasons, suggesting an optimal growing season length for plant survival in this system that balances time for growth with soil moisture levels. Importantly, growing season length and microbes interacted to effect plant survival and growth, such that microbial community composition increased in importance in suboptimal growing season lengths. Further, plants grown with microbes from unvegetated soils grew as well or better than plants grown with microbes from vegetated soils. These results suggest that the rate and spatial extent of plant colonization of
unvegetated soils in mountainous areas experiencing climate change could depend on both growing season length and soil microbial community composition, with microbes potentially playing more important roles as growing seasons lengthen.

**Introduction**

Global environmental change has led to a redistribution of the earth’s flora and fauna, with warmer temperatures typically allowing species to move to higher elevations and higher latitudes (Chen et al. 2011, Settele et al. 2014). Such responses to abiotic conditions do not occur in isolation; rather, they are mediated by complex biotic interactions that can either facilitate or inhibit, and either speed or slow, range shifts (van der Putten 2012). For example, recent research has demonstrated that both plant-pollinator and plant-plant interactions can influence plant range shifts (Meier et al. 2010, Pellissier et al. 2010, Meineri et al. 2012, Hille Ris Lambers et al. 2013, Giannini et al. 2013). Plant-microbe interactions are a ubiquitous and important biotic interaction that has largely been overlooked in the context of plant range shifts in response to climate change, with recent exceptions (Pellissier et al. 2013, Bueno de Mesquita et al. 2015, Van Nuland et al. 2017). Here we first surveyed soil microbial community composition across an unvegetated landscape and then used a manipulative inoculation and transplant experiment to test the importance of these microbial communities in facilitating or inhibiting plant establishment beyond their range as climate changes and growing season lengthens.

Soil microbes are drivers of vegetation productivity, diversity, community assembly, and community structure (Klironomos 2002, Wardle et al. 2004, van der Heijden et al. 2008). The majority of plants rely on mutualistic or beneficial microbes to either mobilize or help acquire nutrients, and cope with abiotic and biotic stressors (van der Heijden et al. 2008). On the other hand, soil borne
microbial pathogens can have devastating impacts on plant fitness (Jackson 2009). Plant interactions with this wide variety of fungal and bacterial enemies, mutualists, and decomposers can contribute to plant-soil feedbacks that have important implications for plant fitness (van der Putten et al. 2016). Importantly, soil fungi and bacteria also have biogeographic patterns based on environmental variables (Fierer et al. 2009, Lauber et al. 2009, Tedersoo et al. 2014), and thus, the microbiomes necessary for plant establishment in new habitats may not necessarily be present.

In mountainous regions, species typically move up in elevation to track a suitable climate (Parmesan and Yohe 2003, Pauli et al. 2007, Parolo and Rossi 2008, Frei et al. 2010, Chen et al. 2011). In many cases, for alpine plants, this requires colonizing areas that are currently unvegetated. Unvegetated soils are characterized by poor development and nutrient limitation (Aide and Cwick 1998, Schmidt et al. 2008a). But these seemingly barren soils are teeming with microbial life long before plant arrival (King et al. 2008). Indeed, complex microbial communities can develop in newly exposed deglaciated soil within four years (Schmidt et al. 2008a). Bacterial taxa that perform nitrogen fixation, mineralization, immobilization, and nitrification are important in governing plant nutrient availability and may be important for “priming” soil for plant colonization (Schmidt et al. 2008a). Bacteria are also capable of solubilizing phosphorus (Rodríguez and Fraga 1999), which was recently suggested to be more limiting than nitrogen in early successional, high-alpine environments (Darcy et al. 2018b). Furthermore, some plant-associated fungi can be present in unvegetated soils. Dark septate endophytes (DSE) are a group of facultative root endophytes that are capable of surviving on organic debris and in biological soil crusts (Caldwell et al. 2000, Menkis et al. 2004, Mandyam and Jumpponen 2005, Green et al. 2008, Mandyam et al. 2010, Day and Currah 2011, Knapp and Kovács 2016). Additionally,
aerially deposited spores of arbuscular mycorrhizal fungi (AMF) and other biotrophic fungi can be present in unvegetated soils (Jumpponen 2003).

In this study, we tested the importance of microbial communities on the success of alpine plant upward range shifts into unvegetated soil as climate changes and snow melts earlier. We first surveyed the distribution of microbes in unvegetated soils and then manipulated the abundance of plant-associated taxa in the soil by collecting soil inocula from eight different locations to simulate the scenario of a plant dispersing into unvegetated soil and encountering different microbial communities. We hypothesized that (1) a longer growing season due to earlier snowmelt enables range expansion of alpine plants into currently unvegetated soils, (2) microbial community composition and differences in the abundance of plant-associated microbes will affect plant survival and growth, and (3) growing season length and microbial community composition will interact such that microbes will most strongly affect plant performance as the growing season lengthens.

**Methods**

**Study Site**

The experiment was conducted in a late-melting snowbed (3900 m.a.s.l.) on the southeast facing slope of Navajo Peak in the Green Lakes Valley, part of the Niwot Ridge Long Term Ecological Research Site in the Front Range of the Rocky Mountains, Colorado, USA (Figure A5.1). The snowbed typically melts out in mid-August and snow starts falling again in September. Average precipitation from 1952 to 2012 was 1090 ± 230 mm yr\(^{-1}\), with a 60 mm yr\(^{-1}\) increase over that time period, driven mostly by increases in winter precipitation (Kittel et al. 2015). Recent mean annual temperatures (2011 – 2014) range from \(-4^\circ C\) to \(-7^\circ C\), while mean daily summer temperatures range from 4°C to 10°C (Losleben 2017). Both annual and
summer temperatures have been increasing over the last several decades (McGuire et al. 2012, Bueno de Mesquita et al. 2018b) leading to increased positive degree days (Caine 2010) earlier lake ice-off dates (Preston et al. 2016), and earlier snowmelt (Bueno de Mesquita et al. 2018b). Over the last several decades, concurrent with this summer warming trend, there have been increases in cover by alpine plants in areas that were previously unvegetated (Bueno de Mesquita et al. 2018b) or dominated by moss (Bueno de Mesquita et al. 2017).

![Experimental design](image)

**Figure 5.1.** Experimental design. We 1) manipulated microbial community composition by collecting eight different inocula across the landscape, 2) inoculated three abundant alpine plant species in the greenhouse, 3) transplanted them beyond their range into unvegetated soils distributed across four blocks, and 4) manipulated the growing season length using black sand to speed snowmelt. At each of the four blocks there were paired control and early snowmelt plots. There were 8 inocula x 8 replicates x 3 species = 192 pots, plus 2 inocula with 8 additional replicates x 3 species = 48 pots, for a total of 240 total pots. Pots in which plants had died were not transplanted, such that a total of 210 pots and a total of 280 Deschampsia, 281 Oxyria, and 106 Silene individual plants were actually transplanted.

We combined a field survey to describe the spatial variation in soil microbial communities that a plant colonizing unvegetated areas might interact with, with an experimental manipulation of the interactive influence of climate change (early snowmelt) and microbial composition (different soil inocula). For the survey, we described soil microbial communities across 20 unvegetated soils (see Porazinska et al. 2018). For the manipulative experiment, we established four experimental blocks of unvegetated soils across an ~50 m area, each of which contained paired 1.5 x 1 m early snowmelt and control plots (Figure 5.1). Three alpine plant species inoculated with one of eight inocula with varying microbial community composition (Figure
5.2A, Figure 5.2B) were transplanted into the plots ((8 inocula x 8 replicates x 3 species = 192 pots) + (2 inocula x 8 additional replicates x 3 species = 48 pots) = 240 total pots)).

Figure 5.2. Principle Coordinates Analysis of Bray-Curtis dissimilarities in bacterial and fungal community composition at the OTU level in the original eight soil inocula used in the experiment and in plant roots in the inocula over time. Four inocula were collected from vegetated areas (V1-V4), and four from unvegetated areas (U1-U4) (Figure A5.1). Inoculum was always a significant driver of community composition (PerMANOVA, p < 0.05), but variation declined over time. Percent variation explained: A = 54.23, 16.14; B = 34.73, 17.11; C = 14.61, 10.42; D = 18.68, 12.70; E = 12.90, 8.41; F = 12.22, 10.21; G = 7.44, 6.36; H = 8.93, 7.83).

Plants

We chose three focal plant species that are abundant at the highest elevation plant communities at our site and likely colonizers of unvegetated soils as climate changes – the generalist bunchgrass *Deschampsia cespitosa* (L.) P. Beauv., the arctic/alpine cushion plant *Silene acaulis* (L.) Jacq., and the talus specialist *Oxyria digyna* (L.) Hill). We collected seed of each of these species in August and September 2015, which were stored at room temperature in paper bags until sowing in March 2016. Plump and likely viable seeds were selected from the seed
collections. We sowed five seeds into open-bottom PVC cylinders 7 cm tall x 7.62 cm diameter containing sterile soil and sand mixed with one of the eight inocula.

_Inocula_

In September 2015 we collected soils from four different unvegetated areas and four different vegetated areas to use as soil inoculum (Figure A5.1). Two quarts of soil at each collection location were collected from within a 2 m radius and thoroughly homogenized by mixing for several minutes in a two-gallon Ziploc bag. Soils were transported on ice to the lab where they were stored at -20˚C until March 2016. We also collected bulk soil from the tundra to use in the greenhouse. The bulk tundra soil was mixed with sand at a 1:1 ratio, similar to the sand content of the unvegetated soils at our site (76% sand, King et al. 2010). One gallon at a time, this mixture was sterilized by autoclaving for 1 hour at 121˚C, remixing, and autoclaving again. Post-autoclaved bulk soil total inorganic nitrogen levels were 74.07 µg g dry soil$^{-1}$, much higher than average unvegetated soils (3.68 µg g dry soil$^{-1}$) and vegetated soils (1.95 µg g dry soil$^{-1}$) near our inocula collection locations (Porazinska et al. 2018), such that minor differences in nutrient concentrations among inocula were overwhelmed. To minimize differences in other soil properties, pots received a 30:1 ratio of sterile bulk soil to inoculum. Each pot was filled with sterile soil until 2 cm from the top, a layer of inoculum was spread, and then 1 cm of bulk sterile soil added on top of the inoculum. This layering of inoculum and sterile soils minimized cross contamination among pots, as well as ensured that roots passed through a layer of inoculum. Pots were watered every day until the first true leaves formed, upon which they were watered every other day. Plants grew from March to July in the alpine room of the University of Colorado greenhouse, with natural light conditions and diurnal temperature cycles ranging from 15˚C during the day to 10˚C at night.

_Field Manipulations_
In July 2016, plants were transported to the University of Colorado Mountain Research Station (2900 m.a.s.l.) where they were exposed to ambient light and temperature conditions outside, but were kept in flats and watered every other day. The goal of this intermediate stage was to start acclimatizing the plants before final transplantation to higher elevations. In August 2016, a total of 212 pots containing 1-5 individual plants were transplanted to the field site where they were distributed among the 4 blocks. Each block was transplanted on a separate day, approximately 1-3 days after the snowbed melted at the block. Holes the size of the PVC cylinders were dug and the cylinders placed into the soil. Since the soils were saturated from snowmelt water, there was no need to water the transplants. To avoid contamination of unvegetated inocula by vegetated inocula, each plot was divided in half vertically such that all of the pots with unvegetated soil inocula were on the top half and all pots with vegetated soil inocula were on the bottom half. Within each half of the plots, plant species and inocula were randomly placed. Pots were 15 cm apart horizontally and 20 cm apart vertically. A HOBO air temperature and light sensor (Onset Corporation, Bourne, Massachusetts, USA) was placed in the center of each plot to help determine snowmelt date.

To manipulate snowmelt timing, a thin layer of inert black sand (composed of primarily silica dioxide, Mission Laboratories, California, USA) was applied to one plot in each block to speed snowmelt (Figure 5.1, Blankinship et al. 2014), and then onto control plots after snowmelt. Sand was spread in a 3 x 3 m area with the plot in the middle (such that there was a 0.75 m buffer zone upslope and downslope, and 1 m buffer zone on each side of the plot), at a rate of 500 g sand m$^{-1}$ as soon as 3 m tall plot marker poles were visible. Survival and leaf number (from which biomass was calculated allometrically) were recorded for each individual in September 2017, and plants were harvested and aboveground biomass weighed in September 2018.

*Staining and Sequencing*
Prior to transplanting, as well as at the end of summer 2017 and the end of 2018, soils and roots were collected for molecular analyses, and a subset of roots were collected for staining and microscopy. Note that for the first two harvests, we carefully extracted one plant from a pot without disturbing the other individuals in the pot, and the decrease in total individual sample size can be found in Figure A5.2. To assess the quantity of fine root endophytes, dark septate endophytes, and arbuscular mycorrhizal fungi, roots were cleared in 10% KOH for 1 hr at 90°C, reacidified in 1% HCl for 20 min, and stained overnight in acidic glycerol trypan blue (modified from Koske and Gemma 1989, Schmidt et al. 2008b). In the morning, roots were destained in acidic glycerol and stored at 4°C until microscopy. Microscopy was done according to McGonigle et al. (1990). Briefly, ~20 cm of roots were placed horizontally on slides and passes were made up and down the slide at random intervals such that 100 intersections between the root and ocular crosshair were observed. The presence or absence of a fungal structure was recorded at each intersection, and percent colonization was calculated.

We used Illumina (MiSeq) sequencing of the 16S and ITS regions of the genome to describe bacterial and fungal communities, respectively, of plant roots, pot soils, the initial inoculum, as well as 20 additional unvegetated soils that we sampled for a previous study (Porazinska et al. 2018). For soils, DNA was extracted from 0.25 g of soil using the Qiagen PowerSoil Kit (QIAGEN, Hilden, Germany) following the manufacturer’s protocols. Roots were first surface sterilized by soaking for 1 minute in 70% ethanol, then 1 minute in 10% bleach, and then triple rinsing with sterile deionized water. Then, 0.1 g wet roots were frozen in liquid nitrogen and ground into a powder with a sterile mortar and pestle. DNA was extracted from this powder using the Qiagen DNeasy Plant Kit (QIAGEN, Hilden, Germany) following the manufacturer’s protocols. Extracted DNA was amplified via Polymerase Chain Reaction, using the 515F/806R primers for 16S (Fierer et al.
2012) and ITS1F/ITS2 primers for ITS (White et al. 1990) following the methods of the Earth Microbiome Project (Amaral-Zettler et al. 2009, Bellemain et al. 2010, Caporaso et al. 2012). Amplified DNA was normalized with the SequalPrep Normalization Kit (Invitrogen Inc., Carlsbad, California, USA), and sequenced (paired-end 2 x 150 bp for 16S, 2 x 250 bp for ITS) at the BioFrontiers Next Generation Sequencing Facility (Boulder, Colorado, USA). Sequencing data was processed with the QIIME (Caporaso et al. 2010) and UPARSE pipelines to demultiplex, merge, quality filter, remove singletons and select operational taxonomic units (OTUs) at 97% similarity, and remove chimeras. OTU tables were rarefied before analysis so all samples within each sample type had the same sequencing depth (Table A5.1). Taxonomy was assigned using the GreenGenes (DeSantis et al. 2006) and UNITE (Abarenkov et al. 2010) databases for bacteria and fungi, respectively. Fungal functional guilds were assigned using the program FUNGuild (Nguyen et al. 2016). Sequencing data is accessible on GenBank via the project accession number PRJNA525120.

*Plant Performance Analyses*

Plant growth data (biomass per individual per year) were analyzed with a linear mixed effects regression (LMER) model (R package ‘lme4’, Bates et al. 2015) with inoculum, growing season length, and their interaction as fixed effects and block as a random effect. We also added number of individuals in the pot (could range from 1 to 5 based on germination and survival) as a random effect. We used growing season length as a continuous variable instead of categorical (control versus black sand additions) because there was also considerable variation in growing season across blocks (Figure A5.3). Note that the field year one biomass data for inoculum V2 includes some contaminants by *Festuca brachyphylla* that were not identified until field year two. Plant survival data were analyzed with generalized linear models with a binomial distribution (GLM, where 1 is a plant
that survived and 0 is a plant that died) with the same fixed effects but no random
effects to avoid overfitting and model errors. Growing season was calculated as the
days from snowmelt until we measured the plants. When a significant inoculum
effect was found, we conducted pairwise comparisons using the emmeans function
from the R package ‘emmeans’ (Lenth 2018). Relationships between fungal root
colonization and pathogen abundance and plant biomass were tested with linear
regressions.

**Microbial Analyses**

Microbial communities were visualized with Principle Coordinates Analysis
and differences between treatments were assessed with permutational multivariate
analysis of variance (PerMANOVA, Anderson 2001, function adonis, R package
‘vegan’, Oksanen et al. 2013) on Bray-Curtis dissimilarity matrices calculated from
Hellinger-transformed relative abundances. Pathogen abundances and root
colonization by potential mutualists were assessed with the same linear mixed
effects regressions as plant growth. To analyze relationships between particular
taxa on plant growth, we conducted linear regressions between relative abundances
and plant growth for dominant (present in 50% of samples, and top 10% in
abundance, Delgado-Baquerizo et al. 2018) soil and root taxa. To further analyze
drivers of unvegetated microbial community composition, we sampled microbes
from 20 additional unvegetated soils (Figure A5.1) and used the envfit function in
‘vegan’ to assess relationships with total dissolved inorganic nitrogen, total
dissolved inorganic phosphorus, dissolved organic carbon, pH, and soil moisture,
which were measured as described in Porazinska et al. (2018). We partitioned
variance of the Bray-Curtis dissimilarity matrix by spatial distance and
environmental variables using the varpart function in ‘vegan’. To test correlations
between space and community composition, we used Mantel tests (package ‘vegan’,
function mantel). R analyses were performed in R version 3.4.4 (R Core Team 2018).
Results

*Deschampsia* had the highest survival in both 2017 and 2018, while both *Oxyria* and *Silene* experienced high mortality in 2017 and additional mortality in 2018 (Figure A5.2). 90% of *Oxyria* individuals were dug up and likely eaten by the American pika (*Ochotona princeps*), which has been observed near the transplant site. Due to the low remaining sample sizes of *Oxyria* and *Silene*, we focused all other analyses on *Deschampsia*.

Snowmelt timing was 2-3 days earlier in the black sand plots in 2017, and 2-10 days earlier in 2018 (Figure A5.3). The entire snowbed melted out earlier and earlier each year from 2015 to 2018 (Figure A5.3). There was a significant positive effect of growing season length on *Deschampsia* survival in year one (GLM, $\beta = 0.59$, $p = 0.001$, Figure 5.3A). In contrast, in year two, there was significantly greater mortality with extended growing season (GLM, $\beta = -0.01$, $p = 0.002$, Figure 5.3B). There was no significant main effect of growing season length on growth in year one (LMER, $\chi^2 = 0.32$, $p = 0.57$, Figure 5.4A) or year two (LMER, $\chi^2 = 2.35$, $p = 0.12$, Figure 5.4B).

![Figure 5.3. Effects of growing season length and soil inocula on Deschampsia cespitosa survival in (A) field year one and (B) field year two. Statistics are from logistic regression models (* < 0.05, ** < 0.01, *** < 0.001, n.s. = not significant). GS = growing season, In = inoculum. The right panels show the main effect of inoculum, as mean (±SE) predicted probabilities across the growing season lengths. Lines represent linear regressions and are depicted for each inoculum (even if the fit is not significant) to demonstrate the significant interactions in each panel. Growing season varied among the four blocks and with the control and black sand (for earlier snowmelt) plots at each block, such that there were six different growing season lengths in year one (two pairs of plots had the same growing season length), and eight different growing season lengths in year two. Points are slightly jittered for visualization.](image-url)
Soil inoculum had a significant effect on *Deschampsia* field survival in both years (GLM, p < 0.05, Figure 5.3A, Figure 5.3B). There were significant differences in survival both between inoculum types as well as within inoculum type (Figure 5.3A, Figure 5.3B). Soil inoculum also significantly affected *Deschampsia* growth in the greenhouse (prior to transplanting) and in both years in the field (Figure 5.4A, Figure 5.4B, LMER, p < 0.05), although the effects changed over time (i.e. inocula with plants with the highest growth).

Growing season and inoculum interacted to affect survival in both field year one and field year two (GLM, p < 0.01, Figure 5.3). There was greater variability in survival among the inocula in plots with the shortest and longest growing seasons (Figure 5.3). In year one, inoculum and growing season interacted to affect growth (LMER, $\chi^2 = 18.67$, p = 0.009, Figure 5.4A), with growing season having either positive, negative, or neutral effects on growth depending on inoculum. There was no significant interactive effect of inoculum and growing season on growth in year two (LMER, $\chi^2 = 8.54$, p = 0.28, Figure 5.4B).

![Figure 5.4](image-url)
Unvegetated soil microbial communities varied significantly across space, and plant-associated taxa showed patchy distributions (Figure A5.4). We captured some of this variability in our inocula (Figure 5.2A, Figure 5.2B). Both fungal and bacterial community composition in roots varied by inoculum after growing in the greenhouse, after one year in the field, and after two years in the field (PerMANOVA, \( p = 0.001 \), Figure 5.2C-H), though differences declined over time. Root colonization by arbuscular mycorrhizal fungi and fine root endophytes each varied significantly by inoculum (LMER, \( \chi^2 = 18.69, p = 0.009 \); \( \chi^2 = 15.56, p = 0.03 \), respectively), while dark septate endophytes did not (LMER, \( \chi^2 = 3.26, p = 0.86 \)). However, only dark septate endophyte colonization was significantly positively correlated with plant growth, and the relationship was weak (Linear regression, \( R^2 = 0.08, p = 0.02 \), Figure A5.5). Fungal pathogen abundance did not significantly differ among inocula (LMER, \( \chi^2 = 11.93, p = 0.10 \)), but tended to be higher in the inocula with the lowest plant growth (Figure A5.6). However, for the subset of plants that were sequenced, there was no relationship between total fungal pathogen abundance and plant growth (Linear regression, \( R^2 = 0.01, p = 0.22 \)), or each individual pathogen’s abundance and plant growth (Linear regression, \( p > 0.05 \)). Five of 67 dominant root bacterial taxa and 24 of 201 dominant soil bacterial taxa were significantly correlated with plant growth (Linear regression, \( p < 0.05 \), Table A5.2). None of the 12 dominant root fungal taxa were significantly correlated with plant growth (Linear regression, \( p > 0.05 \)), while four of 77 dominant soil fungal taxa were significantly correlated with plant growth (Linear regression, \( p < 0.05 \), Table A5.2).

**Discussion**

Our results notably demonstrate that both snowmelt timing and soil microbial communities influence plant survival and growth in areas beyond their
current range. While it was not a focus of our study, we also learned that herbivory can be an important limiting factor for some plant species, which has been the focus of other work (Hille Ris Lambers et al. 2013). Two of the three plants in the study experienced high levels of mortality, suggesting that not all alpine plants will be able to expand into new habitats that are currently unvegetated.

Interestingly, the high mortality in *Oxyria* and *Silene* was driven by different factors. *Oxyria* experienced high herbivory by pika, which suggests that herbivory can be a limiting factor for some plants expanding their ranges. This contradicts other work that has found that range-expanding plants can be released from herbivory pressures, thus facilitating plant establishment (Engelkes et al. 2008). Herbivory has been previously discussed as a factor limiting woody plant expansion into tundra (Cairns and Moen 2004), but further study is needed on tundra expansion into unvegetated areas. It is unknown why so many *Silene* died, as our seedling survival rates are much lower than in a natural Alaskan population (Morris and Doak 1998). One likely explanation is the short growing season at the transplant site, which is well below the optimum growing season length for *Silene* found at other sites (Doak and Morris 2010).

By combining survival data from 2017 and 2018, which had different growing season lengths, we identified an optimum growing season length at this site of 45 – 55 days for *Deschampsia*. As expected, too short of a growing season was detrimental for plant survival. However, the negative effect of extended growing season seen in year two of the experiment contradicts our hypothesis, but is likely due to moisture limitation. Much of the growing season soil moisture in the alpine is supplied by snowpack, and earlier snowmelt can lead to earlier drying out of the soil. Other work in dry and moist meadows on Niwot Ridge has shown that soil moisture is limiting for alpine tundra plants (Fisk et al. 1998, Knowles et al. 2015) and increased moisture limitation later in the growing season during long summers.
can decrease productivity of alpine plants (Berdanier and Klein 2011, Fan et al.
2016) and subalpine forests both at our site (Hu et al. 2010) and elsewhere
(Buermann et al. 2013).

Unvegetated soil microbial communities were patchily distributed across
space (Figure A5.4). However, more work is needed on community assembly in
these ecosystems, as spatial and environmental variables did not explain
substantial amounts of variation in both bacterial and fungal communities. It is
possible that wind deposition and stochasticity play important roles in driving
community composition (Jumpponen 2003), though spatial distance is also
important. In subnival environments, patches of soil habitat are usually surrounded
by an inhabitable matrix of boulders and talus rocks (Figure 5.1). In a recent paper
applying island biogeography theory to microbial community assembly on glaciers,
researchers found that island (cryoconite hole) size was positively correlated with
diversity and that community similarity decayed with distance (Darcy et al. 2018a).
While we did not measure unvegetated soil patch size, we also found spatial
autocorrelation of bacterial and fungal communities in unvegetated soils, similar to
the results of King et al. (2010), who analyzed both unvegetated and vegetated
communities at our site.

The hypothesis that the variability in microbial communities would lead to
differences in plant performance was supported by both the survival and growth
data. While we cannot rule out the possibility that minor differences in other soil
parameters led to some differences in plant growth, these were likely overwhelmed
by homogeneous bulk soil that made up the majority of the pot soil. The differences
in survival among the four unvegetated inocula is an important result that suggests
current microbial distributions can mediate future plant distributions as well as the
rate of distributional shifts. Growth in unvegetated inocula was equal to or
exceeded that in vegetated inocula, which suggests that unvegetated soils can
contain either enough beneficial plant-associated microbes, or fewer pathogens that are more abundant in vegetated soils, thus facilitating plant growth. These results contradict those found for trees and shrubs, whose range expansion can be limited by negative plant-soil feedbacks or a lack of mutualistic fungi in beyond-range soils, (Nuñez et al. 2009, Sedlacek et al. 2014, Van Nuland et al. 2017). Our results are more in line with studies that have found plants escaping belowground enemies in beyond-range soils (Van Grunsven et al. 2007, Engelkes et al. 2008, McCarthy-Neumann and Ibáñez 2012).

The strong interactions between growing season length and microbial communities is a striking result, especially for survival. The interaction suggests that in suitable growing seasons, microbial community composition is not as important for plant survival and growth, but if the growing season is too short or too long, there can be significant differences in plant performance based on microbial community composition. We hypothesized that the magnitude of the effects of microbial communities would be strongest with longer growing seasons, but we also found they are important in shorter growing seasons. More work is needed to conclude whether this result is due to different microbial communities enabling plants to grow rapidly in short growing seasons, or helping them survive moisture limitation late in long growing seasons (Kim et al. 2012). These interactive effects could be particularly important for plants shifting their ranges and for coping with interannual variability in climate. The strong effects of microbes on plant survival in the long growing season of year two is especially relevant as climate continues to warm and growing seasons are predicted to lengthen in the future (Settele et al. 2014).

It remains difficult to identify which microbial taxa are affecting plant performance. Differences in plant survival and growth could be driven by either mutualistic/beneficial microbes, or pathogenic/detrimental microbes (van der Putten
Our data suggest that the dark septate endophyte fungal group could be important, as greater colonization levels by this fungal group were weakly but positively correlated with plant growth. This is particularly interesting because these fungi are more frequently found in unvegetated soil than the other two root endophytic fungal groups. While a metanalysis of dark septate endophyte effects on plant growth demonstrated typically beneficial effects (Newsham 2011), their function is still open to debate (Mandyam and Jumpponen 2015), as some studies have shown that they can negatively impact plant growth (Alberton et al. 2010, Mayerhofer et al. 2013), and have not benefited as wide a range of plants as arbuscular mycorrhizal fungi. However, DSE may play a relatively more important role in alpine ecosystems, where they are found in most plant species (Bueno de Mesquita et al. 2018a). Other important fungal genera identified in the sequencing data include the pathogen *Venturia*, and the saprotrophs *Fontanospora, Pseudogymnoascus*, and *Knufia* (Rikkerink et al. 2011, Crous et al. 2013, Tedersoo et al. 2014, Nguyen et al. 2016). Some of the important bacterial genera include the nitrifier *Nitrospira*, the denitrifier *Rhodanobacter*, the phosphorus-solubilizing *Agrobacterium*, and the anti-microfungus *Pseudonocardia* (Hameed et al. 2005, Kostka et al. 2012, Poulsen et al. 2012, Daims et al. 2015).

Our manipulative experiment provides the first evidence of the role of unvegetated soil microbes facilitating plant colonization as climate changes. We also provide evidence of potential detrimental effects of extended summers on alpine plants. One interesting avenue for future research is to investigate the role of seed-borne microbes in facilitating plant establishment in new ranges. Additional work using cultures and simplified microbial communities can further elucidate which microbial taxa are important for plants. Understanding plant-microbe interactions, and in general, biotic interactions, remains an exciting and crucial area of research for understanding geographic responses to climate change.
CHAPTER VI
SPECIES-SPECIFIC PLANT-MICROBE FEEDBACKS IN A HIGH ELEVATION EARLY SUCCESSIONAL ECOSYSTEM


Abstract

Plant-microbe interactions and feedbacks are crucial components of ecosystem development but are understudied during succession. Here we used a combination of lab and field litter additions in an early successional unvegetated ecosystem in the Front Range of the Colorado Rocky Mountains to examine litter-driven changes in soil bacterial and fungal communities. We then used the plant-trained soil as inocula in a greenhouse experiment to test plant-soil feedbacks. We found species-specific effects of litter additions on bacterial and fungal communities in unvegetated soils, which are likely due to both differences in tissue litter chemistry and differences in the litter microbiome. We also identified a negative feedback between two of the plants, which was likely due to changes in microbial communities that resulted in lowered nitrification rates. Our study demonstrates the importance of plant specificity and potential negative litter-driven feedbacks in primary succession, which could lead to patchy distribution of plant colonists as climate change allows colonization of these areas.
**Introduction**

Plant–microbe interactions are important drivers of broad vegetation patterns in ecosystems, affecting plant species composition, abundance, and diversity patterns (van der Heijden et al. 2008). Further, plant–soil feedbacks via the microbial community, whereby plants affect microbial communities and microbial communities feedback and affect plants, also have broad implications for plant community assembly (Reynolds et al. 2003, Bever et al. 2010, van der Putten et al. 2013). However, plant–soil feedbacks and plant–microbe interactions have been understudied in early successional environments, particularly those with non-leguminous plants (but see van der Putten et al. 1988, 1993), a context in which they could exert even more influence on plant community composition.

Furthermore, while it is well known that plant species can have differential effects on soil microbial communities (Grayston et al. 1998, Kowalchuk et al. 2002, Berg and Smalla 2009, Knelman et al. 2012, Leff et al. 2018), it is unclear in carbon-limited environments such as unvegetated soils whether the carbon released through decomposition could overwhelm any plant species-specific effects (Tscherko et al. 2005). The goal of the present study was to determine how the litter of different colonizer plants affects soil microbial communities, and how this feeds back to affect plant growth.

Ecosystem development begins with the rapid colonization of exposed substrates by microbial communities, which catalyzes the early stages of soil development and nutrient cycling prior to plant arrival (Nemerghut et al. 2007, Schmidt et al. 2008a, Sattin et al. 2010). Studies of microbial communities in early successional environments have shown that they can be limited by carbon (King et al. 2008, Bueno de Mesquita et al. 2017), though nitrogen and phosphorus may also be limiting or co-limiting (King et al. 2008, Knelman et al. 2014, Schmidt et al. 2016, Darcy et al. 2018b). Furthermore, microbes in unvegetated soils are more
carbon limited than microbes in nearby vegetated soils (Porazinska et al. 2018). As plants arrive, they directly interact with soil microbial communities via litter inputs and root exudates (Bardgett et al. 2005), as well as by potentially carrying new microbes with them on their seeds (Schiltz et al. 2015). This can lead to shifts in microbial community composition compared to unvegetated soils, with decreases in phototrophs and nitrogen-fixers and increases in overall biomass, plant-associated taxa, heterotrophs, r-selected bacteria, and fungal to bacteria ratios (Ohtonen et al. 1999, Edwards et al. 2006, Miniaci et al. 2007, Knelman et al. 2012, 2018). While previous studies on the effects of multiple colonizer plants have found species-specific effects on soil bacterial communities (Knelman et al. 2012, 2018), we still need a more mechanistic understanding of bacterial and fungal community responses to plants with different litter traits and how that feeds back on plant growth.

Plant-soil feedbacks in primary succession have been reported as being positive (plants alter microbes in ways that benefit themselves or other plants), whereas in other contexts they are typically reported as negative (Reynolds et al. 2003, van der Putten et al. 2013). This could be due to a bias of a focus on interactions with nitrogen-fixing bacteria in primary succession (Chapin et al. 1994, Titus and Del Moral 1998, Cortí et al. 2002), whereby early colonizer plants associate with nitrogen-fixers and alleviate nutrient limitation for plants arriving later. Yet plants that associate with nitrogen fixers are not always the first colonizers (Cázares et al. 2005), and the nature of plant-soil feedbacks in primary succession without nitrogen-fixers remains unclear. Work in a successional sand dune ecosystem demonstrated positive overall effects of the microbes trained by early successional plant species on later successional plant species, but not due to direct positive effects. Species-specific pathogens increased in the early colonizer plant causing a negative intraspecific feedback, while having neutral effects on

Indeed, pathogen buildup can be a key mechanism for negative intraspecific plant-soil feedbacks, and if the pathogens are host-specific, this may not necessarily translate into a negative interspecific feedback (Reynolds et al. 2003). On the other hand, positive intraspecific and interspecific feedbacks can occur when there is a buildup of mutualists, including mycorrhizal fungi in addition to nitrogen-fixing bacteria, but the nature of these feedbacks is also dependent on how host-specific the mutualisms are (Bever 2002, Reynolds et al. 2003). Another important mechanism of plant-soil feedbacks through the microbial community is mediated through microbes responsible for nutrient cycling that are not necessarily symbionts with plants (van der Putten et al. 2016). For example, feedbacks can be driven by plant litter from conservative plant species that slows down nutrient cycling that would otherwise benefit faster growing plant species (Suding et al. 2008).

Here we used litter additions and inocula collections to analyze the effects of plant litter on unvegetated soil microbial communities, and how this feeds back to affect plant growth. We hypothesized that due to differences in litter chemistry and the leaf microbiome, plant litter would have species-specific effects on soil microbial communities. We hypothesized that changes in the microbial communities would then feedback on plant growth, with negative intraspecific feedbacks due to pathogen buildup, and positive interspecific feedbacks due to increases in mutualists and increases in nitrogen cycling rates.

**Methods**

*Study System*

We collected plant seeds, leaf litter, and soil from a high elevation talus-field subnival ecosystem (3900 m.a.s.l.) on the southeast facing slope of Navajo Peak, in
the Green Lakes Valley, part of the Niwot Ridge Long Term Ecological Research Site in the Front Range of the Rocky Mountains, Colorado, USA. Average precipitation from 1952 to 2012 was 1090 ± 230 mm yr\(^{-1}\), with a 60 mm yr\(^{-1}\) increase over that time period, driven mostly by increases in winter precipitation (Kittel et al. 2015). Recent mean annual temperatures (2011 – 2014) range from -4°C to -7°C, while mean daily summer temperatures range from 4°C to 10°C (Losleben 2017). Both annual and summer temperatures have been increasing over the last several decades (McGuire et al. 2012, Bueno de Mesquita et al. 2018b) leading to increased positive degree days (Caine 2010) earlier lake ice-off dates (Preston et al. 2016), and earlier snowmelt (Bueno de Mesquita et al. 2018b). Over the last several decades, concurrent with this summer warming trend, there have been increases in cover by alpine plants in areas that were previously unvegetated (Bueno de Mesquita et al. 2018b) or dominated by moss (Bueno de Mesquita et al. 2017). We chose three focal plant species that are abundant at the highest elevation plant communities at our site and likely colonizers of unvegetated soils as climate changes – the generalist bunchgrass *Deschampsia cespitosa* ((L.) P. Beauv.), the arctic/alpine cushion plant *Silene acaulis* ((L.) Jacq.), and the talus specialist *Oxyria digyna* ((L.) Hill). Foliar C:N ratios of these species were measured in summer 2017.

**Litter Decomposition and Effects on Microbes**

We assessed litter decomposition rates in the lab and the field and the effects of litter additions on unvegetated soil bacterial and fungal communities. In the lab, 100 ml (~145 g) unvegetated soil was placed into 1 L air tight mason jars and watered to 90% water holding capacity to simulate conditions during decomposition following snowmelt. Two quarts of unvegetated soil were collected from the field site in the fall of 2016, and stored at 4°C for 1 week until being placed in the jars. The starting soil was homogenized in a two-gallon Ziploc bag and subsampled five times for DNA sequencing to determine the initial microbial community composition.
Then 1 g of each species’ litter was added onto the soil surface. There were five replicate jars for each species litter and five control jars with no litter added. Jars were incubated for five weeks at 10°C. Jars lids were fitted with rubber stoppers with two valves. Twice a week, CO₂ measurements were taken with an EGM-4 CO₂ analyzer (PP Systems, Amesbury, MA) by attaching the analyzer to the valves, opening them, and measuring for approximately 30 s. Once a week, after a second weekly CO₂ measurement was taken, the headspace in the jars was cleared to prevent CO₂ buildup and re-equilibrate with the atmosphere. At the end of five weeks, soils were sampled from each jar and stored at -20°C until DNA extraction.

In the field, 1 g of litter of each species was placed in mesh litter bags, with five replicate bags per species and five control bags with no litter. Bags were placed on top of unvegetated soils on October 17, 2017 and staked to the ground with small plastic stakes. Bags were then collected on September 6, 2018 and biomass was dried and weighed. Soils were collected from immediately underneath each bag, placed in sterile bags, transported to the lab on ice, subsampled for DNA extraction (subsample stored at -20°C), and then used as inoculum in a greenhouse experiment (see below).

**Plant-Soil Feedback Experiment**

We added the live inoculum collected from under the litter bags to a 50:50 mixture of sterile bulk tundra and sand at a ratio of 1:30 inoculum to sterile bulk soil. Ammonium and nitrate levels in the four inocula were not significantly different and were overwhelmed by the nutrient pools in the bulk soil, which were on average 29 times higher than the inocula. Furthermore, as inocula were collected from a 2 x 2 m area, differences in other soil properties should be minimal. Bulk soil was sterilized one gallon at a time by autoclaving for 1 hour at 121°C, remixing the soil, and autoclaving again. We grew each of the three species in 10 replicate pots in four different treatments: control, inocula trained with their own litter, and inocula
trained with the litter of the other two species (3 spp. x 4 treatments x 10 replicates = 120 pots). Pots were 8.255 cm wide x 8.255 cm long x 9.525 cm tall. Pots were filled with the sterile bulk soil until ~2 cm from the top, then a layer of inoculum was spread, and then 1 cm of sterile bulk soil was added on top of the inoculum. This minimized cross contamination and ensured that roots passed through a layer of inoculum. Pots were watered every day or every other day depending on solar radiation. Plants grew from September to December in the alpine room of the University of Colorado greenhouse, with natural light conditions and diurnal temperature cycles ranging from 15˚C during the day to 10˚C at night.

At the end of the experiment, aboveground biomass was clipped, dried for 48 h at 60˚C, and weighed, and roots were collected for staining and microscopy. To assess the quantity of fine root endophytes, dark septate endophytes, and arbuscular mycorrhizal fungi, roots were cleared in 10% KOH for 1 hr at 90˚C, reacidified in 1% HCl for 20 min, and stained overnight in acidic glycerol trypan blue (modified from Koske and Gemma 1989, Schmidt et al. 2008b). In the morning, roots were destained in acidic glycerol and stored at 4˚C until microscopy. Microscopy was done according to McGonigle et al. (1990). Briefly, ~20 cm of roots were placed horizontally on slides and passes were made up and down the slide at random intervals such that 100 intersections between the root and ocular crosshair were observed. The presence or absence of a fungal structure was recorded at each intersection, and percent colonization was calculated.

Lastly, we assessed net nitrogen mineralization and nitrification rates in the soils after the greenhouse experiment. After harvesting the plants, the soils in the 40 pots with Deschampsia growing in them were placed in quart sized Ziploc bags and allowed to incubate in the greenhouse for 25 days. We focused on Deschampsia because of its high sample size and to investigate mechanisms of growth differences seen in the greenhouse harvest. 5 g of soil was sampled at the start and end of the
25 days, and inorganic nitrogen pools were extracted by shaking in 25 ml of 2 M KCl for 1 h at 125 rpm. Extracts were analyzed on an OI Analytical Flow Solution 3000 Flow Injection Analyzer at the Colorado State University Soil, Water, and Plant Testing Laboratory.

**Microbial Analyses**

We sequenced the 16S and ITS regions of the genome on an Illumina MiSeq to describe bacterial and fungal communities, respectively, of initial plant litter, initial unvegetated soils, soils after the lab incubations, and soils after the field litterbag additions. DNA was extracted from 0.25 g of soil using the Qiagen PowerSoil Kit (QIAGEN, Hilden, Germany) and from 0.3 g of dry plant litter using the Qiagen DNeasy Kit following the manufacturer’s protocols. Litter was first frozen in liquid nitrogen and ground into a powder with a sterile mortar and pestle. Extracted DNA was amplified via Polymerase Chain Reaction (PCR), using the 515F/806R primers for 16S (Fierer et al. 2012) and ITS1F/ITS2 primers for ITS (White et al. 1990) following the methods of the Earth Microbiome Project (Amaral-Zettler et al. 2009, Bellemain et al. 2010, Caporaso et al. 2012). The amplified DNA was then normalized with the SequalPrep Normalization Kit (Invitrogen Inc., Carlsbad, California, USA), and sequenced (paired-end 2 x 150 bp for 16S, 2 x 250 bp for ITS) at the BioFrontiers Next Generation Sequencing Facility (Boulder, Colorado, USA). Sequencing data were processed with the QIIME (Caporaso et al. 2010) and UPARSE (Edgar 2013) pipelines to demultiplex, merge, quality filter, remove singletons and select operational taxonomic units (OTUs) at 97% similarity, and remove chimeras. OTU tables (except litter 16S) were rarefied before analysis so all samples within each sample type had the same sequencing depth (Table A6.1). Taxonomy was assigned using the GreenGenes (DeSantis et al. 2006) and UNITE (Abarenkov et al. 2010) databases for bacteria and fungi, respectively.
Fungal functional guilds were assigned using the program FUNguild (Nguyen et al. 2016).

Statistical Analyses

Cumulative carbon dioxide production in the lab was analyzed with a repeated measures ANOVA model (function gls, R package ‘nlme’, Pinheiro et al. 2014) followed by a Tukey posthoc test (function glht, R package ‘multcomp’, Hothorn et al. 2008). Microbial communities were visualized with Principle Coordinates Analysis and differences between treatments were assessed with permutational multivariate analysis of variance (PerMANOVA, Anderson 2001, function adonis, R package ‘vegan’, Oksanen et al. 2013) on Bray-Curtis dissimilarity matrices calculated from Hellinger-transformed relative abundances. Following a significant result, we conducted pairwise PerMANOVAs (function pairwise.perm.manova, R package ‘RVAideMemoire’, Hervé 2019). All other continuous variables among the four treatments (foliar C:N, litter mass loss, plant biomass, fungal root colonization, pathogen abundance, mineralization, nitrification) were analyzed with ANOVA followed by Tukey posthoc (functions aov and TukeyHSD, R package ‘stats’, R Core Team 2018). In the case where the assumptions of ANOVA were not met, we used the nonparametric Kruskal-Wallis test (function kruskal.test) followed by Nemenyi posthoc tests (function posthoc.kruskal.nemenyi.test, R package ‘PMCMR’, Pohlert 2014). Binary response variables (germination and survival) were analyzed with logistic regression models (R function glm with family = “binomial”). All analyses were performed with the software R, version 3.4.4 (R Core Team 2018).

Results

Plant Litter
There were significant \((p = 0.058)\) differences in foliar C:N ratios among all three plant species (Nemenyi posthoc, \(p < 0.05\), Figure 6.1A). *Oxyria* had the lowest foliar C:N ratio followed by *Silene* and then *Deschampsia* (Figure 6.1A). The three species’ litter also had significantly different decomposition rates, as evidenced by both CO\(_2\) production in the lab (ANOVA, \(\chi^2 = 256.36, p < 0.001\)) and biomass loss in the field (ANOVA, \(F = 9.94, p = 0.002\)). Differences in CO\(_2\) production by microbial decomposers were driven by significantly higher production in soils with litter compared to unvegetated soils, with *Oxyria* litter having the fastest decomposition rate. Differences in mass loss in the field were driven by significantly higher decomposition of *Oxyria* and *Silene* litter compared to *Deschampsia* litter (Tukey posthoc, \(p < 0.05\), Figure 6.1C). There were also significant differences in the bacterial (PerMANOVA, \(F = 6.03, p = 0.001\), Figure 6.2A) and fungal (PerMANOVA, \(F = 10.986, p = 0.001\), Figure 6.2B) litter microbiomes among the three plant species, with all pairwise comparisons significantly different (Pairwise PerMANOVA, \(p < 0.05\), Table A6.2). In the lab, some of the fungi from the litter contributed to differences in litter-trained soil compared to control soil, but this was not the case in the field, nor did any litter-derived bacteria contribute the most to soil dissimilarities (Table A6.3).

Figure 6.1. Litter chemistry and decomposition. A) Mean (± SE) foliar C:N ratios for the three species, B) mean (± SE) decomposition rates of the three species’ litter as measured by microbial respiration in a lab incubation and C) mean (± SE) decomposition of the three species’ litter as measured by mass loss after one year in the field. Lines in panel B were fit with polynomial functions. As CO\(_2\) was measured weekly and headspace cleared each week, we calculated the rate per day for each of the five weeks of the incubation. Different letters in each panel represent significant pairwise differences (Tukey or Nemenyi posthoc, \(p < 0.05\)).
Microbial Communities

After 35 days incubation in the lab, plant litter had significant effects on soil bacterial (PerMANOVA, $F = 2.42$, $p = 0.001$, Figure 6.3A) and fungal (PerMANOVA, $F = 3.07$, $p = 0.001$, Figure 6.3B) communities, with all pairwise comparisons among the different plant litters significant (Pairwise PerMANOVA, $p < 0.05$).

After 11 months in the field, there was no significant effect of the litterbag additions on unvegetated soil bacterial communities (PerMANOVA, $F = 0.92$, $p = 0.54$, Figure 6.3C), but there was a significant effect on fungal communities (PerMANOVA, $F = 1.62$, $p = 0.001$, Figure 6.3D) and all pairwise comparisons were significant (Pairwise PerMANOVA, $p < 0.05$) except for *Silene*-trained soils and controls (Table A6.3). There were no significant differences in fungal pathogen relative abundance or richness among the four treatments used as inocula (Kruskal-Wallis, $\chi^2 = 5.15$, $p = 0.16$, Figure 6.4A) and pathogens only made up 1% of the fungal community on average. Fungal pathogen richness was not significantly different among the treatments (ANOVA, $F = 2.117$, $p = 0.114$), though litter-trained soils typically contained 2-3 more pathogenic taxa on average. There was
one pathogenic fungal taxon, *Taphrina tormentillae*, which has been reported as a pathogen in other alpine plants (Petrýdesová et al. 2016), that was found in all five *Silene* trained inocula and in no other inocula. Arbuscular mycorrhizal fungi were not detected in the sequencing data, while dark septate endophytes were detected in low abundance (0.3% on average), but did not differ by treatment (Kruskal-Wallis, $\chi^2 = 1.71$ $p > 0.05$). For some of the major bacterial groups, including phototrophs, N-fixers, and plant-associated taxa, there were no significant differences in relative abundances among the treatments (ANOVA, $p > 0.05$), except for Betaproteobacteria, which were significantly lower in *Silene*-trained soil than in *Deschampsia*-trained soils, and Bacteroidetes, which were significantly lower in *Silene*-trained soil than in *Deschampsia* and *Oxyria*-trained soils (ANOVA, $p < 0.05$, Figure A6.1). Actinobacteria and Nitrospirae abundances tended to be higher in litter-trained soils than unvegetated control soils (Figure A6.1).

![Figure 6.3. Principle coordinates analysis of Bray-Curtis dissimilarities for A) soil bacterial communities after the lab litter incubations B) soil fungal communities after the lab litter incubations C) soil bacterial communities from under litter bags after the field experiment, and D) soil fungal communities from under litter bags after the field experiment. Analyses were done separately for each panel. Variation explained by the axes are A) 18.48% and 7.15%, B) 17.08% and 10.67%, C) 31.56% and 10.95%, and D) 11.66% and 11.19%.](image)
Root colonization of plants grown in the greenhouse by arbuscular mycorrhizal fungi, dark septate endophytes, and fine root endophytes was low and there were no significant differences in root colonization among the four treatments (ANOVA or Kruskal-Wallis, $p > 0.05$, Figure 6.4B). Nitrogen mineralization rates did not significantly differ among the four treatments (Kruskal-Wallis, $\chi^2 = 1.15$, $p = 0.76$). On the other hand, there was a marginally significant treatment effect on nitrification rates (Kruskal-Wallis, $\chi^2 = 7.44$, $p = 0.06$), driven by marginally significant differences between the Silene trained soil and both Deschampsia trained soil and controls (Nemenyi posthoc, $p < 0.1$, Figure 6.5).

Figure 6.4. A) Number of sequences (out of 16227) classified as plant pathogens by FUNGuild and B) Combined percent root colonization of Deschampsia cespitosa roots by arbuscular mycorrhizal fungi, fine root endophytes, and dark septate endophytes after growth in the greenhouse.

Figure 6.5. Mean ($\pm$SE) net nitrification rates during a 25-day greenhouse incubation of soils from all pots with Deschampsia plants ($n = 40$). Different letters represent marginally significant pairwise differences (Nemenyi posthoc, $p < 0.1$). Negative values represent net consumption of nitrate.
Plant Performance

In the greenhouse, *Deschampsia* had the highest germination rates, while both *Oxyria* and *Silene* had low germination rates. There were no significant differences in germination or survival among the inoculum treatments for any species (Logistic regression Tukey posthoc p > 0.05, Figures A6.2 and A6.3). There was a significant negative effect of inoculum on *Deschampsia* biomass (Kruskal-Wallis, $\chi^2 = 8.81$, p = 0.03, Figure 6.6A), with significantly lower biomass in *Silene*-trained soil compared to *Oxyria*-trained soil, and marginally significantly lower biomass in *Silene*-trained soil compared to control soils. There were no significant differences in *Oxyria* or *Silene* biomass among the four treatments (Figure 6.6B, Figure 6.6C), though it is important to note that replication was decreased due to low germination, and individuals that did germinate experienced low growth rates.

![Graph showing biomass growth rates for different treatments.]

**Discussion**

Our results demonstrate the species-specific effects of plant litter on unvegetated soil microbial communities, particularly fungi, and how this can lead to negative interspecific plant-soil feedbacks, potentially through microorganisms.
responsible for nitrification, or some as yet unknown mechanism. We do not find strong evidence for other feedback mechanisms that are driven by pathogens or mutualists.

The lab and field litter decomposition experiments demonstrate predictable decomposition rates and microbial responses based on plant traits. *Oxyria* had the lowest C:N ratio and the highest decomposition rates compared to *Deschampsia* and *Silene*, which is consistent with the paradigm that tissue with lower C:N ratios decomposes faster (Enríquez et al. 1993). *Silene* had higher decomposition than *Deschampsia* in the field but not the lab, which could be an artifact of the lab conditions enabling relatively faster *Deschampsia* decomposition. Not only do these differences in litter chemistry drive differences in decomposition, but they also select for certain microbes in the original soils leading to changes in the microbial communities of the litter-trained soil (Leff et al. 2018). Additionally, under controlled conditions, differences in litter fungal composition can further contribute to differences in microbial community composition in litter-trained soil, but this effect is diminished in the field. This stronger influence of litter fungi may also partially explain why fungal communities shifted more than bacterial communities, and sequencing depth was much greater for ITS (mean = 110781) than 16S (mean = 8713) in the litter samples.

We tested mechanistic hypotheses about the roles of pathogens, mutualists, and saprotrophs in driving plant-soil feedbacks (van der Putten et al. 2013). Our results suggest that in this legume-free system, the roles of pathogens and mutualists may be relatively less important than saprotrophs and free-living taxa that are driving nutrient cycling rates. Furthermore, poorly-developed, oligotrophic, unvegetated soils are not amenable for pathogenic or mutualistic taxa that rely on plants, and are dominated by phototrophs that fix their own carbon or heterotrophs that survive off of small sources of aeolian-deposited carbon (Freeman et al. 2009b,
2009a, Mladenov et al. 2012). Indeed, in the unvegetated soils studied here, fungal pathogens and mutualists represented a small percentage of the microbial community (~1%), as did known plant-associated bacteria. This contrasts with work done on plant-soil feedbacks during secondary succession, which have shown that soil-borne pathogens play an important role even early in secondary succession (Kardol et al. 2006, van der Putten et al. 2013). Later in succession, mutualists can also play a more important role as they increase in biomass (Miller 1979, Janos 1980, van der Putten et al. 2013).

With the low abundance of pathogens and mutualists in primary succession, it follows that saprotrophs and other free-living microbes could play a more important role, but results contradicted our hypothesis that litter additions would drive positive plant-soil feedbacks via nutrient cycling. Generally, in unvegetated or other carbon-limited soils, the addition of plant litter should select for increases in saprotrophs performing nutrient cycling, which would then have a positive feedback on plant growth. However, *Silene acaulis* is a slow-growing and long-lived plant, which would benefit from slower nutrient cycling rates. Indeed, previous work on another more conservative forb (*Geum rossii*), compared to the fast growing bunchgrass *Deschampsia*, found slower rates of nitrogen cycling in soils associated with the conservative species (Suding et al. 2008). Our results show that *Silene* litter can slow nitrogen cycling rates, or perhaps increase nitrogen immobilization since we measured net rates rather than gross rates (Fisk et al. 1998), and this can have negative impacts on other plants, particularly fast-growing species. This finding agrees with the idea that the type of plant, either early-successional, mid-successional, or late-successional, can impact the nature of plant-soil feedbacks (Kardol et al. 2006). However, Kardol et al. (2006) found that late-successional soils have positive effects on late-successional conservative plant species, but neutral effects on early-successional fast-growing species, whereas we found that *Silene*
(more characteristic of late-successional species) had negative effects on *Deschampsia* (more characteristic of early-successional species).

Ultimately, as plants colonize unvegetated soils and ecosystems develop, these types of plant-soil feedbacks can determine the establishment of certain plants and the ultimate plant community composition (Bever et al. 2010, van der Putten et al. 2013). At our site, several different plant species are colonizing previously unvegetated soils (Bueno de Mesquita et al. 2017, 2018b), and priority effects mediated by plant-soil feedbacks could be important. For example, if *Silene* establishes in a particular location, it could potentially limit *Deschampsia* colonization there. While we suggest that this was mediated through nitrogen cycling, we cannot rule out potential allelopathic effects of *Silene* litter chemical compounds on *Deschampsia*, which is an avenue for future research. Allelopathy is another mechanism of plant-soil feedback that is difficult to separate from other mechanisms (Lau et al. 2008, van der Putten et al. 2013), although there is evidence of allelopathy by both root and litter-sourced compounds (Padhy et al. 2000, Bais et al. 2003, Callaway and Ridenour 2004, Vivanco et al. 2004, Inderjit et al. 2011). At broader scales in the context of global change and species invasions, plant-microbe interactions are a particularly important type of biotic interaction that can mediate plant success as plants shift their ranges either as invaders (Keane and Crawley 2002, Mitchell and Power 2003, Van Grunsven et al. 2007, Morriën and van der Putten 2013, Yang et al. 2013) or native plants tracking suitable climate (Engelkes et al. 2008, van der Putten et al. 2010, 2016, van der Putten 2012). Future work needs to continue addressing feedbacks in these contexts in more species and at the community level in the field, and also incorporate the effects of root exudates on microbial communities.
CHAPTER VII
CONCLUSIONS

Conclusions

This body of work demonstrates that (1) alpine plants have colonized unvegetated soils at high elevations as summers have warmed over the past several decades, (2) soil bacterial communities have co-occurrence patterns with plants that can improve models of plant distributions, (3) diverse root fungal communities, including key mutualistic taxa, exist in in some of the highest elevation vascular plant communities in Colorado, but are patchily distributed, (4) plant interactions with microbes are important for plant growth and survival as they colonize previously unvegetated soils as snow melts earlier and growing seasons lengthen, and (5) plant species have differential effects on unvegetated soil microbial communities based on their litter chemistry and litter microbial communities, which can feed back and affect plant growth. Importantly, microbial communities and growing season length interacted to affect plant performance such that microbes increased in importance in non-optimum abiotic conditions. This suggests that microbial communities are likely to increase in importance as growing seasons continue to lengthen and plants cope with abiotic stressors.

This research refines early predictions about plant responses to climate change, when plants were expected to shift to track their suitable climate envelopes without consideration of biotic interactions (IPCC 1990). Consistent with literature on other biotic interactions and range shifts, plant-microbe interactions must be considered for a more nuanced understanding about both the rate and spatial extent of a plant range shift (Araújo and Luoto 2007). However, understanding the importance of plant-microbe interactions still remains challenging and there is much future work to be done. The importance of plant-microbe interactions will likely depend on (1) how dependent a plant species is on microbes, (2) how
influenced a plant’s current range limits are by microbes, (3) how changing abiotic conditions affect the interactions between plants and microbes, (4) how microbes are distributed beyond the plant range, and (5) how microbial distributions are affected by changing abiotic conditions (van der Putten et al. 2010, Hille Ris Lambers et al. 2013).

This work demonstrates the importance of plant–microbe interactions at small to medium spatial and temporal scales. Interestingly, this is a key difference in plant–microbe interactions compared to plant–pollinator or plant–herbivore interactions, because microbial distributions can be patchy at smaller spatial scales than animals (King et al. 2010). Other work has shown that biotic interactions are important for predicting broad-scale distributional patterns (Araújo and Luoto 2007). Future work should address plant–microbe interactions at larger spatial scales (Afkhami et al. 2014) and track plant–soil feedbacks over longer time scales.
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Eberhardt, J. E. Edwards, M. S. Elshahed, K. Fliegerova, M. Furtado, M. A.
Garcia, Z.-W. Ge, G. W. Griffith, K. Griffiths, J. Z. Groenewald, M. Groenewald,
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K. Hansen, P. Harrold, G. Heller, C. Herrera, K. Hirayama, Y. Hirooka, H.-M.
Ho, K. Hoffmann, V. Hofstetter, F. Hognabba, P. M. Hollingsworth, S.-B. Hong,
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Johnson, J. E. Johnson, P. R. Johnston, E. B. G. Jones, L. J. Kelly, P. M. Kirk,
D. G. Knapp, U. Koljalg, G. M. Kovacs, C. P. Kurtzman, S. Landvik, S. D.
Leavitt, A. S. Liggenstoffer, K. Liimatainen, L. Lombard, J. J. Luangsarad, H.
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Figure A2.1. The relationship between elevation and percent cover of the three main vegetation types. Curves are from 3rd order (tree, $R^2 = 0.99$), and 4th order (shrub, $R^2 = 0.84$ and tundra, $R^2 = 0.95$) polynomial functions.

Table A2.1. Predictor variables included (noted with an X) in the best models of three different techniques, logistic regression (LR), geographically weighted logistic regression (GWLR), and random forests classification (RFC). Variables for GWLR were forward selected following the procedures of the ‘gwr.model.selection’ function in the GWmodel R package and these were compared to the variables selected in the LR model. Variables for RFC were selected using the mean decrease accuracy metric from the ‘importance’ function in the randomForest R package. The top 3 (open forest and tundra expansion) and 4 (shrub expansion) important variables based on this metric were selected to compare to the LR and GWLR results.

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<th>RFC</th>
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<tr>
<td></td>
<td>Elevation</td>
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Chapter III Appendix

Figure A3.1. The 66 sample sites in the subnival zone of the Green Lakes Valley within the Niwot Ridge LTER study site on the eastern face of the Continental Divide in Colorado USA. The study area is 2 km from northeast corner to northwest corner. These sites are a subset of the sites in our previous studies (King et al. 2010, 2012). The scarcity of plots in the middle of the study area is due to a large cliff face with no soil or plants.

Table A3.1. Taxonomical designations of the clades used in our modeling analyses (italicized) as well as their mean relative abundances across the 66 sampling locations.

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<th>Subgroup</th>
<th>Class</th>
<th>Order</th>
<th>Family</th>
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<td>Deltaproteobacteria</td>
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<td>Sphingomonadales</td>
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*Did not improve any plant SDM

Table A3.2. Elevation average (at our study site), functional group, and specialist type of the modeled species.

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<th>Elevation Average (m)</th>
<th>Functional Group</th>
<th>Specialist/Generalist</th>
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<td>Graminoid</td>
<td>Talus Specialist</td>
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<td>Bryophytes</td>
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<td>Byrophyte</td>
<td>Tundra Generalist</td>
</tr>
</tbody>
</table>
Table A3.3. Model parameters and results (AICc = corrected Akaike Information Criterion; Pseudo $R^2$ = Nagelkerke’s $R^2$; AUC = area under the curve). Error is from 10-fold cross validation, adjusted for not using leave one out. Independence of errors was tested with the Durbin-Watson test (P-value in parentheses) and multicollinearity was tested with VIF statistics (average VIF in parentheses). Bolded numbers are the lowest AICc and error values, and highest $R^2$, $D^2$, and AUC values of the four models for each plant. **Abiotic Factors:** ALT = altitude; Snow = snowpack; Dpinorg = dissolved inorganic phosphorus; DOC = dissolved organic carbon; Dtotal = total dissolved phosphorus; TDN = total dissolved nitrogen; Sand = % sand. **Plant Factors:** desces = Deschampsia cespitosa; hymgra = Hymenoxis grandiflora; kobmyo = Kobresia myosuroides; Carex perglobosa; senfre = Senecio fremontii; carnar = Carex nardina; anggra = Angelica grayi; trispi = Trisetum spicatum; carpha = Carex phaeocephala; geuros = Geum rossii; elyscr = Elymus scriberneri; fesrub = Festuca rubra; antalp = Antennaria alpina; phlsib = Phlox siberica. **Bacteria Factors:** rhodo = Rhodospirillales; acidGP3 = Acidobacteria Gp3; acidGP1 = Acidobacteria Gp1; acidGP7 = Acidobacteria Gp7; oxalo = Oxalobacteraceae; sphiing = Sphingomonadaceae; pseudo = Pseudonocardiales; delta = Deltaproteobacteria; cyano = Cyanobacteria; burk = Burkholderiales; ktedo = Ktedonobacteraceae; actinomy = Actinomyctecales.

Table A3.4. Frequency and direction of abiotic factors included in the best fit ABIOT model of the plants. carnar = Carex nardina, carpha = Carex phaeocephala, desces = Deschampsia cespitosa, elyscr = Elymus scriberneri, fesrub = Festuca rubra, kobmyo = Kobresia myosuroides, trispi = Trisetum spicatum, cirsco = Cirsium scopulorum, geuros = Geum rossii, oxydig = Oxyria digyna, senfre = Senecio fremontii, silaca = Silene acaulis. DOC = dissolved organic carbon, Dpinorg = dissolved inorganic phosphorus, Dtotal = total dissolved phosphorus, TDN = total dissolved nitrogen.
Table A3.5. Frequency and direction of plant-plant interactions. elyscr = Elymus scriberneri, trispi = Trisetum spicatum, silaca = Silene acaulis, carnar = Carex nardina, senfre = Senecio fremontii, desces = Deschampsia cespitosa, carpha = Carex phaeocephala, geuros = Geum rossii, fesrub = Festuca rubra, cirso = Cirsium scopulorum, oxydig = Oxyria digyna, kobmyo = Kobresia myosuroides.

<table>
<thead>
<tr>
<th>Plant Predictor</th>
<th>Positive</th>
<th>Modeled Species</th>
<th>Negative</th>
<th>Modeled Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angelica grayi</td>
<td>3</td>
<td>elyscr, trispi, silaca</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Antennaria alpina</td>
<td>0</td>
<td></td>
<td>1</td>
<td>trispi</td>
</tr>
<tr>
<td>Bryophytes</td>
<td>1</td>
<td>carnar</td>
<td>1</td>
<td>senfre</td>
</tr>
<tr>
<td>Carex nardina</td>
<td>1</td>
<td>descae</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Carex periglobosa</td>
<td>1</td>
<td>carpha</td>
<td>1</td>
<td>bryophytes</td>
</tr>
<tr>
<td>Carex phaeocephala</td>
<td>1</td>
<td>geuros</td>
<td>3</td>
<td>fesrub, cirso, oxydig</td>
</tr>
<tr>
<td>Deschampsia caespitosa</td>
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<td>carnar</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Elymus scriberneri</td>
<td>1</td>
<td>fesrub</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Festuca rubra</td>
<td>1</td>
<td>silaca</td>
<td>2</td>
<td>kobmyo, oxydig</td>
</tr>
<tr>
<td>Geum rossii</td>
<td>2</td>
<td>fesrub, silaca</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Hymenoxis grandiflora</td>
<td>2</td>
<td>carnar, geuros</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Kobresia myosuroides</td>
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<td>carnar</td>
<td>2</td>
<td>fesrub, bryophytes</td>
</tr>
<tr>
<td>Phlox siberica</td>
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<td></td>
<td>1</td>
<td>cirso</td>
</tr>
<tr>
<td>Senecio fremontii</td>
<td>1</td>
<td>carpha</td>
<td>1</td>
<td>descae</td>
</tr>
<tr>
<td>Silene acaulis</td>
<td>1</td>
<td>cirso</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Trisetum spicatum</td>
<td>1</td>
<td>fesrub, silaca</td>
<td>14</td>
<td>silaca</td>
</tr>
</tbody>
</table>
Chapter V Appendix

Figure A5.1. Map of the inocula collection locations (n = 8), plots where plants were transplanted (4 blocks with paired plots), and 20 other unvegetated soils that were surveyed in Porazinska et al. (2018) and analyzed in Figure A5.4.

Figure A5.2. Summary of germination, survival, and sampling numbers for the three species throughout the experiment. Deschampsia had the highest survival, Oxyria were eaten by pika and Silene had high mortality. Greenhouse = number survived after four months growth in the greenhouse, Harvest 1 = number harvested for analysis after the greenhouse portion of the study, Acclimatize = number survived after acclimatizing at the CU Mountain Research Station, Transplanted = number that were actually transplanted up to the field site, Transplant = number survived the transplant shock (2-3 weeks after transplant), Year 1 = survival after one full growing season in the field, Harvest 2 = number harvested for analysis at the end of the first field season, Year 2 = survival after the second field season.

Figure A5.3. Timing of snowmelt in the control and black sand plots over the three years of the experiment. Black sand was applied to plots once poles marking the plots were visible in 2017 and 2018. Additional observations from 2015 suggest that the snowbed melted earlier in 2016 than 2015.
Figure A5.4. Principle coordinates analysis and variance partitioning for unvegetated soil bacterial (A) and fungal (B) communities at the OTU level. Also reported are the mean and maximum Bray-Curtis dissimilarity ("BC") values for all of the pairwise comparisons. Across these 24 unvegetated soils collected at our site (Figure A5.1), there was variation in both bacterial and fungal communities that was driven primarily by soil moisture. Variance partitioning shows that space and environment explain only a small amount of variation in unvegetated soil microbial communities. Bacterial and fungal community composition were both correlated with spatial distance (Mantel test, \( r = 0.17 \) and \( p = 0.04 \); \( r = 0.21 \) and \( p = 0.03 \), respectively). Obligate plant-associated fungi were patchily distributed in unvegetated soils, with arbuscular mycorrhizal fungi present in 5 of the 24 soils. Facultative plant-associated fungi were more widely distributed, with dark septate endophytes present in 22 of the 24, though in some cases in very low abundance. Fungi classified as plant pathogens were present in all 24 soils, though in some cases in very low abundance, and abundances varied by three orders of magnitude.

Figure A5.5. Relationship between percent root colonization by arbuscular mycorrhizal fungi (AMF), dark septate endophytes (DSE) and fine root endophytes (FRE) and aboveground biomass in year two. A line is shown for the only significant linear regression model (\( p < 0.05 \)).

Figure A5.6. Pathogen abundance for each inoculum sorted by year two growth. Pathogens were identified as plant pathogens by the program FUNguild.
Table A5.1. Rarefaction (sequences per sample) for each type of sample used in the project.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Rarefaction</th>
</tr>
</thead>
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<tr>
<td>Soil Inocula 16S</td>
<td>10980</td>
</tr>
<tr>
<td>Soil Inocula ITS</td>
<td>1078</td>
</tr>
<tr>
<td>Unvegetated Soils 16S</td>
<td>21077</td>
</tr>
<tr>
<td>Unvegetated Soils ITS</td>
<td>10613</td>
</tr>
<tr>
<td>Year 2 Roots and Pot Soils 16S</td>
<td>1240</td>
</tr>
<tr>
<td>Year 2 Roots and Pot Soils ITS</td>
<td>5659</td>
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Table A5.2. List of bacterial and fungal OTUs that were significantly correlated with year two Deschampsia cespitosa growth. These taxa were isolated from either the pot soil or root interior and their most detailed taxonomy from a combination of GreenGenes, UNITE, and BLAST is shown.

<table>
<thead>
<tr>
<th>Location</th>
<th>Kingdom</th>
<th>Phylum</th>
<th>Class</th>
<th>Order</th>
<th>Family</th>
<th>Genus</th>
<th>Relationship</th>
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</thead>
<tbody>
<tr>
<td>Root</td>
<td>Bacteria</td>
<td>Actinobacteria</td>
<td>Actinomycetes</td>
<td>Microbacteriaceae</td>
<td>Cryococcus</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>Root</td>
<td>Bacteria</td>
<td>Planctomycetes</td>
<td>Planctomycetes</td>
<td>Pirellulaceae</td>
<td>Pasteuria</td>
<td>Positive</td>
<td></td>
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<tr>
<td>Root</td>
<td>Planctomycetes</td>
<td>Planctomycetes</td>
<td>Pirellulaceae</td>
<td>Pasteuria</td>
<td>Positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Root</td>
<td>Proteobacteria</td>
<td>Alphaproteobacteria</td>
<td>Rhizobiales</td>
<td>Rhizobiales</td>
<td>Agrobacterium</td>
<td>Positive</td>
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<tr>
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<tr>
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<td>Bacteria</td>
<td>Acidobacteria</td>
<td>Acidobacteria-6</td>
<td>III-1-5</td>
<td>ms2524</td>
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<td>Acidobacteria</td>
<td>Acidobacteria-6</td>
<td>III-1-5</td>
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<tr>
<td>Soil</td>
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<td>Cytophagaceae</td>
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<td>Chitinagglomera</td>
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<td>107 W55P</td>
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<td>Pirellulaceae</td>
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<td>Betaproteobacteria</td>
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<td>Oxalobacteriales</td>
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<td>Proteobacteria</td>
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<td>Burkholderiales</td>
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<td>Pseudogyrnosacis</td>
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</table>
Table A6.1. Rarefaction of different sample types. 16S litter was not rarefied in order to keep Oxyria litter in the analysis, which had low sequencing depth (mean = 242).

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<thead>
<tr>
<th>Sample Type</th>
<th>Rarefaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant Litter 16S</td>
<td>Not rarefied</td>
</tr>
<tr>
<td>Plant Litter ITS</td>
<td>45184</td>
</tr>
<tr>
<td>Lab incubated soils 16S</td>
<td>5997</td>
</tr>
<tr>
<td>Lab incubated soils ITS</td>
<td>45184</td>
</tr>
<tr>
<td>Field litterbag soils 16S</td>
<td>9614</td>
</tr>
<tr>
<td>Field litterbag soils ITS</td>
<td>16227</td>
</tr>
</tbody>
</table>

Table A6.2. Similarity percentage analysis (SIMPER) of bacterial (16S) and fungal (ITS) communities in the three species’ litter. Shown are the top three taxa contributing to community dissimilarity, their percent contribution to dissimilarity, and the cumulative percent dissimilarities. Taxonomy was assigned by the GreenGenes and UNITE databases.

<table>
<thead>
<tr>
<th>Pair</th>
<th>OTU ID</th>
<th>Kingdom</th>
<th>Phylum</th>
<th>Class</th>
<th>Order</th>
<th>Family</th>
<th>Genus</th>
<th>Species</th>
<th>% Cont.</th>
<th>Cum. %</th>
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<tbody>
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<td>4.08</td>
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<tr>
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<td>Microbacteria</td>
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<td>Leotiomycetes</td>
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<td>Helotiales</td>
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<td>Helotiales</td>
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<td>Agaricomycetes</td>
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Table A6.3. Similarity percentage analysis (SIMPER) of bacterial and archaeal (16S) and fungal (ITS) communities in soils after litter decomposition in the lab and in the field. Shown are the top three taxa contributing to community dissimilarity, whether they were abundant in plant litter (Yes or No), their percent contribution to dissimilarity, and the cumulative percent dissimilarities. Taxonomy was assigned by the GreenGenes and UNITE databases.

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Figure A6.1. Relative abundances of selected bacterial taxa among the four inocula (C = control empty litterbag addition, D = Deschampsia litter addition, O = Oxyria litter addition, S = Silene litter addition). The taxa shown are some of the dominant phyla as well as some known plant-associated taxa (Rhizobiales, Janthinobacterium, Sphingomonas) (Knelman et al. 2018). Different letters represent significant differences (Tukey posthoc, p < 0.05). nsd = no significant difference among the inocula.
Figure A6.2. Germination of the three species among the four inocula treatments.

Figure A6.3. Survival of the three species among the four inocula treatments.