Nitrogen cycling and microbial communities of alpine soils in the Pacific Northwest

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Abstract

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The alpine Pacific Northwest is an environment of stunning beauty and environmental extremes, with acidic, low-nutrient soils, snow cover often exceeding 4-5 m, and snow periods occasionally exceeding nine months out of the year. Nitrogen (N) deposition from increasing urbanization and intensive agriculture can cause changes in alpine soil chemistry and plant species abundance and increase leaching of inorganic N into streams and lakes. In the state of Washington, N deposition has already exceeded critical loads for shifts in alpine lichen communities (Geiser et al., 2010) and alpine lake microbiota (Sheibley et al., 2014). The effects of climate change, which could include earlier snowmelt, increased fall rains, and even the complete disappearance of permanent snowfields and glaciers, threaten to exacerbate effects of N deposition even further by causing changes in plant phenology and increasing decomposition of soil organic matter.

In this study I used fertilizer treatment of 0, 3, 5 and 10 kg NH_4NO_3-N ha^{-1} yr^{-1} to simulate increased N deposition in three alpine meadow ecosystems of the Pacific Northwest, at Mount Rainier, North Cascades and Olympic National Parks. Using increased soil NO_3-N availability to alpine plants and microbes as an indicator, I define the empirical critical load upper limit for Pacific Northwest alpine meadows to be 6 kg N ha^{-1} yr^{-1}. Increased fall microbial N uptake in these meadows appears to serve as a buffer for inorganic N loss with fall rains. No increases in plant species were observed during the study. In soils with available soil inorganic N from slow depolymerization and mineralization, N pollution accumulated in plots with higher soil N and greater abundance of forbs and graminoid species. In very N-limited soils, N deposition was evenly dispersed among plant communities.

As snowfields and glaciers of the Pacific Northwest are threatened by climate change, I also sampled the soil microbial communities of barren, permanent snowfield soils at Mount Rainier and North Cascades National Parks. I used 16SrRNA metagenomic amplicon sequencing to examine the differences between the microbial communities in samples taken in soil that had only been covered by seasonal snow, and soil underneath permanent snowpack. Photoautotrophic bacteria were not present in samples taken under snowpack and comprised less than 1% of reads in exposed soils. Soils were dominated by Deltaproteobacteria from the genus *Anaeromyxobacter*, which were particularly abundant under snowpack, as well as a number of bacteria from the phylum Gemmatimonadetes. Overall, permanent snowfield soils of the Pacific Northwest contain diverse heterotrophic and chemoautolithotrophic communities of bacteria but have very low overall biomass, comparable to barren soils sampled in the Himalayas. Soil bacterial communities probably depend at least partially on organic matter from atmospheric deposition and carbon fixation from seasonal snow algae for survival in this harsh environment.

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Chapter 1: Introduction and Site Descriptions

Alpine ecosystems occur in the mountains of Pacific Northwest (PNW), especially in the Olympic and Cascade Mountain Ranges of Washington. The three National Parks in Washington (Olympic, Mount Rainier and North Cascades) have extensive areas of alpine areas which are now being threatened by human induced activities, including climate change and excess nitrogen (N) deposition. The National Park Service has great concern about the fate of these ecosystems under an increased N deposition regime, especially with regards to plant and microbial communities and nutrient cycling. To this end the National Park Service provided funds to conduct this research (PMIS#157360). The project also afforded an opportunity to study the influence of the extreme environments in these ecosystems to examine microbial communities in soils underlying permanent snow and ice with respect to the implications for astrobiology.

ALPINE ENVIRONMENTS

Alpine habitats, generally defined as the zones above high-elevation forests and below the zone of permanent snowpack (Körner et al., 2011), are areas of extreme beauty and fragility, and alpine plant communities are some of the most diverse in temperate ecosystems (Gottfried et al., 1998). Appreciation of alpine scenic beauty is one of the top economic benefits these ecosystems provide (Grêt-Regamey et al., 2008). The need to create inflorescences and seeds within such a short growing period, combined with moisture provided from melting snowpack and high sunlight

availability, means that alpine meadows often host a profusion of wildflowers mid-growing season.

The existence of the alpine zone is due to conditions that are too extreme for trees to establish but hospitable for low-lying plants such as grasses, sedges, and herbaceous species. Long snow periods (with snow cover often 9 or 10 months out of the year), harsh winds, low temperatures, and heavy UV radiation create conditions to which alpine plants have adapted using a variety of strategies. Areas with deep, continuous insulating snowpack may support evergreen species and have increased root and soil microbial over-winter survival, while areas with shallow snow and multiple freeze-thaw events may support only annual species (Brooks and Williams, 1999). Many alpine plants are characterized by a slow, steady uptake of N and severe constraints on growth - resulting in dwarfism which allows the plant to reproduce successfully in low-nutrient conditions without exhausting its nutrient supply - and N storage in roots when excess N is available (Friend and Woodward, 1990; Bowman et al., 1995).

Alpine soils are characterized by low pH, high levels of soil organic matter, and occasionally high levels of ash/tephra in volcanic areas (Körner, 2003). Because alpine soils form slowly and accumulate organic matter through low decomposition rates rather than high turnover rates, alpine soils are generally fragile and do not rebound quickly from disturbance (Körner, 2003). Soil pH is often low due to low levels of decomposition and release of base cations in comparison to plant and microbial uptake of nutrients (Nagy and Grabherr, 2009). The lifetime of a leaf during the alpine growing season is short – on average about 60 days. Despite low net primary production due to the brevity of the growing season, low decomposition rates under snowpack cause alpine soils to accumulate vast stores of organic matter over time (Körner, 2003).

THREATS TO ALPINE ECOSYSTEMS

Nitrogen deposition levels in the eastern United States and in the United States as a whole have been steadily decreasing in the last twenty years, due to emissions restrictions and declines in manufacturing (Driscoll et al., 2001). However, N deposition levels are threatening to rise in the previously comparatively pristine western United States as the human population in the west increases. Because sources of nitrogen deposition in the western United States tend to be non-point-source and a product of population increase, N deposition is both widespread and difficult to curtail; any legislation contemplating more restrictive vehicle emissions standards and changes in farming practices over large tracts of the west must have reasonable evidence of ecosystem harm.

The concept commonly used to define levels of harm to ecosystems by pollutants is called the "critical load". Nilsson (1988) defined critical loads as "a quantitative estimate of an exposure to one or more pollutants below which significant harmful effects on specified sensitive elements of the environment do not occur according to present knowledge" – i.e., how much pollution can a species or ecosystem handle before it experiences harm? Critical loads for both sensitive species and sensitive ecosystems are now being defined for reactive N deposition in the western United States; alpine/subalpine ecosystems are considered particularly vulnerable.

THE ALPINE AND ASTROBIOLOGY

The field of astrobiology seeks to understand the nature of life in the universe – whether it can exist or has evolved on other planetary bodies besides Earth, what conditions life can endure, which planetary systems might host life as we understand it, and what forms life might take that differ from life on Earth. Part of this exploration involves understanding the extremes that life on Earth can endure, and even flourish under. Even in the harshest conditions Earth's environments offer – extremes of radiation, temperature, and pressure – metabolic activity has been detected.

Understanding of the limitations of life as we know it can inform astrobiologists and space explorers about where in our solar system we might find life, and what conditions to look for in other solar systems that might have led to the evolution of life. Studies of life's extremes in the field of astrobiology often involve terrestrial analogs, Earth locations studied for their shared condition(s) with an extraterrestrial environment (Sullivan III and Baross, 2007). Studies of terrestrial analogues of extraterrestrial environments have led to the discovery of microbial activity existing beyond the extremes of the conditions thought possible to sustain life (Lineweaver and Chopra, 2012).

Snowfields and glacial/subglacial habitats in alpine areas can serve as terrestrial analogs for a number of possible extraterrestrial habitats in our solar system which are much colder than Earth, including Mars and the icy moons of the outer planets (Sattler et al., 2002). In contrast to Arctic and Antarctic habitats, alpine habitats experience not only freezing temperatures but higher radiation exposure (as the sun's angle is higher) (Perez-Chavez et al., 2000) and lower atmospheric partial pressure due to higher elevation.

However, even lower-elevation alpine meadows are valuable model ecosystems for astrobiologists: under extreme conditions, low nutrient availability, and very low temperatures, plant communities thrive and help support complex grazers and predators (Körner, 2003). Ericoid mycorrhizae allow large-biomass evergreen shrubs to survive highly acidic, low-nutrient environments even when covered in snow for most of the year (Read, 1996), and may prove invaluable tools later in *in-situ* resource utilization for dealing with toxic chemicals and extreme acidity in extraterrestrial soils. Some alpine plants can photosynthesize even under 30 cm of snowpack (Starr and Oberbauer, 2003), and can survive temperatures down to -70ºC for brief periods and even immersion in liquid N_2 (Sakai and Otsuka, 1970; Larcher et al., 2010). Such

adaptations can help us understand how and where multi-cellular life elsewhere in the universe might not only survive but thrive.

ALPINE PACIFIC NORTHWEST

Climate in the alpine Pacific Northwest is characterized by heavy loads of snow and deep snowpack, in contrast to the lighter, shallower snows occurring in continental and eastern mountains of the United States. The winter months of snow-cover typically mid-Oct through May (NOHRSC, 2017). Spring is the period of snowmelt, occurring from May through June/July. Summer, or the growing season, is warm and dry, ranging from July through mid-September (NOHRSC, 2017), with very little precipitation in contrast to alpine areas further inland (Figure 1.3). Fall (late Sept/Oct) is short and characterized by cold temperatures and heavy precipitation in the form of rain or light snow (NOHRSC, 2017).

Because of the relatively high latitude of the Pacific Northwest (46-48ºN) compared to many other montane ecosystems, the delineation of subalpine forest to subalpine parkland to true alpine to permanent snow cover is often fragmented (Körner, 1995). Figure 1.1 shows the altitudinal delineations for alpine vs forest boundaries based on elevational species abundance at Mount Rainier.

The alpine ecosystems of the Pacific Northwest are adapted to well-insulating snowpack for long periods of the year followed by extreme drought after sources from snowpack have been exhausted. The hydrology of these alpine habitats puts them at risk from both N deposition and climate change. Heavy winter precipitation means that large amounts of inorganic N deposition can accumulate in snowpack and, upon snowmelt, enter soils in a few heavily concentrated ion pulse events (Brooks and Williams, 1999). Summer N deposition may accumulate in soils during dry summers only to be flushed into streams and lakes with fall rains rather than taken up by

ecosystem N sinks. The effects of climate change, too, may seriously affect Pacific Northwest alpine meadows (Mote et al., 2005; Salathé et al., 2008). Earlier snowmelt could result in plants being exposed to freeze-thaw cycles early in the growing season (Inouye, 2008), and result in earlier drought before plants have completed their seasonal reproductive cycles.

Alpine glaciers and snowfields, too, are at risk of receding and even disappearing over the long term (Hotaling et al., 2017), threatening rare psychrophilic microbial communities. Microbial action can cause bio-feedback; for example, red algae in snowfields can decrease albedo and increase melting (Lutz et al., 2016), a problem exacerbated by N deposition which can increase red snow algal blooms (Newton, 1982).

Alpine plant and soil microbial uptake, transformations of nitrogen, and soil retention of inorganic and organic nitrogen affect both plant and microbial growth and diversity and the health of downstream and downslope watersheds. Understanding the dynamics of the nitrogen cycle in alpine plant and soil communities, and the effects of increased N deposition on these communities, is important both in defining critical loads and conservation policies to preserve these fragile ecosystems and for protecting the ecosystem services they provide.

OBJECTIVES

This study in general addresses three questions: 1) How do alpine soils of the Pacific Northwest, including both barren snowfield and glacial sediments and vegetated alpine meadows, cycle carbon and nitrogen and support microbial and plant life?; 2) How are vegetated alpine meadows affected by increasing N deposition?; and 3) How do microbial communities under permanent snowfield soils differ from those under annual snowpack, and how might this affect future soil microbial communities and soil formation in the event of permanent snowfield disappearance?

Chapter 2 addresses the effects of N deposition on soil biogeochemistry and microbial uptake in alpine meadows. In Chapter 3, I address whether plant communities changed in response to N deposition in alpine meadows, and how and where these meadows did or did not accumulate N pollution. In Chapter 4, I explore the composition of microbial communities in soils under permanent snowpack vs in barren soils where snow is annual, and address whether and how C and N is accumulating in these soils.

SITE DESCRIPTIONS FOR N DEPOSITION STUDY

Study sites were established on alpine ridges in three national parks in the Pacific Northwest U.S.A.: Paradise Meadows in Mount Rainier National Park (MORA), Sahale Arm in North Cascades National Park (NOCA), and Lillian Ridge in Olympic National Park (OLYM) (Figure 1.2). All research sites were at elevations of approximately 1900 m at each park along ridgelines with a SW/NE aspect (see Table 1 for exact elevations). The treeline in the Pacific Northwest of the United States is approximately >1600 m (Ettinger et al., 2011); however, small stands of conifers can be found up to approximately 150 m below the permanent snowline. Grazing from wild animals at this elevation was observed to be minimal.

Peak snowpack averaged from 3.5-5 m during the study, with MORA experiencing heavier snowpack and higher overall precipitation than the other two sites (Table 1). OLYM had a higher number of snow-free days and ~50% less summer precipitation than MORA and NOCA during the study period (Table 1). At the height of summer, daily minimum soil temperature at 2.5 cm depth was approximately 10-12ºC for all sites. OLYM had the greatest daily maximum soil temperature (up to 24ºC) while NOCA experienced the lowest daily maximum (18ºC).

MORA and NOCA sites are both evergreen shrub/flowering herbaceous meadows, while the OLYM site is drier and comprised of sedges, grasses, and scattered heather with considerable soil crust (see Table 1.2 for dominant species at each site).

Site Soils

The MORA site soils are classified as Lithic or Humic Vitricryands (Andisols) (United States Department of Agriculture et al., 2016) with visible ash, pumice, and some amorphous iron oxides; depth was highly variable, averaging only 20 cm within plots (Table 1.3). The NOCA site soils are classified as Pachic Fulvicryands or Thaptic Haplocryands (Andisols) characterized by layers of fine volcanic ash, very low bulk density, and high organic matter content with a sandy loam texture (Table 1.3) (Briggs et al., 2006; United States Department of Agriculture et al., 2012). NOCA soil depth was often >30 cm. OLYM soils are shallow (average depth-to-rock of 8 cm), gravelly organic-rich Entisols or Inceptisols formed in loess and basaltic parent materials, with a loamy sand/sandy loam texture (Table 1.3) (Kuramoto and Bliss, 1970).

We classified the volcanic ash heather meadows at our two Cascades sites as "younger" (MORA) and "older" (NOCA) based on geologic reports, soil classification, and soil C and N content. NOCA subalpine meadow soils are dominated mainly by tephra from the Mount Mazama eruption that occurred 7,600 years B.P., with much smaller contributions from large eruptions from Mount Saint Helens and Glacier Peak; eruptions from those volcanos tended to be deposited in the opposite direction by prevailing winds (Briggs et al., 2006). MORA subalpine meadow soils, on the other hand, received much more extensive deposits of tephra from eruptions of Mount Saint Helens as well as eruptions of Mount Rainier itself, which deposited fine-grained tephra during an eruption as recently as 1,000 years BP (Mullineaux, 1974; Sisson and Vallance, 2008). Average soil C concentration at the NOCA site was nearly three times

greater than that at MORA (150 vs 56 kg C kg^{-1}), and average soil N was two times greater at NOCA (6 vs 3 g N kg^{-1}). Since the vegetation communities and climatic conditions at these two sites are quite similar, we attribute these differences in soil C and N to the more recent deposition of new parent material (tephra and fine ash) at Mount Rainier.

Table 1.1: Latitude, longitude, elevation, precipitation and temperature data of research sites at MORA, NOCA, and OLYM

			National **Park**	**MORA**	**NOCA**	**OLYM**
			Site Location	Paradise Meadows	Sahale Arm	Lillian Ridge
			Degrees North	46.7977	48.472	47.9146
			Degrees West	-121.7199	-121.0546	-123.3763
			Plot ElevationRange (m)	1905-1951	1891-1909	1916-1932
Mean values for Nov-June (snow months)	Air temp (°C) [a]	Min		-4.5	-7.5	-4.1
		Max		1	-2.4	1
	Soil temp, top 3 cm (excludes early snowmelt) (°C) b			-0.02	-0.02	-0.01
Mean values for July-Sep (non-snow months)	Air temp (°C) [a]	Min		9.4	7.8	9.1
		Max		16.6	15.4	16.2
	Soil temp, top 3 cm (°C) [b]			10	9	10.8
	Snow months snow precip (cm)			135	133	87
	Snow month non-snow precip (cm)[a]			104	31	94
	Non-snow months precip (cm)[a]			17	19	10
	Mean total annual precip (cm) [a]			256	183	191
	Ave. Max Annual Snow Depth (m) [a]			5.5	4	3.5
	Mean snow-free days per year [a]			91	89	103

[a] Data obtained from NOAA's National Operational Hydrologic Remote Sensing Center (NOHRSC 2015); averages based on monitoring data from 2011-2014 (Year 0-Year 3). Monitoring stations were located at Paradise, 1660 m for Mount Rainier; Hart's Pass, 1980 m for North Cascades; and Hurricane Ridge, 1580 m for Olympic.

[b] soil temperature data obtained using iButtons deployed on-site from Summer Year 2-Summer Year 4. Only months with snow cover were used to average winter soil temperatures.

Table 1.2: Dominant vegetation at sites

National Park	Dominant vegetation	Vegetation Community Classification
Mount Rainier	*Phyllodoce empetriformis/ glandulifera,* (Sm.) D. Don/(Hook.) Cov. *Cassiope mertensiana* (Bong.) *G. Don* *Vaccinium deliciosum* Piper *Lupinus spp.*	Heath-shrub [a]
North Cascades	*Phyllodoce empetriformis/ glandulifera,* (Sm.) D. Don/(Hook.) Cov. *Cassiope mertensiana* (Bong.) G. Don *Vaccinium deliciosum* Piper *Lupinus spp.*	Heath-shrub [a]
Olympic	*Carex spectabilis* Dewey *Juncus dromondii* E.Mey. *Cassiope mertensiana* (Bong.) G. Don *Arenaria spp.* *Lupinus spp.*	Mixed dry grass and heath

[a] Henderson 1973, Rochefort and Peterson 1996

Table 1.3: Average site soil properties (±SD). Soil was collected to 30 cm or depth of rock if soil was less than 30 cm deep. BD = Bulk density. Size of C and N pools (Mg C or N ha^{-1}) were calculated using an average depth of 20 cm for MORA and NOCA and 10 cm for OLYM.

National Park	Soil pH	Soil Descript.	Soil C:N	g C kg^{-1}	g N kg^{-1}	Mg C ha^{-1}	Mg N ha^{-1}	Depth (cm)	BD (g cm^{-3})
Mount Rainier	5.1	Andisol; volcanic, shallow, high pumice content, low nutrient	17(3)	56(17)	3(1)	83	4.8	19(8)	0.8(0.2)
North Cascades	4.8	Andisol; volcanic, fine ash, high organic matter	24(3)	150(23)	6(1)	107	4.7	20(5)	0.4(0.1)
Olympic	4.5	Entisol/Inceptisol; very shallow, loamy sand, gravelly, high in nitrogen	15(1)	104(37)	7(2)	77	5.1	8(2)	0.8(0.2)

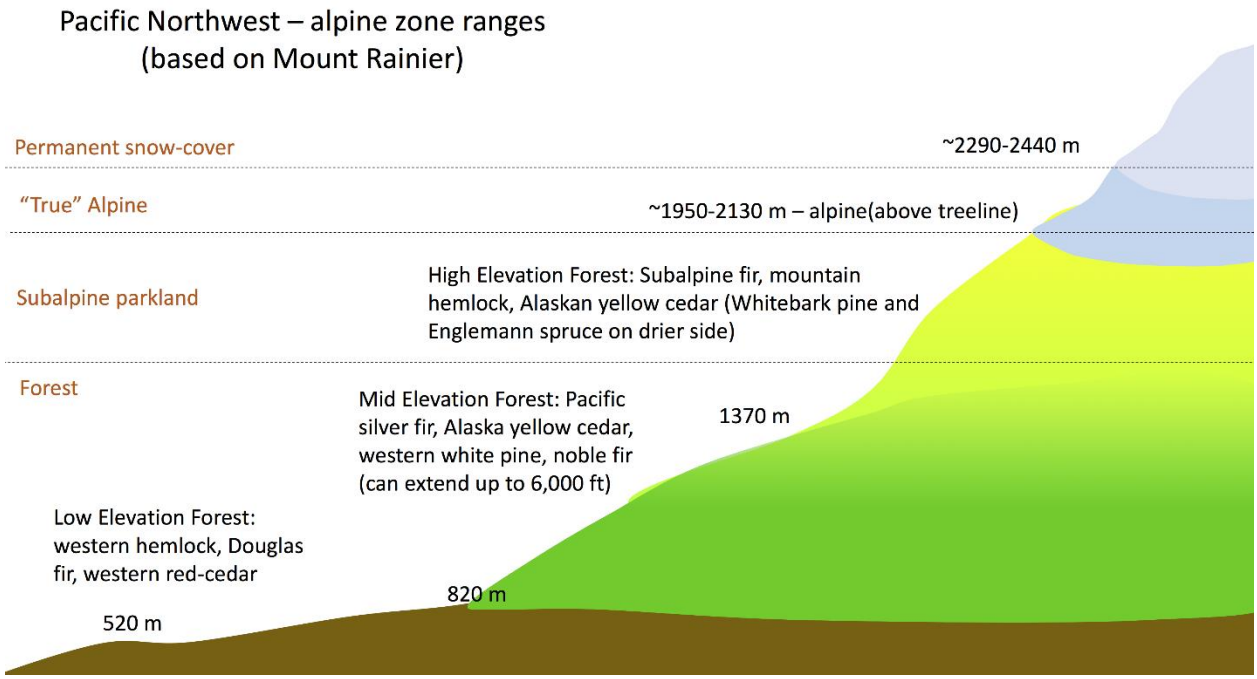

Figure 1.1: Pacific Northwest alpine zone ranges for Western Cascades

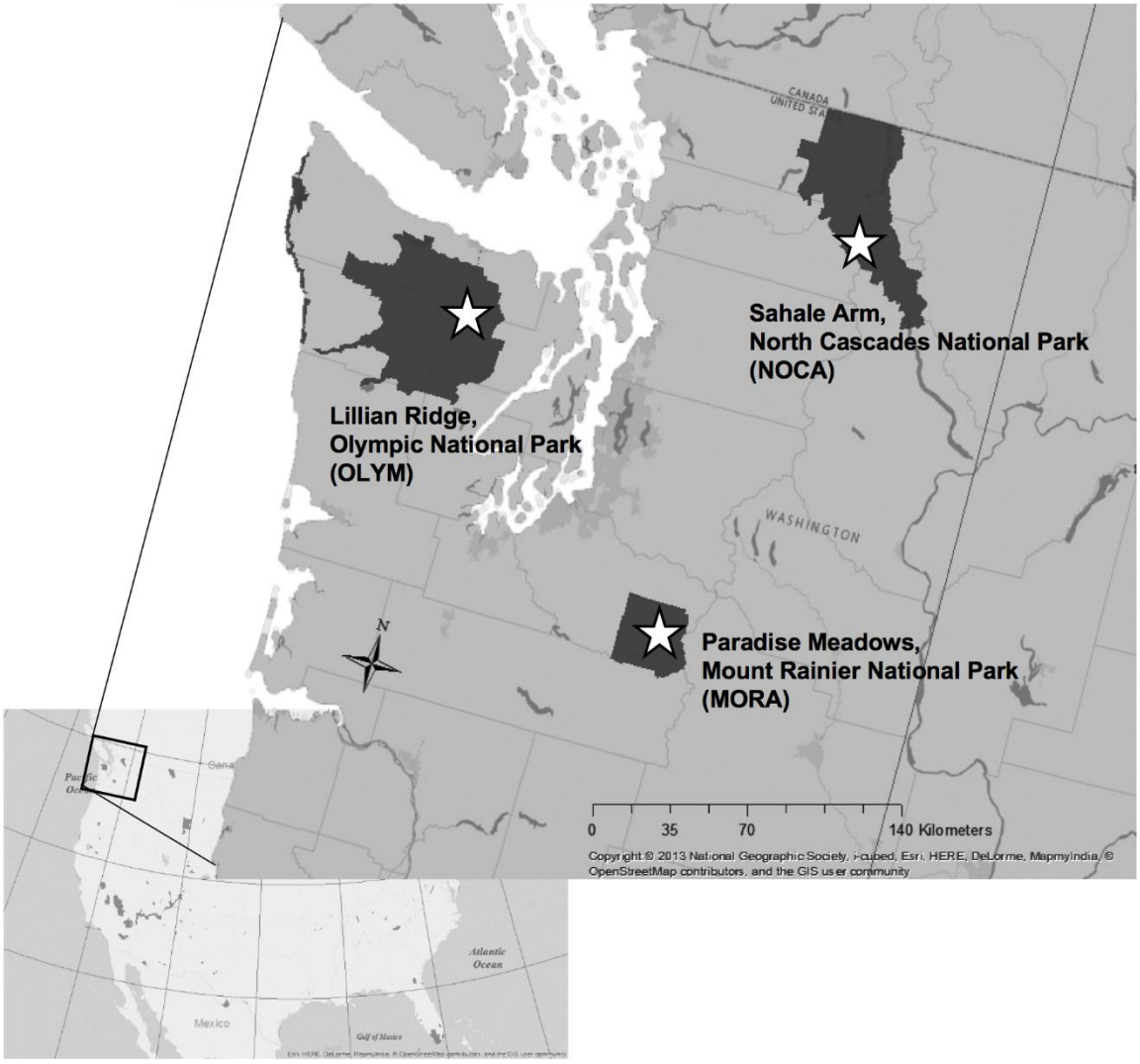

Figure 1.2: Locations of research sites at Mount Rainier (MORA), North Cascades (NOCA) and Olympic (OLYM) National Parks, Washington State, USA.

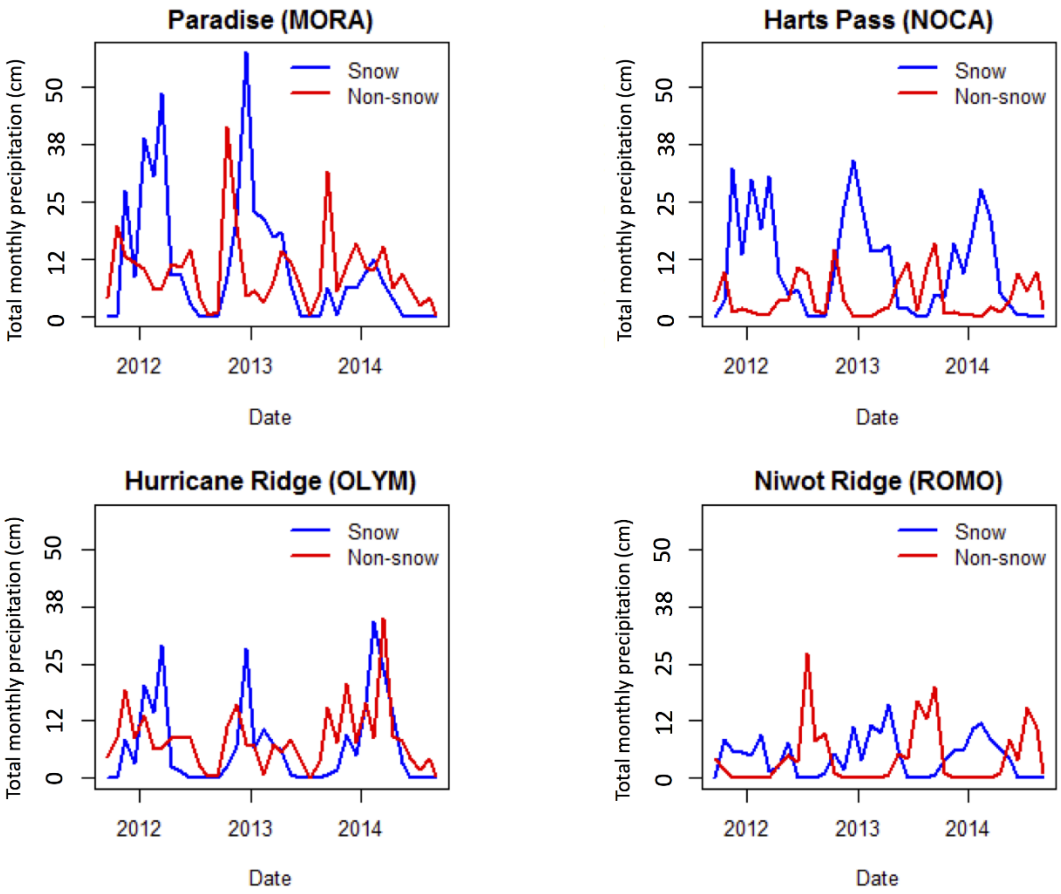

Figure 1.3: Precipitation regimes for the Pacific Northwest alpine in the Cascade Mountains (MORA and NOCA) and the Olymics (OLYM), vs Niwot Ridge in the Rocky Mountains.

Chapter 2: Microbial uptake and plant N availability in response to simulated N deposition

Abstract

As global industry and intensive agriculture increase in response to an expanding human population, oligotrophic ecosystems such as alpine and subalpine habitats are increasingly vulnerable to transported atmospheric nitrogen (N) pollution. Even at low levels, N deposition can alter soil chemistry via changes in decomposition rates and mineralization of N from soil organic matter. I used fertilization to mimic N deposition in three National Park alpine meadow ecosystems in the Pacific Northwest (PNW) of the United States (an area of low ambient N deposition) over a 3-year period. Study sites were two moist heath meadow ecosystems in the western Cascade Mountains and one dry meadow ecosystem in the eastern Olympic Mountains. My objectives were to: 1) Determine a baseline for seasonal patterns of soil and microbial C and N pools and soil solution inorganic N supply, 2) Determine effects of N deposition on alpine soil chemistry, and 3) Suggest empirical critical N deposition load upper limits for inorganic N availability in alpine PNW soils. Soil solution inorganic N supply as measured by resin probes increased in response to N treatment at all sites by Year 3 in plots fertilized at the the 10 kg N ha-1 yr-1 treatment level. At the heath meadow sites, I observed increased soil NO3-N during the summer and decreased extractable organic carbon (C) during the fall in response to applied N. I also observed seasonal increases in the proportion of soil N contained in microbial biomass in

response to treatment. These data indicate season-specific increases in microbial N uptake and mineralization in response to fertilizer treatment. The carbon-rich, fine-ash volcanic soils of the North Cascades were the most sensitive to N treatment with low microbial N uptake. From those soils, I derived an empirical critical load of 6 kg N per ha^{-1} yr^{-1} for increased soil N availability. However, alpine meadow soils in the Western Cascades undergo N limitation in the fall and may have less potential for N leaching with fall rains. In contrast, soils at the dry meadow site had much greater potential for N mineralization, and are temperature- and moisture-limited rather than N limited. Changes in soil chemistry in response to N deposition were site-specific and resulted from differences in plant uptake and soil N mineralization capacity, indicating two very different regimes for response to N deposition for N-limited alpine meadows vs moisture-limited alpine meadows.

INTRODUCTION

The introduction of fertilizer produced by industrial N fixation and the inadvertent oxidation of atmospheric N_2 during fossil fuel combustion has, over the last century, nearly doubled the amount of biologically available N in circulation in Earth's terrestrial ecosystems (Vitousek et al., 1997; Galloway et al., 2008). A portion of this N leaves terrestrial ecosystems through leaching into streams and waterways or through volatilization and particulate transport into the atmosphere (Galloway et al., 2008). Reactive atmospheric N is then deposited elsewhere through either wet or dry deposition, leading to the fertilization of otherwise remote and nutrient-limited ecosystems such as deserts, alpine areas, mires, and boreal forests (Lovett, 1994; Driscoll et al., 2001; Bobbink et al., 2010).

High-elevation ecosystems are particularly sensitive to the effects of air pollution and to climate change. Alpine environments can be subject to large spikes of N deposition not seen at

lower elevations due in part to higher precipitation levels (Bowman et al., 2006; Reddy et al., 2015; NADP, 2017; Williams et al., 2017). Nitrogen deposition in alpine ecosystems has become a special concern at National Parks of the mountainous western U.S., since N fertilization could change vegetation communities (Fenn et al., 2003; Pardo et al., 2011; Cummings, 2014). In addition, it is likely that climate change will result in higher temperatures, more precipitation in the form of rain as opposed to snow in the fall, higher snowlines, and earlier snowmelt (Beniston et al., 1997; Mote et al., 2005; Inouye, 2008; Karl, 2009). These changes could increase alpine soil acidity, weathering rates, decomposition, leaching of NO_3-N into surface waters (Bowman et al., 2014) and the likelihood of invasion by opportunistic plant species (Porter et al., 2012). Since these effects can also result from N deposition, climate change is likely to exacerbate detrimental effects of increasing N pollution, making the need for further research and policy change in this area ever more urgent.

Determining critical N loads, defined as "the highest load that will not cause chemical changes leading to long-term harmful effects on the most sensitive ecological systems" (Nilsson, 1988), is essential if air quality regulations are to protect alpine ecosystems that have not yet been strongly affected by N deposition. Estimates of critical N loads are lacking for many alpine soils and ecosystems. At present, published critical N loads for increased soil inorganic N availability in alpine meadows are 9-10 kg N ha^{-1} yr^{-1} at Rocky Mountain National Park, CO (Bowman et al., 2012), 20 kg N ha^{-1} yr^{-1} at Niwot Ridge, CO (Bowman et al., 2006) (both located in the mountainous western United States), and between 10 and 40 kg N ha^{-1} yr^{-1} on the Tibetan Plateau (Zong et al., 2015).

Nitrogen deposition studies in alpine ecosystems have found a number of different depositional effects on soil chemistry and soil N cycling, including increased decomposition of

particulate organic C (Fang et al., 2014a), changes in CO_2 flux (Fang et al., 2011), increased soil inorganic N pools (Bowman et al., 2006), changes in microbial access to root-derived C and fungal:bacterial ratios (Farrer et al., 2013) and changes in soil pH and in abundance of different microbial phyla (Yuan et al., 2016). One of the most detrimental effects of N deposition on soils is leaching of inorganic N, especially the more mobile NO_3-N, which can lead to eventual eutrophication of streams and lakes (Fenn et al., 2003; Pardo et al., 2011). An increase in soil NH_4-N or NO_3-N, even if ephemeral, means that soil, microbial and plant N sinks are inadequate to retain excess N and that the potential for inorganic N leaching is increased. The time of year at which a change in N chemistry occurs is also significant; N chemistry changes must be coupled with environmental factors such as precipitation and temperature in order to assess leaching potential. For example, ephemeral increases in inorganic N are considerably more harmful if they occur at times of greatest precipitation and lowest plant uptake.

A general model of the soil N processes for an ecosystem is therefore important to provide context for developing critical N loads for that ecosystem. Several studies investigating alpine N dynamics (Jaeger et al., 1999; Lipson et al., 1999; Nemergut et al., 2005) have suggested a model of alpine N availability in the Rocky Mountains: a pulse of high organic N availability upon snowmelt and turnover of the microbial community, followed by mineralization and high plant uptake, followed by late-summer and fall microbial immobilization of N. However, availability of labile C and N and microbial processing of N in alpine soils is strongly influenced by snow depth and snowpack duration (Brooks et al., 2011) and the very heavy snowpack and dry summers (NOHRSC, 2017) of the maritime-influenced alpine Pacific Northwest (PNW) may cause alpine N cycling to differ from more continental alpine regions where the previously mentioned Rocky Mountain studies were focused. For instance, deep, unbroken and long-term snowpack in winter

allows for greater plant root and microbial survival (Brooks and Williams, 1999).providing advantage to evergreen shrubs over graminoid and forb species (Starr and Oberbauer, 2003) and altering N uptake and retention patterns in soil,

I used artificial application of NH_4NO_3-N at relatively low levels of 0, 3, 5 and 10 kg N ha^{-1} yr^{-1} to simulate increased N deposition at three alpine sites. My study sites were located in the alpine meadows of Mount Rainier (MORA), North Cascades (NOCA), and Olympic (OLYM) National Parks. Specific objectives of this study were to: 1) Determine a baseline for seasonal patterns of soil and microbial C and N pools and soil solution inorganic N supply, 2) Determine effects of N deposition on alpine soil chemistry, and 3) Suggest empirical critical N deposition load upper limits for inorganic N availability in alpine PNW soils.

METHODS

Research was carried out at three alpine sites within national parks of the PNW: Paradise Meadows at MORA, Sahale Arm at NOCA and Lillian Ridge at OLYM (Fig 1). For detailed site descriptions see Section Chapter 0: (Site Descriptions). Plots were established in summer 2012 and maintained for three years. Sampling periods were designated as Year 1: Summer 2012-Spring 2013; Year 2: Summer 2013-Spring 2014; and Year 3: Summer 2014-Spring 2015. Areas with no slope or gentle slope were selected to prevent runoff of N from plots. Three out of 5 blocks at NOCA and 2 out of 5 blocks at MORA and OLYM were located in areas with less than 5% slope and all other blocks were located on flat ground.

Experimental Design and Nitrogen Application

A 0.1-0.2 km^2 area of each ridgeline was designated for this study by park staff. Five blocks were randomly selected within each study area. Four plots were established at each block, for a total of 20 plots per site (60 plots total for study). Within each block, plots were spaced at least

1.5 m apart. Plots were 1 m x 2 m, with 1 m^2 of the plot dedicated to monitoring and the other 1 m^2 used for soil sample collection. A 0.5 m buffer around each plot was also treated, for a total fertilized area of 6 m^2 at each plot, ensuring that the rooting area of all plants within the 2 m^2 plot area would receive treatment. In every block, one plot was randomly designated for each treatment level: 0, 3, 5, and 10 kg N ha^{-1} yr^{-1}. These levels were recommended by an interagency panel on alpine vegetation response to N deposition (E&S Environmental Chemistry 2009).

Nitrogen was applied as NH_4NO_3-N in aqueous solution (1 L water per application) twice a year (at the beginning and middle of the growing season). One L of untreated water was applied to control plots at the same time as treated plots received application. Plots were established late in the summer of 2012, and received a 25% application. All plots received full N applications in Years 2 and 3. The solution was applied using hand-pump sprayers.

Soil Sample Collection and Analysis

Soil samples were collected using a 3 cm diameter soil corer, down to 30 cm. Where soil was shallower than 30 cm, soil was collected to depth of bedrock. All soil samples were sieved to 2 mm and analyzed for pH and gravimetric soil moisture. In Years 1 and 2, one soil core per plot was collected for a total of 15 soil samples per treatment level per year. In Year 3, multiple cores per plot were collected to ensure sufficient soil for all samples (2-3 cores at MORA and NOCA, an average of 6 cores at OLYM) and homogenized before sieving. All soil samples were either entirely or predominantly A horizon. OLYM soils included no visible B horizon; MORA and NOCA samples sometimes included B horizon below 10-20 cm.

In all three years of the study, soil samples were collected in the fall (late September/early October) and analyzed for potential N mineralization and nitrification. Samples were extracted with 2 M KCl and analyzed for NH_4-N and NO_3-N before and after 30-day aerobic lab

incubations at field capacity and 20°C (Binkley and Matson, 1983) using a Perstorp 500 Model Autoanalyzer at the Analytical Service Center, University of Washington, Seattle. These samples were also ground and analyzed for total C and N on an Elementar Vario EL Cube or Micro Cube elemental analyzer at the Stable Isotope Facility at the University of California, Davis. In Year 3, soil was also collected during mid-summer (mid-August), and both those soils and soils collected in the fall were extracted with 0.5 M K_2SO_4 and used as a measure of soil C availability (De Luca, 1998; Chantigny et al., 2000). Potassium sulfate-extractable organic C and N were determined on a Shimadzu TOC-V Analyzer at the Analytical Service Center at the University of Washington, Seattle. Microbial biomass was determined using the chloroform-fumigation extraction method, with K_{EC} values (fractions of biomass C and N mineralized) of 0.45 and 0.54 used to calculate microbial C and N respectively (Brookes et al., 1985; Beck et al., 1997).

Ambient N Deposition, Soil Inorganic N Availability and Soil Temperature

Soil solution NH_4-N and NO_3-N availability was determined using Plant-Root Simulator (PRS) probes (Western Ag Enterprises, Inc., Saskatoon CA), which are ion-exchange membranes used to collect soil solution cations and anions (Qian and Schoenau, 2002). Plant-Root Simulator probes capture the flow of inorganic N through soil solution, which is a measure of the difference between release of inorganic N into soil via deposition or mineralization, and uptake of inorganic N via plant roots, microbes, and adsorption onto soil colloids. Nitrogen collected by PRS probes is referred to as "soil solution inorganic N supply" and indicates N which is in soil solution and therefore can be potentially leached from soil. Two cation and two anion probes were placed below the surface of the soil in each plot at a 45° angle. The center of each PRS probe's angled resin strip was ~10 cm below the soil surface at MORA and NOCA

and at ~7 cm at OLYM (due to very shallow soils). To determine soil solution inorganic N supply during the summer and fall months, probes were placed in all plots at the beginning of each growing season (late July) and were collected during the fall (late September or early October) and processed by Western Ag (Saskatoon, CA) using 2 M KCl extraction followed by analysis on a Technicon Autoanalyzer II (Hangs et al., 2004) for all 3 years of the study. A second set of PRS probes was placed in control and 10 kg N ha^{-} yr^{-1} treatment plots in early October and retrieved in late July in order to measure soil solution inorganic N supply in the winter months and during spring snowmelt.

Ambient N deposition during the snow-free summer and autumn (July through early October) was monitored at NOCA and OLYM using three bulk deposition collectors at each site during the summer and fall (Fenn and Poth, 2004). Nitrogen deposition during winter and spring, including late fall non-snow precipitation, snowfall and snowmelt (October-June), was measured using resin bags deployed in PVC pipes situated ~3 cm above the surface of the soil during the winters of Year 2 and Year 3. A PVC pipe measuring 10 cm in length and 2.5 cm in diameter was placed in a hole created by previous soil sampling in the control plot of each block (thus, 5 measurements at each site). A nylon bag containing mixed ion exchange resin was suspended ~2 cm from the top of each PVC pipe. Each bag contained 7.5 g of resin, more than enough to completely fill the diameter of each pipe and reduce side-flow of water down the edges of the pipe to a minimum. The top of each pipe was covered in a plastic netting to prevent large particles from infiltrating the resin. Resin bags and netting were pre-treated with 0.5 M HCl, 2 M NaCl, and DI water according to Allison et al. (2008). One resin bag per site was also suspended in a PVC tube but wrapped in sealed plastic as a control, in order to account for any amine release in the resin from freeze/thaw cycles. Resin bags were extracted with 2 M KCl and

analyzed on a Perstorp 500 Model Autoanalyzer (Analytical Service Center, University of Washington, Seattle) for NH_4-N and NO_3-N. Ambient N deposition data for MORA were available from monitoring studies at Paradise Meadows conducted by researchers at Central Washington University, Ellensburg, WA and were used to provide all summer and Year 1- Year 2 winter/spring ambient N deposition data for this study (Reddy et al., 2015). Methods for calculating seasonal deposition loads and National Atmospheric Deposition Program monitoring criteria were provided by Dr. Jason Williams, Washington State University, later published in Williams et al. (2017). Soil temperature was monitored in control and 10 kg N ha^{-1} yr^{-1} plots using iButtons (Maxim Integrated), buried 2-3 cm in soil.

Statistical Analyses

I used R to perform all statistical analyses (R Core Team, 2014). Levene's Test was used to test for equal variances among groups (fertilization levels) across all combinations of site, time, and data type (Schultz, 1985). For C and N pools in Year 3, paired t-tests were used to assess differences between summer and fall. Data were log-transformed where necessary to meet the assumptions of the models.

To test the effects of treatment on the soil extractable C and N pools I tested for (including NH_4-N, NO_3-N, microbial C and N, and extractable organic C and N) , I divided the data into separate datasets for each combination of site and season using linear mixed-effects models with block as a random effect (Pinheiro et al., 2016). A p-value of 0.05 was used to test for significance.

I tested the effects of fertilization on soil solution inorganic N supply and soil total C and N for years 1, 2 and 3 with a linear mixed-effects model using the nlme package (Pinheiro et al., 2016).I treated both year and treatment level as fixed effects because I expected the effects of N

treatment to be cumulative over time. The interaction between time and treatment level was assessed for significance. Because I used a randomized blocked design for this study, each block, nested within each site, was set as the random effect.

RESULTS

Background Alpine Nitrogen Cycling

Ambient N deposition

Ambient inorganic N deposition was dominated by NH_4-N at MORA in all seasons and years of the study (Figure 2.1). At NOCA and OLYM, the dominant inorganic N molecule tended to be NO_3-N when deposition was low and NH_4-N when deposition was high (Figure 2.1). Dominance of NH_4-N in deposition was significantly correlated with higher total deposition (R^2=0.60, df=10, $p<0.01$). N deposition at NOCA was greatest during summer/fall (July-September). At MORA it was highest during winter/spring and at OLYM there was no seasonal pattern. There were significant differences in levels of seasonal deposition among sites. For all three years of the study, summer/fall deposition was significantly lower at OLYM than at MORA and NOCA , and winter/spring deposition was significantly higher at MORA than at NOCA or OLYM.

Average ambient inorganic N deposition during the study period was 1.5 kg N ha^{-1} yr^{-1} for MORA, 1.0 kg N ha^{-1} yr^{-1} for NOCA, and 0.4 kg N ha^{-1} yr^{-1} for OLYM with a maximum seasonal value of 1.8 kg inorganic-N ha^{-1} at NOCA during summer and fall months in Year 2 (Figure 2.1). Total annual N inputs (ambient deposition + treatment) for the fmy application levels used in this study were therefore 1.5, 4.5, 6.5 and 11.5 kg N ha^{-1} yr^{-1} for MORA, 1, 4, 6 and 11 kg N ha^{-1} yr^{-1} for NOCA, and 0.4, 3.4, 5.4 and 10.4 kg N ha^{-1} yr^{-1} for OLYM.

Background soil temperatures

Soil temperatures during winter and spring (before total snowmelt) were significantly higher at NOCA. Between December and May of Year 2 (period of complete snow cover before melting begins), soil temperatures for MORA, NOCA and OLYM averaged 0.0, 0.1 and -0.1°C respectively. NOCA soils never reached a temperature significantly below zero. During the winter of Year 3 (2015, a year characterized by extremely low snowpack in the PNW) soils at all sites averaged below 0.0°C; however, NOCA soils were still warmer compared to MORA and OLYM.

During the summer and fall when plots were snow-free, there were no site differences in minimum soil temperatures, but there were significant differences in maximum soil temperatures. For the month of August, for example, daily maximum temperature averaged 17, 15, and 19°C and highest temperatures reached were 22, 20, and 25°C for MORA, NOCA, and OLYM respectively. For the entirety of the snow-free season, NOCA had the lowest daily maximum soil temperature and OLYM had the highest daily maximum soil temperature. Average daily differences between minimum and maximum soil temperature at this time of year were 7.4°C for MORA, 5.7°C for NOCA, and 10.0°C for OLYM ($p<0.001$).

Background soil carbon and nitrogen

Total soil C and N content varied considerably between sites, though all sites had substantial organic matter content (Table 1.3). For MORA, NOCA (heath meadows) and OLYM (dry meadow) respectively, soil C and N concentrations were 56, 150 and 104 g C kg^{-1} and 3, 6, and 7 g N kg^{-1}, with average soil C:N ratios of 17, 24 and 15 (Table 1.3). Soil microbial biomass C and N, extractable inorganic N, and extractable organic C and N concentrations were all higher at the dry meadow site compared to the heath meadow sites. At the dry meadow site, 3-4% of soil C

and 3-6% of soil N was contained in microbial biomass, compared to 1-2% of soil C and ~2% of soil N at the heath meadow sites.

Microbial biomass C:N ratios (which ranged from a minimum of 10 at the dry meadow site to a maximum of 17 at the younger heath meadow site) were higher than average for a bacteria-dominated grassland (Cleveland and Liptzin, 2007), indicating substantial presence of fungi. At the heath meadow sites, microbial biomass C and N was strongly negatively correlated with pH, while the opposite was true at the dry meadow site, also suggesting that the heath meadow soil microbial biomass may be more dominated by fungi and dry meadow soil microbial biomass more dominated by bacteria.

The dry meadow (OLYM) soils contained high levels of mineralizable soil N while the heath meadow (MORA and NOCA) soils showed low to no mineralizable soil N after aerobic incubation (Figure 2.2). For the dry meadow soils, N mineralization was positively correlated with initial N conditions, including initial EIN (Figure 2.3), initial microbial N content, and total soil N. However, for MORA soil N mineralization was negatively correlated with initial EIN (Figure 2.3), and for NOCA soils N mineralization was negatively correlated with soil C:N ratio but had no correlation to initial N conditions.

Seasonal shifts in soil C and N

I assessed differences in soil extractable C and N compounds and microbial biomass C and N between summer (the height of the growing season when plant demand is high) and fall (when many plant species have senescent above-ground tissue and are no longer photosynthesizing). Soil extractable inorganic N decreased significantly to near-zero levels at the two heath meadow sites from summer to fall, although at the younger heath meadow site inorganic N were quite low in summer as well(Figure 2.4, Figure 2.5). The dry meadow site soil also decreased in inorganic

N from summer to fall. but fall soil still contained comparatively more inorganic N (Figure 2.5). Other seasonal soil nutrient shifts included fall increases in EOC and microbial biomass C and N, and decreases in EON (Figure 2.5).

The microbial biomass C:N ratio at MORA and OLYM was significantly lower in the fall than during mid-summer (decreased from 17 to 14 at MORA and 14 to 10 at OLYM), indicating a proportionally greater uptake of N than C compared to uptake levels in summer biomass. NOCA had lower microbial biomass C and N pools (Figure 2.5) and showed no significant change in the microbial C:N ratio from summer to fall (11 to 12).

Mean soil solution inorganic N supply (±SE) during winter/spring (~9 months out of the year) was 10.2 (2), 6.5 (0.4) and 11.5 (4) μg N 10 cm^{-2} burial $period^{-1}$ for MORA, NOCA and OLYM, respectively, with no significant differences among sites. Soil NO_3-N supply during this time was significantly higher at the dry meadow site, (15.6±5 μg N 10 cm^{-2} burial $period^{-1}$) than at MORA or NOCA, which averaged 4±1 and 4.9±1 μg N 10 cm^{-2} burial $period^{-1}$, respectively. Winter/spring soil solution inorganic N supply was highest at OLYM in years of late snow-melt and highest at MORA in years of early snowmelt.

Background (control plot) soil solution inorganic N supply during summer/fall (July-September) was highest at OLYM in Year 2, but no difference in ambient soil solution NH_4-N supply was observed in Year 3 among the sites. Control plot soil solution inorganic N supply at NOCA was lowest among the three sites for every season for the entire study, despite the strong increase in soil solution inorganic N supply with treatment (Figure 2.6).

Soil carbon and nitrogen response to N application

At all sites, soil solution NO_3-N supply significantly increased in response to nitrogen application by Year 3 at the 10 kg N ha^{-1} yr^{-1} level (Figure 2.6, Table 2.1). At NOCA, soil

solution inorganic N supply for both NO_3-N and NH_4-N increased at the 5 kg N ha^{-1} yr^{-1} treatment level (Figure 2.6, Table 2.1). Mid-summer soil KCl-extractable NO_3-N also significantly increased in response to applied N at the 10 kg N ha^{-1} yr^{-1} level (Figure 2.4, Table 2.1).

Fall levels of soil extractable organic C (EOC) at NOCA and MORA significantly decreased with treatment in Year 3 of the study at the 10 kg N ha^{-1} yr^{-1} level ($p<0.05$) (Figure 2.4, Table 2.1). Soil EOC during the summer and extractable organic N (EON) in both summer and fall showed a trend of decrease with increased N application but this was only significant at the $p<0.10$ level. No change in response to N application was observed in total soil C and N, microbial biomass C, or in mineralization and immobilization of inorganic N.

I found no direct relationship between treatment and microbial biomass N expressed as a concentration, but did find that treatment significantly affected microbial N when it was expressed as a proportion of total soil N (Figure 2.7) or as the residuals of the relationship between microbial N and soil total N. Microbial biomass N at all three sites was dependent on total soil nitrogen, which masked treatment effects. When this relationship was accounted for, I found that at the younger heath meadow and the dry meadow, more soil N was present in microbial biomass at higher treatment levels. This treatment effect was significant at the 10 kg N ha^{-1} yr^{-1} treatment level at MORA and the 5 kg N ha^{-1} yr^{-1} treatment level at the dry meadow in the summer, and at the 10 kg N ha^{-1} yr^{-1} treatment level at the dry meadow in the fall.

DISCUSSION

This study addressed the increased risk of alpine meadow inorganic N availability and leaching with N deposition, which is a major concern for alpine land managers and ecologists (Cummings, 2014). My goal was not only to assess the effects of N deposition on soil chemistry

but to also place those effects within the context of seasonal alpine N cycling at these sites.

In this study, soil solution NO_3-N supply measured by PRS probes was the most responsive to increased deposition at all three sites. However, potential for N leaching must be evaluated in terms of when greatest risk of N deposition occurs, when greatest risk of excess soil inorganic N occurs and when greatest chance of soil water percolation (snow melt or precipitation) occurs. I was particularly concerned with whether forms of plant- and microbe-available soil N increased or decreased from summer to fall during the non-snow months, as non-snow fall precipitation is generally high in the region. I interpreted soil responses to N treatment as showing two distinct response patterns to N deposition: increased storage of total soil N in microbial biomass at the dry meadow site where vegetation was patchy, and increased microbial uptake of soil labile organic C at the heath meadow sites where vegetation was continuous (Figure 2.8). Where there was strong seasonal microbial N uptake, this functioned as a buffer against changes in soil inorganic N.

Alpine Seasonal N Availability

As a first step to determining N leaching risks, I investigated seasonal availability of inorganic N in dry versus mesic meadow soils. I wanted to establish whether seasonal changes in soil C and N compounds fit within the model proposed by studies of alpine meadow N cycling in the Rocky Mountains: high N availability early in the growing season due to labile N release from microbial turnover, followed by N limitation in the fall due to plant uptake. Authors of these studies found that later in the growing season, microbial biomass increased as microbes received less resource competition from plants, and labile soil C increased due to root turnover (Lipson et al., 1999; Nemergut et al., 2005). Because of the volcanic parent material at the two heath meadow sites I expected that soil properties and N cycling might differ somewhat from

studies in the Rocky Mountains. Soil C and N concentrations at the young heath meadow and the dry meadow sites (Table 1.3) did not differ substantially from other published values for alpine A horizons. At Niwot Ridge, an extensively studied alpine meadow in the Rocky Mountains, A horizon soil concentrations were 81 g C kg^{-1} and 8 g N kg^{-1} with a C:N ratio of 11 (Raab et al., 1999; Lipson and Schmidt, 2004). In a review of C and N concentrations for alpine permafrost soils, soils in A horizons averaged 82 g C kg^{-1} and 5 g N kg^{-1} with a C:N ratio of 17 (Bockheim and Munroe, 2014). Values for the dry meadow soils were similar to other published values for alpine meadows, most likely because many of the published values are for Inceptisols and Entisols. Soils at the older heath meadow in particular had much higher C and a higher C:N than average; there are few published values for soil characteristics of well-established Andisol-type alpine meadows.

The model proposed for alpine soil N cycling in the Rocky Mountains at least partially explained my results - soils at all three of my sites underwent a seasonal decrease in inorganic N from summer to fall. Soils at the two heath meadow sites experienced N-limiting conditions in the fall, probably due to steady plant uptake of N during the growing season. I based this mainly on two observations of the two heath meadow soils: the strong decrease in soil inorganic N from summer to fall (Figure 2.5), and the net immobilization/ very small net mineralization of N (Figure 2.2). Soils at the younger heath meadow site may be N-limited to some extent in the summer as well, as levels of EIN were also extremely low earlier in the growing season (Figure 2.4).

In contrast, soils at the dry meadow site appeared to be moisture- and temperature-limited rather than N-limited. The dry meadow soils had a much higher concentration of total N and other forms of N (Figure 2.6, Figure 2.4, Table 1.3). Although dry meadow soils underwent a

decrease in extractable N in the fall, the decrease in NH_4-N was small and ambient NH_4-N levels were comparable to mid-summer levels at the heath meadow sites (Figure 2.5e). Vegetation at the dry meadow site was sparser and more dominated by soil biological crust, with less overall plant biomass compared to the more mesic heath meadow sites and greater daily variability in soil temperature. Each year I observed vegetation at the dry meadow site senescing several weeks earlier than at the heath meadow sites, as soils dried out during the PNW summer. In addition, once provided with moisture and steady temperature, fall soil samples from the dry meadow site were strongly mineralizing (Figure 2.2).

The fall increases in microbial biomass and labile C, and decreases in labile N, in the soils of the dry meadow (OLYM) and the younger heath meadow (MORA) fit within the Niwot Ridge/Rocky Mountains model of alpine N cycling . However, these changes were less pronounced at the younger heath meadow site and were not observed at the older heath meadow site. It is possible that because of the very high levels of organic matter and high water-holding capacity in the volcanic ash soils at NOCA, both plant and microbial communities are more insulated from changes in temperature and moisture, delaying root senescence and buffering against the seasonal changes seen at the other soils and in other studies. This is supported by soil temperature data – soils at the older heath meadow site are significantly cooler during summer months and significantly warmer during months of snow-cover compared to the other sites (Section 3.1.2).

Ultimately, my conclusions were that the heath meadows have lower availability of N and undergo fall N limitation, while the dry meadow has available labile N year-round and is not N-limited (Figure 2.8). Although reduced N availability may be attributed to greater plant uptake at the more vegetated heath meadow sites, a higher mineralization rate can also explain this. The

plant community at the dry meadow site is patchy but contains a higher proportion of plants (flowering forbs, graminoids) that have high N uptake and produce easily-decomposed high-N plant litter compared to evergreen shrubs (Wookey et al., 2009). I suggest that the presence and depolymerization of N-rich organic matter at the dry meadow site by the microbial community is also a main driver of N availability (Figure 2.8). Lower plant root N concentrations and higher lignin content in soil organic matter likely suppress N mineralization at the heath meadow sites.

Fall soil N mineralization capacity: mesic vs dry alpine meadows

One of the expected outcomes of climate change is a shift from snow to non-snow precipitation (Karl, 2009), exacerbating risk of alpine N leaching in response to N deposition. In particular, more non-snow precipitation instead of snow is locally predicted in the PNW for the autumn (Salathé et al., 2008), a time of heavy precipitation for the ecoregion in general. In contrast to alpine spring, when snow is melting but plant demand for N is relatively high (Jaeger et al., 1999; Bilbrough et al., 2000; Larsen et al., 2007), plants may senesce during the alpine autumn, leaving microbial uptake as the dominant form of N retention in soils. For this reason I was interested in whether inorganic N was immobilized or released in these alpine soils during the autumn and how active plant and microbial soil N sinks were at that time of year. Given the low C and N levels at the MORA site and the high C:N ratio of the NOCA site soils (24:1) I expected that these heath meadow soils would be immobilizing (Manzoni et al., 2010).

This was indeed the case - soils from both heath meadow sites immobilized N during fall. MORA soils were strongly immobilizing, and had a negative relationship between initial and final EIN during aerobic incubations (Figure 2.3). At NOCA I found that only one block (located on a ridge where volcanic topsoil appeared to have eroded) showed any capacity for mineralization. Lack of mineralization at the heath meadow sites may be due in part to Andisol

soil properties. Amorphous volcanic minerals can provide physical protection of SOC within nanopores (Sollins et al., 1996; Huang et al., 2014) and within soil aggregates (Huygens et al., 2005), protecting OM from microbial decomposition (Sollins et al., 1996).

In contrast, the dry meadow site (OLYM) soils had significant potential for mineralization and leaching in the fall, and were mainly limited by moisture and temperature in their capacity for releasing inorganic N (Figure 2.2). Dry meadow soil solution inorganic N supply was correlated with soil total N concentration, a trend not seen at other sites and indicative of strong microbial reliance on decomposition of the soil N pool (Manzoni et al., 2010). Large stores of organic N have built up at the OLYM site soils, likely through fine root turnover, N-fixation, limited leaching and lack of plant uptake during the latter half of the growing season, and possibly historic N deposition (Figure 2.8).

Microbial uptake protects against fall inorganic N leaching

Microbial N uptake during the fall can act as a buffer against excessive N leaching – as evidenced by the increased proportion of soil N within microbial biomass at the dry meadow site (Figure 2.7). Percentage of soil N within microbial biomass at the dry meadow site increased from 3.5% to 6% . Microbial biomass N increased from summer to fall at the dry meadow site by an average of 134 kg N ha^{-1} (in contrast to an average increase of 17 kg N ha^{-1} at MORA and a small decrease in fall microbial N at NOCA) (Figure 2.5). Nitrogen released through decomposition in summer is likely the origin of this N, as ambient deposition at this site averaged 0.4 kg N ha^{-1} yr^{-1} and the upper range for contribution of N fixation to alpine environments published thus far is approximately 10 kg N ha^{-1} yr^{-1} (Cleveland et al., 1999).

Thus, microbial N uptake at the dry meadow site appears mainly dependent on stores of easily-decomposed N in the soil. Microbial N in fall soils at the dry meadow site was strongly

correlated with total soil N (R^2= 0.78, n=20, $p<10^{-6}$ 1e-6) but not EON, indicating that de-polymerization of complex soil O.M. rather than mineralization of labile N is the controlling factor for microbial N uptake in these soils (Schimel and Bennett, 2004).

Response to Increased N Deposition

Because the dry meadow site soils were more N-rich and had less abundant vegetation cover than the heath meadow sites, I expected the dry meadow site to be most sensitive to changes in N chemistry with treatment (Figure 2.6). The dry meadow site was the first to respond with increased soil solution NO_3-N supply at the 10 kg N ha^{-1} yr^{-1} treatment level in Year 2, but this response did not increase in Year 3 of the study. The only other treatment response at the dry meadow site was an increase in the proportion of soil N contained within microbial biomass.

I expected that at the two heath meadow sites, plant uptake and low soil mineralization capacity would buffer against increases in soil inorganic N in response to treatment. However, I did not find this to be the case – soil inorganic N supply was just as, or more, sensitive to treatment at the heath meadow sites compared to the dry meadow site. In addition I observed increased extractable NO_3-N during the summer and decreased EOC during the fall in response to treatment, which I did not observe at the dry meadow site (Figure 2.6, Figure 2.4).

It is likely that increased N deposition during the fall stimulates microbial activity and subsequent uptake of organic C, resulting in the observed decrease in fall EOC at these sites with treatment (Figure 2.4). Similar results were found in a study of N deposition effects on the Tibetan Plateau, where N treatment significantly increased decomposition of particulate organic C (while increasing retention of large-fraction organic C) (Fang et al., 2014b). However, in a study of a different meadow ecosystem in the area, applied N had the opposite effect (Seok et al., 2016). At that site, C was considered the limiting factor, not N, and labile C inputs increased soil

respiration. In my own study, increased labile C uptake in response to treatment may be contributing to the increased variability in soil solution inorganic N supply observed from the 5 to the 10 kg N ha^{-1} yr^{-1} treatment level. It appears that in the heath meadows, microbial communities were stimulated by increased N availability to increase uptake of labile C. I suspect that the microbial population of the older heath meadow site may have undergone a community shift to one better suited to make use of inorganic N, actually decreasing NO_3-N leaching at the highest treatment level, although I saw no significant changes in microbial biomass N with treatment. These alpine soils in mesic meadows may be a sink for N deposition in the autumn.

Response to increased N deposition: mesic meadows vs dry meadows

My proposed explanation for the differences between the sites in response to N treatment is that in alpine ecosystems with continuous soil and vegetation, such as those found at the more mesic heath meadow sites (MORA and NOCA), N becomes highly limiting at some point during late summer or fall due to growing season plant uptake and low soil mineralization potential. Nitrogen deposition during this time of year primes previously N-limited r-selected (copiotrophic) microbes to make use of labile organic C (Figure 2.8). In alpine systems where ample soil organic N is available for mineralization, such as the dry meadow site of this study, there is much greater potential for fall N leaching in that there are still appreciable levels of NH_4-N in fall soils (Figure 2.5). In such non-N-limited systems, microbial uptake plays a much greater role in retention of N in soils.

I found that microbial N uptake did control leaching of N in the case of the dry meadow ecosystem at Olympic, but only when soil N concentration was already accounted for. Deposition at the dry meadow site would have to exceed 70 kg N ha^{-1} yr^{-1} for treatment effects alone to cause a significant change in microbial N in my model. This is unsurprising as the

seasonal variation in ambient average microbial biomass N was nearly two orders of magnitude higher than the highest level of N fertilization used in this study (10 kg N ha^{-1} yr^{-1}). That I observed any treatment effect on microbial biomass N is surprising, given that such effects have only been seen at very high treatment levels in other studies of cold-environment soil N cycling. For example, increases in the microbial N pool with N application, independent of other factors, were observed at Niwot Ridge at a treatment level of 25 g N m^{-2} yr^{-1} (250 kg N ha^{-1} yr^{-1}) (Fisk and Schmidt, 1996), and in arctic soils at an application rate of 10 g N m^{-2} yr^{-1} (100 kg N ha^{-1} yr^{-1}) (Buckeridge et al., 2009).

Sensitivity of mesic North Cascade heath meadow soils to N addition

I expected the demand for N by plants and microbes at the heath meadow sites to buffer effects of N additions. This was the case for the relatively nutrient-poor soils at MORA. However, the fine-ash Andisols at the NOCA site were by far the most sensitive to changes in soil chemistry in response to N treatment among all three sites, despite the high C:N ratio of these soils.

I was particularly surprised by the mid-summer increase in the soil NO_3-N pool at NOCA, 3 weeks after N application and after a heavy precipitation event (Figure 2.4). It is doubtful that the ephemeral increase in soil NO_3-N concentration I observed was the original N application remaining unchanged as NO_3-N in the soil. The mean residence time of solution NO_3-N in soil has been shown to be 24 hours or less in a wide variety of soils (Stark and Hart, 1997; Booth et al., 2005). And although volcanic soils have potential for NO_3-N adsorption which might prevent uptake by plants, NOCA soils contain large amounts of organic matter complexed with volcanic ash that would inhibit nitrate absorption capacity (Nambu and Yonebayashi, 2000; Strahm and Harrison, 2007). Thus I must attribute the change in NO_3-N to increased microbial cycling of N

including temporarily increased nitrification, possibly combined with low plant uptake (though doubtful, given the lush and continuous vegetation at this site).

Critical loads for PNW alpine meadows

Despite differences in sensitivity and response to N deposition, all three sites experienced changes in C and N cycling at the highest application level of 10 kg N ha^{-1} yr^{-1}. The average yearly ambient N experienced by any of the sites in this study was 0.5 kg N ha^{-1} yr^{-1} (at OLYM). Thus, after 3 years of N application, I suggest a critical N load of inorganic N for PNW alpine meadow soils of between 5.5 and 10.5 kg N ha^{-1} yr^{-1}. For NOCA soils, which were more sensitive to N application, I suggest a critical load upper limit of 6 kg N ha^{-1} yr^{-1} - the lowest level of ambient N deposition plus treatment at which change in N availability was detected.

Leaching Risk

The times of highest leaching potential in alpine meadows are during snowmelt in spring (usually June and July in the PNW) and during fall rains before temperatures are low enough for a snowpack to form (usually mid-to-late October in the PNW) (NOHRSC, 2017). Of the three sites only one of the heath meadow sites (MORA) received the majority of ambient N deposition during the months of snowpack cover (Oct-June) (Figure 2.1). Soil N stores appeared to be a much larger contributor to spring N flux in soils than N deposition as snow. I saw no relationship between soil solution inorganic N supply and N deposition through snowmelt. For example, soil solution inorganic N supply (as measured by PRS probes inserted in late fall and retrieved after snowmelt) was significantly higher at the dry meadow site despite this site having the lowest levels of N deposition. As the PRS probes measure both inorganic N from precipitation leaching through the soil and inorganic N supply resulting from decomposition, this data suggests that ambient microbial inorganic N soil supply (the mineralization of organic matter and nitrification

of ammonium) is the dominant control over inorganic N leaching potential at current levels of deposition in the PNW.

Plants and soil microbial communities of PNW alpine meadows appear to have strong seasonal N uptake that buffers against leaching at the times when waterflow through soils is most likely (snowmelt and fall rains), as long as levels of ambient N deposition remain low. Accumulation of N in the soil system, rather than leaching, may be the cause of shifting species composition in in the future at these sites.

CONCLUSIONS

My three objectives were to: 1) Assess background C and N cycling in alpine meadow soils of the Pacific Northwest in the United States, 2) Study the effects of simulated increased N deposition on these meadows and assign a critical load for N deposition in these soils, and 3) Put my findings into the larger context of precipitation patterns and alpine plant uptake in the Pacific Northwest to give recommendations regarding the greatest ecological dangers of N deposition.

1. I found overall that while background soil N cycling partially conformed to models of alpine N cycling developed in the Rocky Mountains, differences in soil N cycling and in response to simulated N deposition were site-specific.
2. I suggest that the critical load for alpine N deposition in PNW meadows is between 5.5 and 10.5 kg N ha^{-1} yr^{-1}. However, the lowest level of deposition (ambient + treatment) at which I observed in soil N chemistry was 6 kg N ha^{-1} yr^{-1} at North Cascades National Park, suggesting that the upper limit for the N critical load should be set at 6 kg N ha^{-1} yr^{-1}. This indicates much greater N sensitivity than soils studied at Niwot Ridge, where N cycling response to N deposition was detectable at an application level of 20 kg N ha^{-1} yr^{-1} (Bowman et al., 2006), but is similar to the 10 kg N ha^{-1} yr^{-1} value found at Rocky

Mountain National Park (Bowman et al., 2012).

3. Plants and microbes in dry graminoid/forb alpine meadows of the PNW appear limited by moisture rather than N and have high N availability even into fall, and so have a higher probability of N leaching at that time of year. However, a microbial community adapted to high levels of N can serve as a buffer against N leaching in the short term. In contrast, moist heath meadows dominated by evergreen shrubs appear to be N sinks in the fall, with N application stimulating microbial uptake of organic C. However, during the growing season N is still readily available in soils after snowmelt. Atmospheric N additions are at greater risk of leaching during this season because the microbial community at these sites is adapted to low N and cannot compensate in the short term. Changes in alpine soil chemistry in response to N treatment can be site-specific and result from differences in plant uptake and soil N mineralization capacity, indicating different regimes for response to N deposition.

Table 2.1: Significant soil responses to nitrogen addition. Significant soil C and N responses to treatment with no other covariates in linear mixed models: ↑ or ↓ indicates whether response variable increased or decreased in response to treatment, number indicates treatment level at which response to treatment was significant ($p<0.05$) in kg N ha^{-1} yr^{-1}.

Response variable	MORA	NOCA	OLYM
Soil NO_3-N supply	↑10	↑5	↑10
Soil NH_4-N supply		↑5	
Extractable soil organic C	↓10	↓10	
Extractable soil NO_3-N		↑10	
Microbial N % of Total N	↑10		↑5

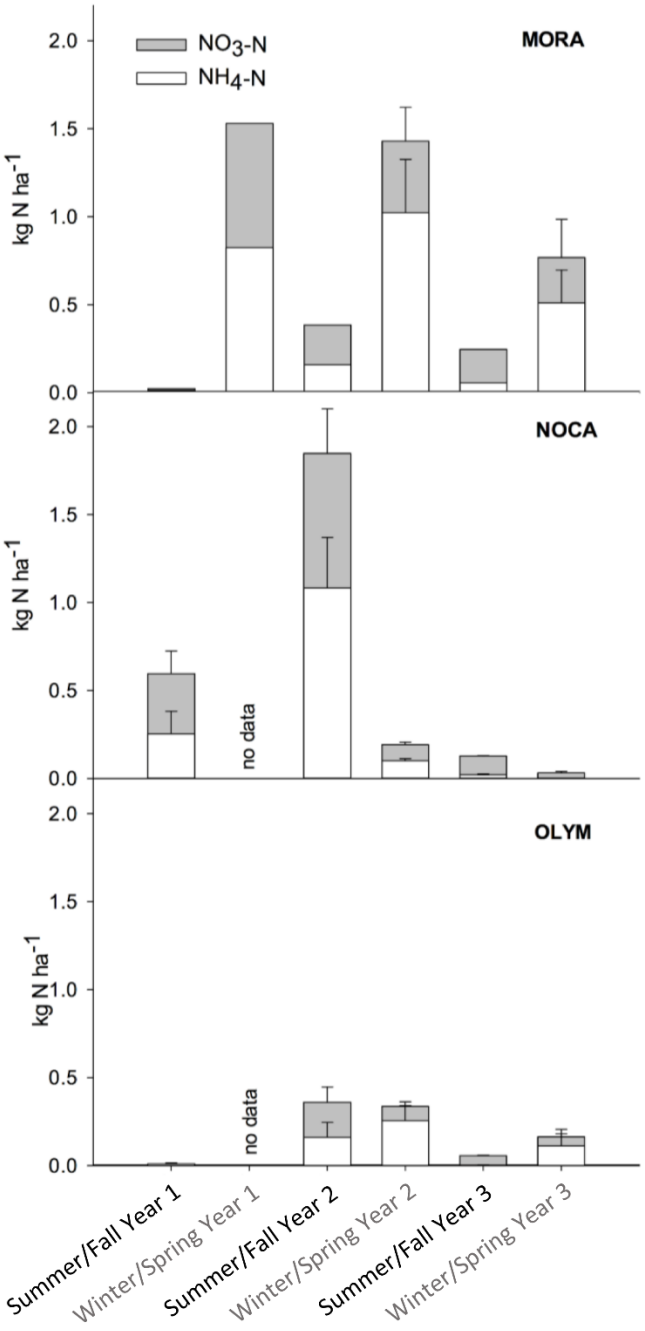

Figure 2.1: Background inorganic N deposition during the study period (±SE). N deposition data are divided into the months of July through early Oct (summer/fall, when snow has melted and snow precipitation = 0), and the months of late Oct through June (winter/spring, months of snow cover until snow melt). Data from winter of Year 1 are not available for NOCA and OLYM due to problems with resin tubes.

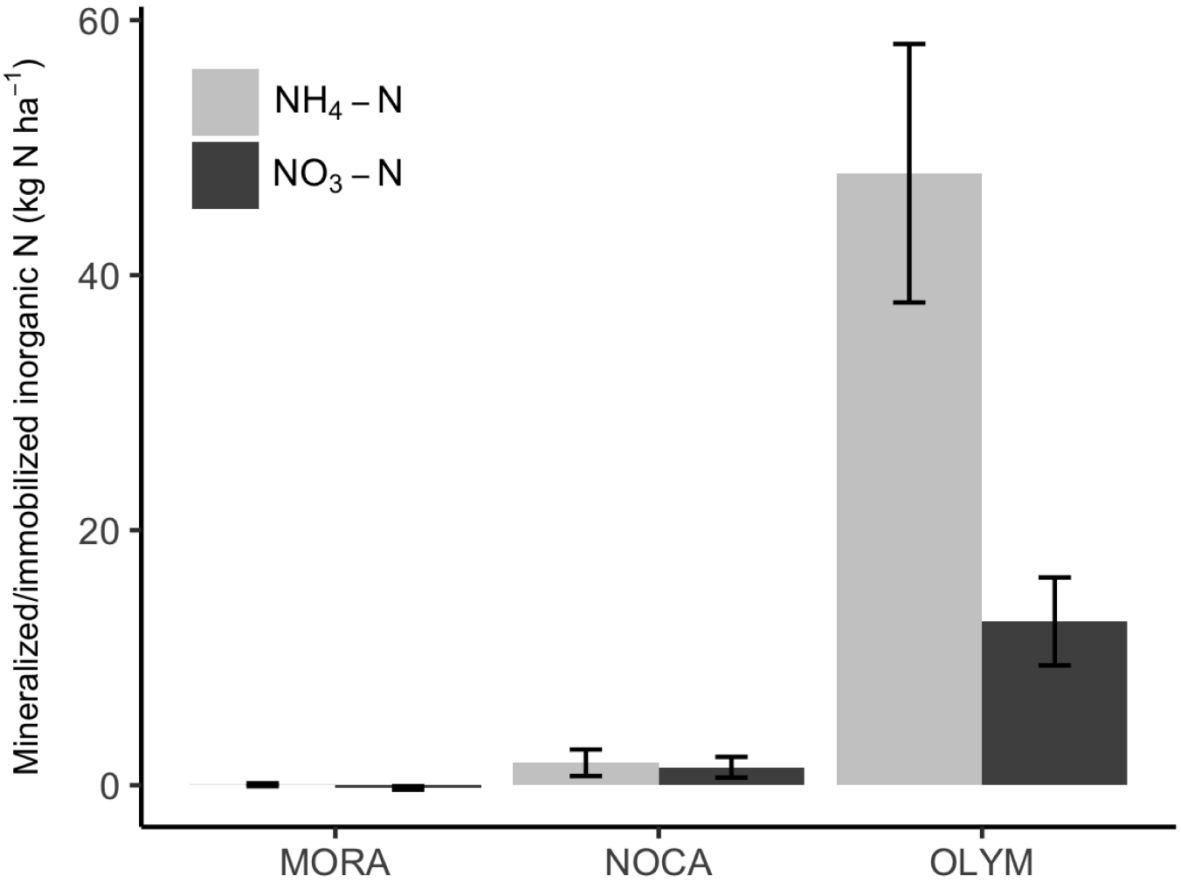

Figure 2.2: Average change in NH_4-N and NO_3-N after 30-day room-temperature aerobic incubation at field capacity for fall Year 3 soil samples (+/- SE). MORA showed very low levels of immobilization and mineralization. NOCA showed moderate mineralization of NH_4-N and NO_3-N. OLYM were strongly mineralizing for both NH_4-N and NO_3-N.

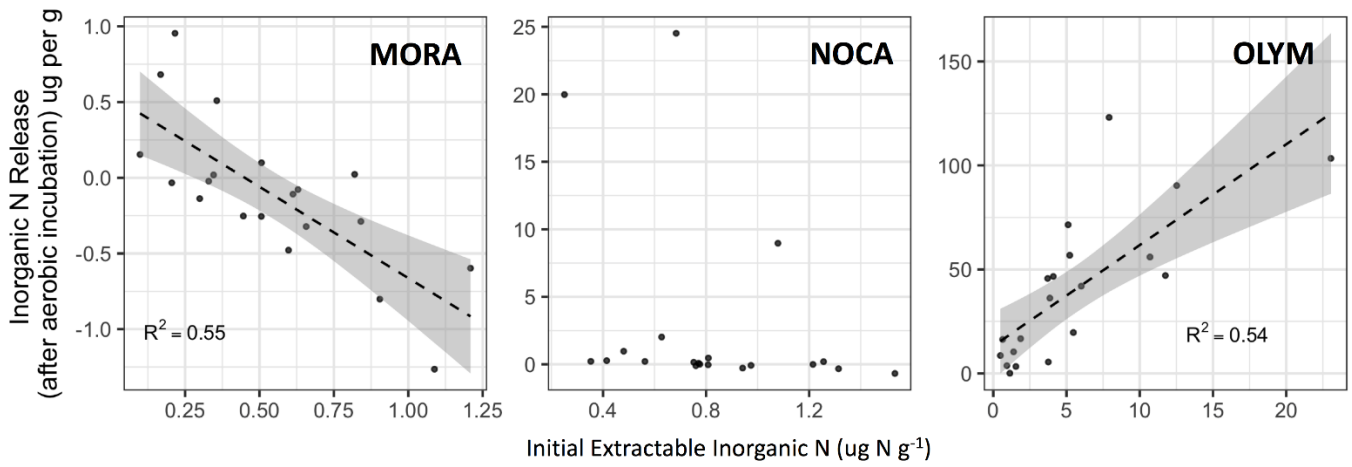

Figure 2.3: Relationship between initial total soil extractable inorganic N and soil inorganic N release after 30-day in-lab aerobic incubation at each site with 95% confidence interval. Soil samples were collected at the end of the growing season (late September/early October) in Year 3. There was a negative correlation in MORA soils between initial organic N and N soil release, vs a positive correlation at OLYM (also, note different scales of axes). Mineralization and N release in NOCA soil samples had no relationship to the initial inorganic N pool.

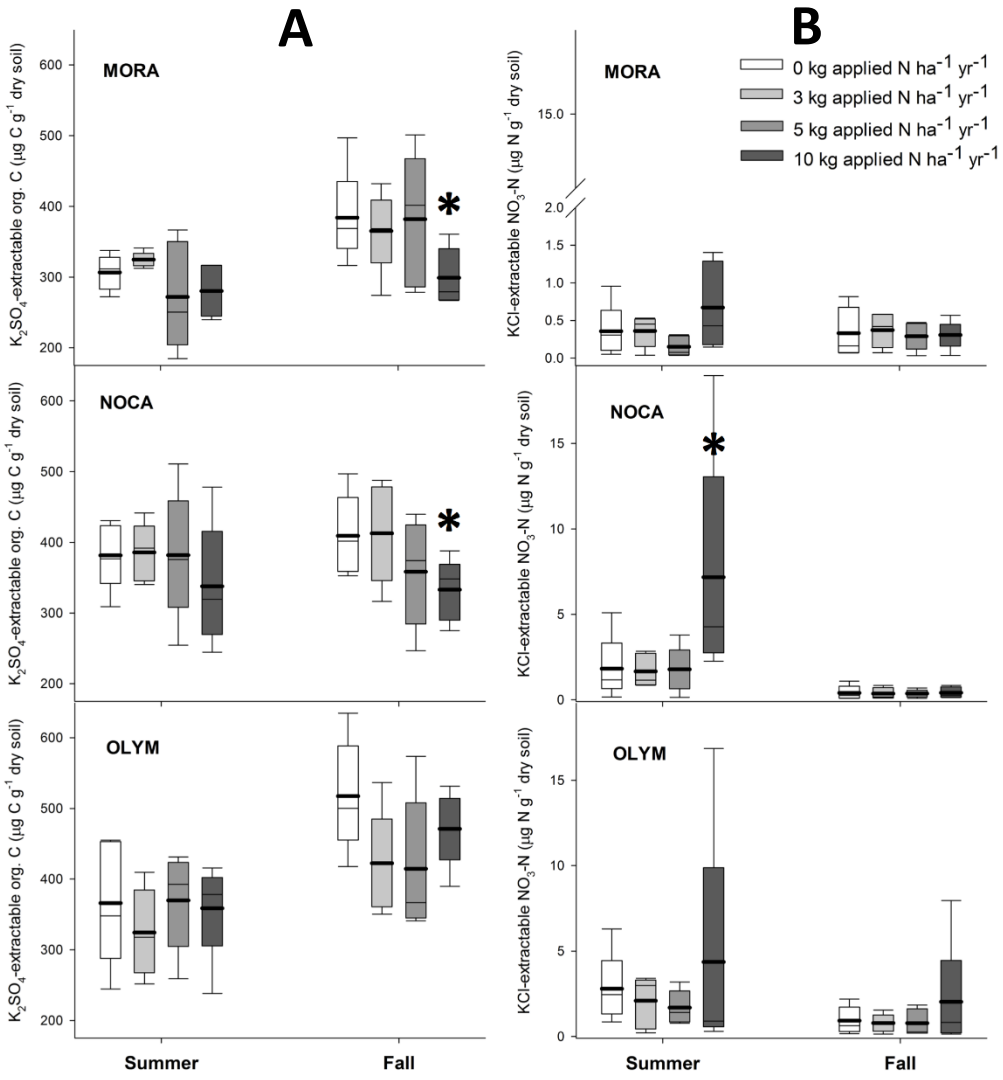

Figure 2.4: End-of-study (Year 3) soil pool responses to increased application of N for A) K_2SO_4-extractable organic C and B) KCl-extractable NO_3-N. Soils were collected and analyzed in summer (mid-growing season, in this case mid-August), and again in the fall (late September/early October). "*" indicates significant difference from control plot ($p<0.05$).

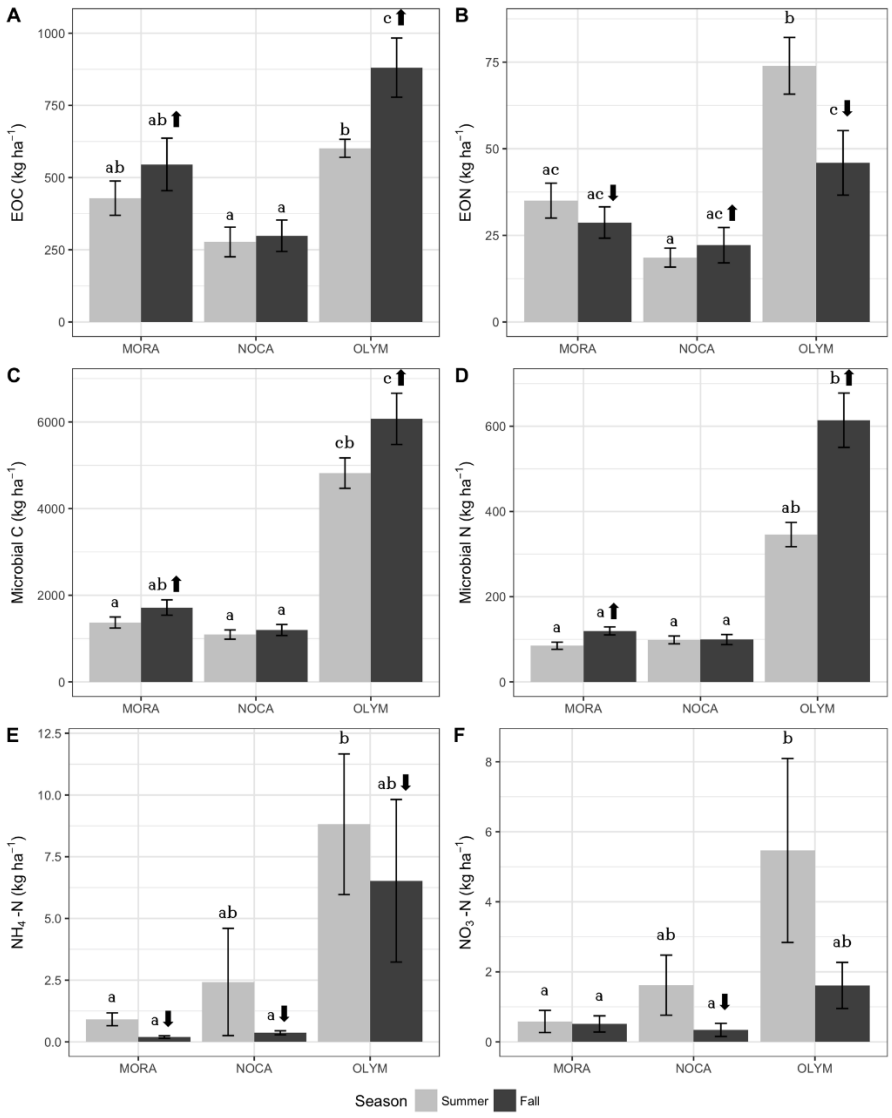

Figure 2.5: Site-ambient extractable soil C and N pool from control plots. A: EOC, B: EON, C: Microbial C, D: Microbial N, E: NH_4-N, and F: NO_3-N from Year 3, averaged across season and site (±SE.). Letter codes indicate significant groupings using Tukey's HSD Test ($p<0.10$), and arrows indicate significance in paired t-tests comparing summer and fall values in each plot ($p<0.05$). Soils were collected and analyzed in summer (mid-growing season, in this case mid-August), and again in the fall (late September/early October). For microbial C and N, all treatment levels are shown, since fertilizer treatment did not independently affect those soil variables.

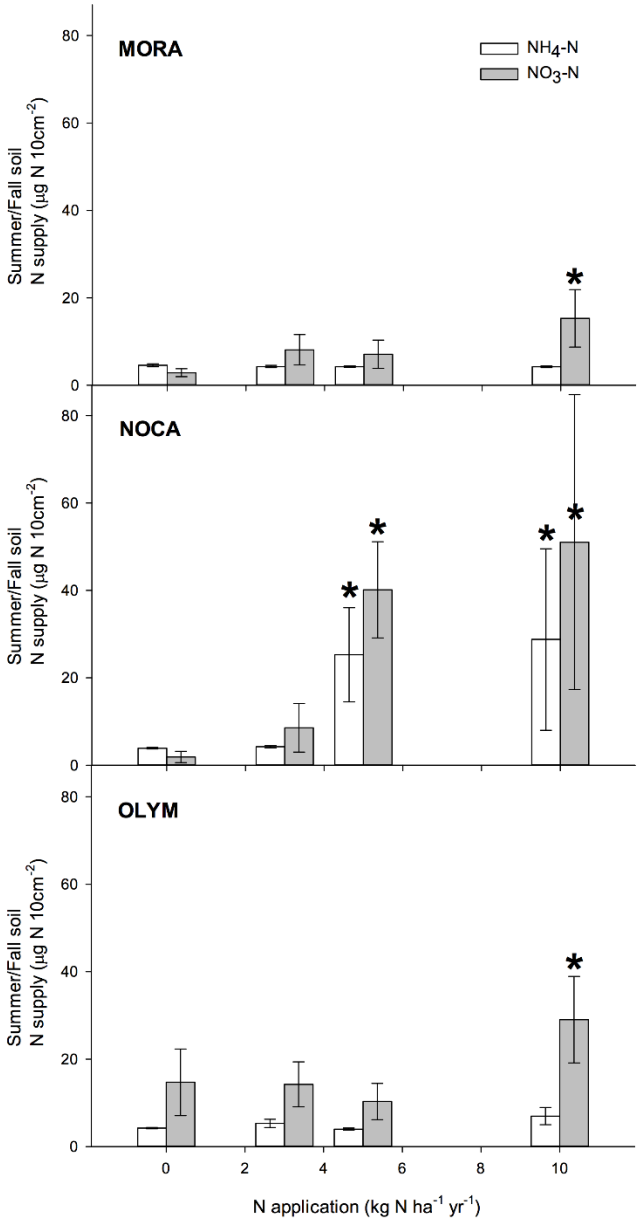

Figure 2.6: Soluble, plant-available soil inorganic N supply in response to increased N application for Year 3 (±SE) as measured by PRS probes. "*" indicates that N supply was significantly higher than control levels ($p<0.05$). PRS probes were in place from late July to early October. Burial periods for Year 3 for MORA, NOCA, and OLYM were 72 days, 64 days, and 66 days respectively.

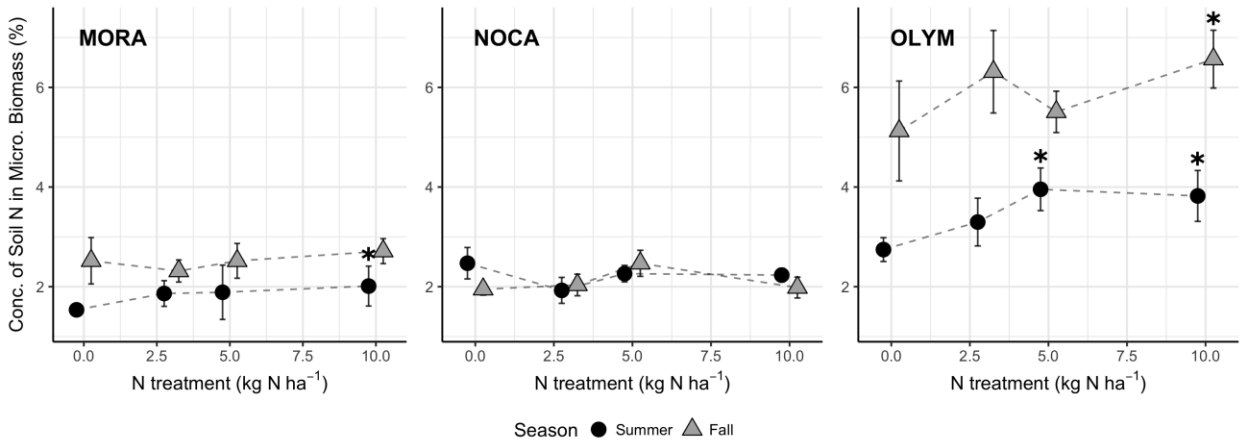

Figure 2.7: Fertilizer treatment effect on percentage of total soil N contained within microbial biomass. "*" indicates significant difference of the treatment level from control plots in a linear mixed model with block as random effect. MORA soils had elevated % microbial N of total N during the summer at the highest level of treatment; OLYM soils had elevated % microbial N of total N at the 5 and 10 kg N ha^{-1} yr^{-1} level during the summer at the 10 kg N ha^{-1} yr^{-1} level during the fall. Fertilizer treatment had no effect on percentage of soil N within microbial biomass at NOCA.

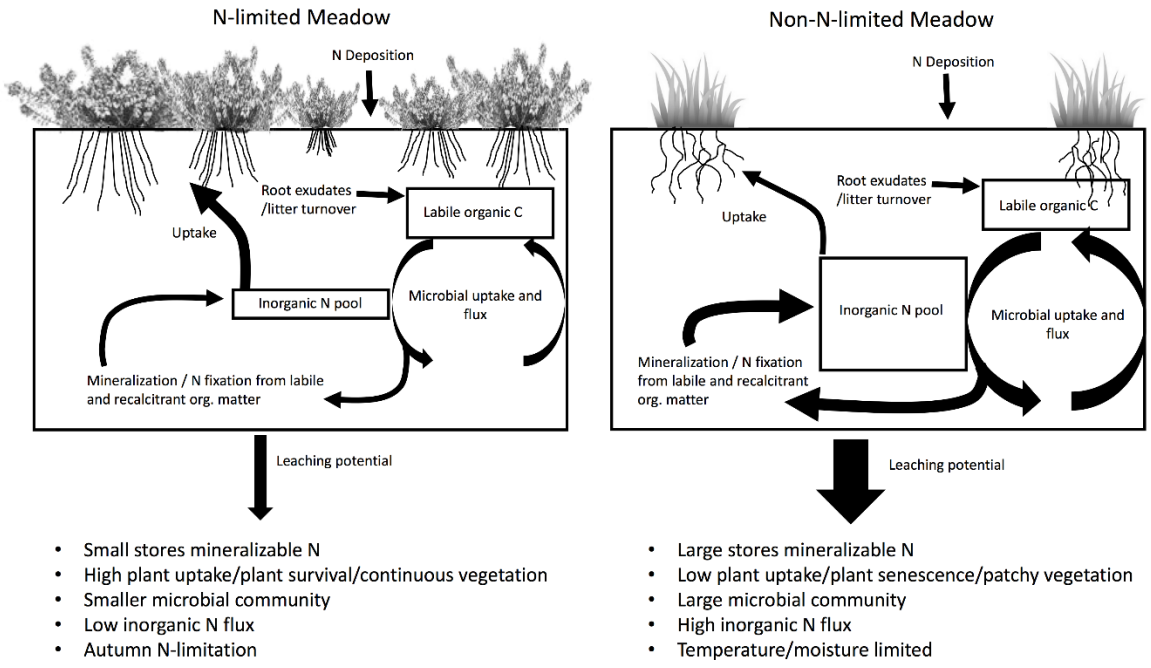

Figure 2.8: N-limited vs Non-N-limited alpine meadow PNW fall N cycling. High ambient levels of inorganic N flux at OLYM/non-N-limited sites lead to little stimulation of the microbial community with addition of inorganic N, and subsequent danger of N leaching.

Chapter 3: Nitrogen limitation and vegetation community effects on accumulation of N pollution in alpine meadows

Abstract

Nitrogen (N) pollution is encroaching on remote and pristine ecosystems such as alpine and subalpine meadows. Deposition of reactive N threatens to alter high-elevation plant community composition. Mitigation of N pollution in previously oligotrophic ecosystems is extremely difficult. In previously polluted areas where N depositions has decreased to below critical loads, N pollution has left a lasting effect on soil chemistry and plant species composition. Land managers are increasingly focusing on identifying and protecting pristine ecosystems not yet affected by N pollution. Nitrogen-limited alpine ecosystems should be assessed for background N cycling and response to reactive N where levels of ambient N deposition are still relatively low. To address this issue, I used N fertilization to mimic increased deposition at three alpine meadow sites in the Pacific Northwest (PNW) of the United States: a young, N-limited heath meadow, an older, seasonally N-limited heath meadow, and a comparatively N-rich dry meadow dominated by sedges and grasses. Fertilization levels ranged from 0 to 10 kg NH_4NO_3-N ha^{-1} yr^{-1}. Each plot was randomly assigned a 5% ^{15}N label on either the NH_4^+ or NO_3^- ion in the applied fertilizer. My objectives were to: 1) Examine any changes in plant species abundance and diversity in response to N treatment; 2) Examine preference for NH_4^+ or NO_3^- in the foliar tissue of three high-biomass target species (*Lupinus* spp., *Cassiope mertensiana*, *Phyllodoce*

empetriformis); and 3) Explore how background soil N cycling and plant species composition affected recovery of applied ^{15}N in soil and root tissue.

I found no effects of N fertilization on plant community composition or diversity at any site after three years of treatment, despite significant increases in inorganic soil N. Heather species showed a preference for uptake of NO_3-N over NH_4-N. The dry meadow site had much greater soil and root retention of applied N compared to the more mesic heath meadow ecosystems. Site N availability had a strong effect on root retention of applied N. Soils where plants could depend on steady mineralization of soil N for uptake (non-N-limited soils) showed strong relationships between plant species and fertilizer retention in root tissue. Soils with very limited stores of N had fairly even retention of fertilizer in root tissue across species. More applied N was recovered in soils and roots in areas dominated by flowering herbaceous and graminoid species, and less was recovered in areas dominated by evergreen shrubs. Mid-growing season soil moisture availability and root biomass were the strongest predictors of fertilizer recovery in soil, with drier soils and greater root biomass positively predicting recovery. Where plant and microbe N limitation is extreme, N deposition is evenly accumulated. Where N limitation is not extreme, forbs and graminoids appear to accumulate low level N deposition in alpine soils before any measurable change in species abundance.

INTRODUCTION

Increasing nitrogen (N) deposition has been identified as a key factor causing changes to ecosystems worldwide (Bobbink et al., 2010; Pardo et al., 2011; WallisDeVries and Bobbink, 2017). In areas with low levels of ambient N deposition, periodic excess N may be incorporated into soils, stored in plant roots, or used for temporarily increased plant growth without any long-term effects on plant community composition or the soil microbiome. However, over many years

of deposition, N can accumulate in soil, roots, and microbial biomass until levels of N are high enough to cause changes in the plant community (Bowman et al., 2014). Ideally, mitigation efforts would take place long before plant community change.

However, the variability of alpine plant species strategies for acquiring N makes it difficult to identify plant response to N deposition. Alpine plants are most often limited by N, whether due to low levels of plant available N or to adaptive self-limitation (Bowman and Seastedt, 2001). But high N concentrations in alpine leaves are necessary in order to build photosynthetic capacity to support reproduction in the short weeks of the alpine growing season (Körner, 2003). Thus many alpine plants favor reproductive growth above vegetative growth, resulting in adaptations of dwarfism or early flowering and senescence in some alpine species (Bliss, 1962; Billings, 1974; Körner, 2003). These growth strategies do not allow many alpine plants to take full advantage of available excess N.

Alpine plant species with more opportunistic growth strategies that take advantage of available N are expected to increase in cover and biomass in response to N deposition (Bobbink et al., 2010; Bowman et al., 2012; Jin et al., 2015), which could lead to these species dominating an ecosystem at the expense of other plant species less adapted to quick growth and N uptake. This is not limited to alpine habitats; for example, a recent meta-study of 189 long-term N addition sites found that increased N deposition universally decreased plant species richness in both terrestrial and aquatic habitats (Soons et al., 2017).

Because of the wide range of growth strategies of alpine plants, the results of N fertilization studies in alpine plant communities are highly variable and dependent on species composition (Farrer et al., 2013). Studies conducted at Niwot Ridge and at Rocky Mountain National Park, CO (ROMO) found that *Carex* (sedge) species responded opportunistically to increased N and

Carex cover increased over the course of the study at Niwot Ridge (Bowman et al., 1995; Bowman et al., 2006; Bowman et al., 2012). In the alpine tundra of the Changbai Mountains in China, the fast-growing grass species *Deyeuxia angustifolia* quickly made use of available applied N via rapid growth and an increased number of tillers (Zong et al., 2016).

Heath species also appear to benefit in the short-term from increased N deposition; for example, the heath plant crowberry (*Empetrum nigrum*) underwent increased growth under heavy N deposition (Tybirk et al., 2000). In European heath meadows, *Calluna vulgaris* increased in biomass in response to N deposition over the short term (Edmondson et al., 2013). However, over long periods of N exposure (critical load 10-20 kg N ha^{-} yr^{-}) these meadows decrease in heath and bryophyte abundance and shift to a grass-dominated regime (Bobbink and Heil, 1993; Bobbink, 2003; Edmondson et al., 2013).

Other studies have found no changes or contradictory changes in alpine plant species abundance in response to N deposition. One study in the Alps of Switzerland documented a seven-fold increase in plant biomass following fertilization, while other studies in the same area found that increased N did not stimulate plant growth and concluded that those communities were not nitrogen-limited (Körner, 2003). Nitrogen fertilization studies at Niwot Ridge/ROMO found the treatment response of forbs to be relatively small and to consist of an increase in root biomass that took several years to manifest (Bowman et al. 2006; Bowman et al. 2012; Bowman, Theodose, and Fisk 1995).

This response is thought to be due to the adaptive strategy of most alpine forb species. For example, a case study of *Bistorta bistortoides* (American bistort) response to N fertilization showed accumulation of N in rhizomes but no corresponding response in plant growth – bistort plants simply relied less on soil uptake of N after N had been sufficiently accumulated in

rhizome tissue (Monson, 2006). Many alpine plant species grow potential flowers/leaf buds/shoots years in advance, and so possible growth in the current year can reflect conditions from several years previous (Bliss, 1962; Aydelotte and Diggle, 1997; Meloche and Diggle, 2001; Körner, 2003).

Because of this wide variety of alpine plant growth and N use strategies, studies of alpine plant response to N deposition must be conducted over many years and at very high levels of N deposition in order to see a potential response. This has meant that N deposition studies on alpine meadows have been extensively carried out at just a few locations, leaving large gaps in the N deposition literature. However, using low levels of N fertilization over shorter time periods, we can study applied N partitioning in soil, root and foliar tissue to predict which factors cause N deposition to accumulate within an ecosystem.

Due to increasing urbanization and agricultural production, N deposition is now a special concern to alpine ecosystems in the mountainous western U.S. (Fenn et al., 2003; Pardo et al., 2011; Cummings, 2014). In order to address this problem, I applied either $^{15}NH_4NO_3$-N or $NH_4{}^{15}NO_3$-N to plots in three different alpine meadow ecosystems in the Pacific Northwest of the U.S. to simulate increasing levels of N pollution. My objectives were to: 1. Examine any changes in plant species abundance and diversity in response to N treatment; 2. Examine preference for NH_4^+ or NO_3^- in the foliar tissue of three high-biomass target species (*Lupinus* spp., *Cassiope mertensiana*, *Phyllodoce empetriformis*); and 3. Explore how background soil N cycling and plant species composition – in particular, heather abundance - affected retention and recovery of applied N treatment in soil and root tissue.

METHODS

Experimental Design and Treatment

Plots were established at Paradise Meadows at Mount Rainier National Park (MORA), Sahale Arm at North Cascades National Park (NOCA), and Lilian Ridge at Olympic National Park (OLYM). Five blocks of four plots were randomly established on each ridge. Plots were 2 m x 1 m, established on areas of gentle or no slope. A $1m^2$ subplot was designated for vegetation monitoring, while the other $1m^2$ subplot was used for soil, root and vegetation sampling. At NOCA and OLYM three blocks were established on areas of 2-5% slope and two blocks in flat areas; at MORA two blocks were established on 2-5% slopes and three blocks on flat areas.

Nitrogen Treatment and Background N Deposition

Each plot within a block received a different treatment: 0, 3, 5 or 10 kg NH_4NO_3-N ha^{-1} yr^{-1} dissolved in 1 L of water, with two half-applications per year. The NH_4NO_3 was enriched with 5% ^{15}N, either as $^{15}NH_4NO_3$-N or $NH_4{}^{15}NO_3$-N. The location of the ^{15}N label (on either the ammonium or nitrate ion) was randomized by block and by plot. At each site, nine plots received $^{15}NH_4NO_3$-N and six plots received $NH_4{}^{15}NO_3$-N.

Plots were sprayed with 1 L of either ^{15}N-labeled NH_4NO_3-N solution or water twice during the growing season – once in early summer (late July) after snowmelt, and once in mid-summer (August). Precipitation was zero on days where plots were treated. Plots at OLYM were sprayed using DI water; plots at MORA and NOCA were sprayed using on-site water from snowmelt after determining that snowmelt N levels were negligible. A 0.5 m buffer around each plot was also sprayed to ensure that the rooting area of plants within the plots would be fully treated.

Ambient N deposition was measured in the summer using resin deposition collectors (Fenn and Poth, 2004) and in winter using resin balls within empty PVC pipes buried above the level of soil to collect deposition from rain and snowmelt.

Plant Community Monitoring

I monitored vegetation cover using 100-point-counts in mid-summer (mid- to late August) each year of the study. A 1 m^2 grid evenly divided into 10 cm^2 squares was placed on the vegetation monitoring plot, and a rod was dropped into the upper-right-hand corner of each square. Each species touching the rod was recorded. Because the last fertilizer treatment occurred after the Year 3 vegetation monitoring, I also performed 100-pt-counts during the summer after Year 3.

Measuring Recovery of N Treatment in Foliar Tissue

Vegetation samples were collected in late August/early September in Years Two and Three of the study (after full N application but before plant senescence). Highest biomass species among all three sites were *Cassiope mertensiana* (white mountain heather) and *Lupinus spp.* (lupine). At the two heath meadow sites, *Phyllodoce empetriformis* (pink mountain-heath) also comprised a large portion of biomass. I chose these three species for sampling.

For each sampling plot that contained one or more of the target species, several ramets/stems of *C. mertensiana* and *P. empetriformis.* and several leaves from different *Lupinus spp.* were removed. In the lab, new growth tips were removed from *C. mertensiana* and *P. empetriformis* stems, and lupine leaflets were removed from their petioles. New growth for *C. mertensiana* was identified using information from Rozema et al. (2009) and Rayback et al. (2012). The new growth tips and leaflets were oven-dried at 60ºC, individually ground and sent to the U.C. Davis Stable Isotope Facility for C, N, $^{13}C/^{12}C$, and $^{15}N/^{14}N$ analysis. In Year Two, foliar samples from

only the controls and highest treatment plots (10 kg N ha^{-1} yr^{-1}) samples were sent for analysis; in Year Three, samples from all N application levels were analyzed.

Measuring Effects and Recovery of N Treatment in Soil and Root Tissue

Soil and root samples were obtained using a 3 cm-diameter corer down to a maximum depth of 30 cm or to depth of rock. In Years One and Two, one core was taken from a random location in the sampling plot in early fall (late Sept/early Oct). In Year 3, cores were collected in mid-growing season (mid-August) and again in early fall (late Sept/early Oct). I collected multiple cores per plot in Year 3 in order to have sufficient soil for multiple C and N analyses. At MORA and NOCA, 2-3 cores were collected per sampling plot; additional cores were collected per plot at OLYM due to extremely shallow soil depth (Table 3). Only an A horizon was observed at OLYM; at MORA and NOCA some B horizon was evident near the bottom of cores (>15cm deep).

Soil samples were sieved to 2 mm, and soils >2 mm were placed in a 375-micron sieve and washed thoroughly under distilled (DI) water to remove excess soil. The resulting roots, rocks, and larger pieces of O.M. were air-dried. Roots were picked by hand, weighed, and ground. The <2 mm soil fraction was air-dried and ground.

Sieved <2 mm soils were analyzed for pH using a Sartorius combination electrode. Gravimetric moisture content was measured on a dry weight basis; soils were weighted, incubated at 105°C for 48 hours, and weighed again. Soil subsamples in all years of the study were extracted with 2M KCl and analyzed for NH_4-N and NO_3-N. Subsamples from all soils collected each fall were incubated for 30 days at field capacity under aerobic conditions, and reanalyzed using 2M KCl extractions for NH_4-N and NO_3-N. Soils collected during summer and

fall of Year 3 were also analyzed for microbial biomass C and N, and for K_2SO_4-extractable C and organic N (Brookes et al., 1985; Beck et al., 1997).

Root samples were sent for $^{15}N/^{14}N$ analysis and <2mm soil samples were sent for $^{13}C/^{12}C$ and $^{15}N/^{14}N$ analyses using an isotopes ratio mass spectrometer (U.C. Davis Stable Isotope Facility). In Year Two, only the control and 10 kg N ha^{-1} yr^{-1} soil and root samples were sent for stable isotope analysis; in Year Three, samples from all treatment levels were analyzed.

Calculation of Recovery and Retention of Applied N

I calculated total retention of applied N in kg N ha^{-1} and % recovery of applied N for root, soil, and new growth increment of target species *C. mertensiana*, *Lupinus*. spp., and *P. empetriformis* over the course of the study using equations modified from Akkal-Corfini et al. (2009):

$$\text{Retention} = [S_N * (\%^{15}N_{sample} - \%^{15}N_{control})] / [100*(T_{15N}/ T_N)]$$

$$\%\ \text{Recovery} = [S_N * (\%^{15}N_{sample} - \%^{15}N_{control})] / T_{15N}$$

where S_N = total measured amount of sample N (kg N ha^{-1} of root, soil or foliar tissue), T_{15N} = total amount of treatment ^{15}N applied during the study (kg N ha^{-}), and T_N = total amount of treatment N applied during the study (kg N ha^{-}).

To calculate S_N for new foliar tissue, I randomly selected 100cm^2 subplots in the sampling section of control and 10 kg N ha^{-1} yr^{-1} treatment level plots at the North Cascades site. I measured abundance of all species within each subplot, and then clipped all vegetation within the subplot. I trimmed annual foliar growth increments from my target species, oven-dried the tissue and weighed it. I then converted grams of new foliar tissue of target species per subplot to kilograms of foliar tissue ha^{-1} using values for cover of subplots and total abundance per plot as measured by 100-point-counts.

Verification of Mycorrhizal Status in Target Species

I wanted to be able to interpret uptake and recovery of inorganic N treatment in foliar and root tissue in terms of whether our target species had mycorrhizal associations that allowed for uptake of organic N. In order to verify that the mycorrhizal fungal associations of my target species were consistent with what other studies have found, I collected soil cores within the rooting zone of *C. mertensiana*, *P. empetriformis*, and *Lupinus spp.* at MORA and NOCA sites. Roots were separated from soil core samples by manual removal using tweezers. Roots of each species were examined under a dissecting microscope (Leica EZ4D) and qualitative data was collected - the morphological characteristics for each species were noted and pictures were taken using the built-in camera of the dissecting microscope.

C. mertensiana, *P. empetriformis*, and *Lupinus spp.* roots were stained to examine the mycorrhizal fungi for each species (Vierheilig et al., 1998). Before staining, root samples were cleaned by incubation in 10% KOH solution for 15 minutes, then boiled in 5% ink diluted in vinegar (5% acetic acid) for 3 minutes. After staining, the roots were rinsed with slightly acidic DI water (addition of a few drops of acetic acid). Roots of each species were examined under compound microscope for the presence of mycorrhizal associations by identifying hyphae, arbuscules, and/or tips.

Statistical Analysis

R was used to carry out all statistical analyses (R Core Team, 2014). I used one-way ANOVAs followed by Tukey's Honestly Significant Difference tests to determine differences in plant species richness and Shannon's and Inverse Simpson's diversity indices among sites.

Linear mixed-effects models were used to test for changes in individual species cover over the course of the study, as well as changes in $\delta^{15}N$ signature in roots, soil and leaves, in response to treatment in Year 3 (Pinheiro et al., 2016). PERMANOVAs with randomization set within

block (using the adonis function in R) were used to test for changes in plant community structure with fertilization over time, while PERMANOVAs with randomization set within site were used to relate vegetation community to recovery of applied N in soil and root tissue (Oksanen et al., 2016).

I used Pearson's and Spearman's rank correlation coefficients to examine relationships between environmental variables and percent recovery of applied N separately for each site. I selected environmental variables that I felt were either important to determining which plant species or soil conditions caused greater retention or loss of applied N or important explanatory variables that might mask treatment responses.

Selected independent variables were: number of species per plot, species diversity (Inv. Simpson's) in each plot, total soil N content, soil C:N ratio, seasonal change in microbial N, average root biomass, and soil moisture content (as measured in mid-summer). I included number of species per plot and species diversity because other studies (Miller and Bowman, 2002; Zhu et al., 2016) have found that plants with different rooting strategies can access different N pools in different locations in soil horizons, and therefore plots which are more diverse or have greater species richness may retain more N. I chose total soil N content and C:N ratio because they are indicators of the availability of inorganic N to plant roots. Seasonal change in microbial N was included as a proxy for microbial N uptake. I chose root biomass because plant uptake is generally the greatest mechanism for long-term N retention in soils. Finally, I included soil moisture content because soils that are dry may have limited N uptake, and therefore N might be flushed out of soil during fall rains rather than retained in foliar or root tissue.

RESULTS

Changes in Plant Species Abundance and Diversity in Response to N Treatment

Characterizing pre-treatment plant communities

Plant community composition was significantly different among all three sites using pairwise multivariate comparisons (PERMANOVA, df 1,38, $p<0.001$). The two heath meadow sites (MORA and NOCA) were dominated by heather (40 and 55% cover respectively) with secondary dominance of *Vaccinium* spp. and forbs, while the dry meadow site (OLYM) had a fairly even distribution of forbs, non-vascular ground cover (lichen and moss) and graminoid species (Table 3.1). See Appendix for species list.

Species richness per plot and Shannon's and Inverse Simpson's diversity indices were all significantly lower at the older heath meadow than at the younger heath meadow or the dry meadow (Table 3.1) ($p<0.001$, Tukey's HSD). The heather species *C. mertensiana* was a significant predictor of low plot-level diversity at all three sites and was almost always the most abundant species in any plot it was found in. Small graminoid and herbaceous species were, as might be expected, correlated with increased species diversity.

After root staining to confirm the presence of mycorrhizae, I found evidence of ericoid-like coiled hyphae but no presence of arbuscules or ectomycorrhizal tips in roots of *C. mertensiana* and *P. empetriformis*, indicating the presence of solely ericoid mycorrhizal fungi. In *Lupinus* spp. roots, I found arbuscules but no other signs of other types of mycorrhizal fungus.

Plant community response to N treatment

I found no significant changes in plant community composition, plant species diversity (Simpson's and Shannon's diversity indexes), species richness, or individual species cover with fertilization (Figure 3.1). Lupine cover significantly decreased in Year 4 (2015), presumably in response to significantly reduced snowpack and early snowmelt in Spring 2015

N Uptake in Target Species Foliar Tissue

Background trends in foliar N and ^{15}N

Of the three dominant plant species I selected to test for foliar uptake – two heather species, *C. mertensiana* and *P. empetriformis*, and one forb, *Lupinus sp.*, there were no significant site differences in foliar N concentration (Figure 3.2). Foliar N concentration was significantly higher in lupine leaves than in the new growth increments of the heather species *C. mertensiana* and *P. empetriformis* (Figure 3.2). There was no significant difference in N content between the two heather species. Foliar N content of *C. mertensiana* and *P. empetriformis* was greater in plots with greater species diversity. There were no significant differences in the background $\partial^{15}N$ values of foliar tissue among species or among sites.

Foliar uptake recovery of applied N

All three of my target species had significantly higher $\partial^{15}N$ with ^{15}N-labeled N treatment. Overall, the two heather species had significantly greater $\partial^{15}N$ response to treatment than lupine. Heather species had higher foliar $\partial^{15}N$ in nitrate-labeled plots, while lupine had higher foliar $\partial^{15}N$ in ammonium-labeled plots (Figure 3.3). Among the three sites, NOCA had significantly lower ^{15}N response in heather foliar tissue compared to MORA and OLYM, and OLYM had significantly higher ^{15}N signature in lupine foliar tissue compared to MORA and NOCA.

I recovered significantly more ^{15}N in the new foliar tissue of heather species than in the foliar tissue of lupine species. Applied ^{15}N recovery in *C. mertensiana* in the annual foliar increment was significantly higher at the dry meadow site than the heath meadow sites (Figure 3.4).

Soil and Root Retention and Recovery of N Application

Background C, N, ^{13}C and ^{15}N in soils and roots

Nitrogen and ^{15}N concentrations indicate an N gradient among sites, with the young heath meadow (MORA) as the most N-limited and the dry meadow (OLYM) as the most N-rich site.

The young heath meadow had significantly lower soil and root N concentrations than the other two sites (Figure 3.2). The dry meadow soils had the highest soil N and highest background $\delta^{15}N$ in soils and roots among all three sites; the background ^{15}N enrichment of the soil indicates significantly more soil microbial N processing at that site (Figure 3.2). Plant roots in control plots were significantly depleted in ^{15}N compared to plant foliar tissue and soil at all sites (Figure 3.2). The dry meadow site, and to a lesser extend the older heath meadow site, showed strong positive linear relationships between soil and root N, while the younger heath meadow showed no relationship between soil and root N (Figure 3.5), further indicating N-limitation at the younger heath meadow.

I tested the relationship between soil N and root N concentrations and vegetation community, using both PERMANOVA and distance-based redundancy analysis for each site. At all three sites, root N concentration was significantly associated with plant community. However, only the dry meadow site had a significant relationship between plant species abundance and soil N. In addition, after visual inspection of distance-based redundancy analysis graphs, vectors of soil and root N concentrations at NOCA and OLYM share the same general direction, while the relationship is orthogonal in MORA soils (Figure 3.6).

I found a negative correlation between evergreen shrub species abundance and root N concentration (Figure 3.7). At all three sites, analyzed both together and separately by site in linear mixed effects models, plots with greater abundance of heath species had lower bulk root N concentrations. This has been observed in other studies of heath meadows - for example, a study of two heather species and one grass species found that heathers had both significantly less N contained in root tissue and significantly less proportional recovery of N from senescing root tissue (Aerts, 1990).

Retention and recovery of N treatment in soils and roots

Bulk soil and bulk root tissue ^{15}N increased with increasing levels of ^{15}N treatment (Figure 3.8, Figure 3.9). Root tissue $\partial^{15}N$ and soil $\partial^{15}N$ were significantly higher at the dry meadow sites compared to the heath meadow sites at all treatment levels (Figure 3.8, Figure 3.9). The older heath meadow site (NOCA) was the only site to show an interaction effect between label type and treatment; soil $\partial^{15}N$ at NOCA was significantly higher in plots treated with ^{15}N-labeled ammonium than in plots treated with ^{15}N-labeled nitrate (Figure 3.9). Plots treated with ^{15}N-labeled nitrate had a significantly lower soil:root $\partial^{15}N$ compared to plots treated with ^{15}N-labeled ammonium, independent of site effects, presumably because nitrate more easily diffused to plant roots.

Effects of site soil properties and plant communities on recovery of N treatment in soils and roots

I recovered more applied N in both roots and soil at the dry meadow site compared to the heath meadow sites (Figure 3.10). Average applied N recovery in roots was 1.2, 1.1, and 2.4%, and in soil was 18.2, 26.1, and 40.8% respectively for MORA, NOCA and OLYM. The only significant difference in % recovery of NH_4^+ vs NO_3^- was in root % recovery at OLYM: I recovered more nitrate than ammonium in roots.

Overall, plant community was weakly related to root recovery of applied N ($p=0.09$), but not to soil recovery of applied N. When I looked at each site individually, root recovery of N was significantly related to vegetation community at NOCA at the $p<0.1$ level and at OLYM at the $p<0.05$ level; this relationship was non-significant at MORA.

To explore patterns in ecosystem retention of applied N fertilizer in soils and roots, I calculated Pearson's and Spearman's rank correlation coefficients between percent recovery and plant species groupings/environmental variables. Percent recovery of applied N in soils was for

the most part not correlated with any plant group (Table 3.2). I found a positive correlation between forbs (herbaceous flowering species) and soil N retention at the older heath meadow (NOCA) and the dry meadow (OLYM) sites. Non-vascular ground cover was a significant predictor of soil retention at the young heath meadow site and a negative predictor at the dry meadow site.

In contrast, recovery of applied N in roots was strongly associated with plant groups (Table 3.2). At the older heath meadow site and the dry meadow site, heath species were negatively associated with root recovery of N, and graminoid and forb species were positively associated with root recovery of N. *Vaccinium deliciosum*, an ericoid mycorrhizal species (Cázares et al., 2005) only present at the heath meadow sites, was also correlated with root N recovery, but negatively so at the young heath meadow and positively so at the older heath meadow.

Environmental variables showed some site-specific correlations with soil and root recovery of applied N (Table 3.2). Only the older heath meadow showed any relationship between species richness/diversity and retention of N: species diversity/richness was positively correlated with recovery of N in roots and negatively correlated with recovery of N in soil. This is probably due to the influence of forbs and graminoids on increased species diversity at this site, where often *C. mertensiana* completely dominates the plant community.

Background soil N properties had little influence on soil retention of applied N but a strong effect on root retention of applied N (Table 3.2). Although soil N concentration was directly used in calculating recovery of applied N in soil, it ultimately had no correlation with soil N recovery. At the young heath meadow, soil N negatively predicted root recovery of N, while the opposite was true at the older heath meadow and the dry meadow. Soil C:N negatively predicted recovery of applied N in root tissue at the older heath meadow site.

Unsurprisingly, root biomass was strongly positively correlated with root N recovery, as it was used to calculate root N recovery. Root biomass also had a significant effect on soil retention of N. Root biomass positively predicted treatment N recovery in soil at the younger heath meadow site and dry meadow site, and negatively predicted treatment N recovery in soil at the older heath meadow site. This may be because the older heath meadow site was more dominated by heather, and so root samples at this site taken plots with high root biomass were more likely to be dominated by heather roots. However, there was no significant relationship between recovery of treatment N in soil and presence of heather.

Seasonal change in microbial N was not associated with any differences in N treatment recovery in soil, but was associated with treatment recovery in root tissue at the dry meadow site. Soil moisture had a strong negative association with N treatment recovery in soil at the older heath meadow site.

DISCUSSION

Lack of Plant Community Response to N Treatment

The main ecological issue that this study addressed was whether, and how, N deposition is accumulated in Pacific Northwest alpine meadows, and whether any plant species are sensitive enough to N deposition to change in abundance over the course of a three-year study. As I expected over such a short time period and low application rate (0-10 kg N ha^{-1} yr^{-1}, plus average background deposition of 1.5, 1.0 and 0.4 kg N ha^{-1} yr^{-1} for MORA, NOCA and OLYM respectively), there were no changes in percent cover for any species or species grouping. However, I did recover significantly more applied N in soils at the dry meadow compared to the heath meadows, and significantly more applied N in root tissue in areas dominated by forb and graminoid species as opposed to heather species.

Foliar Preference for Nitrate vs Ammonium

I expected to see a preference for nitrate-^{15}N in the foliar tissue of lupine and preference for ammonium-^{15}N in the foliar tissue of heather species. Nitrate is more mobile in soils and a more likely candidate for diffusion towards plant roots, particularly given the likely allophane/imogolite and organic matter complexation in these volcanic soils (Strahm and Harrison, 2007) which would lead to very few anion exchange sites. In that case nitrate might be even more available in soil solution than in a more typical non-volcanic soil. But many species of the Ericaceae show extreme preference for ammonium (de Graaf et al., 1998), exhibit almost no nitrate reductase activity (Read, 1996), and in fact have retarded growth in the presence of nitrate (Havill et al., 1974). Thus I did not expect much nitrate uptake in the two heather species.

Instead, I found the opposite: a strong preference for nitrate-^{15}N uptake in the two heather species and a weak preference for ammonium-^{15}N uptake in lupine (Figure 3.3). Lupine preference for ammonium-^{15}N may be explained by the relative cost of nitrate reduction in plant roots versus stems and by peculiarities of lupine root exudates. Legumes, and lupines in particular, perform a large portion of nitrate reduction in root tissue, whereas many other plant species are adapted to perform nitrate reduction in stems where it can be directly coupled to photosynthesis (Andrews et al., 1984; Loss et al., 1994; Gavrichkova and Kuzyakov, 2008). The proportion of nitrate reductase activity located in the stem is directly linked to more efficient nitrate uptake in legumes (Andrews et al., 1984). Lupines in particular do not invest in stem nitrate reductase until they have been exposed to high levels of NO_3^- availability; as well, they do not excrete OH^- in any large quantities and instead rely on excretion of organic acids to maintain charge balance, limiting their capacity to take up NO_3^- (Loss et al., 1994). A study of *Lupinus albus* found that the energy cost of assimilating nitrate by reducing it to ammonium in plant roots was over twice the energy cost of assimilating ammonium, and that lupine preferentially took up

^{15}N-labeled ammonium (Gavrichkova and Kuzyakov, 2008). Therefore I assume a similar case for the lupine species at my sites: lupine species were not exposed to high enough nitrate levels to trigger investment in more efficient nitrate reduction, or this study was not long enough for those changes to occur (especially given the tendency of alpine plants to pre-form the cells for new growth up to several years in advance). The comparatively weak ^{15}N signal in lupine compared to heather is probably due to the contribution of biologically-derived fixed N with a δ^{15}N of near zero (Delwiche and Steyn, 1970).

The heather preference for nitrate-^{15}N is puzzling. I saw no pattern of preference for ammonium-^{15}N or nitrate-^{15}N soil or root retention in plots dominated by heather vs plots dominated by forbs/graminoid species. Given the lack of nitrate-^{15}N signal in heather roots, it is possible that *Cassiope mertensiana* and *Phyllodoce empetriformis* are taking up nitrate through leaves and stems, similar to canopy uptake of nitrate in red spruce (Bowden et al., 1989) and Douglas-fir (Fenn et al., 2013).

Environmental and Plant Species Effects on Retention and Recovery of Applied N

Background availability of soil N to plants and soil microbes in these alpine meadows strongly affected how N treatments were retained in soil and roots. At the strongly N-limited younger heath meadow, I saw very little relationship between plant species type and retention of N in roots and soil. Plants at this site may be so N-starved that all plants are operating at maximum N-use efficiency, and N diffusion rather than comparative root physiology governs uptake.

At the comparatively more N-rich older heath meadow and dry meadow, however, applied N accumulated in plots where forbs and graminoid species were dominant and root biomass was high. At both of these sites, forb abundance predicted higher treatment recovery in soils, and

heath abundance predicted lower recovery in roots. At the dry meadow, forb abundance predicted higher treatment recovery in roots. Recovery of N in roots was linked to areas of high soil N, and recovery of N in soil was linked to high root biomass. In these cases, plots already primed for higher N level usage accumulated N. I propose that in PNW alpine meadows without severe N limitation, N deposition accumulates in areas with high root N and high root biomass. In contrast, areas with extreme N-limitation accumulate N evenly across species and soil conditions.

Soil conditions and N recovery

I expected that seasonal change in microbial N (a proxy for microbial N uptake) would positively predict soil recovery of applied N, because microbial N uptake would function as a mechanism for N storage in soils. This did not occur. Seasonal increase in microbial N did positively predict *root* recovery of N at the dry meadow site, which was unexpected because microbial N uptake is generally in competition with plant N uptake. However, it may simply be that both plant roots and microbes in areas of high N availability were better primed to take up applied N when it became available.

PNW summers have very little precipitation and areas of greater soil moisture retention would have greater potential for root and microbial growth, so I expected soil moisture to positively predict retention of applied N. However, the only relationship I found between soil moisture and N recovery was at the older heath meadow – and soil moisture strongly negatively predicted soil N recovery. The older heath meadow site, with its organic-rich fine ash soils, had much higher water-holding capacity than soils at the other two sites and so at high moisture levels at that site leaching or denitrification of nitrate may have occurred.

Heather species and alpine N cycling

Many of the studies which have compared different alpine meadow types and their different responses to N deposition have been in geographical areas (the Rocky Mountains in the United States, the Tibetan Plateau in China) where 'dry' meadows are considered more N-limited. At my sites, the dry meadow site was similar in its soil C and N concentrations, and soil mineralization capacity, to meadow sites considered N-limited in some other studies (Bowman et al., 2006; Bowman et al., 2012; Schleuss et al., 2015; Zong et al., 2015). However, I did not consider this site to be N limited compared to the evergreen shrub meadows of the Western Cascades where the heath meadow sites are located; these ecosystems have significantly lower levels of soil total N and inorganic N. Relatively few studies have considered the effects of N deposition on alpine evergreen shrubs (Ackermann et al., 2015); research on N deposition effects on heath and heather species have mainly taken place in the Arctic or in lowlands which do not experience the same extremes of alpine climate. Even in the Scottish Highlands, where extremes of wind and temperature create a harsh environment in which heath species are dominant, snowpack rarely remains on the ground for more than two to three months of the year (Harrison et al., 2001).

Alpine heath meadows endure particularly harsh conditions compared to lowland heath meadows or Arctic tundra. Heathers are adapted to low-nutrient conditions and have high nutrient-use efficiency (productivity per unit of nutrient), but part of their growth strategy is to utilize their evergreen photosynthetic tissue year-round in order to compete with fast-growing deciduous species (Aerts, 1990). In lowland areas heather may be exposed to sunlight (not snow-covered) for a full 9-10 months out of the year (Aerts, 1990), or while in the high-sunlight summer months in the Artic heather may receive 19-20 hours of sunlight a day (4-5 more hours of sunlight than they receive at the latitude of the PNW). In both of those cases, heath species

can “break even” with deciduous species, in the same way that conifers match the NPP of deciduous trees by continuous low-level photosynthesis during winter months, when they can access soil N stores during a time when deciduous species are senescent or dormant.

However, the heather species in Pacific Northwest alpine meadows are only exposed to sunlight for approximately three to four months out of the year (~July-September/October) because PNW snowpack is deep and takes months to melt. In addition, PNW alpine heather do not receive the benefit of the long summer days of the Arctic. From my observations, deciduous plant senescence took place in early September (earlier for the dry meadow at OLYM). This gives alpine heathers of the PNW only a few weeks to a month of time in September-October to take advantage of lower deciduous plant uptake – a time of year when daylight and warmth are decreasing and meadows lack moisture before fall rains. This leads to extremely slow growth for heather in lower-latitude alpine meadows. For example, a dendrochronological study at Mount Rainier of the heather species *C. mertensiana* found that average growth of the species was less than 1 mm per year (Rayback et al., 2012).

Heather species survival, including *C. mertensiana* and *P. empetriformis*, depends on forming associations with ericoid mycorrhizal fungi which produce proteolytic extracellular enzymes and allow heathers and other ericaceous species to take up simple organic N (Read, 1996; Johansson, 2000). Some heather species, including *Cassiope tetragona,* a close relative of *Cassiope mertensiana* that is found in the Arctic, use ericoid mycorrhizae to gain advantage under snowpack. *Cassiope tetragona* actually increases in growth with deeper snowpack (Blok et al., 2015) because it has better access to N compounds that are released from slowly decomposing organic matter under deep insulating snow. *Cassiope tetragona* and other evergreen shrubs are able to use this N during late snowmelt; when snow is <30cm deep, light

can sufficiently penetrate for photosynthesis to take place, and up to 20% of evergreen shrub gross primary production can take place before snow has even melted (Larsen et al., 2007).

Thus it is likely that both of the heather species found at my sites begin photosynthesis before snow has fully melted; in addition, both showed ericoid mycorrhizal associations. A study of the subalpine forefront of a nearby retreating glacier in the North Cascades Mountains found that 97% of the *C. mertensiana* and *P. empetriformis* roots the authors collected contained ericoid mycorrhizal fungi (Cázares et al., 2005). My own investigations of heather roots from my field sites found evidence of ericoid mycorrhizal in all collected roots, although my sampling was not comprehensive.

Investigations of N sources in ericoid mycorrhizal dwarf evergreen shrubs in meadows and fell-fields has found that ericoid mycorrhizae rely on uptake of simple organic N compounds from fresh litter, rather than from slow mineralization of recalcitrant N (generally the largest store of N in a soil system) (Michelsen et al., 1996). Thus I hypothesized that plots with higher species diversity – i.e. plots with greater numbers of diverse, small herbaceous species – would, via root turnover and litterfall, introduce simple, easily-decomposed litter that would provide increased N. Heather species utilizing ericoid mycorrhizal fungi to take up organic N would therefore have higher foliar N content in plots with increased species diversity.

I found this to be the case: plots with higher foliar N in new growth increments also had higher species diversity. I therefore more closely examined the relationships between plant community and root and soil retention of N.

Soil and root retention of applied N

Nitrogen-limitation status of each site was linked to whether plant species abundance affected retention and recovery of artificial N pollution. Overall, I found that plant species

abundance at the older heath meadow and the dry meadow, which I considered to be less N-limited, had a significant effect on the amount of applied ^{15}N recovered in soil and root tissue. However, there was very little relationship between plant species composition and ^{15}N recovery at the highly N-limited young heath meadow site.

I expected that plots with higher root biomass would have higher soil N recovery because plant uptake and root turnover would function as soil N sinks. This was true for the young heath meadow and the dry meadow. However, root biomass negatively predicted soil N recovery at the older heath meadow site. This may be due to the prevalence of heather species at the older heath meadow; heather roots were associated with lower ^{15}N recovery and appear to contribute equally to root biomass at that site.

At the older heath meadow and the dry meadow, root ^{15}N recovery was higher in plots dominated by graminoid and forb species, and lower in plots dominated by heath species (Figure 3.11). I found that abundance of heather species at all three sites was correlated with lower root N (Figure 3.7), and so this trend can partially be attributed simply to the root N concentrations natural to heath vs forb/graminoid species. However, I saw no relationship between plant community and recovery of ^{15}N in roots at the younger, N-limited heath meadow site.

The linear relationships between root and soil N at the older heath meadow and dry meadow (Figure 3.5) suggests that the plant communities at those sites rely on soil stores of mineralizable N, while the plant community at the younger heath meadow may rely more on the early-season release of N from snowpack melt and microbial turnover, and on biologically fixed N. If all three sites shared this relationship, it might suggest that plant species in these communities that have higher N requirements (and subsequently greater N storage in roots) are found in areas of higher soil N concentrations. However, plant species preference for soil N does not account for the

relationship between soil and root N at the older heath meadow and not the younger heath meadow, since these two sites share similar plant communities.

I suggest that at the younger heath meadow site, plants rely heavily on uptake of labile organic N or biologically-fixed N due to severe N limitation and very low levels of available inorganic N. This organic N is mostly taken up during microbial turnover at snowmelt (Lipson et al., 1999), rather than being the product of breakdown of soil organic material. I base this conclusion on the relationships at these three sites between bulk soil N and bulk root N (Figure 3.5). At the older heath meadow and dry sites, bulk root N increased with bulk soil N, indicating that roots are accumulating N from mineralized soil N. I observed no such relationship at the younger heath meadow site, despite equivalent or higher levels of root N compared to the other two sites. Thus, the plant community at the younger heath meadow sites is probably relying mostly on another N source besides slowly mineralized N.

Increased N uptake efficiency can help explain the lack of relationship between soil N and root N at the younger heath meadow site. Severe N limitation can stimulate increased N uptake efficiency via investment in root transporters with higher affinity for N species (Kiba and Krapp, 2016). If all or most plant species at this site are investing heavily in root N transporters, effects of comparative species N demand and uptake ability may be muted. Plant investment in N uptake efficiency could also explain the negative linear relationship between soil N and recovery of N in plant roots at the N-limited young heath meadow site, which contradicts the positive linear relationship between bulk soil N and recovery of applied ^{15}N in roots that I observed at the other two sites (Table 3.2, Figure 3.12). Since only the dry meadow site plant community has any link to soil N, the selective preference of high-N-use plants for high-N soils is not sufficient to explain this trend either.

The negative relationship between soil N and ^{15}N recovery in root tissue at the N-limited young heath meadow site (Figure 3.12) may be explained, then, by extreme N limitation. Simply, in areas with extremely low soil N availability, plants made very heavy use of applied ^{15}N due to high demand and higher N uptake efficiency, while this ^{15}N signal was somewhat diluted in areas with more available soil N.

So, why did I see increased root recovery of N in areas with higher soil N at the other two sites? I propose that at those sites, soil N supply to plant roots is sufficiently high that it overwhelms the relatively small amount of N treatment applied to plots at all levels of soil N. Plant roots that have spent resources on greater N uptake capacity to take advantage of higher N conditions – through higher biomass, higher nitrogen transportation enzyme activity, etc. – take up more applied N as well. N in fine roots and foliage is thus retained and eventually recycled within the soil system. This means that in non-N-limited circumstances, N accumulates in areas where N is already high (Figure 3.13).

Results of studies of alpine plant and soil systems are often difficult to parse, because of the extreme variations in soil, temperature and moisture conditions that occur in alpine habitats over small scales (Körner, 2003). Nitrogen in particular is highly variable in alpine soils, with nutrient-limited conditions interrupted by N 'hot spots' (Darrouzet-Nardi and Bowman, 2011). Evidence from my study suggests that in PNW alpine meadows where N is already available to plants, N will accumulate most strongly in areas of high N availability. Therefore, even though overall conditions may be N-limiting for at least part of the year in alpine soils, these hot spots may have the capacity to leach N or to provide enough N for eventual plant community change.

CONCLUSIONS

Nitrogen (N) pollution threatens to alter alpine meadow plant community composition, and some alpine meadow sites in the United States have already experienced increased cover of graminoid and forb species in response to increased N. My objectives for this simulated N deposition study in Pacific Northwest alpine meadows were to observe whether any plant species changed in percent cover with treatment, to observe whether high-biomass plant species preferentially took up ammonium or nitrate, and to examine how, where and why applied N accumulated in soils and root tissue. I found the following:

1. There were no significant changes in plant species cover or diversity over the course of a three-year N application study.
2. I recovered more applied N in the foliar tissue of heather than the foliar tissue of lupine. New growth of heather accumulated more applied NO_3^- while new growth of lupine accumulated more applied NH_4^+. We suspect capacity for foliar uptake of NO_3^- in Pacific Northwest heather species.
3. There was a significant relationship between recovery of applied ^{15}N and the presence of forbs and graminoids, but only where N was not extremely limiting.

Severe N-limitation led to a more even distribution of applied N across plots. In contrast, at sites where plant roots had access to mineralized N and where root N was associated with soil N availability, N deposition accumulated in areas with high background root N concentrations. This trend may not only lead to eventual plant community change in high-N soils, but may explain why plant community change in response to N deposition is so difficult to identify in comparison to changes in soil nutrient availability. Land managers should be concerned with which topographic conditions are most likely to lead to ‘hot spots’

of N and which may be nuclei for future plant species shift in the face of increasing N deposition.

Table 3.1 : Average % abundance, species diversity and species richness at MORA, NOCA and OLYM sites, ±S.E. of the mean, averaged across treatment and year of study.

Plant % Abundance	**MORA**	**NOCA**	**OLYM**
Evergreen shrubs	40	55	9
Other flowering, herbaceous plants	17	6	25
Lupine spp.	13	5	4
Vaccinium spp.	10	15	0
Non-vascular (lichen, moss, soil crust)	10	9	33
Graminoid spp. (sedge, rush, grass)	6	3	24
Non-plant (bare ground, rock, O horizon)	3	7	5
Inverse Simpson's Diversity Index	4.6(0.3)	3.2(0.3)**	4.7(0.3)
Shannon's Diversity Index	1.8(0.1)	1.3(0.1)**	1.7(0.1)
Species Richness	11.2(0.5)	7.9(0.5)**	9.8 (0.4)

* indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$

Table 3.2 : Pearson's (ρ) and spearman's (r_s) correlation coefficients showing relationships between soil and root % recovery of applied N with plant groups or environmental variables (n=15 for each value). All data used to calculate these values was Year 3 of the study. Variables that were significant at the p<0.1 are bolded. Shaded rows indicate where an environmentalvariable was directly used to calculate percent recovery. *Arenaria* spp. (sandworts) were not included as forbs because they are woody.

	Soil						Root					
	MORA		NOCA		OLYM		MORA		NOCA		OLYM	
	ρ	r_s	ρ	r_s	ρ	r_s	ρ	r_s	ρ	r_s	ρ	r_s
Graminoids	0.02	0.17	-0.09	-0.07	0.34	0.25	0.04	0.36	**0.55***	0.4	**0.44**	**0.45**
Evergreen shrubs	0.32	0.27	-0.23	-0.28	0.04	0.01	0.14	0.01	**-0.51***	**-0.48**	**-0.50***	**-0.55***
Lupine	0.1	0.19	-0.32	-0.35	0.14	0.08	0.05	**0.45**	0.24	0.29	0.23	0.19
Vaccinium	-0.27	-0.07	-0.43	-0.38	NA	NA	**-0.45**	**-0.57***	**0.62***	**0.68***	NA	NA
Forbs (other)	-0.29	-0.38	**0.72****	**0.54***	**0.56***	0.41	0.03	0.15	0.31	**0.51***	**0.50***	**0.50***
Non-vascular ground cover	**0.48***	0.37	0.27	0.44	**-0.52***	-0.36	0.13	0.23	0.06	0.05	-0.3	-0.28
Species Richness	-0.19	-0.15	0.17	-0.04	0.23	0.11	0.34	0.29	**0.48***	0.41	0.41	0.37
Species Diversity (Inv Simpson's)	0.17	0.15	**-0.47**	**-0.58***	0.15	0.09	-0.04	0.06	**0.65****	**0.62***	0.41	0.39
Soil % N	-0.12	-0.06	0.42	0.36	0.16	0.22	**-0.61***	**-0.72****	**0.62***	**0.69****	**0.55***	**0.46**
Soil C:N	**0.50***	**0.48**	-0.40	-0.36	0.28	0.15	-0.31	-0.27	**-0.67****	**-0.66****	0.29	**0.25**
Seasonal Change in Microbial N	0.09	0.15	0.34	**0.48**	**0.46**	0.43	0.08	-0.15	0.12	0.14	**0.56***	**0.57***
Root biomass	**0.61***	0.36	**-0.47**	**-0.57***	**0.65****	**0.53***	0.24	**0.52***	0.41	**0.67****	**0.77*****	**0.83*****
Soil moisture	0.12	0.18	**-0.82*****	**-0.82*****	0.15	0.27	-0.08	-0.23	-0.2	-0.25	0.24	0.43
Soil depth	**-0.73****	**-0.57***	**-0.71****	**-0.70****	-0.16	-0.14	-0.28	**-0.46**	-0.01	0.06	-0.27	-0.3

* indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001

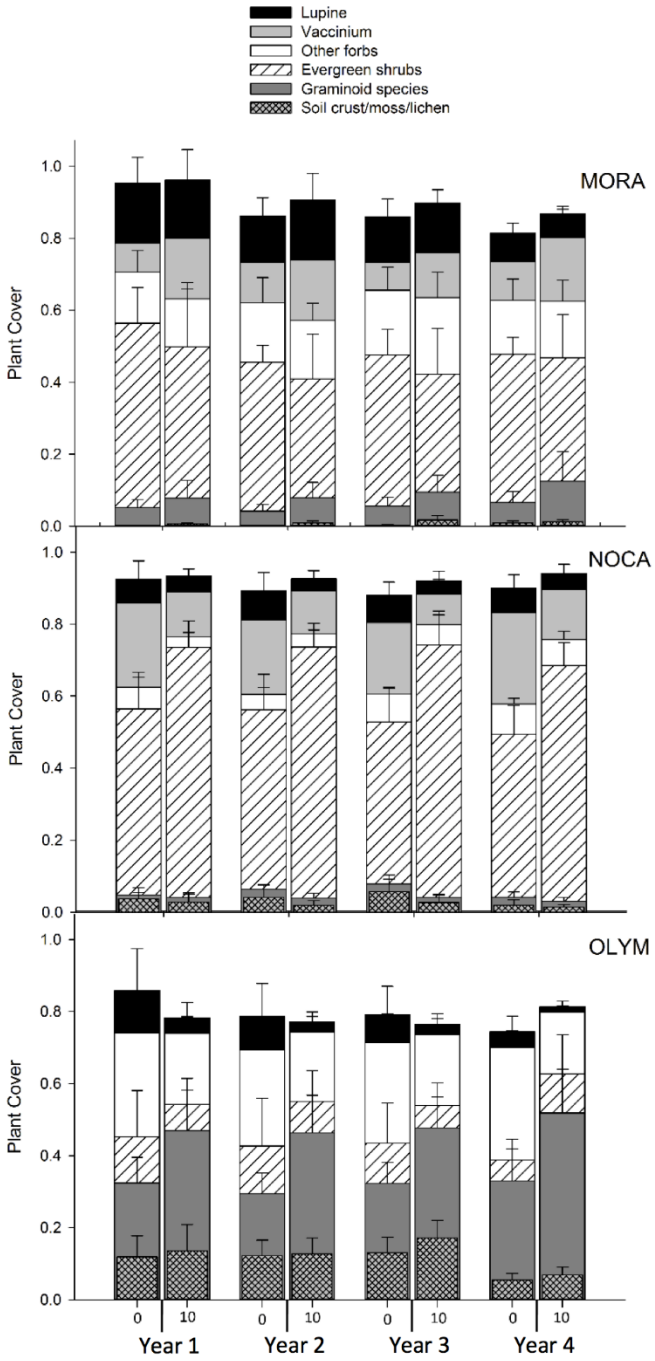

Figure 3.1: Mean plant cover data of dominant vegetation types, at treatment levels of 0 vs 10 kg N per ha per year, for all years of vegetation monitoring, ±S.E.

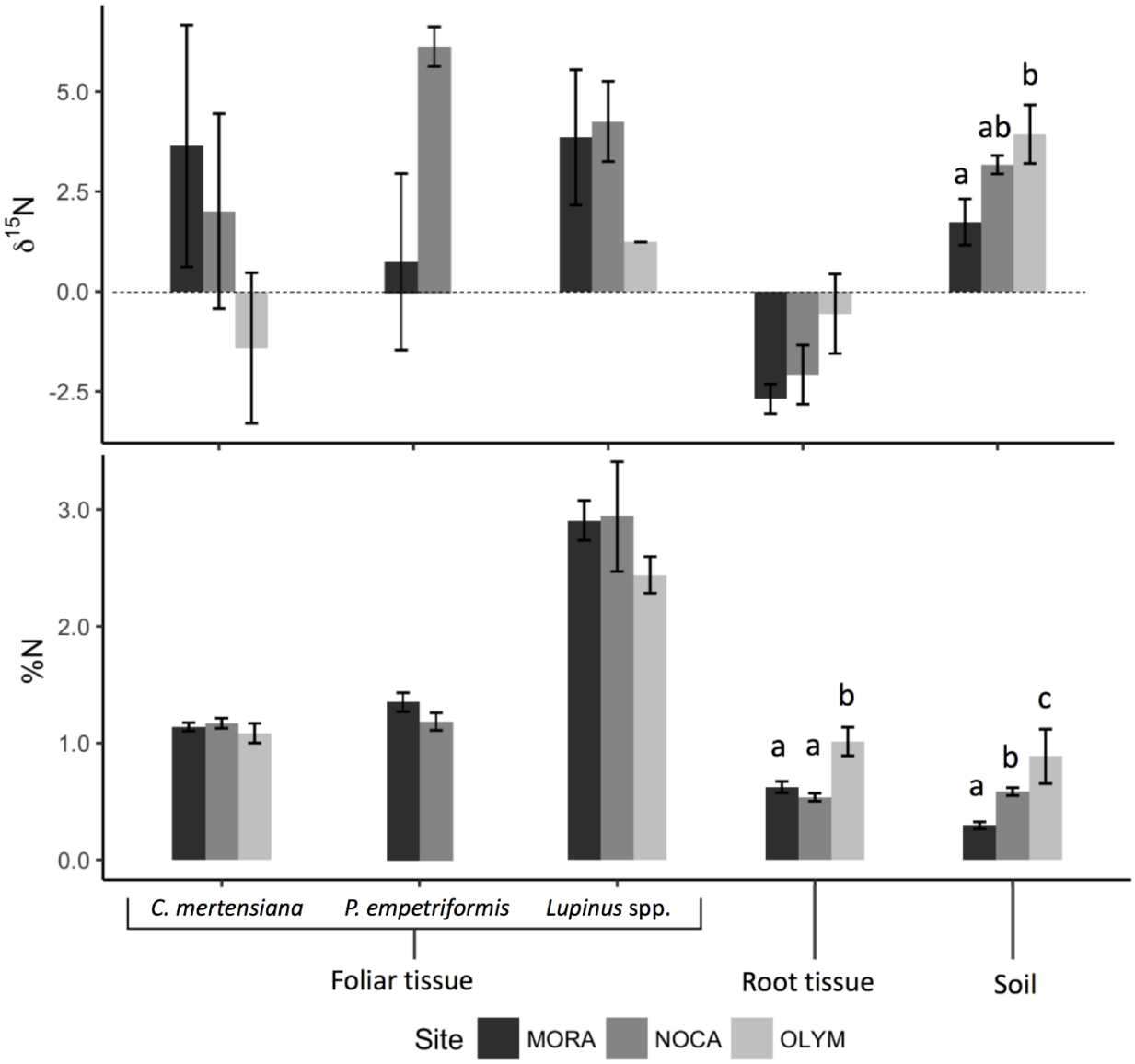

Figure 3.2: Background ∂^{15}N and %N values for bulk soil, bulk root tissue, and foliar tissue from target species at all three sites (control plots) (±SE). Letters indicate significant site difference at $p<0.05$ among each factor. Where no letter codes are provided, there were no significant differences among sites.

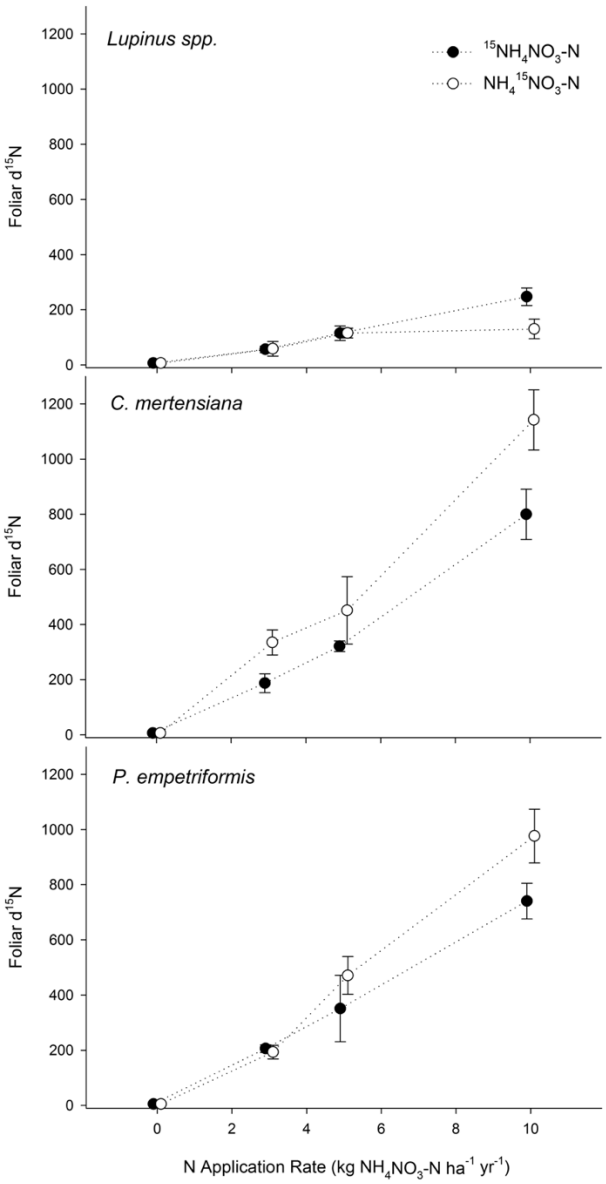

Figure 3.3: Average δ15N (change in 15N/14N ratio from atmospheric ratio) for plant foliar tissue for the heather species *C. mertensiana* and *P. empetriformis*, and the forb *Lupinus spp* (±SE). Note: *P. empetriformis* was not found at OLYM site and *C. mertensiana* and *Lupinus* spp. were only found in 20% of plots at OLYM.

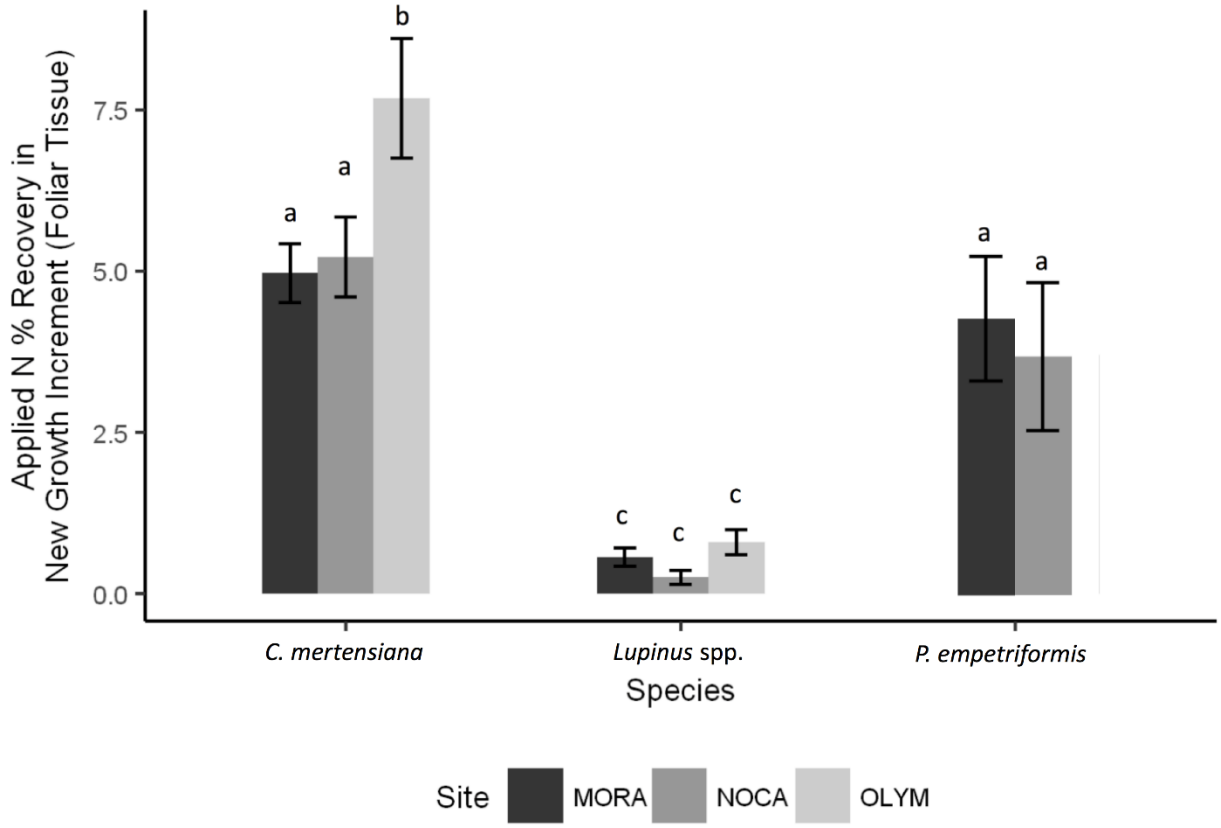

Figure 3.4: Average percent recovery of total applied N in Year 3 new growth increments for the species *C. mertensiana*, *Lupinus* spp., and *P. empetriformis* (±SE). *P. empetriformis* was not present at OLYM. Letters indicate significant site difference at $p<0.05$.

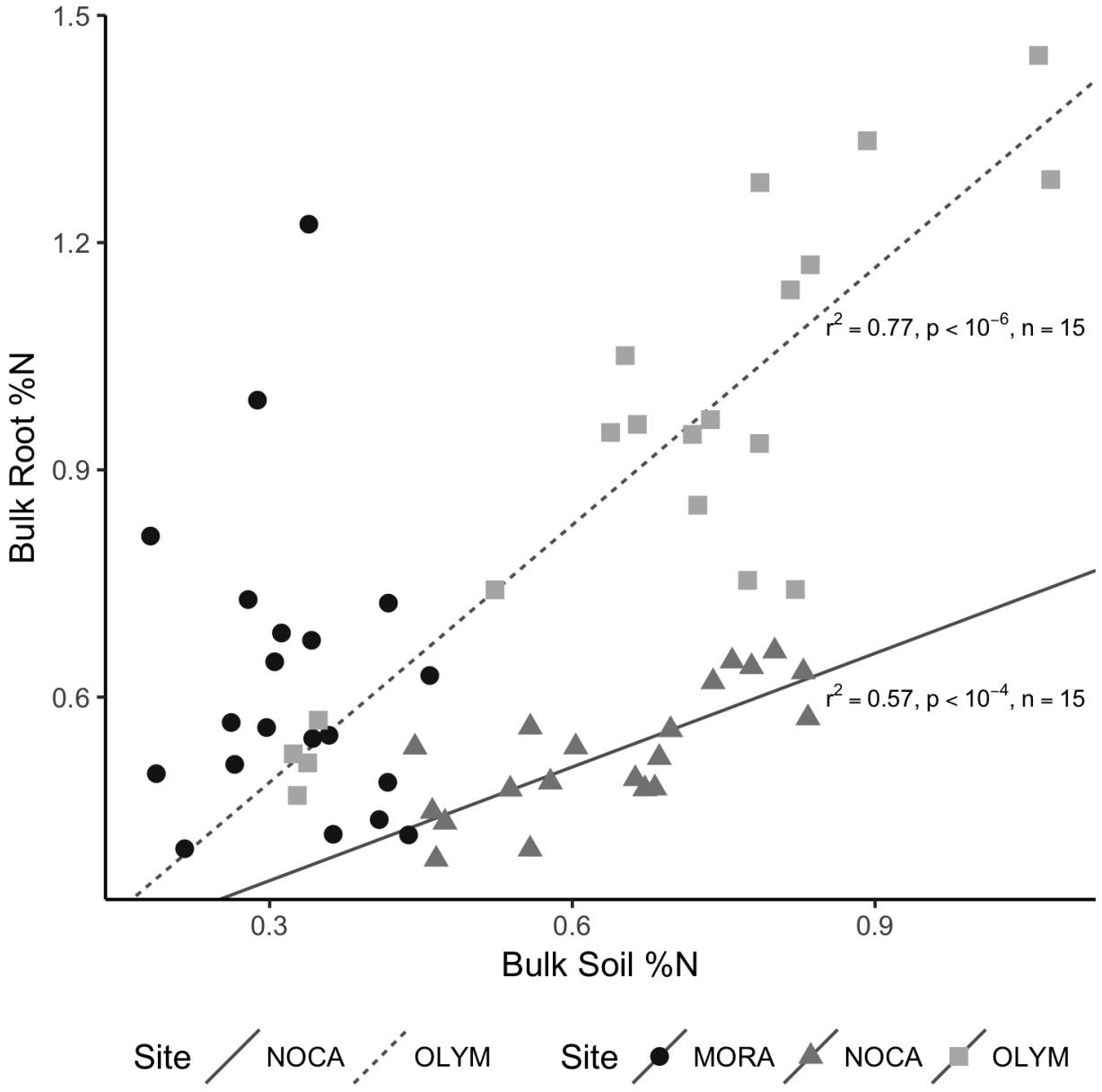

Figure 3.5: Bulk soil vs bulk root % N content.

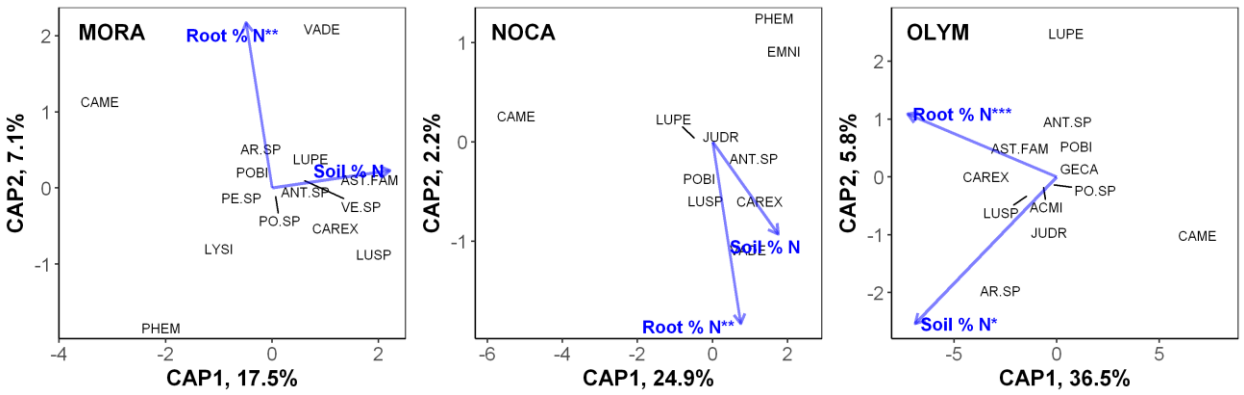

Figure 3.6: Distance-based redundancy analysis of vegetation cover constrained by soil and root N. Species with a distance of less than 0.1 from the origin were excluded for purposes of readability. *C. mertensiana* (CAME), *P. emptetriformis* (PHEM), *L. pectinate* (LUPE), *Lupinus spp.* (LUSP) and *V. deliciosum* (VADE) were the most important species in terms of differences in plant community structure. For other plant species abbreviation codes see Appendix.

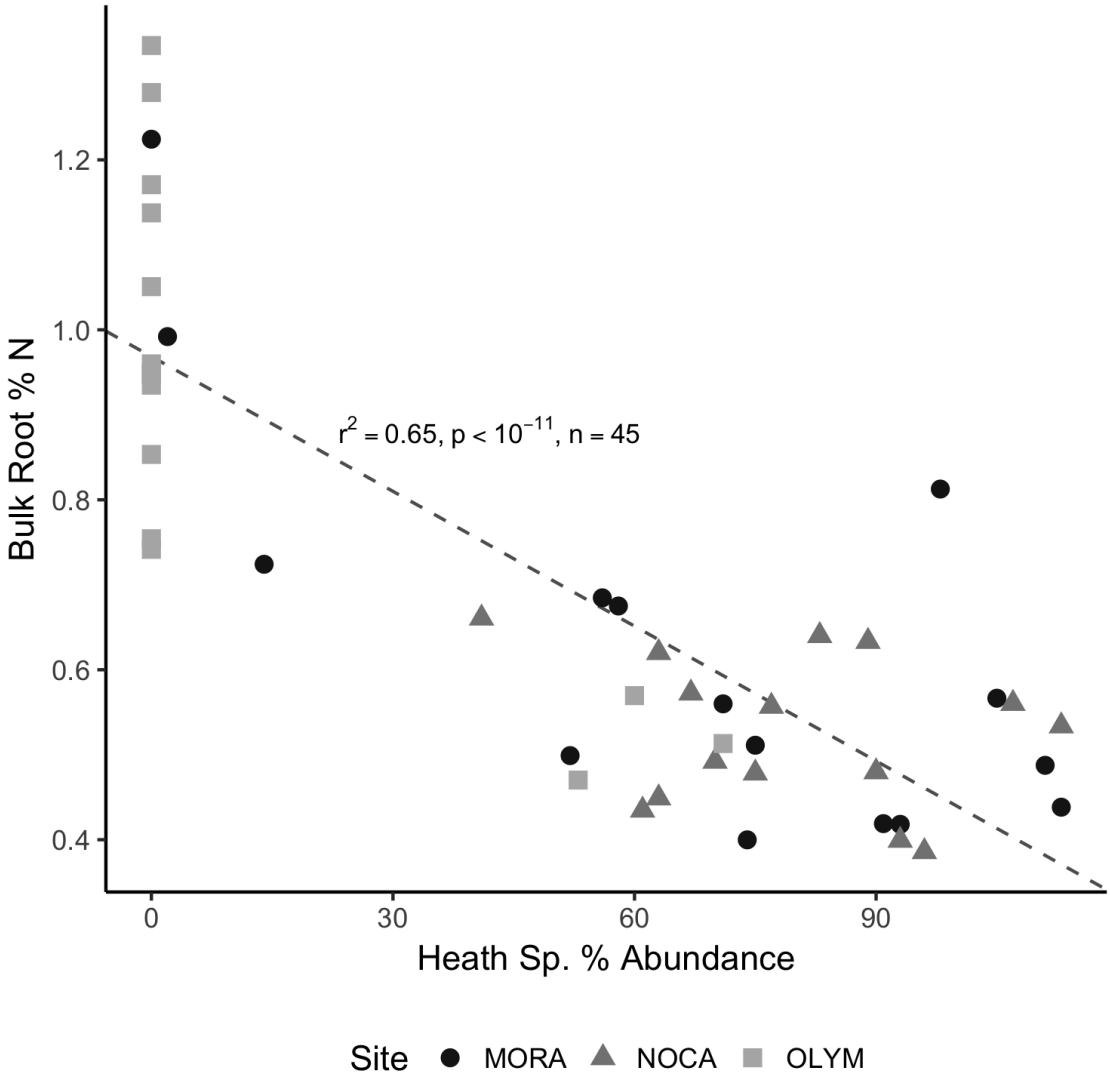

Figure 3.7: Negative correlation between heath/evergreen species abundance and root N concentration. Heath species percent abundance is the combined abundance of *C. mertensiana* (white mountain heather), *P. empetriformis* (pink mountain heath), and *E. nigrum* (crowberry). In both a linear mixed model where site and block were random effects, and simple linear models for each individual site, root N concentration was significantly negatively correlated with heath species abundance.

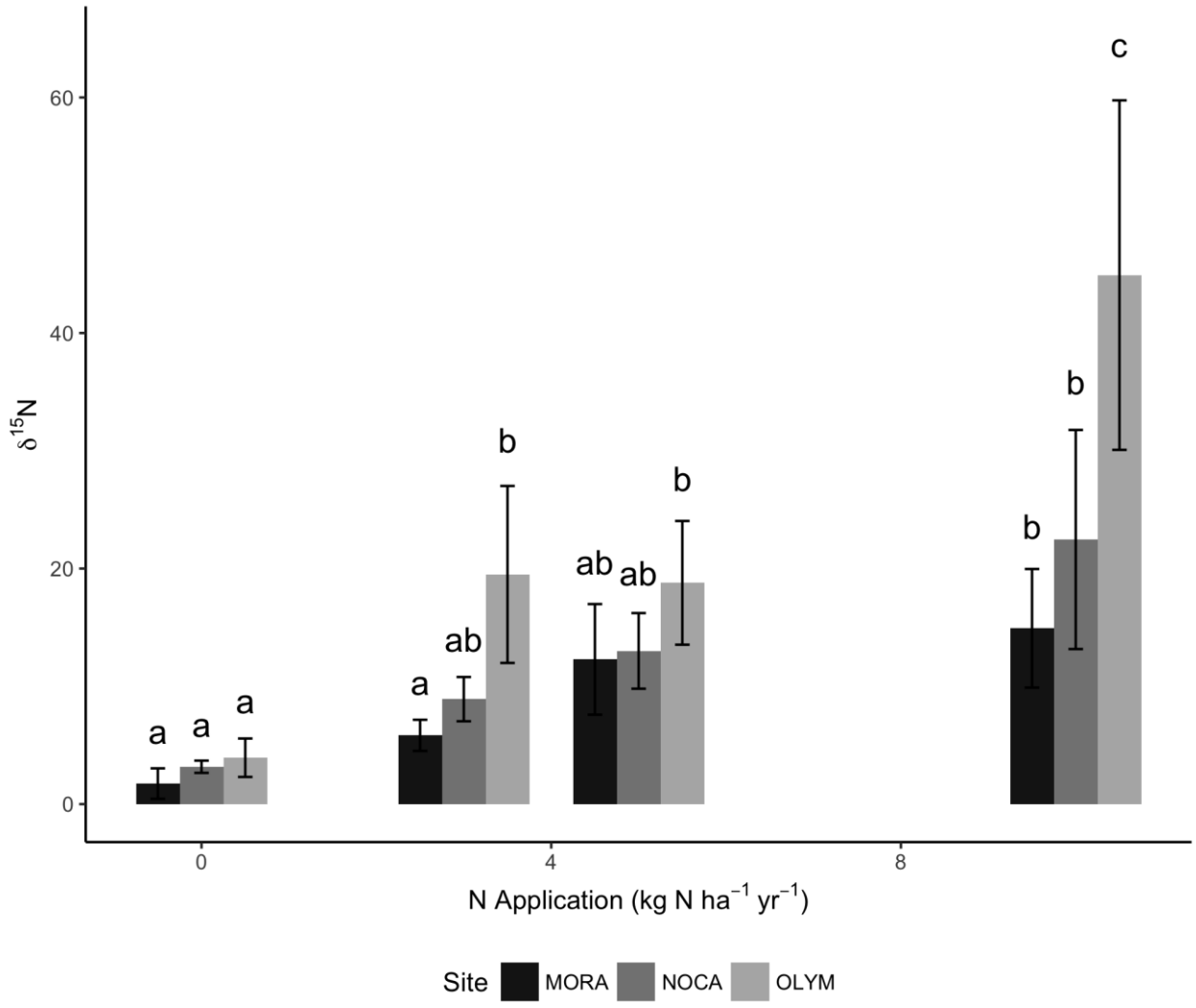

Figure 3.8: Average $\delta^{15}N$ of root tissue harvested in Year 3 (±SE) The dry meadow site (OLYM) had significantly higher root tissue $\delta^{15}N$ in response to treatment compared to the two heath meadow sites. Letters indicate significant difference at $p<0.05$ between each combination of site and treatment level.

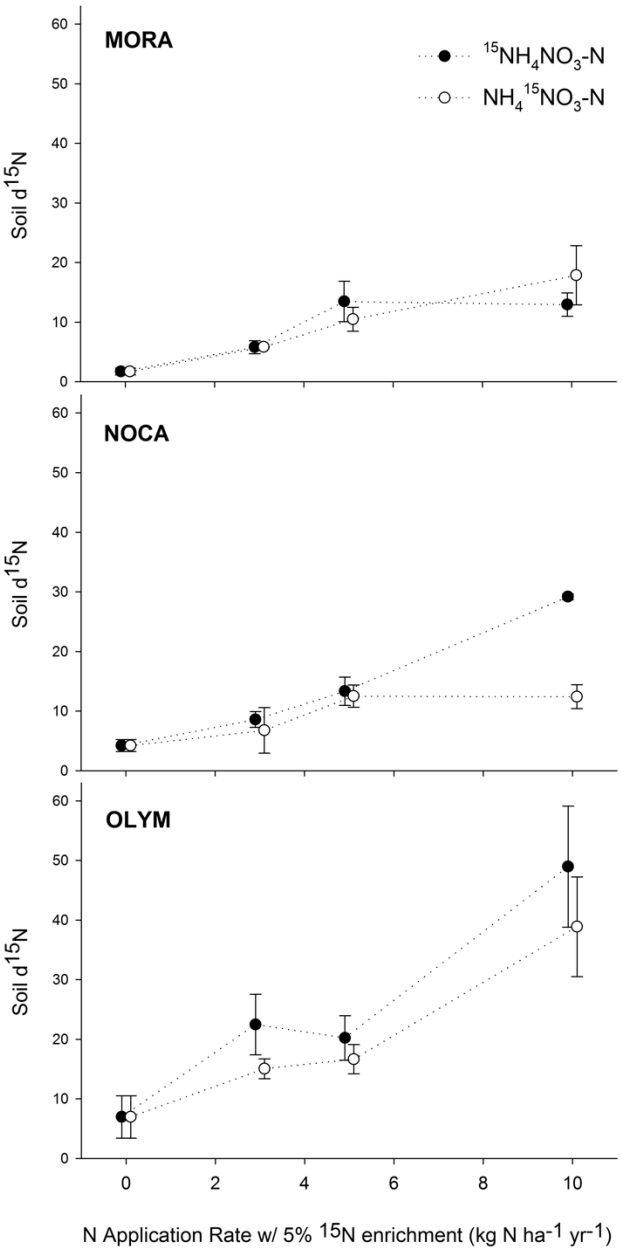

Figure 3.9: Average Year 3 soil ∂^{15}N after 3 years of N application at 5% 15N enrichment (±SE).

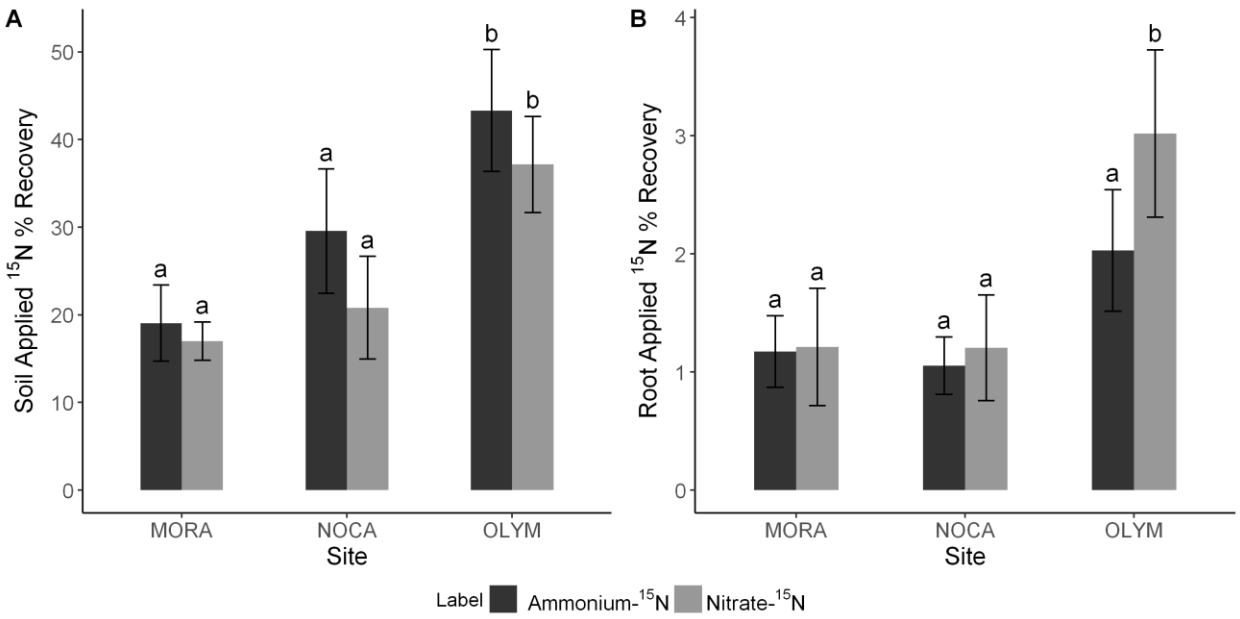

Figure 3.10: Average percent recovery of applied ^{15}N (±SE). Letters indicate significant site difference at $p<0.05$.

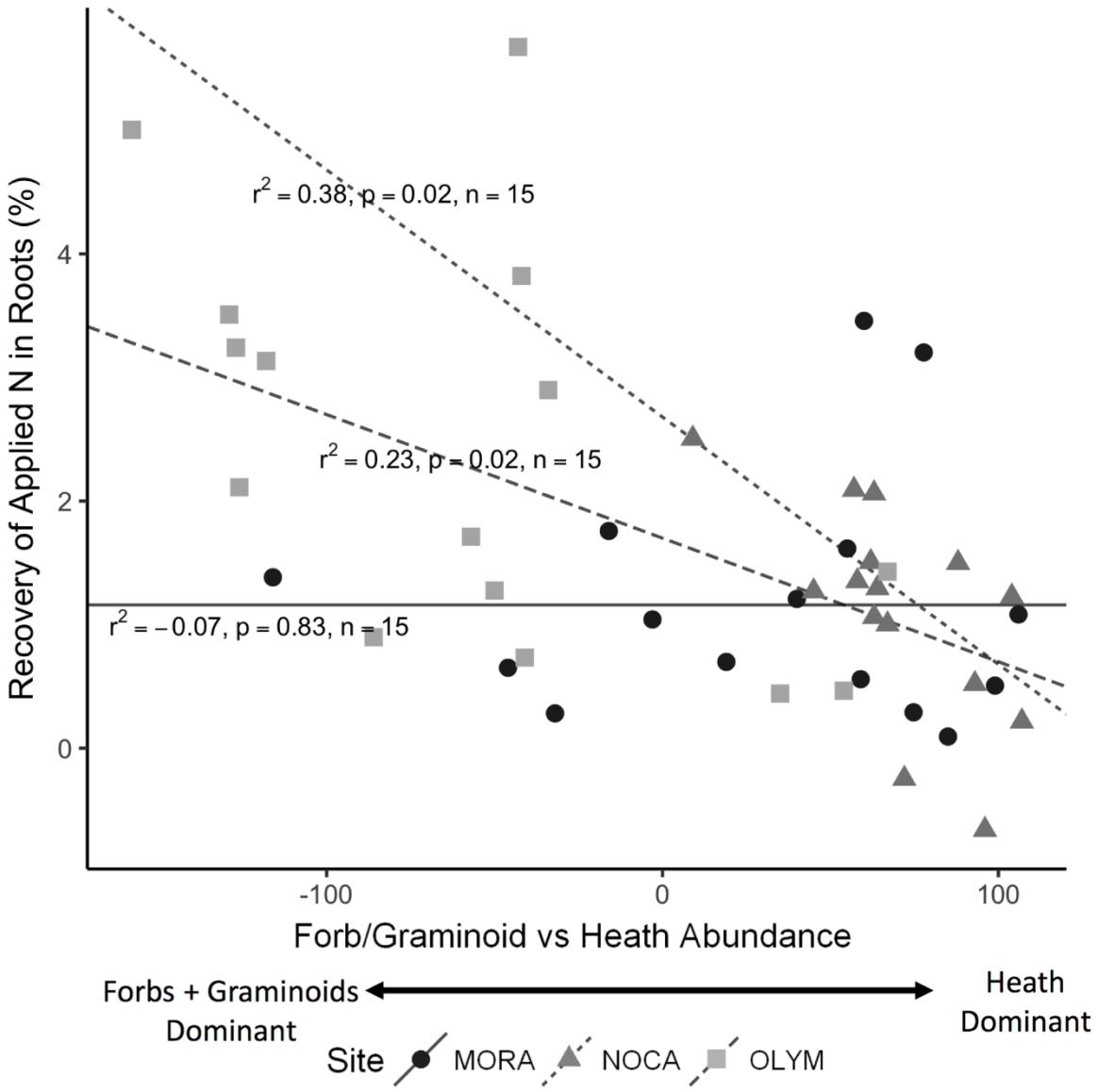

Figure 3.11: Root tissue % recovery of N pollution decreased along a gradient from graminoid/ forb species to heath species at the seasonally N-limited heath meadow (NOCA) and the dry meadow (OLYM), but not at the highly N-limited heath meadow (MORA). Total graminoid and forb abundance was subtracted from total heath and heather species abundance for all plots. Points at '0' had equal abundance of graminoids/forbs and heath. Because multiple heather or graminoid/forb species may have been present at each point in 100-point-counts, total heather or graminoid/forb abundance sometimes exceeded 100%

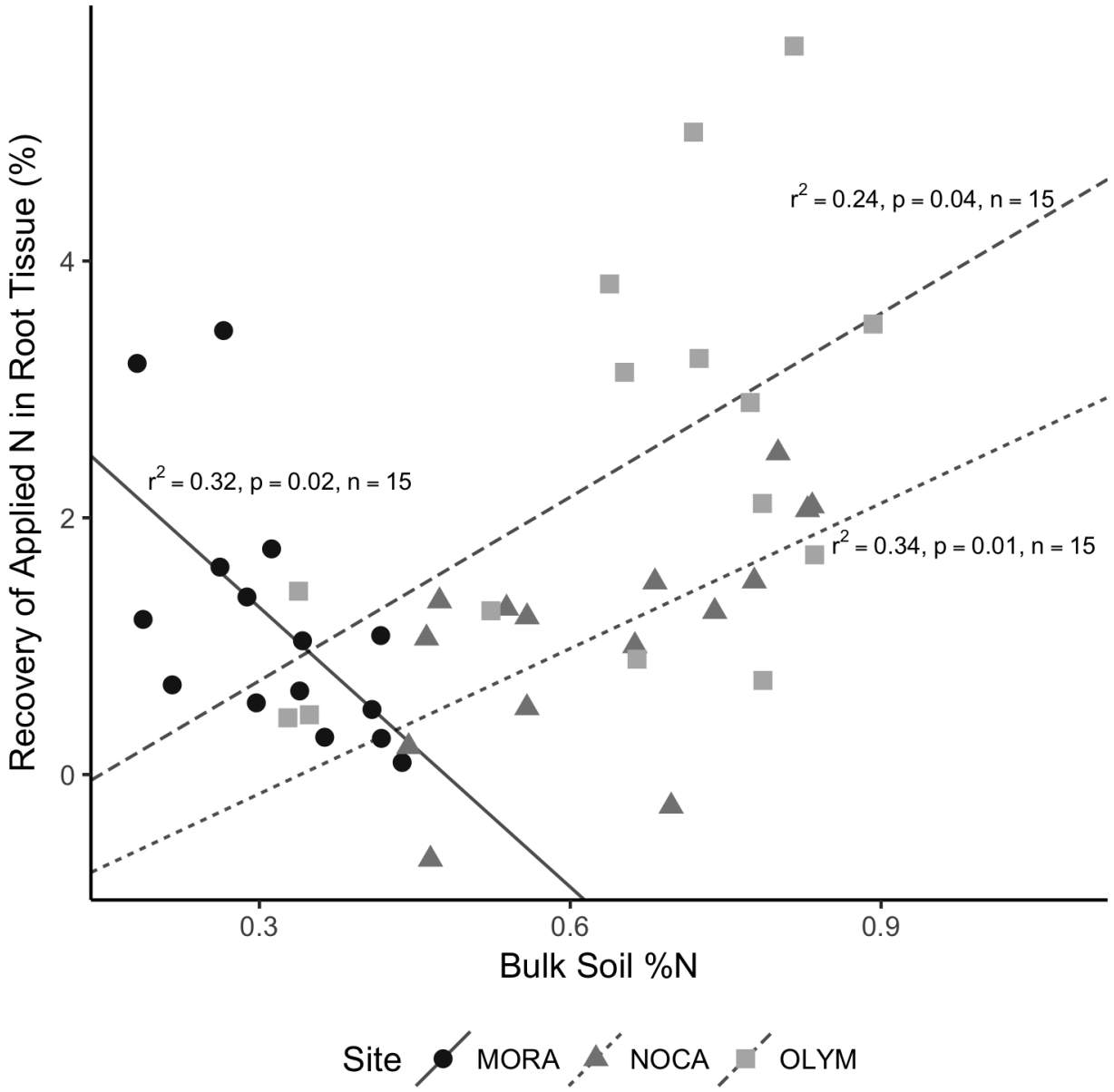

Figure 3.12: Relationship between soil N concentration and recovery of applied N in root tissue. The older heath meadow and dry meadow showed positive relationships between soil N and applied N recovery in roots, while the young heath meadow showed a negative relationship between soil N and root recovery of N.

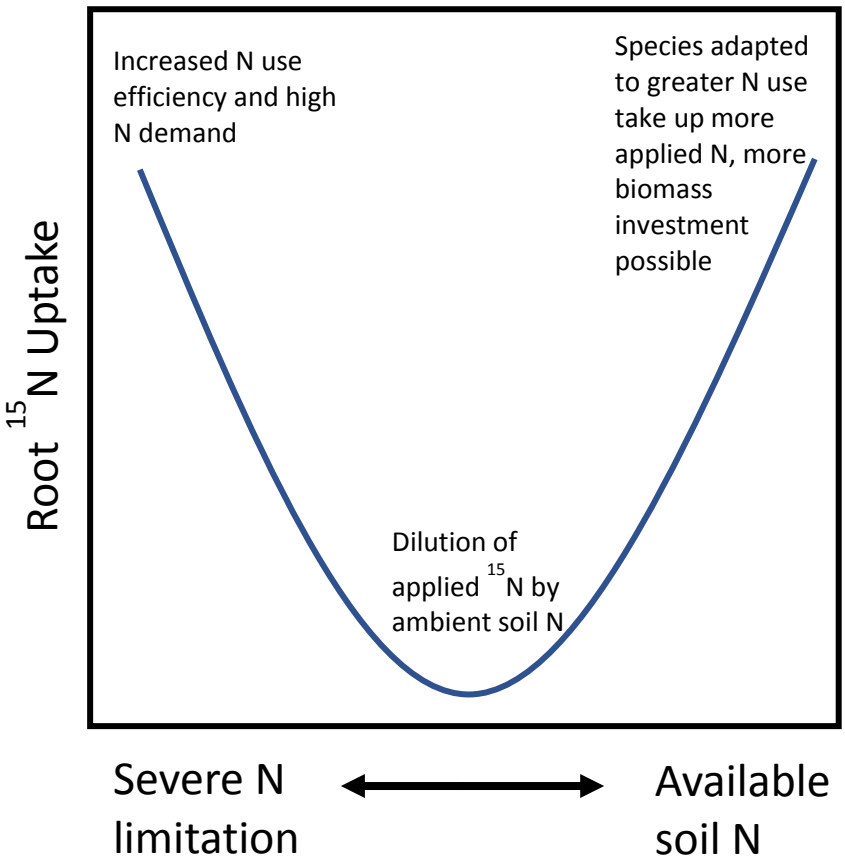

Figure 3.13: Diagram of my proposed model of ^{15}N recovery in root tissue in alpine sites of the PNW. At very low levels of soil N, high plant demand and increased N uptake efficiency increase the amount of ^{15}N recovered in roots. As soil N availability increases, applied ^{15}N is diluted or substituted for by ambient soil N.

Chapter 4: Microbial communities of permanent alpine snowfield soils and implications for Astrobiology

Abstract

Environments with extremes of temperature, pH, and pressure are often studied as potential analogues for extraterrestrial environments. As the planets and moons most likely to harbor life in our solar system are all considerably colder than Earth, microbial communities in alpine and polar environments are under particular scrutiny. Understanding microbial survival, metabolic activity, and nutrient availability and cycling in and under ice and snow is important for several reasons. It helps us understand where life could exist in our solar system and on extra-solar icy bodies, how life in and below frozen water obtains the necessary nutrients to survive, and how we might adapt Earth life to similar conditions on other planets. In order to determine how microbial communities differ in frozen vs non-frozen conditions in cold glacial environments, I measured soil nutrient status, bacterial community structure, and bacterial abundance both under snowpack and in barren exposed soil along an elevation gradient at two permanent snowfields in the Pacific Northwest of the United States.

The Proteobacteria, Gemmatimonadetes, and Actinobacteria were the most abundant phyla. The main differences between microbial communities under snowpack and those in exposed soils were lack of Cyanobacteria under snowpack and differences in the abundance, community structure and diversity of the Deltaproteobacteria. Two 16S rRNA amplicon sequence variants

(ASVs) identified at the genus level comprised 20% of all reads – an unknown species of the genus *Anaeromyxobacter* in the class Deltaproteobacteria, and an unknown species of the genus *Gemmatimonas* in the phylum Gemmatimonadetes. Many of the most abundant bacteria were most closely related to bacteria found in alpine glacial, Arctic, and Antarctic soils across the world. They are not found in mesic environments, suggesting that environmental selection for psychrophiles in frozen soil conditions rather than specific geographic location is the driving factor in microbial community assembly in these environments. Microbial communities in extreme environments on Earth may not depend on seeding from more mesophilic and organic-rich ecosystems.

INTRODUCTION

Alpine glaciers and 'permanent' snowfields are beginning to recede in many parts of the world due to warmer temperatures and decreased snowpack (Beniston et al., 1997; Xu et al., 2016; Huss et al., 2017; Woo and Young, 2017). This has resulted in new soil formation and presumably alteration of the soil microbial community to one presumably adapted to more mesic conditions (Brown and Jumpponen, 2014; Liu et al., 2016; Rime et al., 2016; Zeng et al., 2016; Hotaling et al., 2017).

A number of recent studies have found that active microbial communities exist in soils underneath permanent snowpack, ice, and even glaciers (Brooks et al., 1996; Cary et al., 2010; de Pascale et al., 2012; Bajerski and Wagner, 2013; Larose et al., 2013; Stres et al., 2013; Zumsteg et al., 2013; Maccario et al., 2015; Ansari, 2016; Learman et al., 2016; Liu et al., 2016; Makhalanyane et al., 2016; Seok et al., 2016; Anesio et al., 2017; Bore et al., 2017; Hotaling et al., 2017; Choe et al., 2018). These microbial communities operate at low temperature and have

low metabolic rates, but are diverse and abundant enough to be the main driving force of bacterial community assemblages after ice melt.

As Earth's climate continues to change in the coming centuries due to anthropogenically elevated greenhouse gas concentrations, permanent snowlines are expected to recede upward and more rain is expected in previously frozen environments (Beniston et al., 1997; Salathé et al., 2008; Huss et al., 2017). This will result in the formation of new microbial communities, which will in turn aid in soil formation and increased carbon (C) and nitrogen (N) accumulation in these new soils. One recent study found that the source for these new microbial communities is more likely the under-snow and under-glacier soil itself rather than the microbes living on the snow surface or bacterial and fungal spores deposited through wind or precipitation (Rime et al., 2016).

The defined edge of a permanent snowpack makes it an ideal habitat in which to test microbial succession from under snowpack to barren exposed soils. These sites can be accessed during summer months, when annual ephemeral snow has melted away to expose layers of solidified snow from previous years. Soils underneath the edges of these snowfields are subjected to numerous freeze/thaw cycles during the brief summer afforded to alpine tundra, and thus are a much harsher environment than soils insulated by yearly uncompacted snowpack (Brooks et al., 2011).

To address the question of differences in microbial community assemblage between soils that are under snowpack/freeze-thaw conditions and soils that have recently been exposed to sunlight/more mesic conditions, I evaluated 16SrRNA amplicon sequences from soil samples collected in barren and under-snowpack conditions at two permanent snowfields in the Pacific Northwest of the United States. My study addressed the following four objectives: to determine

(1) The soil nutrient conditions and core microbiome of the barren alpine soils at these locations, (2) The main factors driving differences in bacterial community composition and how the presence of permanent snowpack affects community composition, (3) Which phyla, classes, and specific amplicon sequence variants (ASVs) cause the largest differences in microbial community structure in exposed soils vs soil under snowpack, and what this tells us about environmental conditions at these sites, and (4) What inferences can be made about how C and N accumulate in soils in permanent snowfields, based on bacterial community composition and bacterial abundance relationships to soil nutrient conditions.

METHODS

Site Descriptions

Mount Rainier is an active, Andesitic volcano in the Central Cascade Range at latitude 46.8523° N, hosting approximately 30 official glaciers and multiple permanent snowfields. At its highest point it is 4392 m above sea level. High-elevation soils at Mount Rainier are dominated by tephra, volcanic ash, and physically weathered (via freeze-thaw and glacial grinding) volcanic rock (Roberts, 2014; United States Department of Agriculture et al., 2016). Muir Snowfield is located on the southern face of Mount Rainier, between the Nisqually and Cowlitz glaciers. Permanent snowpack begins at approximately 2200 m. Organic matter content in these soils is extremely low, on par with other cold soils such as those found in Antarctica which are used as Martian analog environments (Figure 4.1).

Sahale Peak, located at latitude 48.4892° N, is part of the granitic Chilliwack Batholith in the Cascade Pass family (less than 20 Ma) in the North Cascade Mountains, and is 2646 m in height (USGS, 2016). The peak and its neighbors have received considerable fine ash deposits from volcanic eruptions of Mount Mazama, Mount Rainier, and Mount Saint Helens over the past 10,000 years, and at lower elevations ash layers from these eruptions are still present and

discernable in the soil (United States Department of Agriculture et al., 2012). However, there are no currently erupting volcanoes in the region. Probably due to the greater age of the high elevation parent material at Sahale Peak, soils at this site have accumulated considerably more carbon (C) than the high elevation soils of Mount Rainier (Figure 4.1), though soil C is several orders of magnitude below that of a typical vegetated soil in the area. Sahale Glacier and its snowfield begin at approximately 2,250 m and end at approximately 2,530 m, with separated patches of semi-permanent snow up to 2,750 m.

I refer to high-elevation barren soils of Muir Snowfield at Mount Rainier as "younger" than those of Sahale Glacier at Sahale Peak based on geological reports of tephra content in soils at North Cascades and Mount Rainier National Parks. Soils at North Cascades National Park are dominated by tephra from the Mount Mazama eruption 7,600 years before present (B.P.). The western slopes of the Northern Cascades are positioned relative to Mount Saint Helens and Mount Rainier such that they are not in the direct path of ash fall for smaller eruptions (Briggs et al., 2006), and therefore have received mostly fine ash from large eruptions further in the past. Mount Rainier, by contrast, is an active volcano and is also directly in the path to receive tephra from Mount Saint Helens. Soils at Mount Rainier National Park have received much more extensive deposits of tephra, and more recently – the most recent large tephra deposits being from the eruption of Mount Rainier 1,000 years B.P. (Mullineaux, 1974; Sisson and Vallance, 2008). Because parent material at Mount Rainier is younger and more frequently deposited, these soils have accumulated less carbon.

Sample Collection

Soil samples were collected at the Muir Snowfields on Mount Rainier in Mount Rainier National Park and at the snowfields above and around the Sahale Glacier on Sahale Peak in

North Cascades National Park (Figure 4.2). Both parks are located in Washington State, the United States. Samples were collected in in early September.

At each site, samples were taken in intervals of ~150 m elevation gain, starting at 2,286 m (Figure 4.3). At Mount Rainier samples were taken up to 2,745 m. At Sahale samples were taken up to 2,591 m, as Sahale Peak is 2,652 m high. Two areas identified as having no evidence of human contamination were selected at each chosen elevation, and in each area I sampled both soil underneath snowpack and dry soil 1-2 m away from the edge of the snowfield.

Soil samples for DNA extraction were obtained with pre-sterilized metal scoops from the top 1-2 cm of the soil profile and placed in sterile WhirlPak bags. Average snow depth at the edge of the snowfield ranged between 0.2-0.6 m. To obtain sub-snowpack soil, snowpack was loosened with an ice axe, then sterile metal scoops were used to clear away remaining snow. I also took bulk soil samples for C and N analyses. Latitude and longitude were obtained for each sampling location.

Samples for DNA extraction were packed with snow in the field, and frozen at -20˚C at the School of Environmental and Forest Sciences at the University of Washington within 8 hours of collection in the case of Muir Snowfield and within 24 hours in the case of Sahale Glacier.

Assessing Background Soil C and N

Soil analyses

Within 48 hours of collection, bulk soils samples were analyzed for gravitational moisture content, extractable NH_4-N and NO_3-N, extractable organic carbon (C) and nitrogen (N), and microbial biomass C and N. All analyses were conducted at the soils joint use lab in the School of Environmental and Forest Sciences at the University of Washington, Seattle.

One 10 g sub-sample was extracted with 2M KCl and analyzed for NH_4-N and NO_3-N using a Perstorp 500 Model Autoanalyzer (Analytical Service Center, University of Washington,

Seattle). Another 10 g subsample was extracted with 0.5M K_2SO_4; total K_2SO_4 was analyzed for organic C and total N using a Shimadzu TOC-V Analyzer (Analytical Service Center, University of Washington). The chloroform-fumigation extraction method (Beck et al., 1997) was used to determine microbial biomass using a third 10 g subsample. These samples were incubated with chloroform for 3 days and extracted with 0.5M K_2SO_4 to determine total extractable organic C and total N. Pre-incubation levels of extractable C and N were subtracted from post-incubation levels. K_{EC} values (fractions of biomass C and N mineralized) of 0.45 and 0.54 were used to convert to microbial biomass C and N respectively (Brookes et al., 1985; Beck et al., 1997).

A portion of each bulk soil sample was air-dried and ground to a fine powder using a ball grinder (Analytical Service Center, University of Washington, Seattle). Samples were analyzed for total C, N, and hydrogen (H) on a CHN Analyzer 2400 Model, Perkin Elmer Co at the Analytical Service Center at the School of Forest Sciences at University of Washington, Seattle. Samples from Mount Rainier were also analyzed for $\delta^{13}C$ and $\delta^{15}N$ at the Isolab in the department of Earth and Space Sciences at University of Washington, Seattle via a Costech elemental analyzer and Thermo MAT253 isotope ratio mass spectrometer.

Nitrogen fixation

I used the acetylene reduction method (Schöllhorn and Burris, 1967) to estimate biological fixation of N_2 in soils. Within 24 hours of collection, bulk soil was weighed out into 20 ml glass vials, occupying not more than 50% of vial volume, and sealed with a rubber septum. I removed 10% of headspace within the vial and replaced that headspace with injected acetylene using a syringe. I also injected several empty vials with acetylene or mixed ethylene and acetylene as controls. Vials were incubated at 18°C for 24 hours. Ethylene generated by reduction of acetylene by the enzyme nitrogenase was measured using a Thermo Scientific Trace Gas

Chromatograph at the University of Washington, Seattle. From ethylene partial pressure of incubated samples after incubation I calculated nmol ethylene per gram dry soil per 24 hour period.

Assessing Soil Microbiome

DNA extractions

DNA was extracted from soil samples using MOBIO's Powersoil DNA extraction kits. Bead beating was carried out on a Mixer Mill 200 from Retsch using 2 ml tube adapters in 5 minute intervals; maximum frequency (25 Hz) was used. Extraction methods according to manufacturer's instructions were modified for low yield samples according to directions from Hale and Crowley (2015). Briefly, 200 µl of bead solution was removed and replaced with 200 µl of 25:24:1 phenol:chloroform:isoamyl alcohol, C2 solution was reduced to 100µl, and 100% ethanol was used to increase precipitation and to aid in membrane washing. Silica membranes were dried for 2 minutes prior to DNA elution, and the eluting solution was incubated on the membranes for 5 minutes before spinning down.

To concentrate DNA sufficiently for downstream analysis, solution from three separate extraction tubes was eluted onto one silica membrane for each sample.

Sequencing and sequence pre-processing

DNA samples were sent to Molecular Research LP in Shallowater, TX for 16S library prep and sequencing. The 16SrRNA V4 hypervariable region was amplified using the primer set 515/806 (Caporaso et al., 2011) and the HotStarTaq Plus Master Mix kit (Qiagen, USA). Libraries were sequenced on an Ion Torrent PGM according to the manufacturer's instructions.

All sequence filtering and statistical analyses were carried out in R (R Core Team, 2014). Sequences were pre-processed using the DADA2 package in R (Callahan et al., 2016). Reads were truncated at 250bp, as recommended for Ion Torrent reads, and the first 15bp of each

sequence were removed. A maxEE value of 2 was used to filter out low-quality reads. After dereplication and error modeling, sequences were denoised to create amplicon sequence variants (ASVs) using a homopolymer gap penalty of -1 and a band size of 32, as recommended for pyrosequencing reads. Taxonomy was assigned using the RDP's naïve Bayesian classifier via DADA2 with the Silva reference database (Wang et al., 2007; Quast et al., 2012). Sequences were aligned using the DECIPHER package (Wright, 2016) and tree-building and optimization was performed using the phangorn package (Schliep, 2011) (method: Maximum Likelihood). Taxonomy, abundance, phylogenetic tree, and sample data were passed to phyloseq (McMurdie and Holmes, 2013) for downstream analysis.

I removed two samples from the dataset due to very low amplicon sequence copy number compared to all other samples. I also pruned chloroplast and mitochondrial sequences. After rarefaction curves were generated, sequences were standardized by the minimum number of sequences among remaining samples and rounded down to get whole numbers of sequences.

Statistical Analyses

I used t-tests with Benjamini-Hochberg correction for multiple p-values (Benjamini and Hochberg, 1995) to test for differences in alpha diversity. Diversity measurements used were: species richness, Shannon-Wiener index (which emphasizes rare species), Inverse Simpsons index (which emphasizes dominant species), and Faith's Phylogenetic Distance index (Faith, 1992). I divided the sequences into separate datasets by phylum to see if specific phyla had strong differences in diversity for under-snowpack vs exposed soil samples, using t-tests with Benjamini-Hochberg correction ($p<0.05$ used to test for significance).

I used Wilcoxon rank sum tests to test for test for differences in microbial abundance at the phylum level and class level between sites and between exposed vs under-snowpack soil. I used

the Kruskal-Wallis test (Kruskal and Wallis, 1952) to look at differences in abundance between combinations of site and exposed vs. under-snowpack.

To test for differences in microbial community species composition and abundance between snow vs non-snow samples and between sites, I used PERMANOVA tests with the distance measurement weighted unifrac (Lozupone et al., 2011) using the adonis function in R (Oksanen et al., 2016). I also tested each phylum individually for differences in community composition. To confirm PERMANOVA results I also used two forms of ordination. I removed ASVs that occurred in fewer than 10% of samples (in this case, 2 samples) and performed distance-based redundancy analysis (using a weighted unifrac distance matrix) and NMDS analysis (using a Bray-Curtis distance matrix) using the vegan package (Oksanen et al., 2016). For the distance-based redundancy analysis I tested for significant sorting by environmental factors, and for the NMDS I regressed environmental variables against ordination coordinates using the envfit function in vegan.

RESULTS

Soil C and N at Muir Snowfield and Sahale Glacier

Levels of soil total C and N and extractable organic C and N were all higher at Sahale than at Muir. Levels of soil extractable inorganic N under snowpack at Sahale were similar to those found at Muir but exposed soils had significantly higher NO_3-N and significantly lower NH_4-N (Figure 4.4).

Muir Snowfield and Sahale Glacier soils had opposite trends in terms of soil C and N with elevation (Figure 4.5). At Muir Snowfield, soil total C and N concentrations and soil extractable organic C and N (i.e., labile, small-fraction organic C and N) all significantly decreased with elevation. Soil C concentration was significantly lower in samples taken under snowpack at lower elevations, but at 2590 m soil C concentration in barren vs under-snowpack samples

converged. Extractable inorganic N (NH_4-N and NO_3-N) had no relationship to elevation or snow vs non-snow samples.

At Sahale Glacier, in contrast, soil total C and N and extractable organic C and N all increased with elevation. Soil total C and N tended to be lower under snowpack than in exposed soils but the difference was not significant ($p=0.08$); there were no differences between snow vs non-snow samples for extractable organic C and N. Like Muir Snowfield, extractable soil inorganic N (NH_4-N and NO_3-N) had no relationship to elevation. However, soil NO_3-N significantly increased with NH_4-N in dry samples, while in snow-covered soils NO_3-N showed no increase with NH_4-N, suggesting that nitrification is only occurring in exposed soils (Figure 4.6).

Nitrogen fixation was patchy; fixation vs non-fixation did not have any relationship to soil nutrient content, snow vs. non-snow, site or elevation. Among samples that did show any level of N fixation, samples taken from exposed soils had higher rates of N fixation than samples taken from under snowpack (Figure 4.7).

Soil Bacterial Community Composition

After error correction, chimera removal, and amplicon sequence variant (ASV) identification using the dada2 package, my pooled samples contained a total of 2,904 unique ASVs. Number of ASVs for each sample ranged from a minimum of 176 to a maximum of 387. After scaling sequences to the lowest number of sequences per sample (minimum 26,313, maximum 44,904), this made for 654,136 total sequences. The most abundant bacterial phyla were the Proteobacteria (35% of sequences), Gemmatimonadetes (18% of sequences), Actinobacteria (13% of reads) and Bacteroidetes (13% of sequences) (Table 4.1, Figure B.3). The Proteobacteria were dominated by the classes Deltaproteobacteria (19% of sequences),

Alphaproteobacteria (13% of sequences), and Gammaproteobacteria (3% of sequences). Chloroflexi made up 8% of reads; however, the classification of the Chloroflexi may be changing in the near future. Many of my sequences from the Chloroflexi were classified under the class "AD3" in the Silva database (Quast et al., 2012), also considered Candidate Phylum AD3 by the GreenGenes database (McDonald et al., 2012).

The two most abundant ASVs overall were an ASV classified in the genus *Anaeromyxobacter* in the class Deltaproteobacteria, accounting for 13% of all reads, and an ASV classified in the genus *Gemmatimonas* from the phylum Gemmatimonadetes, accounting for 6% of all reads (Table 4.2). These two genera were also the most abundant overall; ASVs from the genus *Anaeromyxobacter* accounted for 16% of reads at Muir and 18% of reads at Sahale (average 16%), and ASVs from the genus *Gemmatimonas* accounted for 18% of all reads at Muir and 13% of reads at Sahale (average 16%) (Table 4.1). BLAST hits for the most abundant ASVs were generally all from soil and many were from cold environments such as glaciers or Antarctic soil (Table 4.2). In particular, ASVs from the genera *Gemmatimona*s (phylum Gemmatimonadetes) and *Sphingomonas* (phylum Alphaproteobacteria) had mostly significant hits to sequences found in glacial/Arctic/Antarctic environments.

Snowpack and other environmental variable effects on bacterial community composition

Bacterial alpha diversity

Alpha diversity t-tests showed significantly higher diversity in exposed soils vs under-snowpack soils using alpha=0.05 (Shannon's and Inverse Simpson's indices). When I tested each site individually, the alpha diversity was significantly different between snow and non-snow samples at Muir Snowfield but not Sahale Glacier (Figure 4.8). There was no significant difference in number of observed consensus sequences (species richness) or Faith's PD at Muir vs Sahale or under snowpack vs barren soil. I tested significance between bacterial alpha

diversity in under snowpack vs barren soil and between sites using t-tests with Benjamini-Hochberg (Benjamini and Hochberg, 1995) correction for p-values.

When I calculated the number of unique ASVs for each sample, I found that at both sites the average number of ASVs unique to each sample increased with elevation (Figure 4.9). At Muir Snowfield, the samples taken at the highest elevation sampled (2743 m) had nearly twice the number of unique ASVs compared to lower elevation samples.

Phylum-specific diversity

When sequences were subset by phylum, Cyanobacteria, Actinobacteria, and Proteobacteria showed significant differences in biodiversity between exposed and under-snowpack soils. Actinobacteria and Cyanobacteria showed a higher observed number of ASVs and a higher value for Faith's PD in exposed soil samples compared to under-snowpack samples ($p.adj << 0.05$). Proteobacteria were associated with higher Shannon's diversity index in exposed soils. Because Proteobacteria were dominant, I also tested for differences in alpha diversity in the proteobacterial class; I found that there were a larger number of observed species of Deltaproteobacteria in samples collected under snowpack than samples collected from exposed soils, but only when all samples were included. When I tested each site separately for significant differences in diversity in proteobacterial classes, I found that there were more representative ASVs in the Alphaproteobacteria in exposed soils at Sahale ($p.adj < 0.05$).

Beta diversity

I found that both the unconstrained NMDS ordination using Bray-Curtis distances (Figure 4.10) and the distance-based redundancy analysis using weighted Unifrac distances and constrained by environmental variables (Figure 4.11) showed similar trends: samples from the lower elevations at Muir Snowfield grouped together closely with samples taken at 2,438 m at

Sahale Glacier, while samples taken at 2,743 m at Muir Snowfield and some samples taken at 2,286 m or 2,590 at Sahale Glacier diverged considerably from the core community.

The NMDS ordination showed four orthogonal microbial associations for phyla that had significant linear relationships with Bray-Curtis distances between samples: the Gemmatimonadetes and Bacteroidetes at the positive end of NMDS1, the Chloroflexi and Acidobacteria at the negative end of NMDS1 (associated with extractable organic N), the Actinobacteria and Gammaproteobacteria at the negative end of NMDS2 associated with NO_3-N, and the Deltaproteobacteria on the positive end of NMDS1, associated with soil moisture (Figure 4.10).

I tested the associations revealed by the NMDS in simple linear models, and found that Deltaproteobacteria abundance was negatively associated with Actinobacteria and Gammaproteobacteria abundance, and Chloroflexi abundance was negatively associated with Gemmatimonadetes and Bacteroidetes abundance (Figure 4.12).

Microbial Community Relationships to Environmental Variables

I found several different relationships between abundance of individual phyla in soil and environmental variables (Table 4.3). In linear mixed-effects models with site and snow vs non-snow as random effects, I found that the Gemmatimonadetes were strongly negatively associated with soil total and organic extractable C and N and soil DNA concentration, while the Firmicutes, Deinococcus-Thermus, Planctomycetes, and Acidobacteria were positively associated with soil C and N and/or soil DNA concentration. Correlations between abundance of individual phyla with soil total N and extractable organic N appeared to have no relationship to correlation with inorganic N. The Gammaproteobacteria and Actinobacteria were strongly associated with increased NO_3-N. Actinobacteria were also strongly correlated with lower

elevations and low soil moisture. Deltaproteobacteria were correlated with high soil moisture and snowpack (these two environmental variables were strongly correlated with one another as well). Levels of nitrogen fixation (ARA) had no relationship to any phylum.

Actinobacteria were positively correlated with NO_3-N at both sites, though most strongly in exposed soils (Figure 4.13). At Sahale Glacier (but not Muir Snowfields) NH_4-N levels were also correlated with higher Actinobacterial abundance, and this relationship was much stronger in samples taken from under compared to samples taken from exposed soils. Ammonium levels were higher under snowpack than in exposed soils at Sahale Glacier as well.

Differences in Microbial Abundance Under Snowpack vs Exposed Soils

Snow vs non-snow did not predict microbial community in a PERMANOVA testing prediction of community composition by site and snow vs non-snow using each ASV as a separate species. However, after pooling sequences at the phylum/proteobacterial class level, difference in abundance between samples was significantly predicted by both site and snow vs non-snow at the $p<0.05$ level.

Kruskal-Wallace tests of each bacterial class for differences between each combination of site and snow vs non-snow yielded only a few significant differences in snow vs. non-snow samples (Figure 4.14). Oxyphotobacteria from the phylum Cyanobacteria were both scarce in general and almost completely absent in samples under snowpack, while the basal heterotrophic lineages of the Sericytochromatia and Melainabacteria were more evenly distributed (Figure 4.15). Sericytochromatia in the phylum Cyanobacteria and Thermoleophilia in the phylum Actinobacteria were also more abundant in exposed soils but the difference was less significant. Deltaproteobacteria were also more abundant under snowpack. The Chthonocomanadetes in the phylum Armatimonadetes, the Planctomycetacia in the phylum Planctomycetes, the

Alphaproteobacteria, and the Verrucomicrobiae in the Verrucomicrobia all shared the same trend: decreasing abundance from Muir barren samples to Sahale under-snowpack samples.

When site was accounted for, Deltaproteobacteria were more abundant under snowpack, while Alphaproteobacteria and Verrucomicrobia were associated with barren soil, in agreement with the results of the Kruskall-Wallace tests. In order to independently test microbial abundance in under-snow vs exposed soils while still accounting for site differences, I used a mixed effects model where site was the random effect (Table 4.3). I also found that the phyla Alphaproteobacteria, Verrucomicrobia, and Planctomycetes were all more abundant at Muir Snowfields, while the phylum Firmicutes was more abundant at Sahale Glacier.

Based on the results from my mixed models and Kruskall-Wallace tests I expected that ASVs from the Deltaproteobacteria, Alphaproteobacteria, Verrucomicrobia, and Cyanobacteria would be the greatest predictors of snow vs exposed soil samples.

However, I found that the presence of several ASVs from the Gemmatimonadetes and the Gammaproteobacteria were by far the greatest predictors of whether a sample came from soil under snowpack vs exposed soil. A bacterium from the genus *Noviherbaspirillum* in the class Gammaproteobacteria had the largest log-2-fold difference from the general population predicting exposed soil, while two bacteria from the family *Gemmatimonadaceaea* in the phylum Gemantimonadetes were the greatest predictors of a sample coming from under snowpack.

Soil N relationships to bacterial phyla

I observed several different relationships involving the Actinobacteria and Gammaproteobacteria and extractable soil NO_3-N. Because of the opposing relationships between the Deltaproteobacteria (which are associated with snowpack) and the Actinobacteria and Gammaproteobacteria (Figure 4.10, Figure 4.12), the strong association between

Actinobacteria and Gammaproteobacteria and NO_3-N levels (Table 4.3, Figure 4.10, Figure 4.13), and the interaction effects between NO_3-N and NH_4-N under snowpack (where NO_3-N does not increase with NH_4-N) vs in exposed soils (where NO_3-N increases with NH_4-N) I built a model to predict soil NO_3-N based on abundance of Gammaproteobacteria and Actinobacteria and whether the sample was taken from under snowpack or exposed soil. I already knew that Gammaproteobacteria and Actinobacteria both independently predicted higher NO_3-N in my mixed-effects models (Table 4.3).

Where Actinobacteria and Gammaproteobacteria were found together in abundance, NO_3-N was likely to be higher; where Actinobacteria were located under snow, NO_3-N was likely to be lower. Soil extractable NO_3-N was significantly predicted in the model NO_3-N = Gammaproteobacteria + Actinobacteria + Snow + Gammaproteobacteria:Actinobacteria - Actinobacteria:Snow (adj R^2 = 0.88, F = 33.12 on 5 and 18 df).

DISCUSSION

This study addressed the broad ecological question of how soil microbial communities change in response to environmental variables in extreme environments, in order to gain information about the adaptation of microbial life in extreme conditions on Earth and also in extraterrestrial conditions. I specifically investigated the background nutrient levels and bacterial community composition in these soils, and how soil microbial communities under snowpack exposed to freezing temperatures differed from soil microbial communities in warmer areas. I tested whether soil microbial communities changed along an altitudinal gradient. I also addressed whether extremes of temperature and elevation were in fact the primary drivers of community diversity, or whether other environmental factors such as nutrient availability better predicted bacterial community. A fourth question I wanted to investigate was how C and N enter these

soils (through chemoautotrophy, N-fixation, or photoautotrophy) and whether these soils are composed mainly of primary producers or a complex network of heterotrophs, in order to address the issue of increasing soil formation in recently deglaciated soils.

Bacterial Communities in Permanent Snowfield Soils

Proteobacteria, Gemmatimonadetes, and Actinobacteria were the dominant phyla in soil samples. I found that soil nutrient concentrations rather than elevation, snowpack, or site were the strongest predictors of microbial community composition. Only two strong phylum/class level associations with presence of snowpack were found: Deltaproteobacteria were more abundant under snowpack, and Cyanobacteria, although low in abundance, were almost exclusively found in exposed soil. Bacteria from the Gemmatimonadetes were dominant and played a surprisingly important role in microbial community composition given that most soil metagenomics studies have not found this phylum to be dominant or important in explaining environmental factors.

Overall, I found very little evidence of photoautotrophy as the main source of C in these soils; it is likely that chemoautotrophy and atmospheric deposition are the main source of carbon in these environments. However, these soil communities were diverse and despite the low levels of C contained a large number of presumed heterotrophic bacteria.

Microbial Communities in Cold Environments

We are now finding that snow, ice, and subglacial and sub-snowpack environments, once thought to be environments too extreme for life to thrive, are in fact habitats for prokaryotic communities (Maccario et al., 2015). However, although archaea are abundant in extremes of low nutrient availability, acidity, or high temperature, in cold environments archaea are rarely detected in more than trace numbers – it is bacteria that dominate ice and icy sediments

(Maccario et al., 2015). Many bacteria otherwise adapted to mesic environments have the capacity for psychrotolerance, particularly the ability to produce polysaccharides and charged particles to prevent ice crystallization and membrane rupture (Hoover et al., 2004), the ability to modify the cell membrane to include more unsaturated, flexible lipids (Mirete et al., 2016), and the evolution of proteins that include fewer stabilizing bonds (De Maayer et al., 2014), so that they can remain functional under cold conditions. What were once considered 'extreme' environments of cold temperature, low pressure, low oxygen, and low light availability such sea ice, glacial ice or the soil underneath have now been discovered to be teeming with microbial life.

Despite the number of microbes that can survive in cold temperatures, microbial community assemblages in cold environments do tend to share many characteristics. Several different studies in glacial ice and soils found that the Proteobacteria, Actinobacteria and Bacteroidetes are generally the dominant phyla (Maccario et al., 2015), with Chloroflexi and Firmicutes as secondary phyla (Seok et al., 2016; Anesio et al., 2017; Hotaling et al., 2017; Choe et al., 2018). Acidobacteria are also often to be dominant in tundra soils, particularly low pH soils (Mannisto et al., 2013). Microbiomes in extreme desert soils such as the Atacama also share similar distributions of bacteria, with dominance of the Bacteroidetes, Firmicutes, and Proteobacteria (Mandakovic et al., 2018).

A major difference between this study and many others in alpine, glacial, and polar cold soils is the presence and dominance of the Gemmatimonadetes. Gemmatimonadetes are a common phylum found in soil but in many studies of alpine and polar soils they appear only as small parts of the microbial community (1-5%) (Frey et al., 2016; Learman et al., 2016; Rime et al., 2016). One study, however, did find a microbial community very similar to that found in my

Cascade alpine snowfield samples – a study of two different glacial forefields in Antaractica found widespread Gemmatimonadetes which were associated with increasing soil depth (Bajerski and Wagner, 2013).

Why these bacterial phyla are more abundant in soils that experience extremes of temperature, pressure and pH is still being investigated. Some traits are known: the Actinobacteria, for example, have high G+C content (Qin et al., 2016) and can maintain DNA repair and metabolic activity in freezing temperatures (Makhalanyane et al., 2016).

Snow-Covered Soils vs Non-Snow-Covered Soils

Overall I found that Deltaproteobacteria abundance was positively correlated with soils sampled under snowpack and with moister soil (Table 4.3), but that this comprised only a part of the differences in community structure between samples. At the phylum/Proteobacterial class level, bacteria fell into four main groups: the Deltaproteobacteria group, associated with snowpack and moisture, the Actinobacteria/Gammaproteobacteria group, associated with drier soils, NO_3-N, and higher elevations, the Chloroflexi/Acidobacteria group, associated with more mesic conditions and available organic N, and the Gemmatimonadetes/Bacteroidetes group, associated with low nutrient levels.

The relationship between Deltaproteobacteria and snowpack may be one of adaptability in general rather than specific adaptation to cold environments. *Anaeromyxobacter*, the genus that made up over 80% of all sequences in the Deltaproteobacteria in my samples, are facultative anaerobic spore-forming, gliding bacteria known to possess a wide variety of metabolisms, including using halophenols, iron, magnesium, nitrate, nitrite, nitrous oxide, and fumarate as electron acceptors and H_2 and acetate as donors (Sanford et al., 2002; Petrie et al., 2003; Treude et al., 2003). Some species of *Anaeromyxobacter* adapt easily to environments that fluctuate

regularly between oxic and anoxic conditions (Treude et al., 2003). Many of the most abundant ASVs in this study were classified as *Anaeromyxobacter*. When sequences for these ASVs were blasted against NCBI records, many matched with 100% identity to environment samples found around the world in different soil and freshwater environments.

In contrast, although Gemmatimonadetes abundance in general was not associated with samples under snowpack, ASVs in that phylum were the strongest indicators of snow-covered soil. In addition, abundant ASVs from the Gemmatimonadetes were most likely to have 99% or 100% identity to sequences from other polar and alpine/glacial soils around the world, as opposed to identity to a large variety of environmental samples, as I found for many other of my ASVs (Table 4.2).

Primary Production

Photoautotrophy by Cyanobacteria does not appear to be a large contributor to the soil carbon pools at Muir Snowfield and Sahale glacier. Cyanobacterial sequences made up less than 1% of all reads and were found only in exposed soils; cyanobacterial communities may re-establish themselves every year in these soils rather than carrying over the snow-covered winter months.

However, C is clearly present in these soils. Given the extremely young soils at Muir Snowfield in particular (<1,000 years), the lack of vegetation, and the lack of any real difference in soil C content in soil under snowpack vs in exposed soil, there must be considerable chemoautotrophy taking place. There is also possible contribution from atmospheric deposition of organic matter or from deposition of snow algae after annual snowmelt.

Other Functional Traits

Data from 16S rRNA amplicon libraries can only go so far in describing the functional capacity of a microbial community, and so I can only examine the functional traits of organisms closely related to the dominant ASVs I have identified at the genus level and discuss them qualitatively. I expected, and found, a number of facultative anaerobes capable of using alternative electron acceptors, as well as highly adaptable heterotrophic aerobes (Table 4.2). However, some traits are common amongst the dominant ASVs I have identified: gliding motility, and the ability to reduce nitrate and nitrous oxide (Table 4.2). I also see strong evidence for active ammonia oxidation in my samples (see pg 108). Genes for ammonia oxidation and denitrification have been detected in many environmental samples taken in cold environments, though these processes have been found to not necessarily be active in winter when temperatures are lowest (Makhalanyane et al., 2016). Why organisms that possess these genes are so common in cold, low-N environments where these genes are not necessarily functional is still an open question.

Gemmatimonadetes

I found that ASVs from the phylum Gemmatimonadetes, the most abundant of which are all classified in the genus *Gemmatimonas*, played a significant role in microbial community structure at my sites. Four out of the ten most abundant ASVs were in the genus *Gemmatimonas* (Table 4.2), and *Gemmatimonas* was the second-most abundant genus after *Anaeromyxobacter*.

Bacteria from the phylum *Gemmatimonadetes* are common in soils worldwide but only a few species have been isolated, and little is known about their function or taxonomy. Isolated species so far are *Gemmatimonas aurantiaca* (Zhang et al., 2003); *Gemmatimonas phototrophica* (Zeng et al., 2015), *Gemmatirosa kalamazoonensis* (Debruyn et al., 2014), *Longimicrobium terrae* (Pascual et al., 2016), and *Roseisolibacter agri* (Pascual et al., 2018). Most of these have

been aerobic chemoheterotrophs sharing the common traits of poly-phosphate accumulation and the ability to grow under micro-oxic conditions. *Gemmatimonas aurantiaca* can use nitrous oxide as an alternative electron acceptor (Park et al., 2017). *Gemmatimonas phototrophica* has a fully functional type 2 photosynthetic reaction center similar to that found in purple phototrophic bacteria, gained from horizontal gene center from same, which absorbs in the infrared and uses carotenoids to absorb light in the visible (Zeng et al., 2014). Biomarkers surveys in a number of different available environmental metagenomes such as biofilms, lake water and soil have found that purple phototrophic *Gemmatimonas* bacteria make up to 11.9% of all phototrophs in these communities.

Correlations Between Inorganic N and Bacterial Phyla

Given the strong positive relationships I observed between abundance of Actinobacteria and Gammaproteobacteria and NO_3-N (Figure 4.13), it seems likely that some of the abundant bacterial species from these phyla are either nitrifiers or denitrifiers. Some species of Actinobacteria, in the genus *Frankia*, are known to fix N and form root nodules with specific plant species (Raymond et al., 2004). However, none of my ASVs were classified in that genus, and these soils are barren and unvegetated. Also, I would expect that if N fixation was a major contribution of one of these phyla, soil N in general would be higher in their vicinity.

Instead it appears as though bacteria in the Actinobacteria and Gammaproteobacteria are either depending strongly on NO_3-N in particular or are producing it. Bacterial species in the Gammaproteobacteria and Betaproteobacteria perform chemolithotrophic ammonia oxidation (Weidler et al., 2008; Christman et al., 2011), and so it is possible that the Gammaproteobacteria in my soils are oxidizing ammonium. I found no references in the literature to ammonia or nitrite

oxidation by Actinobacteria. However, species of both Actinobacteria (particularly *Streptomycetes*) and Gammaproteobacteria are known to be common denitrifiers in soil.

Both Actinobacteria and Gammaproteobacteria species found in these soils could be obligate or facultative denitrifiers using nitrate as an electron acceptor. Alternatively, ammonia-oxidizing Gammaproteobacteria could be supplying denitrifying Actinobacteria with nitrate. However, neither of these possibilities make total sense given the conditions in which I found Actinobacteria to be most abundant – drier soils – and given that Actinobacteria appear to be correlated some kind of decrease in NH_4-N when comparing snow-covered (high NH_4-N) to barren (low NH_4-N) soil. This relationship was not observed in conjunction with Gammaproteobacteria. In short, Actinobacteria appear to be related to oxic conditions, drier soils, and appear to drive the balance of NH_4-N to NO_3-N in exposed oxic soils.

Implications for Astrobiology

Studies of the patterns in microbial communities in cold environments have greatly increased in number in the last few years, but these communities are complex and many species appear to be mesotrophic but able to survive in extreme environments. Soils under or beside glaciers, in Antarctic dry valleys, or in Arctic permafrost may not be as 'extreme' as believed before the advent of metagenomic sequencing.

It is likely that if we find life in our own solar system, it will be unicellular and possible similar to prokaryotes on Earth. However, in an extreme environment such as the surface of Europa or brine channels on Mars, a 'mesotroph' would be considered a rare extremophile on Earth. In order to study and learn from organisms and systems which are truly extreme in their adaptation to freezing, lack of oxygen, etc., we must first differentiate which organisms are

markers for extreme conditions and which organisms are simply able to adapt to a wide variety of conditions.

I suggest that the Gemmatimonadetes, a poorly characterized phylum which may contain a wide variety of unknown metabolisms and which has recently been found to contain a phototroph with a unique Type 2 reaction center (Dachev et al., 2017), may have a number of species which are true extremophiles rather than exceptional adapters, given the association of many ASVs in my study with snowpack and matching sequences from other studies of glaciers and ice, while not matching with samples taken from more mesic environments.

CONCLUSION

Alpine and polar environments are the closest analogs we have to the planets and moons most likely to harbor life in our solar system, and studying the microbial communities that survive in these environments may give us clues as to where and how to look for life in extraterrestrial environments. In order to study microbial community adaptation to freezing conditions, I sampled the microbial communities in soil under snowpack and in soil that was exposed to sunlight along an elevational transect at two permanent snowfields in the Cascade Mountains of the Pacific Northwest of the United States.

I found direct relationships between different phyla and environmental characteristics. Deltaproteobacteria were associated with higher moisture and the presence of snowpack. Actinobacteria and Gammaproteobacteria were associated with each other and with higher soil extractable NO_3-N, and Gemmatimonadetes were associated with lower soil DNA and microbial biomass, lower total C and N concentrations, and lower extractable organic C and N. Specific ASVs from the Gemmatimonadetes were the strongest indicator species for soil samples taken under snowpack, and ASVs from that phylum were strongly associated with other sequences on

NCBI from glacial and polar environments. Cyanobacteria were few and chemolithoautotrophy is likely the main form of primary production.

Differences in microbial communities between samples, however, were actually fairly low, even between soils underneath the snowpack vs soils exposed to sunlight. This is further support for the hypothesis proposed by Bajerski and Wagner (2013) in their study of glacial forefields in Antaractica: that microbial communities are highly diverse and fairly undifferentiated when habitat formation is in its early stages. A community of single-celled alien organisms on Europa, however, might be fully adapted to conditions we consider to be extreme, and show a much reduced diversity and clearer functional relationships. While the conditions prevailing in terrestrial analogs may be similar to icy moons or Mars, the communities behave differently. Thus a study of bacterial species that are most adapted to cold conditions, rather than the entire community, may prove more beneficial to the field of astrobiology.

Table 4.1: Total abundance of bacterial phyla and genera in soil at Muir Snowfield and Sahale glacier. Proteobacteria and Gemmatimonadetes were the most abundant phyla, and Deltaproteobacteria were similar in abundance to Gemmatimonadetes. Genera were included if their abundance rounded up to 1% of all sequences.

Phylum	**%**	**Class**	**%**	**Genus**	**%**
Proteobacteria	35	Deltaproteobacteria	19	*Anaeromyxobacter*	16
				Haliangium	1
		Alphaproteobacteria	13	*Sphingomonas*	3
				Acidiphilium	3
				Rhodovastum	1
		Gammaproteobacteria	3		
Gemmatimonadetes	18			*Gemmatimonas*	16
Actinobacteria	13			*Oryzihumus*	5
				Conexibacter	1
Bacteroidetes	10			*Solitalea*	2
				Flavisolibacter	1
				Ferruginibacter	1
				Hymenobacter	1
Chloroflexi	8				
Acidobacteria	5			*Granulicella*	1
				Bryobacter	1
				Candidatus_Solibacter	2
WPS-2	4				
Verrucomicrobia	2			*Candidatus_Udaeobacter*	2
Planctomycetes	2				
Deinococcus-Thermus	1				
Cyanobacteria	1				
Other	2				

Table 4.2: Taxonomy and closest BLAST hits for the 25 most abundant ASVs for both cultured and uncultured environmental samples.

Phylum	Class	Genus	%	Hits to Cultured Samples >96%	% Identity	Cultured Sample Origin	Hits to Uncultured/Environmental Samples >96%	% Identity
Proteobacteria	Deltaproteobacteria	*Anaeromyxobacter*	13.4	None	NA	NA	Soil (8), Freshwater biofilm (1)	100%
Gemmatimonadetes	Gemmatimonadetes	*Gemmatimonas*	5.4	*Gemmatinomas sp., Gemmatimonas phototropica*	94%	Brackish water, floodplain soil, lake water, grassland soil	Periglacial soil (1), post-volcanic pyroclastic surface in Alaska (1), glacial forefield in Antarctic soil (2), glacial sediment (1)	99%
Actinobacteria	Actinobacteria	*Oryzihumus*	2.9	*Tetrasphaera duodecades*	100%		>100 100% identity matches, all soil (prairie, glacial, mine drainage site)	100%
Proteobacteria	Alphaproteobacteria	*Sphingomonas*	2.6	*Sphingomonas sp.* (5), *Sphingomonas naphthae* (2)	99%		post-volcanic pyroclastic surface in Alaska(1), subsurface soil in former uranium mine(1), wetland soil (2), glacier forefield (1), high latitude soil under soil crust (5), glacier ice (1), glacier surface snow (1), Antarctic microbial mat (1)	100%
Gemmatimonadetes	Gemmatimonadetes	*Gemmatimonas*	2.1	None	NA	NA	periglacial soil (1), post-volcanic pyroclastic surface in Alaska(1), Antarctic glacial forefield(3), glacier sediment(1)	99%
Gemmatimonadetes	Gemmatimonadetes	*Gemmatimonas*	1.9	None	NA	NA	periglacial soil (2), boreal lake water(1), leachate from copper mine (1), Kellogg Biological Station soil (3), desert stream sediment (1)	100%
Chloroflexi/ Candidate Division AD3	AD3		1.4	None	NA	NA	fermentation starter (1), high-elevation forest soil (3), acid mine drainage soil (3), general soil (9), wetland soil (2), boreal lake water (1), crayfish cuticle (1), river biofilm (1), acidic high-Arctic wetland permafrost soil (3), acidic high-Arctic wetland active layer soil (1), alpine tundra soil (1), dune soil (2), forest soil (4), grassland soil (1), rhizosphere soil (4)	100%
Actinobacteria	Actinobacteria	*Oryzihumus*	1.7	*Tetrasphaera* sp., *Oryzihumus* sp., *Tetrasphaera duodecadis*, *Intrasporangiaceae* sp.	98%	soil (2), grassland soil (1), desert steppe soil (1), mountain soil (1), volcanic rock (1), salamander skin (1), permafrost soil (1)	Acid mine drainage soil (1), Periglacial soil (1), Freshwater biofilm (1), Antarctic soil (3), Cold desert soil (Norway) (1), High-Arctic permafrost soil (7), Glacier ice (1), Glacier soil (10), Arctic microbial mat on ice shelf (4)	100%
Chloroflexi/ Candidate Division AD3	AD3		0.9	None	NA	NA	Fermentation starter Daqu (1), acid mine contaminated sediment (2), Mountain soil (4), Grassland soil (2), Forest soil (5), Dune field (2), Alpine soil (2), acidic high-Arctic wetland soil (2), acidic high-Arctic wetland permafrost soil (2), acid mine drainage (1), Wetland soil (1), Undescribed soil (9), River biofilm (1), crayfish cuticle (1), Lake water (1)	100%
Gemmatimonadetes	Gemmatimonadetes	*Gemmatimonas*	0.9	None	NA	NA	soil or post-volcanic pyroclastic surface	99%
Bacteroidetes	Bacteroidia	*Solitalea*	0.9	None	NA	NA	surface of Byron glacier (Alaska)	100%
Bacteroidetes	Bacteroidia		1.0	*Sphingobacteriaceae* bacterium	98%	soil from Baekdu Mt.	grassland soil (2), grassland winter soil (2)	99%
Chloroflexi/Candidate Division AD3	AD3		0.7	Unidentified clones	99%	soil	acidic alpine stream sediment (1)	100%
Proteobacteria	Deltaproteobacteria	*Anaeromyxobacter*	0.7	None	NA	NA	ice core from Austre Lovenbreen glacier, Ny-Alesund	100%
Bacteroidetes WPS-2	Bacteroidia		0.8	*Sphingobacteriia* bacterium (2), *Flavihumibacter* sp. (4), *Flavihumibacter stibioxidans* (2), *Sphingobacteriaceae* bacterium (1)	97%	freshwater sediment (4), undescribed sediment (1), grassland soil (2), grassland winter soil (2) antimony mine soil (2), soil from Baekdu Mt. (1)		99%
			0.7	None	NA	NA	alpine soil (3)	100%
Proteobacteria	Alphaproteobacteria	*Acidiphilium*	0.7	*Acetobacteraceae* bacterium (2)	97%	Antarctic soil (2)	forearm skin (1), rainwater (1), carbonate soil (1), air in human environment (1), water column during cyanobacteria bloom (1)	99%
Proteobacteria	Deltaproteobacteria	*Anaeromyxobacter*	0.6	None	NA	NA	undescribed Montana soil (1)	99%
Proteobacteria	Alphaproteobacteria	*Acidiphilium*	0.6	*Acetobacteraceae* bacterium (2)	97%	Antarctic soil (2)	river biofilm (1), glacier sediment (1), Iceland volcanic rock (2), alpine soil (1)	99%
Gemmatimonadetes	Gemmatimonadetes	*Gemmatimonas*	0.7	None	NA	NA	undescribed soil (1), basaltic glass (1), periglacial environment (1)	100%
WPS-2			0.6	None	NA	NA	undescribed soil (4), soil of a tobacco plantation (1), glacial forehead soil (1), high mountain unvegetated soil (1), ferralsol, Madagascar (2), soil under biological soil crust (1), Antarctic soil (1)	100%
Proteobacteria	Alphaproteobacteria	*Acidiphilium*	0.6	None	NA	NA	rock-colonizing lichen (1), glacial snow (3), Al-rich rock coating (1), glacier forefield soil (2), obsidian rock in iceland (1), alpine soil (1)	98%
Gemmatimonadetes	Gemmatimonadetes		0.4	Unidentified clone (2)	98%	Soil (1)	Top 10: plant root (1), rhizosphere soil (9)	100%
Actinobacteria	Thermoleophilia		0.6	Unclassified Verricomicrobia clone (1)	99%	Soil (1)	Antarctic glacial forehead soil (1), fly gut/reproductive organs (1)	100%

Table 4.3: Simple linear mixed-effects models regressing phylum or Proteobacterial class abundance against environmental variables, with 1. site and 2. snowpack vs barren sample as random effects (exception: testing site preference and barren vs snowpack, see footnotes).

Phylum	Barren vs Snowpack[a]	Muir vs Sahale[b]	Elevation	Moisture	Soil DNA Conc.	Microbial Biomass	% C	%N	TOC	TON	NH_4-N	NO_3-N	ARA
Deltaproteobacteria	Snow *			↑ **									
Alphaproteobacteria	Barren **	Muir ***											
Gammaproteobacteria										↓ *		↑ *****	
Gemmatimonadetes					↓ *	↓ *	↓ **	↓ **	↓ **	↓ **			
Actinobacteria			↓ *	↓ *****								↑ *****	
Bacteroidetes						↑ *							
Chloroflexi													
Acidobacteria								↑ *	↑ *	↑ *			
WPS-2										↑ *			
Verrucomicrobia	Barren *	Muir **											
Planctomycetes		Muir *						↑	↑ *	↑ *			
Deinococcus-Thermus					↑ ****	↑ *	↑ ***	↑ **					
Cyanobacteria													
Firmicutes		Sahale *			↑ *	↑ ****	↑ *	↑ *	↑ *	↑ *			
Fibrobacteres			↑ *										
Chlamydiae			↑ *	↓ *									
Dependentiae				↓ *							↑ **		
Rokubacteria											↑ **		
Patescibacteria				↓ *									

[a] Main effect: Barren vs Snow, Dependent variable: Phylum abundance, Random effect: Site

[b] Main effect: Site, Dependent variable: Phylum abundance, Random effect: Barren vs Snow

* indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$, **** indicates $p<0.0001$

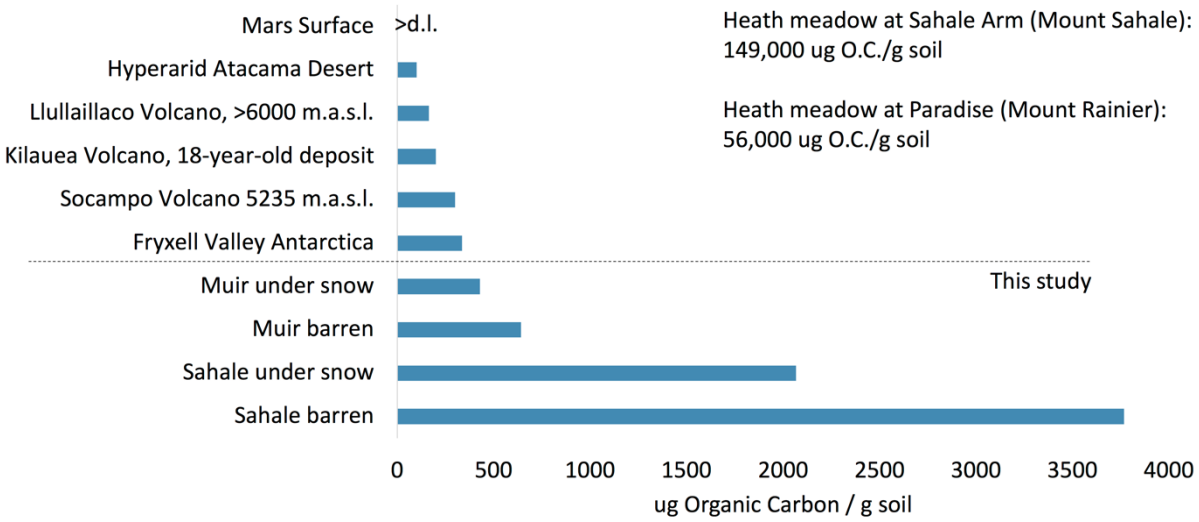

Figure 4.1: Comparative organic carbon concentrations for various barren cold-environment soils that have been used as Mars or icy moon analogues (modified from Lynch and Neufeld (2015)). Values for vegetated lower-elevation alpine meadows in the same general area are included for comparison.

Figure 4.2: Sampling locations in the state of Washington, United States of America.

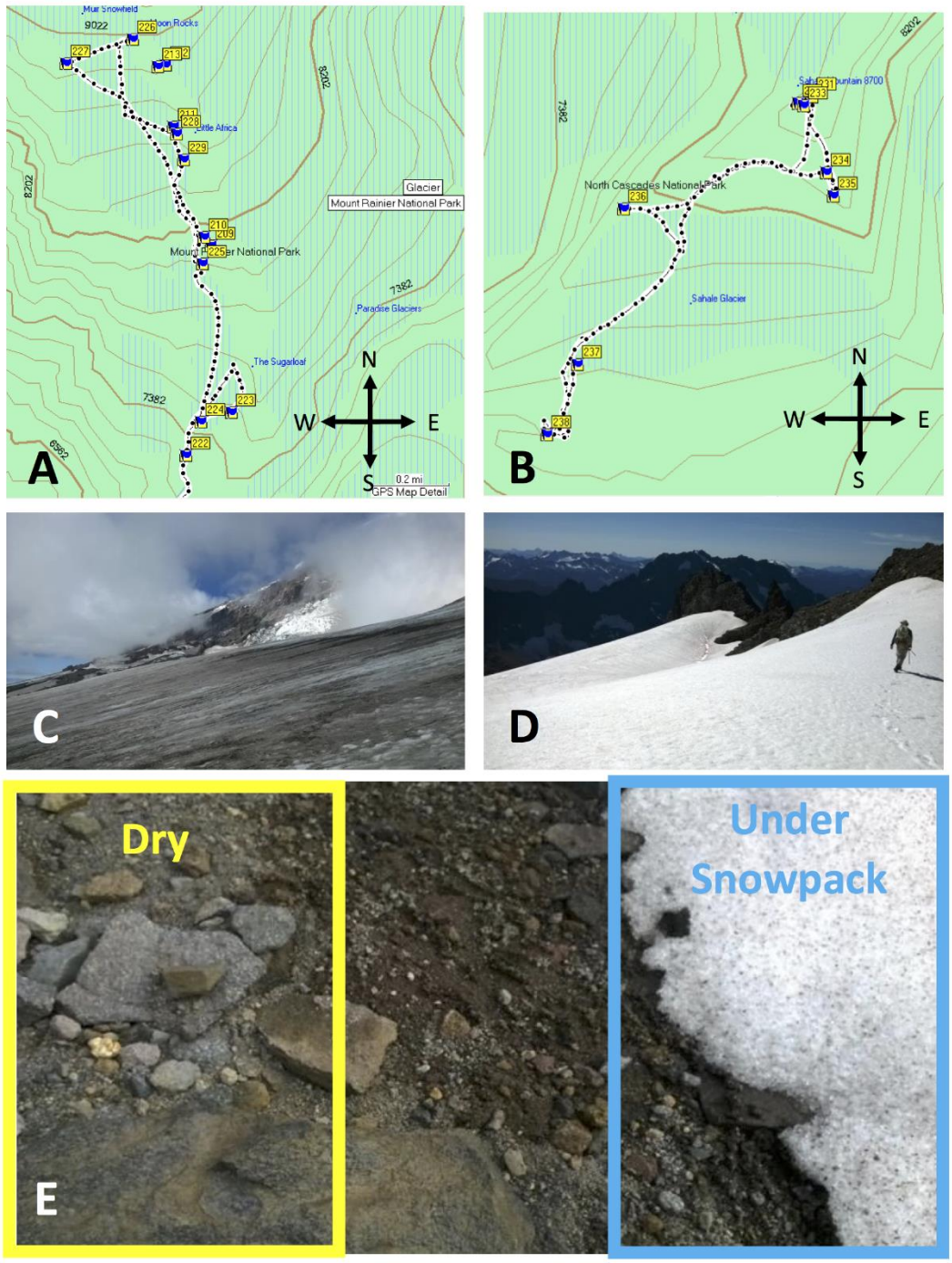

Figure 4.3: Sampling location transects, site pictures, and example of site sampling location. A) Muir Snowfield sampling route and locations at Mount Rainier National Park and B) Sahale Glacier sampling route and locations at North Cascades National Park and C) Muir Snowfield, D) Sahale Glacier, E) Example of sampling location/sampling scheme.

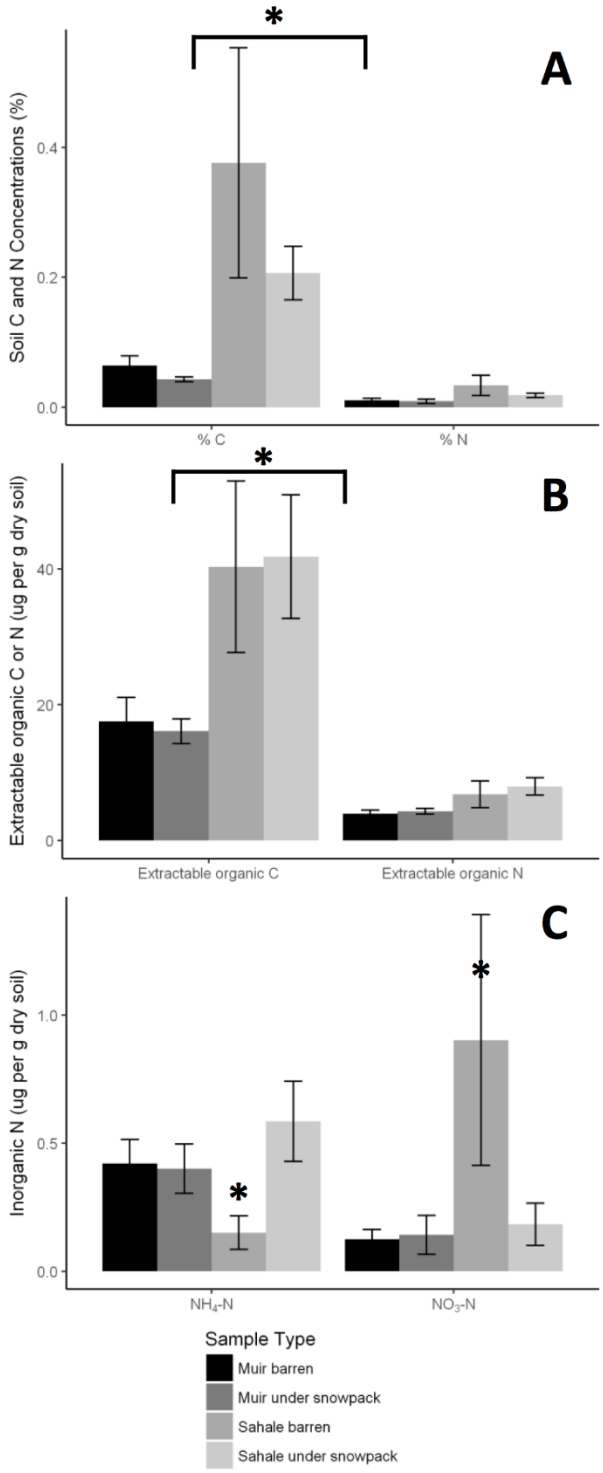

Figure 4.4: Average soil total C and N, extractable organic C and N, and inorganic N concentrations for Sahale Glacier and Muir Snowfield barren and under-snowpack soil samples.

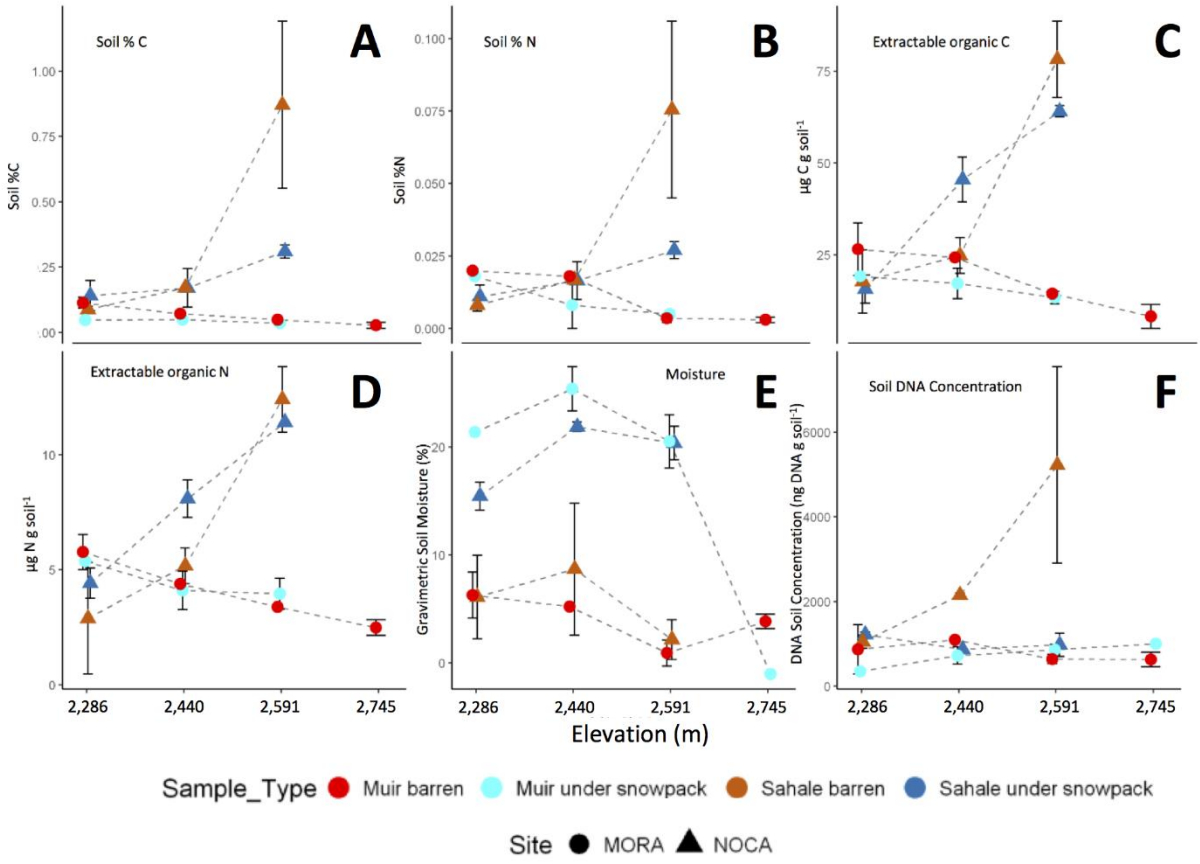

Figure 4.5: Soil carbon (C) and nitrogen (N) properties at Muir Snowfield and Sahale Glacier sampling sites. Sahale Glacier was in general more nutrient-rich and had higher soil C to N at higher elevations. Because Sahale Peak was sampled up to its highest elevation (2,621 m) it is likely that snowmelt and microbial photosynthesis at higher elevation contributed to this trend. The reverse was true at Muir, with soil total and extractable organic C and N decreasing with elevation.

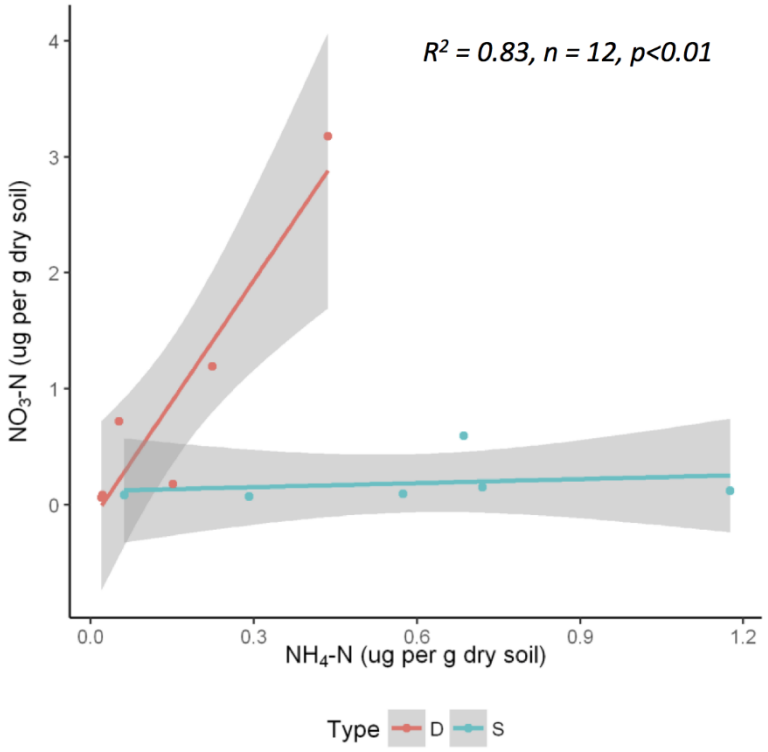

Figure 4.6: Relationship between extractable NH_4-N and NO_3-N at Sahale Glacier. NO_3-N increased with NH_4-N in exposed samples but not in samples take form under snowpack, suggesting that nitrification is only occurring in exposed soils.

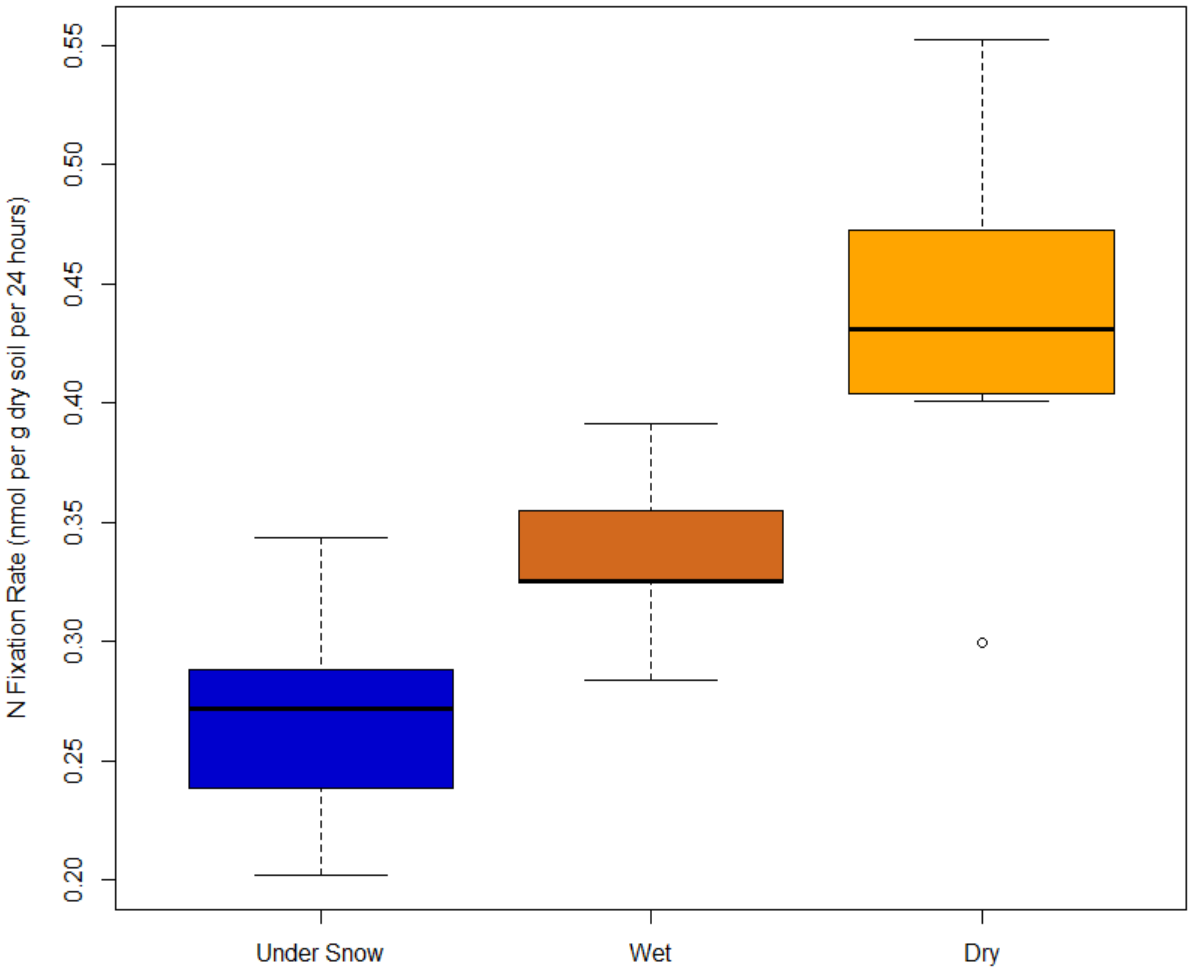

Figure 4.7: Nitrogen fixation rate of soil samples taken from under snowpack, the moist zone of soil that has recently melted, and exposed dry soil further from the snowfield. Not included are samples that registered no fixation; fixation vs non-fixation had no relationship to snowpack, site, or elevation.

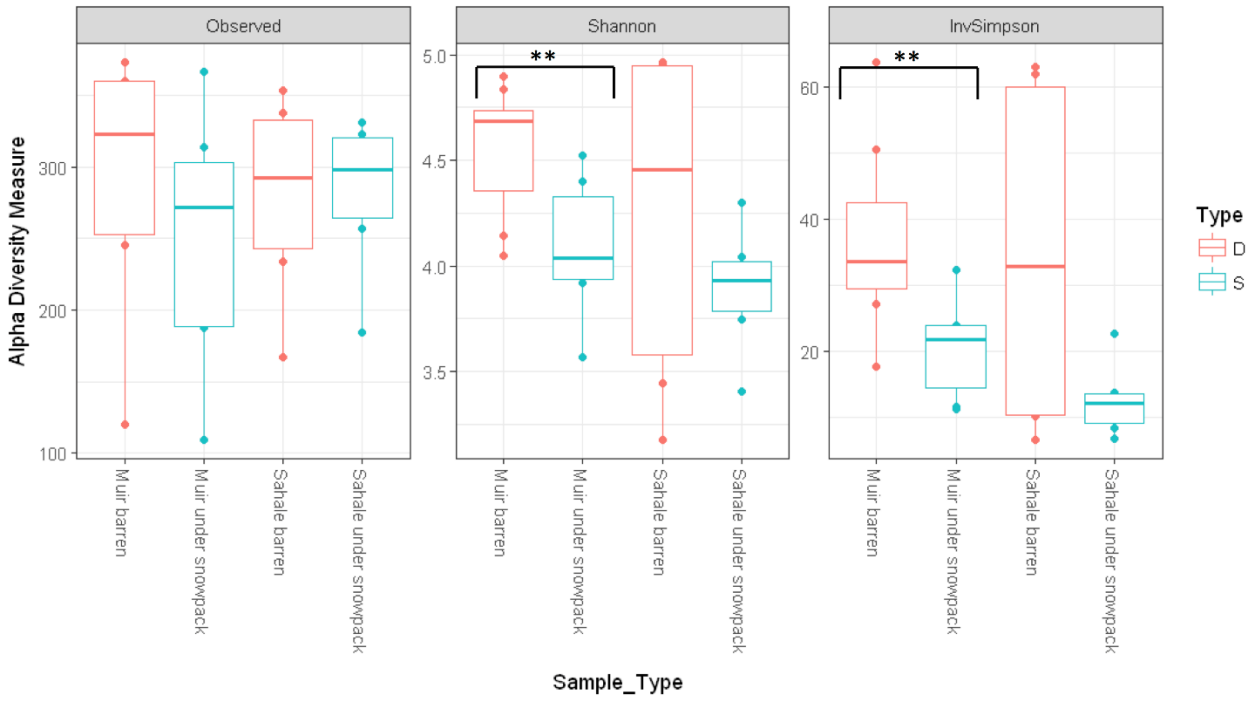

Figure 4.8: Alpha diversity measurements for consensus sequences from under-snowpack vs barren soils at Muir Snowfield and Sahale Glacier. Faith's Phylogenetic Distance index values are not shown. Diversity as measured by Shannon's and Inverse Simpson's indices was significantly lower in samples from underneath snowpack at Muir snowfields, but not at Sahale glacier, due to low levels of diversity at the lowest elevation samples at Sahale.

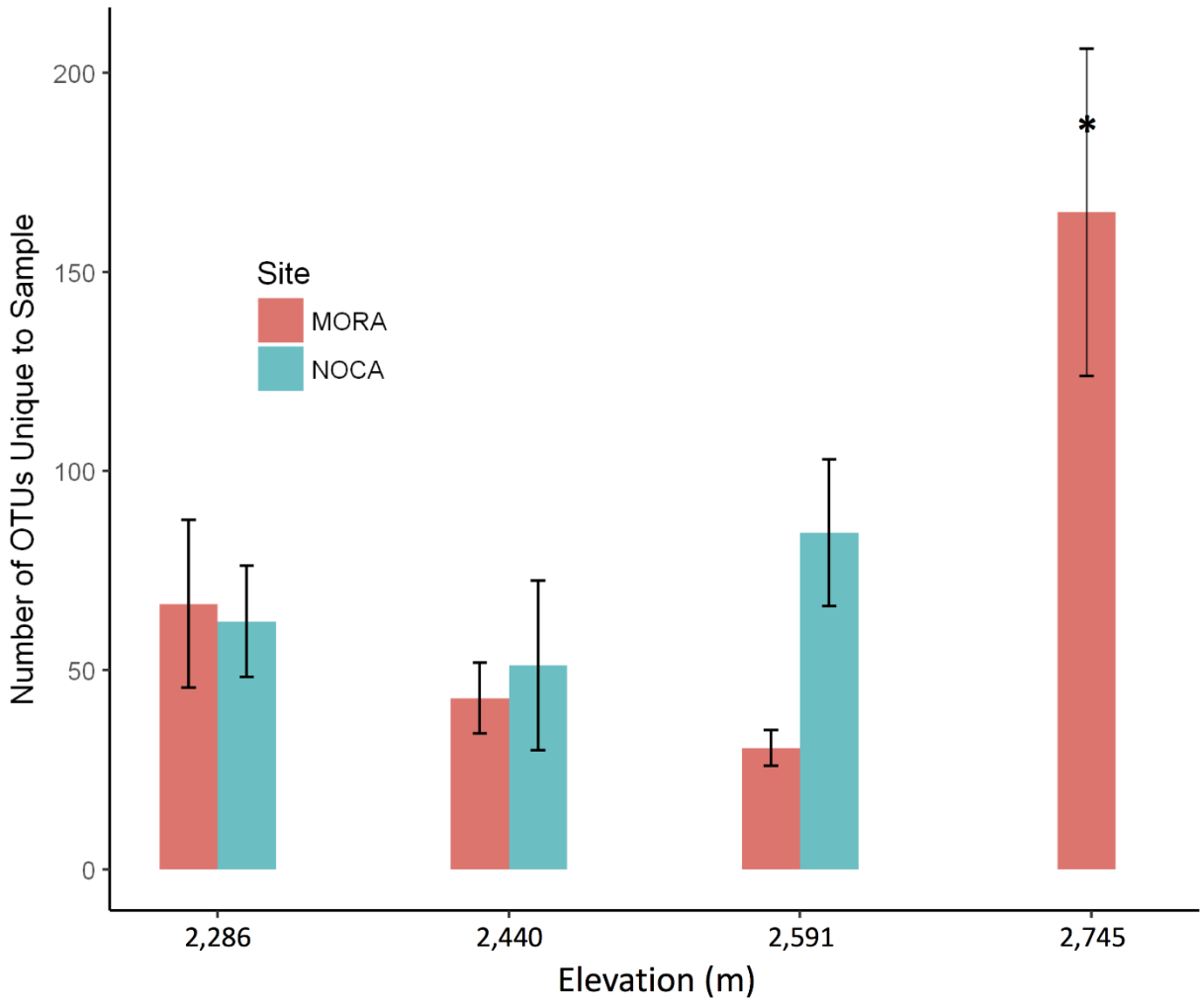

Figure 4.9: Number of unique ASVs per sample along an elevation gradient. There were significantly more ASVs unique to a single sample at 9,000ft elevation at Muir Snowfield.

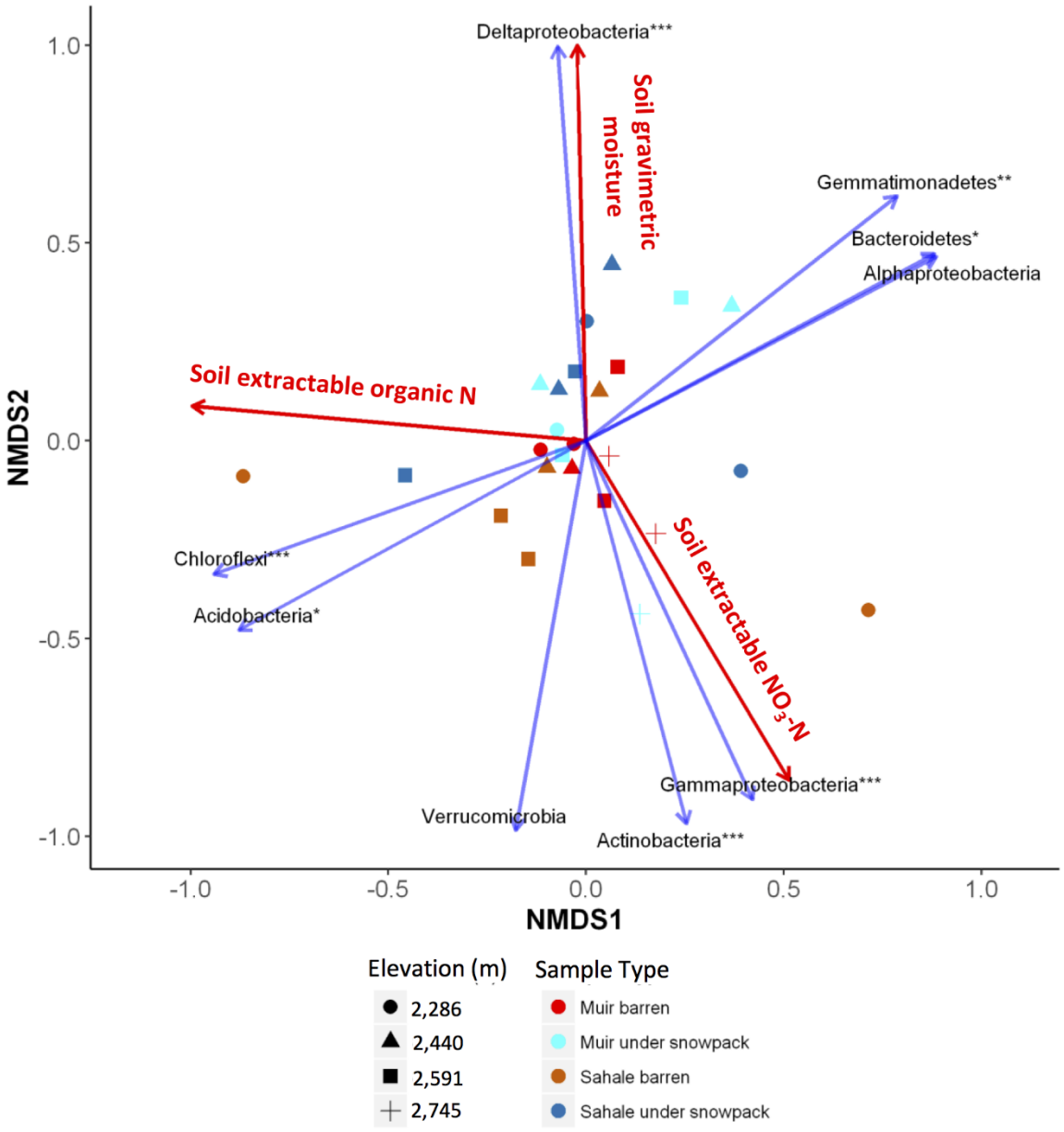

Figure 4.10: NMDS ordination (Bray-Curtis distance) using total abundance of each phylum per sample. All phylum abundances and environmental variables were transformed to fall between a range of 0 and 1. Only environmental factors significantly associated with beta diversity (envfit, $p<0.05$) are shown.

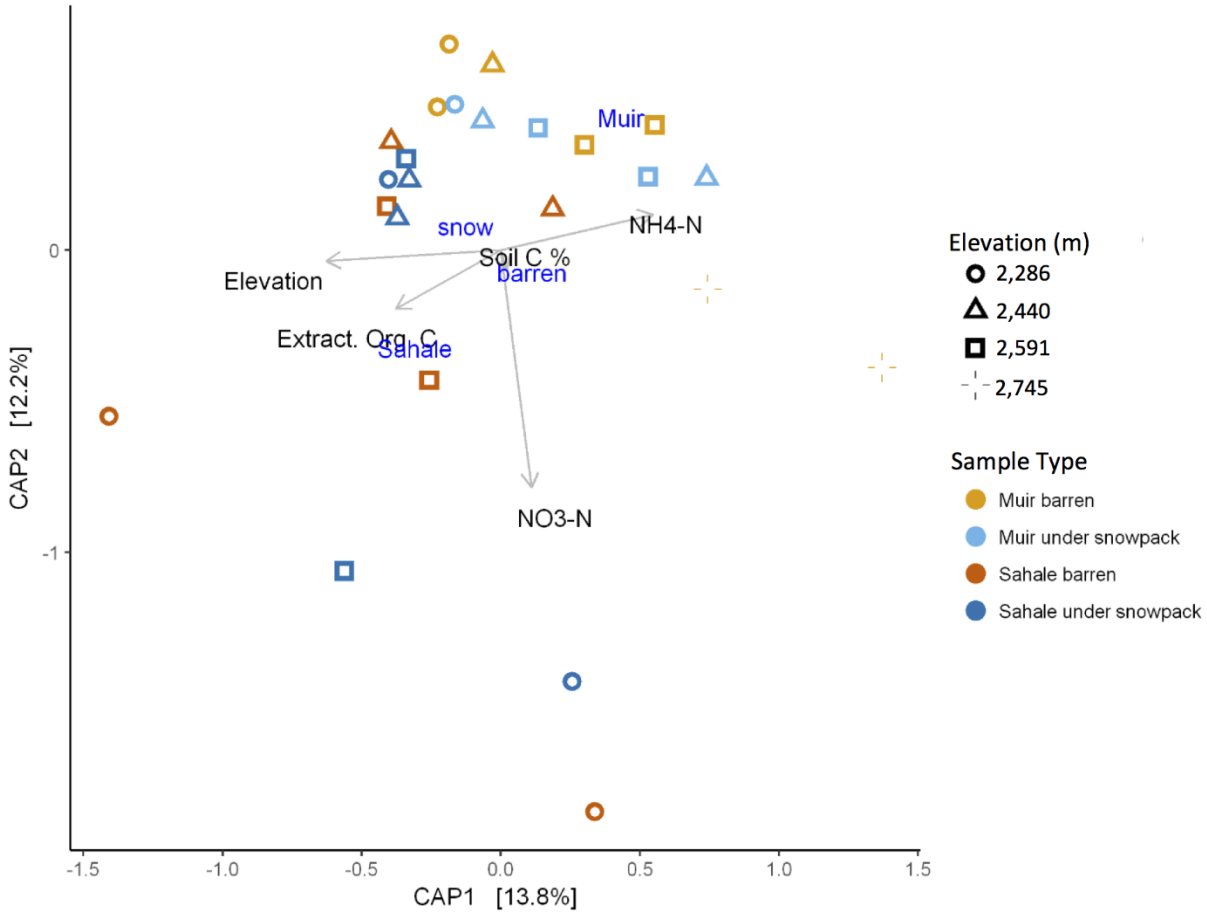

Figure 4.11: db-RDA ordination of scaled community 16S data with weighted unifrac distances. Soil nutrient status, rather than site, snow vs non.snow, or elevation, appears to be the largest determiner of difference in the microbial community at these sites.

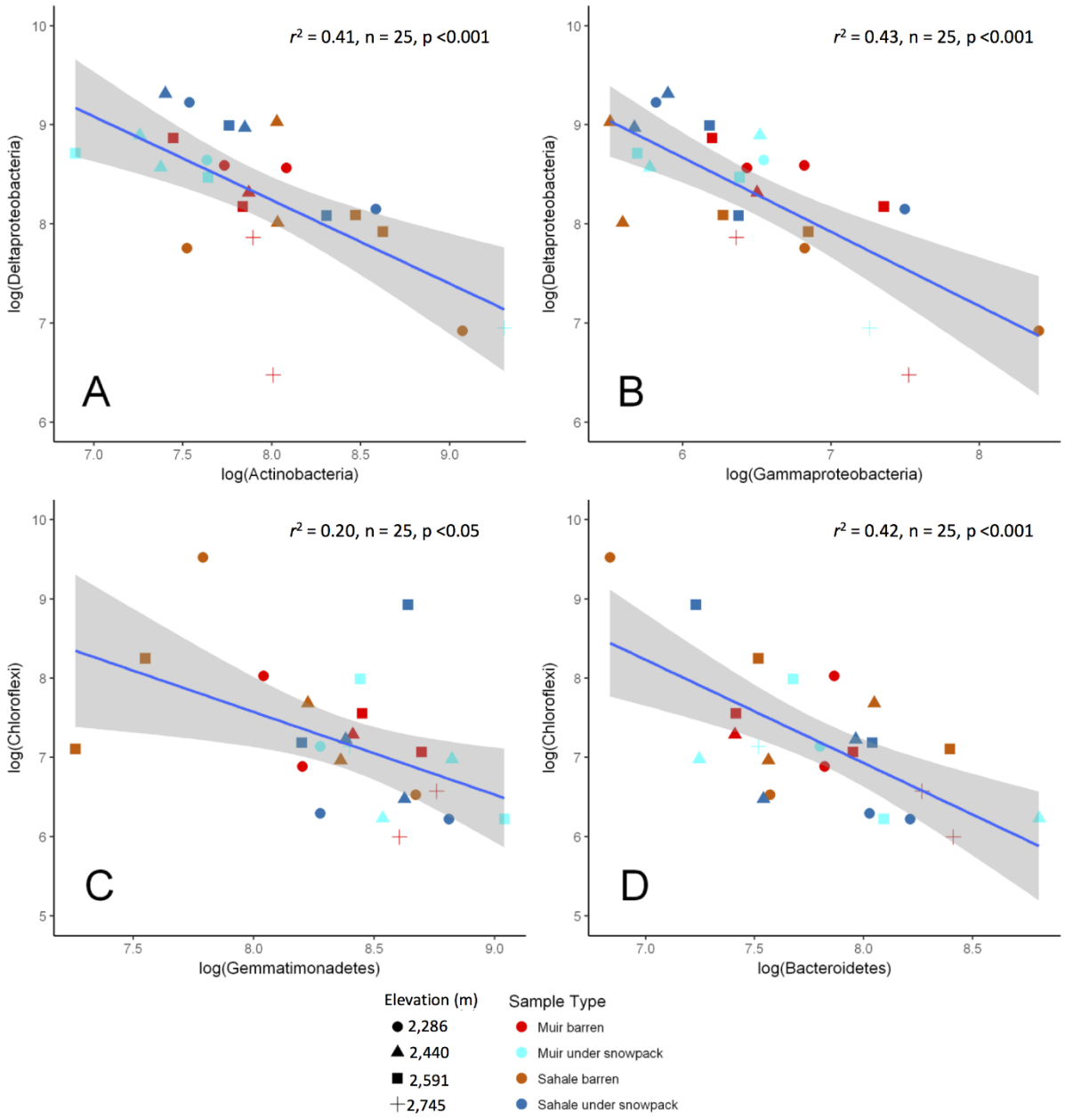

Figure 4.12: Relationships between negatively correlated phyla. Chloroflexi abundance is negatively correlated with the abundance of Gemmatimonadetes, Bacteroidetes, and Alphaproteobacteria, which are all positively associated with each other (Adjusted R^2=0.66). Deltaproteobacteira abundance is negatively correlated with Actinobacteria and Gammaproteobacteria abundance.

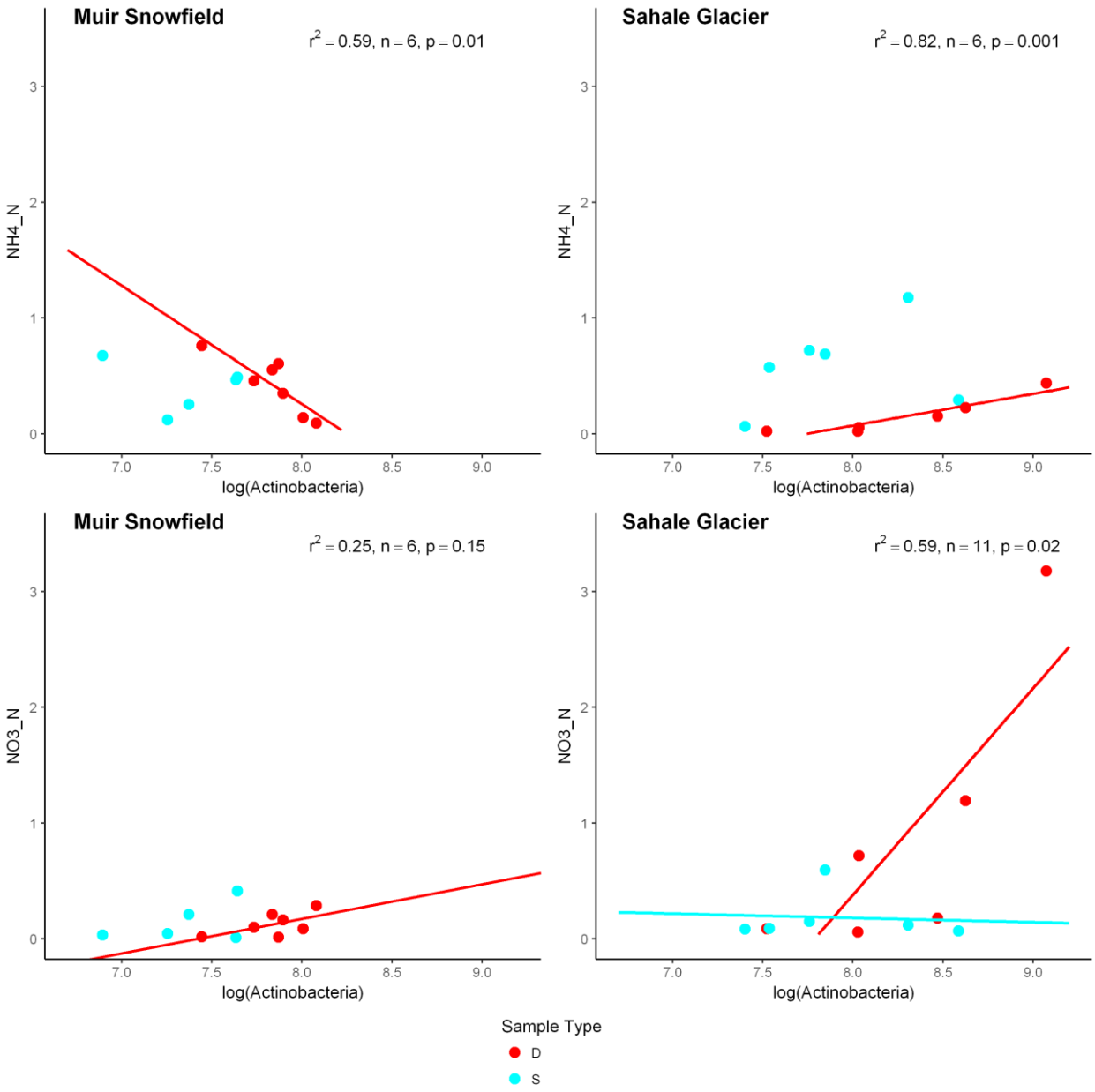

Figure 4.13: Relationship between Actinobacterial abundance and extractable NH_4-N and NO_3-N in exposed and below-snowpack soil at Sahale Glacier and Muir Snowfield. There was an overall significant positive linear relationship between Actinobacterial abundance and NO_3-N availability (NO_3-N~Actinobacteria R^2=0.63).

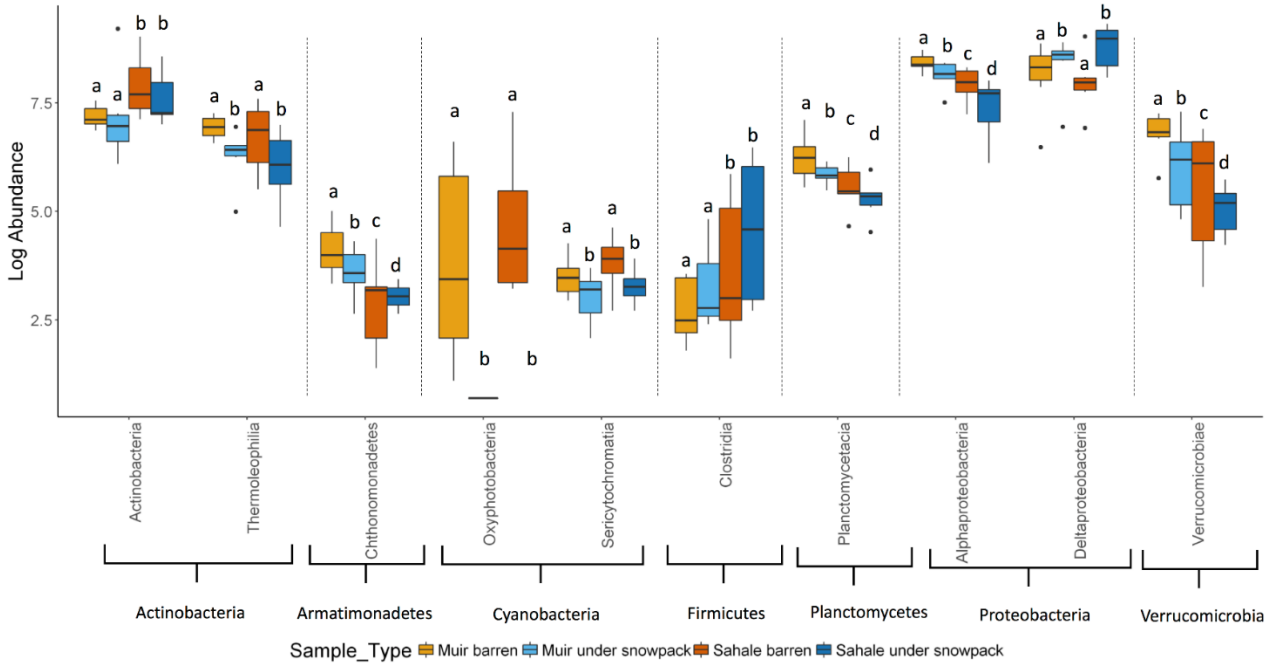

Figure 4.14: Bacterial classes with significant abundance differences by site and by sample type, according to the Kruskal-Wallace test with $p<0.05$. There were significantly more bacteria in the classes Actinobacteria and Clostridia at Sahale than at Muir. The Chthonomonadetes, Planctomycetacia, Alphaproteobacteria, and Verrucomicrobiae all exhibited the same pattern of decreasing abundance: Muir barren → Muir under-snowpack → Sahale barren → Sahale under-snowpack. The Thermophilia, Oxyphotobacteria, and Sericytochromatia were all more abundant in exposed, barren soils regardless of site, while the Deltaproteobacteria were more abundant under snowpack.

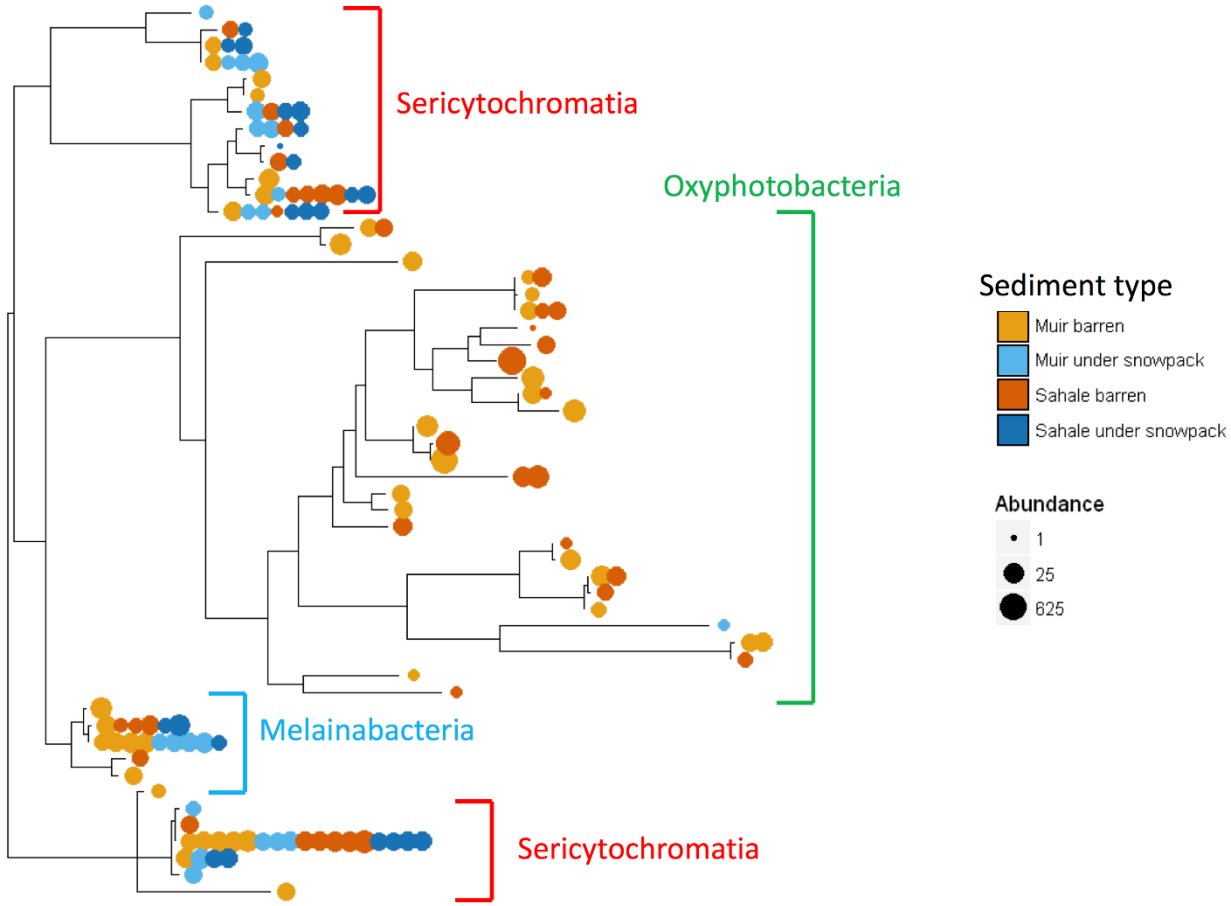

Figure 4.15: Phylogenetic tree of cyanobacteria 16SrRNA sequences with chloroplast sequences removed. Basal, non-photosynthetic classes of the cyanobacteria were fairly evenly distributed between under-snowpack vs barren soil samples, while oxyphotobacteria were strictly confined to samples collected in barren soils. The number of cyanobacterial sequences was quite low in comparison to most other phyla, but the signal of photosynthetic cyanobacteria upon snowmelt is preserved even in the face of probable high levels of soil-preserved extra-cellular DNA.

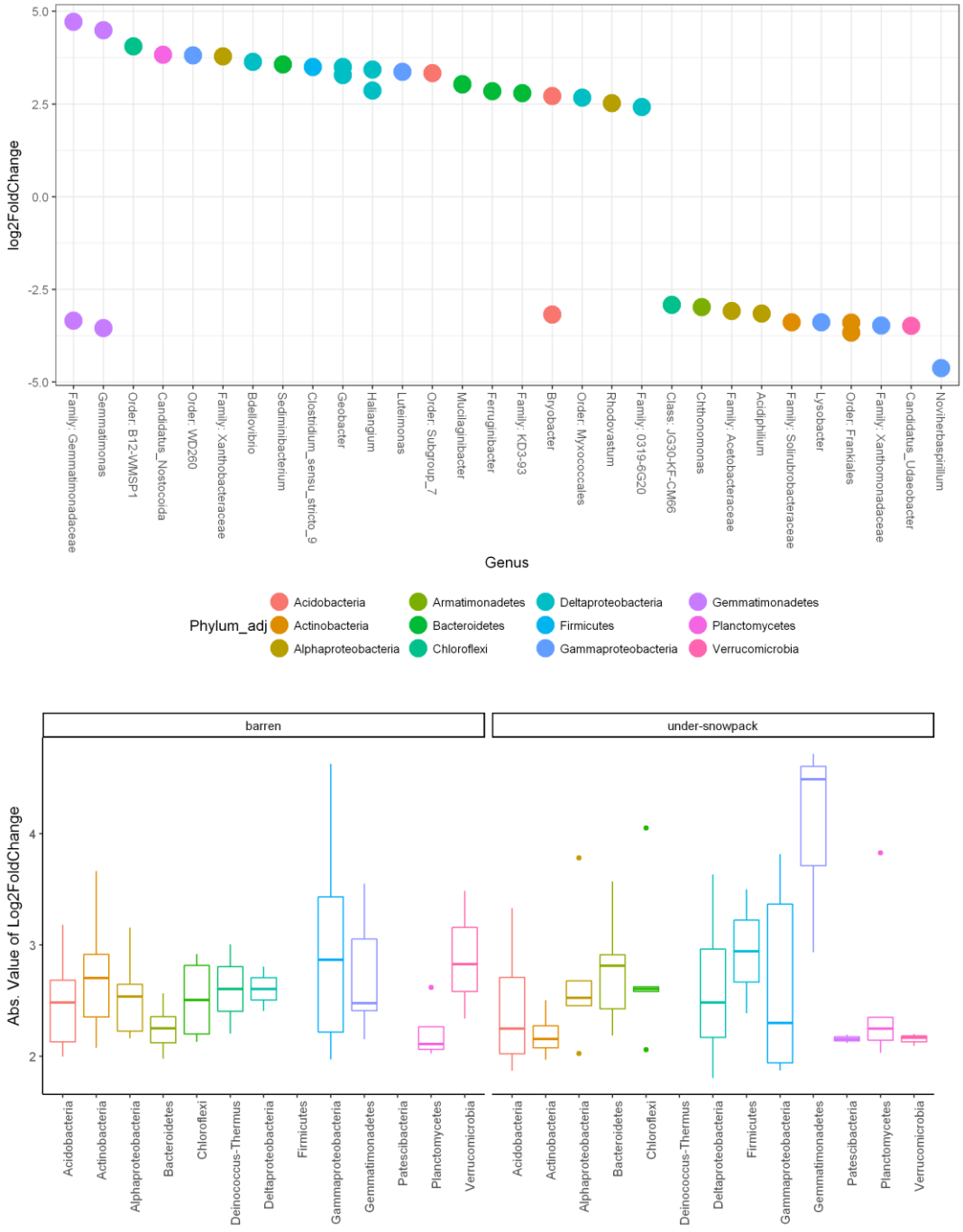

Figure 4.16: Log 2-fold change values from DESeq analysis of barren vs under-snowpack soils. Consensus sequences that were significantly more represented in barren or under-snowpack communities are here grouped by phylum and category.

Chapter 5: Conclusions

This course of research involved two field studies in the alpine Pacific Northwest of the United States. The first study was a three-year N fertilization experiment in three vegetated meadows at treatment levels of 0, 3, 5, and 10 kg NH_4NO_3-N ha^{-1} yr^{-1} at Paradise Meadows at Mount Rainier National Park, Sahale Arm at North Cascades National Park, and Lilian Ridge at Olympic National Park. The second was an investigation of the microbial communities present in the barren soils of permanent snowfields at higher elevation, conducted at Muir Snowfield at Mount Rainier National Park and Sahale Glacier at North Cascades National Park.

NITROGEN FERTILIZATION EXPERIMENT

My objectives for the N fertilization study were to

1) assess background C and N cycling in alpine meadow soils of the Pacific Northwest in the United States
2) study the effects of simulated increased N deposition on plant species abundance and soil nutrient cycling
3) study where and how applied N accumulated in soils and plant tissue
4) assign a critical load for N deposition in these soils
5) put my findings into the larger context of precipitation patterns and alpine plant uptake in the Pacific Northwest to give recommendations regarding the greatest ecological dangers of N deposition.

I found overall that while background soil N cycling partially conformed to models of alpine N cycling developed in the Rocky Mountains, differences in soil N cycling and in response to simulated N deposition were site-specific. Background N deposition levels

Using increased availability of NO_3^- to plant and microbes as an indicator, I suggest the upper limit for the N critical load should be set at 6 kg N ha^{-1} yr^{-1}. This indicates much greater N sensitivity than soils studied at Niwot Ridge, where N cycling response to N deposition was detectable at an application level of 20 kg N ha^{-1} yr^{-1} (Bowman et al., 2006), but is similar to the 10 kg N ha^{-1} yr^{-1} value found at Rocky Mountain National Park (Bowman et al., 2012). There were multiple other soil C and N responses to N treatment, but there were no significant changes in plant species cover or diversity over the course of the study.

Plants and microbes in dry graminoid/forb alpine meadows of the PNW appear limited by moisture rather than N and have high N availability even into fall, and so have a higher probability of N leaching at that time of year. However, a microbial community adapted to high levels of N can serve as a buffer against N leaching in the short term.

In contrast, moist heath meadows dominated by evergreen shrubs appear to be N sinks in the fall, with N application stimulating microbial uptake of organic C. However, during the growing season N is still readily available in soils after snowmelt. Atmospheric N additions are at greater risk of leaching during this season because the microbial community at these sites is adapted to low N and cannot compensate in the short term. Changes in alpine soil chemistry in response to N treatment can be site-specific and result from differences in plant uptake and soil N mineralization capacity, indicating different regimes for response to N deposition.

I recovered more applied N in the foliar tissue of heather than the foliar tissue of lupine. New growth of heather accumulated more applied NO_3^- while new growth of lupine accumulated more applied NH_4^+. We suspect capacity for foliar uptake of NO_3^- in Pacific Northwest heather species.

There was a significant relationship between recovery of applied ^{15}N and the presence of forbs and graminoids, but only where N was not extremely limiting.

Severe N-limitation led to a more even distribution of applied N across plots. In contrast, at sites where plant roots had access to mineralized N and where root N was associated with soil N availability, N deposition accumulated in areas with high background root N concentrations. This trend may not only lead to eventual plant community change in high-N soils, but may explain why plant community change in response to N deposition is so difficult to identify in comparison to changes in soil nutrient availability. Land managers should be concerned with which topographic conditions are most likely to lead to 'hot spots' of N and which may be nuclei for future plant species shift in the face of increasing N deposition.

MICROBIAL COMMUNITIES OF PERMANENT SNOWFIELD SOILS

My objectives for this study were to assess:

1) soil nutrient conditions and core microbiome of the barren alpine soils at these locations
2) main factors driving differences in bacterial community composition and how the presence of permanent snowpack affects community composition
3) which phyla, classes, and specific amplicon sequence variants (ASVs) cause the largest differences in microbial community structure in exposed soils vs soil under snowpack, and what this tells us about environmental conditions at these sites

4) what inferences can be made about how C and N accumulate in soils in permanent snowfields, based on bacterial community composition and bacterial abundance relationships to soil nutrient conditions.

The soils of permanent snowpack soils in the Pacific Northwest are low in microbial biomass, comparable to soil samples taken in the Himalayas, and low in C and N. Carbon concentrations in these soils are at the same order of magnitude as sites that have been used as Martian analogs, such as the Atacama or the dry valleys of Antarctica.

I found direct relationships between different phyla and environmental characteristics. Deltaproteobacteria were associated with higher moisture and the presence of snowpack. Actinobacteria and Gammaproteobacteria were associated with each other and with higher soil extractable NO_3-N, and Gemmatimonadetes were associated with lower soil DNA and microbial biomass, lower total C and N concentrations, and lower extractable organic C and N. Specific ASVs from the Gemmatimonadetes were the strongest indicator species for soil samples taken under snowpack, and ASVs from that phylum were strongly associated with other sequences on NCBI from glacial and polar environments. Cyanobacteria were few and chemolithoautotrophy is likely the main form of primary production.

Differences in microbial communities between samples, however, were actually fairly low, even between soils underneath the snowpack vs soils exposed to sunlight. This is further support for the hypothesis proposed by Bajerski and Wagner (2013) in their study of glacial forefields in Antaractica: that microbial communities are highly diverse and fairly undifferentiated when habitat formation is in its early stages. A community of single-celled alien organisms on Europa, however, might be fully adapted to conditions we consider to be extreme, and show a much reduced diversity and clearer functional relationships. While the conditions prevailing in terrestrial analogs may be

similar to icy moons or Mars, the communities behave differently. Thus a study of bacterial species that are most adapted to cold conditions, rather than the entire community, may prove more beneficial to the field of astrobiology.

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Appendix A

Table A.1: Plot locations in UTM coordinates

Block Name	Easting	Northing
MORA		
Block 1	597686	5183481
Block 2	597574	5183606
Block 3	597731	5183386
Block 4	597748	5183384
Block 5	597644	5183579
NOCA		
Block 1	643785	5370588
Block 2	643806	5370545
Block 3 East	643744	5370586
Block 3 West	643707	5370599
Block 4	643881	5370532
Block5 East	643840	5370569
Block5 West	643831	5370592
Fenn collectors:	643789	5370555
	643714	5370591
	643878	5370509
OLYM		
Block 1	471887	5306873
Block 2	472106	5306221
Block 3	472050	5306493
Block 4	472083	5306146
Block 5	71958	5306554
Fenn collectors:	472115	5306217
	472060	5306410
	471887	5306873

[1]Locations are presented in Universal Transverse Mercator projection (UTM), Zone 10 North, WGS84

Table A.2: Species list of plants found within study plots at MORA, NOCA, and OLYM field sites. Note: we did not attempt to identify non-vascular plants at the species or genus level and they are not included here. Species codes are used in distance-based redundancy analysis (Figure 3.6).

Scientific Name	Common Name | CODE	MORA	NOCA	OLYM
Abies lasiocarpa	Subalpine fir | ABLA	x		
Achillea millefolium	Yarrow | ACMI			x
Antennaria spp.	Rosy pussytoes | ANT.SP	x	x	x
Arenaria spp.	Sandwort | AR.SP	x		x
Aster spp.	Aster family | AST.FAM	x	x	x
Campanula rotundifolia	Common Harebell | CARO			x
Carex spp.	Sedges | CAREX	x	x	x
Cassiope mertensiana	White mountain heather | CAME	x	x	x
Castilleja parviflora/miniata	Magenta or Scarlet Paintbrush | CA.SP	x		x
Empetrum nigrum	Crowberry | EMNI		x	
Gentiana calycosa	Mountain Bog Gentian | GECA	x		x
Juncus dromondii	Drummond's rush | JUDR	x	x	x
Lupine spp.	Lupine | LUSP	x	x	x
Lutkea pectinata	Partridgefoot | LUPE	x	x	x
Lycopodium sitchenses	Club moss | LYSI	x		
Pedicularis ornithorhyncha	Bird's Beak Lousewort | PE.SP	x		
Penstemon davidsonii var. Menziesii	Menzie's Penstemmon | PEN.SP	x		
Phyllodoce emptreformis	Pink mountain-heath | PHEM	x	x	
Poaceae spp.	Grasses | PO.SP	x	x	x
Polygonum bistortoides	American bistort | POBI	x	x	x
Potentilla flabellifolia	Fan-leaf Cinquefoil | POFL		x	
Vaccinium deliciosum	Cascade huckleberry | VADE	x	x	
Valeriana sitchensis	Sitka valerian | VASI	x		
Veronica wormskjoldii/ cusickii	Alpine Speedwell/ Cusick's Speedwell | VE.SP	x	x	

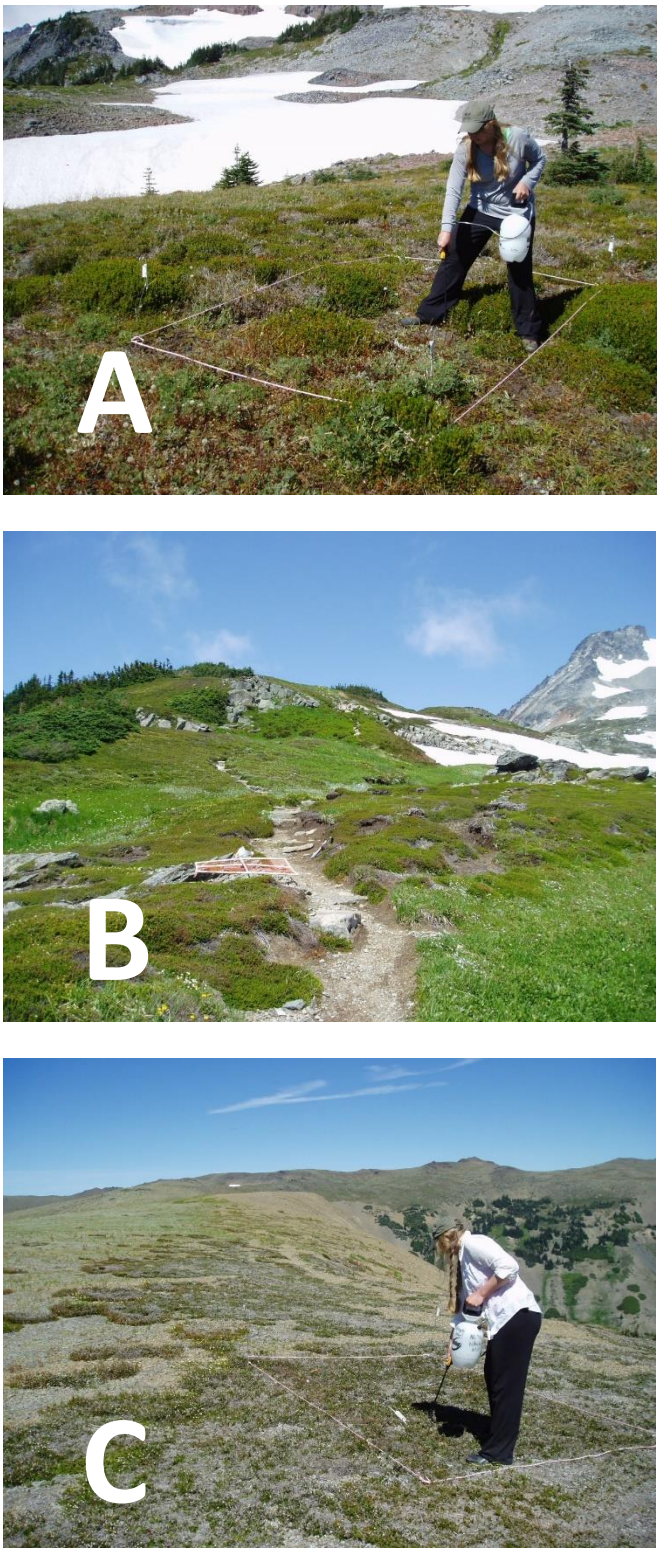

Figure A.1: Site photos at A) Paradise Meadows at MORA, B) Sahale Arm at NOCA, and C) Lilian Ridge at OLYM

Appendix B

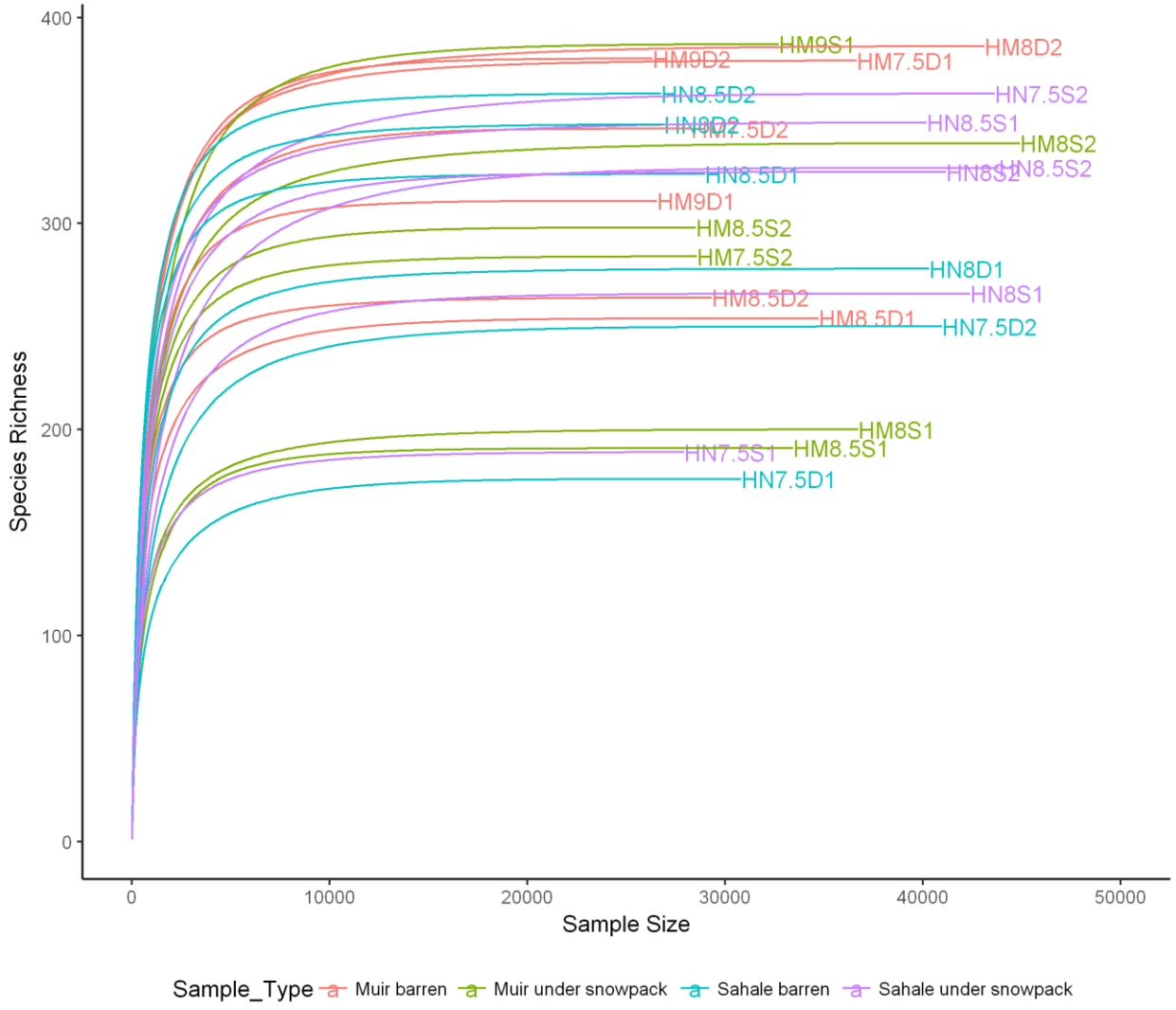

Figure B.1: Rarefaction curves for each sample (pre-correction for sample size)

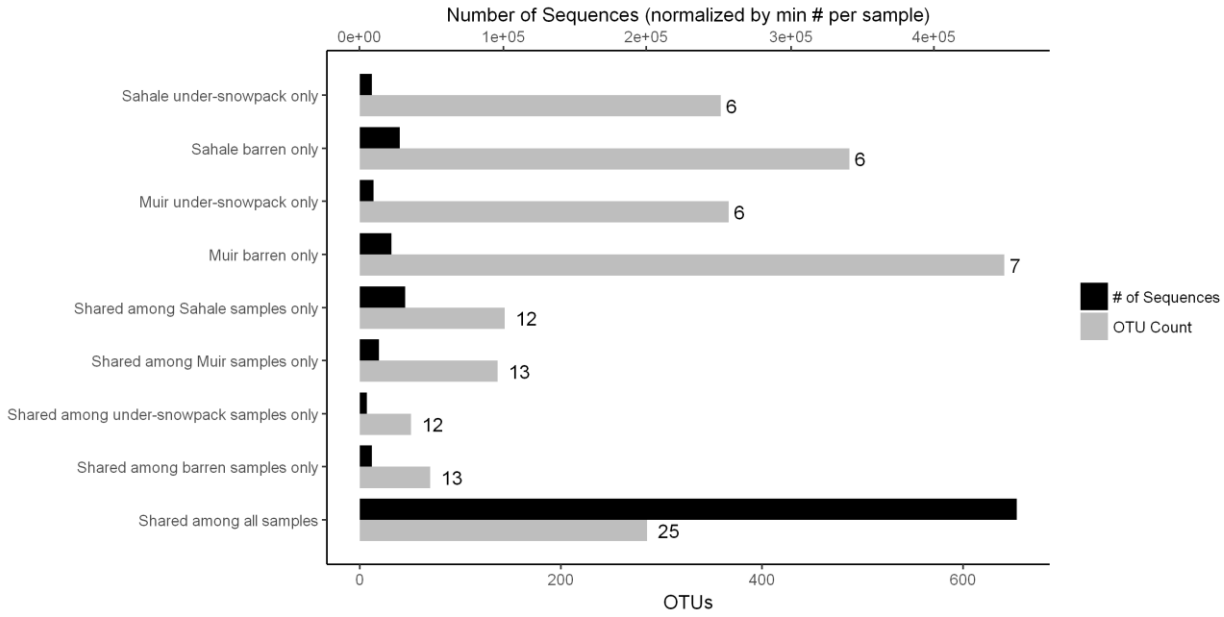

Figure B.2: Number of unique ASVs per group and number of sequences associated with those ASVS. While ASVs uniquely shared among each specific group of Site/snow vs barren were most numerous in terms of species richness, the majority of sequences belonged to ASVs that were shared among all samples.

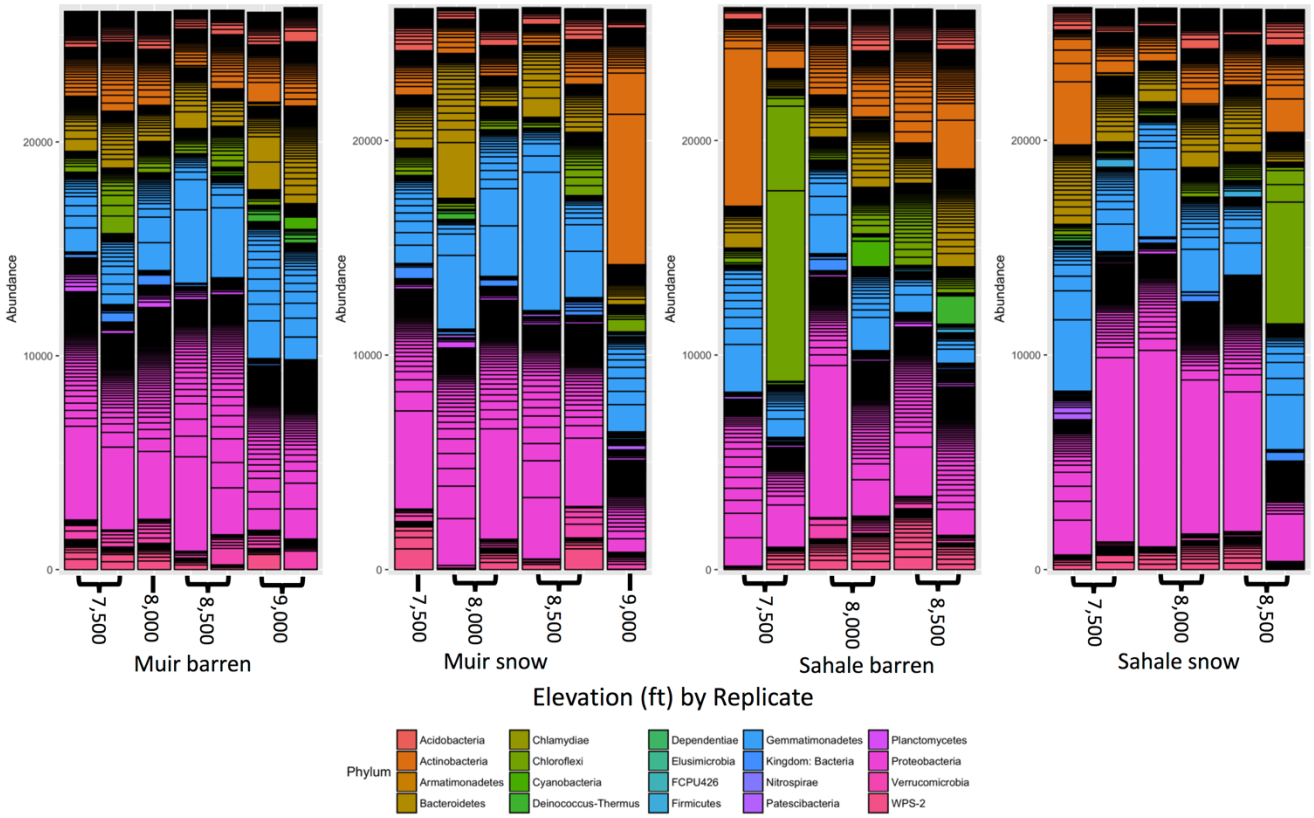

Figure B.3: Consensus sequence abundance counts by phylum, normalized by the minimum number of sequences per sample, versus elevation. Proteobacteria, Gemmatimonadetes, Acidobacteria and Bacteroidetes dominate these microbial communities. Each consensus sequence is represented by its own square, so that ASVs represented by very few sequences not discernable.