PATTERNS AND PROCESSES UNDERLYING SOIL MICROBIAL COMMUNITY SUCCESSION

by

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Thesis directed by Associate Professor Diana R. Nemergut

ABSTRACT

Given the vast diversity of microorganisms and their relevance for environmental and human health, there remains a need to better understand the patterns and processes that fundamentally underlie the structure and function of microbial communities through space and time. In particular, soil bacterial communities are vital to ecosystems and agriculture as they largely control soil fertility, plant community dynamics, and global biogeochemical cycles. To this aim, my dissertation work builds understandings regarding the development of soil microbial communities and their function. First, I demonstrate the paramount role of nutrient limitation in controlling the assembly of autotrophic microbial communities through succession (Chapter 2), based on a nutrient manipulation experiment. This work shows that nitrogen (N) and phosphorous (P) fertilization act as a major control on microbial community succession. In Chapter 3, I examine the connections between environmental, community, and functional properties of microbes in post-fire successional soils. This work builds empirical evidence that relationships between resource environment and ecosystem function shift in relative strength across succession. This work also suggests the role of rRNA operon copy number as a trait that varies across succession and may serve future research in describing processes of microbial

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community assembly that connect environment, trait, and function. In Chapter 4, I continue to examine plant-microbe interactions, assessing how post-fire revegetation processes may drive post-fire secondary succession with implications for ecosystem function. This work also reveals that the abiotic context of the post-fire landscape may impact when and to what extent biotic factors, such as plant-microbe interactions, matter in structuring microbial communities and their function. The Appendix provides additional research that describes plant-microbe interactions in succession, assessing how the plant root environment may select for particular bacteria in early succession that range in generalist to specialist character. Overall, my work builds knowledge on the controls that may underlie changes in microbial communities, and ecosystem processes. My work advances our knowledge of how microbial communities assemble through succession and the resulting patterns in their composition and function, which can have immense implications for humans to ecosystems.

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CHAPTER 1

INTRODUCTION

Background

Ecological succession has long been studied to understand the processes and patterns that underlie the development and function of ecosystems. The details of disturbance (e.g. from fires, volcanoes, or receding glaciers), and the subsequent processes of ecosystem development, have long held implications for human society. Flooding of the Euphrates and Nile and subsequent succession, for example, acted to restore soil fertility and give rise to the "cradle of civilization," while current advancements in the study of microbial succession are poised to improve a variety of fields from human medicine to terraforming practices of the future. Today amidst new environmental challenges and a changing climate, understanding how the increasing prevalence of disturbance and successional responses will unfold is of certain relevance to the future of Earth and all its occupants.

As a centerpiece of theory in ecology, ecological succession has been framed by ideas ranging from Frederic Clements to Henry Gleason. Clements proposed a deterministic framework by which succession progressed to a stable climax stage and generally advanced a view of holism in describing ecological succession as the development of a superorganism (Clements, 1916, 1920; Walker and del Moral, 2003). In contrast, Gleason proposed a reductionist approach that argued that stochasticity and the separate life history of species could lead to variable outcomes (Gleason, 1927; Walker and del Moral, 2003). In all, disputation surrounding elements of these two perspectives has resulted in continued study of succession and the examination of questions at the intersection of community and ecosystem ecology, yielding a

variety of models considering controls on how ecosystems assemble and develop in structure and function over time across a variety of disparate environments (Walker and Chapin, 1987; Connell and Slatyer, 1977; Tilman, 1985; Grime, 1977; Ferrenberg *et al.*, 2013). These various models of succession have proposed the importance of organismal life history strategy, resource availability, biotic interactions, and stochastic assembly processes; thus, this work seeks to examine assembly from such varied perspectives in its evaluation of the role of microbial traits, macronutrients, and plant-microbe interactions in driving the patterns of microbial succession which underlie ecosystem development and function.

While past work has generally viewed ecosystem succession as a process beginning with the colonization of substrate by plants (Chapin et al., 1994) more recent work has demonstrated that dynamic processes of succession occur with microbial communities long before plant establishment. Advancements in DNA sequencing and analyses have allowed for great strides in revealing the role that microorganisms play in the structure and function of ecosystems (Hugenholtz et al., 1998). Assisted by such improvement in technology, study of the microbial world provides a new context in which to understand ecosystem and community ecology. In soil environments, for example, microbial succession results in turnover of bacterial communities of particular structures over time that mediate vital chemical and physical transformations of soils. It is now understood that the succession of microbial communities can strongly alter the physical composition of the soil (Treseder et al., 2004; Schmidt et al., 2008), nutrient availability (Zackrisson et al., 2004; Brankatschk et al., 2010; Knelman et al., 2014), soil carbon (Zak et al., 1990; Schmidt et al., 2007; Sattin et al., 2009), and plant community ecology (Reynolds et al., 2003; Kardol et al., 2006), and thus proves an integral consideration in our understanding of ecological succession as a whole. While such patterns of microbial community succession have

come to light, a better understanding of the controls or processes that underlie these patterns is much needed. Particularly with the vast biodiversity contained within microbial communities, there is a need to develop generalizable understandings of controls on how microbial succession may unfold, beyond previously observed phylogenetic, taxonomic, and biogeochemical patterns. Thus, the ability to study both ecosystem and community ecology in microbial systems now allows the wedding of these two fields to understand when and where different processes may preside over community assembly and how community assembly may influence outcomes in ecosystem development and function. Exploration of this connection between ecosystem and community ecology will ultimately provide a mechanistic understanding of how ecosystems, both managed and natural, may respond in structure and function amidst a rapidly changing world and allow for better predictions and management of ecosystem properties ranging from global nutrient cycles and soil fertility to plant productivity.

My work herein adds to our understanding of deterministic processes that may control the assembly of microbial communities through ecological succession. As well, I examine patterns in relationships between environment and ecosystem function that may be influenced by different assembly processes governing temporal succession, and therefore vary predictably across succession. Here, I examine the role of nutrients and plant colonization as selective filters driving microbial community succession and I more broadly assess how relationships among environmental parameters and ecosystem function may vary across succession.

Nutrients

Emblematic shifts in nutrient availability have been shown as a prominent feature of ecological succession (Walker and Syers, 1976; Crews et al., 1995; Ugolini, 1968; Walker and del Moral, 2003). With soil development in primary succession, decreases in P availability and increases in N prove to be limiting to early and late successional plants respectively (Walker and Syers, 1976; Vitousek et al., 1993), and nutrient limitations have been explored as dominant controls on plant community succession (Chapin et al., 1994; Richardson et al., 2004). In secondary succession as well, studies have revealed nutrients to dominantly control the development of plant communities (Van Wijnen and Bakker, 1999; Tilman, 1987), leading to models and perspectives that place nutrients in a central role in plant community dynamics (Tilman, 1985). For example, Van Wijnen and Bakker (1999) found a convergence of plant community composition in early successional salt marshes with those of later succession when N and P additions were applied. Additional research has also illustrated that in addition to having direct effects plant community composition, nutrients may modulate the relative importance of stochastic processes and assembly history (Kardol et al., 2013) or biotic interactions (Cramer et al., 2008; van de Voorde et al., 2012) in community assembly and succession.

Despite the prominent role of nutrients in structuring plant communities across succession, little is known on the effect of nutrients in microbial succession. Past studies have noted the influence of nutrients along with concomitant shifts in a variety of other factors such as pH and soil C, though those studies that observe changes in microbial communities with nutrient availability do not distinguish if communities are responding to or causing the measured nutrient pools (Zumsteg *et al.*, 2012; Edwards *et al.*, 2006). Nonetheless, some evidence suggests that nutrients can play a strong role in microbial community structure and function in early successional soils (Yoshitake *et al.*, 2007; Göransson *et al.*, 2011). However, other factors which vary directionally over succession such as pH and soil C are also known to exert strong selective filters on microbial communities at a global scale (Fierer and Jackson, 2006) and stochastic processes may also mute ecological selection by abiotic factors in microbial succession, particularly at incipient stages (Ferrenberg *et al.*, 2013; Meola *et al.*, 2014). Thus, there remains a need to more clearly understand the role of nutrients as a control on microbial community succession, and the applicability of macrobiotic theory on the topic in a microbiological context.

Environment-ecosystem function relationships

Successional sequences provide a study system that pass through well-defined changes such as in environmental heterogeneity, disturbance, resource availability, biotic interactions, and edaphic properties (Chapin *et al.*, 1994; Crocker and Major, 1955; Matthews, 1992; Walker and del Moral, 2003). Interestingly, changes in the relative importance of these various factors have been recently revealed to strongly influence community assembly processes (Chase, 2010, 2007; Chase *et al.*, 2009; Ferrenberg *et al.*, 2013; Kardol *et al.*, 2013; Lepori and Malmqvist, 2009), and can impact other properties of communities including functional redundancy and microbial trait abundance (Tscherko *et al.*, 2003; Sigler and Zeyer, 2004; Yin *et al.*, 2000; Shade *et al.*, 2012). Interestingly, these factors may also influence the relationship among environment, and microbial mediated function, though their importance has only been recently acknowledged and scarcely investigated in relation to microbial ecology (Knelman and Nemergut, 2014). For example, while both stochastic and deterministic processes may contribute to the assembly of microorganisms through succession, recent research has suggested definied patterns in their influence with advancing succession (Ferrenberg *et al.*, 2013; Dini-Andreote *et al.*, 2015) (Figure 2). Other aspects of microbial community structure, such as phenotypic plasticity, traits, and functional redundancy may change across succession, therein influencing the relationships between environmental factors, microbial community structure, and ecosystem function (Yin *et al.*, 2000; Lennon and Jones, 2011). Therefore, there is a need to develop theory and empirically examine how shifts in such community attributes across succession may also alter the relationships between environment and microbial mediated ecosystem function (Knelman and Nemergut, 2014; Nemergut *et al.*, 2013).

For example, increases in determinism across succession (Ferrenberg *et al.*, 2013), could lead to a coupling of environmental variables, community composition, and function, whereas more stochastic early succession may engender a decoupling of environmental variables and ecosystem function (Nemergut *et al.*, 2013). Likewise, a shift in the traits of organisms associated with early and late succession, such as the ability of organisms to form spores (Ferrenberg *et al.*, 2013; Lennon and Jones, 2011) or rRNA copy number (Shrestha *et al.*, 2007; Klappenbach *et al.*, 2000), or the relative level of functional redundancy could too alter this coupling (Shade *et al.*, 2012).

Traits underlying selection may provide a generalizable and mechanistic approach to understanding community assembly of microbial communities (Coleman and Chisholm, 2010; Cottenie, 2005; Edwards *et al.*, 2013; Green *et al.*, 2008; Lennon *et al.*, 2012; Macalady *et al.*, 2013; Treseder *et al.*, 2011). For example, in terms of ecological succession, Shipley et al. (2006) demonstrated the ability to predict relative abundance of plant species across succession based on variety of traits, and studies of microbial traits have too revealed a mechanism to describe community assembly in early succession (Burke *et al.*, 2011). Other metagenomic and genomic studies of microbes have also found trait approaches to contain valuable information on ecological niches (Lauro *et al.*, 2009; Mulkidjanian *et al.*, 2006), and r vs. K-selected life history traits have been implicated in biodiversity-ecosystem function relationships (Bohn *et al.*, 2014; Mora *et al.*, 2014). The generation of biodiversity through the addition of more K-selected species to a community of r-selected character could underlie concave up patterns of BEF curves observed in nature (Mora *et al.*, 2014), indicating that microbial traits related to these life history strategies may also act as an important factor in changing relationships between environment, microbial community structure and ecosystem function across succession, thus providing a promising avenue to study such connections across succession.

Plant-Microbe Interactions

Central questions in ecology surrounding processes that underlie ecosystem development have historically focused on succession as it involves plant communities, lending important insights in an ongoing debate regarding the factors that control ecosystem development in both structure and function (Chapin *et al.*, 1994; Matthews, 1992). Increasingly, while research is uncovering the role of soil microbial communities in overall ecosystem development across primary succession (Nemergut *et al.*, 2007; Schmidt *et al.*, 2008; Miniaci *et al.*, 2007; Tscherko *et al.*, 2003; Sigler and Zeyer, 2002), studies now are connecting belowground and aboveground communities in the context of succession (Reynolds *et al.*, 2003; Cline and Zak, 2015; van de Voorde *et al.*, 2011; Kardol *et al.*, 2007).

Indeed, plants may exert species-specific effects on microbial communities through litter inputs, rhizodeposition, and the unique chemical and physical attributes of the root compartment (Grayston *et al.*, 1998; Hartmann *et al.*, 2008; Dean *et al.*, 2015). In turn, plant-influenced microbial communities may then feedback on plant performance through direct effects of plantmicrobe symbioses and indirect effects via changes in microbial-mediated biogeochemistry (Van Der Heijden *et al.*, 2008; Kardol *et al.*, 2007; Bever *et al.*, 2010). Such plant-microbe interactions in themselves have been shown to act as drivers of succession through positive and negative feedbacks on both host and competitor plants (Kardol *et al.*, 2007, 2006; Van der Putten *et al.*, 1993; van de Voorde *et al.*, 2011; Kulmatiski *et al.*, 2008; Middleton and Bever, 2012; Herzberger *et al.*, 2015). In total, feedbacks between plants and soil microbial communities are integral to plant performance, plant community ecology, and ecosystem development (Wardle, 2004; Bardgett *et al.*, 2005; Van Der Heijden *et al.*, 2008).

Amidst climate change, shifts in aboveground vegetation and ecosystem properties are increasingly prevalent. Fire disturbance, for example, dramatically alters plant communities, and Colorado and the Western U.S. provide an example of environments that now face increasing fire frequency amidst climate change (Westerling, 2006). The dramatic alterations to plant communities resulting from fire, and subsequent regrowth and secondary succession, may profoundly impact soil microbes and the vital ecosystem processes they mediate. While a growing body of research has described ecosystem consequences of fire (Bond *et al.*, 2004; Bond and Keeley, 2005), few have addressed the impact of revegetation on the assembly of microbial communities after fire and the recovery of integral ecosystem functions that they mediate (Hart

et al., 2003). In particular, plant reconlonization of soils is a pivotal stage of secondary succession/ecosystem recovery and is also a common technique for post-fire land management, reinforcing the importance of understanding how initial stages of revegetation can alter post-fire ecosystem processes and aid in ecosystem recovery.

Previous work in soils after disturbance has indicated that vegetation can have strong effects in early succession through carbon inputs via litter and/or root exudates, for example (Knelman *et al.*, 2012; Miniaci *et al.*, 2007). In such environments, it is often the case that that low soil C constrains heterotrophic microbial structure and activity (Sattin *et al.*, 2009) and new plant carbon inputs can therefore strongly alter microbial community structure and activity. In high carbon soils, however, differences in plant communities have been noted to effect less change in microbial communities (Jangid *et al.*, 2013). After fire, soil carbon can be burned off depending on fire severity: high severity fire sites result in little soil carbon while low severity fire sites have carbon remaining in the soil (Keeley, 2009). Thus, questions remain as to in what abiotic contexts plant recolonization of post-fire landscapes during secondary succession may act as a selective filter on microbial community succession and the ecosystem properties and functions that they mediated.

Study Systems

In order to assess patterns and processes of microbial succession, I completed my dissertation fieldwork at 4 different sites. Two sites represented primary successional systems, or early successional landscapes with little biotic legacy, and two study sites represented

secondary successional systems, early successional landscapes of preexisting biotic legacy. Primary successional sites included the Puca Glacier forefield in Perú (Chapter 2), and the Mendenhall (Aak'wtaaksit) Glacier forefield in Alaska, USA (Appendix). The Puca Glacier is a low latitude, high elevation glacier (~5,000m) located in La Cordillera de Vilcanota of Perú. The Mendenhall Glacier is a high latitude, low elevation outlet glacier of the Juneau Icefield. Secondary successional systems located in the Colorado Front Range were located outside of Boulder and Ft. Collins, CO. These sites included areas resulting from the Four Mile Canyon Fire, a burn that began on September 6, 2010, (Chapter 3) and the High Park fire, which began on June 9, 2012 (Chapter 4). Each of these study sites is described in more detail within relevant chapters.

Research Outline

The first half of my dissertation focuses on details of the processes that may control microbial community assembly through succession (Chapters 2-3). Chapter 2 asks questions about the influence of nutrients, a well-known control in plant community succession, on microbial community succession. Here, the effect of this potential environmental filter that varies emblematically across succession was evaluated in isolation from a multitude of other factors that change across succession as well. This work considers how nutrients may act as a primary, deterministic control on microbial community assembly. Chapter 3 assesses how relationships between environmental properties and ecosystem function (enzyme activity) may change over the course of secondary succession. Here we examine post-fire soils, where changes

in the relative influence of stochastic and deterministic assembly have been previously observed. This work demonstrates how the linkages between edaphic factors and enzyme activity change across succession and considers factors, including assembly process and microbial traits, which may lead to the relative coupling or decoupling of environment and function across succession. Further, this chapter examines rRNA operon copy number as a microbial trait related to life history strategy as it may connect environment, microbial communities, and enzyme activity to inform understandings of microbial community assembly across succession. The second half of the dissertation (Chapter 4, Appendix) evaluates plant-microbe interactions through succession, given the importance of such relationships in driving major biotic and abiotic successional patterns. In Chapter 4, I examine the role that plant-microbe interactions play in driving microbial and biogeochemical attributes of secondary succession after a major wildfire. Here I assess how plant-microbe interactions may influence microbial communities both in their structure and decomposition enzyme activity that they mediate. This work also generates understandings of when changes in aboveground communities may impact belowground communities, showing that abiotic contexts for plant colonization may alter the relative importance of plant-microbe interactions in secondary succession. The Appendix provides additional evidence of in what way early successional plants provide a selective environment in and around the roots for microbial communities to develop. Past research has shown that early plant colonizers may be strongly influenced by associated microbes, and that plant effects on microbial community assembly may also drive the development of biogeochemical cycling through primary succession. As such, we seek to understand how these root environments provide a unique, and potentially selective microhabitat for bacteria in early succession, and

whether bacterial colonizers of roots are specialized or more generalized in their habitat occupancy.

Ecological succession, a fundamental concept in ecology that has commanded the attention of ecologists for over a century, provides an opportunity to understand both how communities assemble and the resulting implications for ecosystem function. Indeed, the very concepts of what constitutes an ecological community and how it assembles emerged from disputation surrounding ecological succession, and studies of succession have long focused on how ecosystem function changes over time and whether deterministic or stochastic processes largely govern the assembly of ecological communities (Clements, 1916; Gleason, 1927). Succession offers a testing ground to examine community assembly and ecosystem function. In particular, resulting insights of how ecological communities develop and/or respond to perturbations in structure and function caries special relevancy in a changing world in which ecosystems are responding to climate change and increasing rates of disturbance (Walther *et al.*, 2002). Central questions in ecology surrounding processes that underlie ecosystem development lend important insights in an ongoing debate regarding the factors that control ecosystem development in both structure and function (Chapin et al., 1994; Matthews, 1992). Overall, this work serves to ask fundamental questions in ecology regarding the biotic interactions and abiotic edaphic factors that may control the development and ultimately the function of an ecosystem.

CHAPTER 2

NUTRIENT CONTROLS ON MICROBIAL PRIMARY SUCCESSION Adapted from: Knelman, Joseph E., et al. "Nutrient addition dramatically accelerates microbial community succession." (2014): e102609. PLOS ONE.

Abstract

The ecological mechanisms driving community succession are widely debated, particularly for microorganisms. While successional soil microbial communities are known to undergo predictable changes in structure simultaneous with shifts in a variety of edaphic properties, the causal mechanisms underlying these patterns are poorly understood. Thus, to specifically isolate how nutrients – important drivers of plant succession – affect soil microbial succession, I established a full factorial nitrogen (N) and phosphorus (P) fertilization plot experiment in recently deglaciated (~3 years since exposure), unvegetated soils of the Puca Glacier forefield in Southeastern Peru. I evaluated soil properties and examined bacterial community composition in plots before and one year after fertilization. Fertilized soils were then compared to samples from three reference successional transects representing advancing stages of soil development ranging from 5 years to 85 years since exposure. I found that a single application of +NP fertilizer caused the soil bacterial community structure of the three-year old soils to most resemble the 85-year old soils after one year. Despite differences in a variety of soil edaphic properties between fertilizer plots and late successional soils, bacterial community composition of +NP plots converged with late successional communities. Thus, my work

suggests a mechanism for microbial succession whereby changes in resource availability drive shifts in community composition, supporting a role for nutrient colimitation in primary succession. These results suggest that nutrients alone, independent of other edaphic factors that change with succession, act as an important control over soil microbial community development, greatly accelerating the rate of succession.

Introduction

Deglaciated forefields have been a valuable model systems for developing and testing theories of succession and have greatly enhanced our understanding of the relationship between community structure and function during ecosystem development (Chapin *et al.*, 1994; Walker and del Moral, 2003; Matthews, 1992). Shifts in soil nutrient pools, including increases in available nitrogen (N) and phosphorus (P), have been well documented along early primary successional chronosequences (Walker and Syers, 1976; Crews *et al.*, 1995; Ugolini, 1968) and have been shown to correlate with changes in plant community succession (Vitousek *et al.*, 1993; Chapin *et al.*, 1994; Richardson *et al.*, 2004). Recently, studies in such systems have revealed that – like plants – microbial communities also progress through successional stages (Nemergut *et al.*, 2007; Brown and Jumpponen, 2013; Schütte *et al.*, 2009). However, the forces that control microbial succession are not well understood.

Some evidence suggests that shifts in nutrient availability may also, in part, drive microbial community succession. For example, in primary successional ecosystems, research has corroborated relationships between natural gradients in soil nutrients and microbial community composition (Zumsteg *et al.*, 2012; Edwards *et al.*, 2006). Such correlations can be difficult to

interpret, however, as changes in microbial community composition could be both a cause and consequence of shifts in soil fertility. Furthermore, the mechanisms underlying correlations between standing nutrient pools and microbial communities may be temporally disconnected, in that current soil biogeochemical status may not accurately reflect the historical nutrient conditions that structured the microbial community. Thus, manipulation experiments are essential in evaluating the direct impact of nutrients and their limitations on microbial communities. Indeed, fertilizer treatments are known to elicit changes in soil microbial community structure and function in more developed ecosystems (Ramirez *et al.*, 2010; Nemergut *et al.*, 2008) suggesting that nutrient availability may also be important in controlling successional changes in microbial community composition.

Yet, it would be surprising if nutrients alone drove microbial community succession for several reasons. First, other edaphic properties also undergo concomitant shifts with microbial community structure and function during succession, some of which are known to more strongly correlate with microbial community structure than nutrient pools in developed soils. For example, organic carbon (C) pools and pH, which typically show dramatic changes across primary successional chronosequences (Walker and del Moral, 2003), are key determinants of soil microbial community composition at regional to global scales (Fierer and Jackson, 2006; Deiglmayr *et al.*, 2006; Noll and Wellinger, 2008; Fierer *et al.*, 2007). Second, soil microbial community structure can correlate with plant community composition (Jangid *et al.*, 2013; Knelman *et al.*, 2012), which can show strong spatial gradients in early succession (Matthews, 1992). Third, stochastic processes can be key in shaping early successional communities where the importance of dispersal events may be accentuated, (Ferrenberg *et al.*, 2013; Cline and Zak, 2013; Meola *et al.*, 2014) and arrival order may influence assembly through priority effects

(Fukami and Morin, 2003). Given the large functional and phylogenetic diversity of microbial communities, it is possible that succession is influenced by a diverse combination of such factors (Nemergut *et al.*, 2013).

Thus, the extent to which nutrients themselves influence microbial community assembly outside of the myriad of factors that change over succession is unknown. To specifically isolate the effects of nutrients, I performed a full factorial N × P fertilization experiment in soils that had been exposed for ~3 years in the forefield of the Puca Glacier in Southeastern Peru. I analyzed soil bacterial communities before and one year following nutrient additions and compared them with soils sampled from three different positions over an 85-year section of the Puca Glacier chronosequence. The Puca Glacier soils constitute an autotrophic successional sequence (Fierer et al., 2010), and both photosynthesis and respiration respond strongly to P additions in microcosms (Schmidt et al., 2012, 2011). Nitrogen appears to be limiting in this system as well and N-fixation rates in 4 year old unvegetated soils are comparable to rates measured in developed soil crusts (Schmidt et al., 2008). Thus, given work that demonstrates relationships between nutrients and microbial community composition, I hypothesized that fertilizer additions to early successional soils would drive communities to be compositionally different than unfertilized (control) soils. However, due to the potential influence of other edaphic (e.g. pH, organic C, soil moisture) and stochastic factors on microbial succession, I hypothesized that fertilized communities would be unique from communities found along the natural chronosequence.

Methods

Study site description, fertilization, and sampling

The study site is located in the forelands of the Puca Glacier in the Cordillera Vilcanota of Peru ($13\circ46^{2}4^{"}S$, $71\circ04^{'}17^{"}W$, ~5,000 m.a.s.l.). Mean annual precipitation is roughly 100 cm and mean annual temperature is ~5 °C. Moraine rocks at this site have high quartz and calcite mineral content. Further details of this site can be found in previous work (Nemergut *et al.*, 2007; Schmidt *et al.*, 2008) and soil characteristics are presented in Table 2-4.

I established permanent plots (1 m²) near the terminus of the glacier, in soils that had been deglaciated for approximately 3 years at the time of initial sampling. Corners were marked with long nails (approximately 15 cm shank length) to guide resampling. Sampling occurred in August 2010 (pre-treatment) and August 2011 (post-treatment). All of the plots were unvegetated and no mosses and lichens were present at the time of establishment. Each of the 16 plots was randomly chosen to receive one of three nutrient amendments (nitrogen addition (+N), phosphorus addition (+P), the combination of the two (+NP)) or to serve as controls, resulting in a total of four plots per treatment and four control plots.

Pre-weighed amounts of fertilizer were dissolved in glacier-melt stream water and fertilizer solutions were applied with handheld sprayers. Each sprayer was designated for a particular treatment to avoid cross contamination. For the +N plots, nitrogen was added in the form of ammonium nitrate (NH4NO3) resulting in 15 g of NH4NO3 and 5.25 g of N/m². The +P plots received 0.5 g of phosphorus in the form of 2.2 g of potassium dihydrogen phosphate

(KH₂PO₄). +NP plots received 15 g of NH₄NO₃ and 2.2 g of KH₂PO₄. For controls, stream water from the same source was sprayed onto the plots. These levels of nutrient addition were designed to result in a pulse of nutrients that would greatly overcome any possible natural limitations.

Plots were sampled prior to the application of fertilization treatment. In each plot surface soil was collected (0-5 cm) from 2 locations, and samples were composited to generate one sample per plot. Samples were obtained in the same manner one year following the fertilization treatment. Ethanol and paper towels were used to sterilize the tools before sampling each individual plot.

Samples were collected in a similar manner along three transects of varying age across the glacial forefield both years; molecular analyses were done on the samples collected in 2011. These reference soils represented advancing stages of succession: soils that had been exposed for approximately 5 years, soils with biological soil crust formation (approximately 20 years after exposure), and soils with 25-50% vegetation cover (approximately 85 years after exposure). At the field site, samples were kept in a cooler on ice for transport to Boulder, CO. Soils were sieved (to 2 mm), and then stored at 4°C for soil characterization. A subsample was immediately archived in a -80°C freezer for molecular analysis and later used for KCl extractions.

Soil Analysis

Gravimetric soil moisture and pH (using a ratio of 2 g soil to 4 mL DI H₂O) were calculated based on standard methods (Nemergut *et al.*, 2007). For total organic C analysis,

carbonate (inorganic C) removal was first performed on dried, ground soils (Nemergut et al., 2007). 50 mg of these processed soils were packed into tin capsules; %C and %N were determined using a Thermo Finnigan EA 1112 Series Flash Elemental Analyzer (Thermo Fisher Scientific, Inc., Waltham, Massachusetts, USA) (Matejovic, 1997). Bio-available P concentrations were measured on air-dried and sieved soil (2 mm x 2 mm) by extracting 3-5 g of soil with 0.5 M sodium bicarbonate for 30 minutes (Jeannotte et al., 2004). Extracts were filtered and analyzed colorimetrically using the ammonium molybdate-malachite green method (Station et al., 1999) adapted for microplate analysis. NH₄⁺ and NO₃⁻/NO₂⁻ extractable N were analyzed from soils using 2M KCl with 1 hour shaking and a 22 hour extraction period (Weaver et al., 1994). This analysis was performed on soils that were frozen at -80°C. Although not fresh samples, these soils typically withstand extreme fluctuations in temperature (Schmidt et al., 2009) and the data presented here are intended for within study comparison only. NH_4^+ and NO3⁻/NO2⁻ were measured on a Lachat QuikChem 8500 Flow Injection Analyzer (Lachat Instruments, Hach Company, Loveland, CO) and BioTek Synergy 2 Multidetection Microplate Reader (BioTek, Winooski, VT) respectively.

DNA Extractions for 454 pyrosequencing

Mo Bio PowerSoil[™] DNA Isolation kits were used as per the manufacturer's instructions for DNA extractions of the soil samples (Mo Bio Laboratories, Inc., Carlsbad, CA). PCRamplified bacterial 16S rRNA genes from the genomic DNA of the soil samples were generated using a universal bacterial 27F and 338R primer set as described by Hamady *et al.* (2008), and reaction conditions followed those described by Fierer *et al.* (2008), though modified to 25 PCR cycles. Primers included a 2 bp linker, the 454 Roche Titanium A/B primer, and a unique, 12 base pair error-correcting Golay barcode for pyrosequencing as detailed by Knelman *et al.* (2012). 454 Life Sciences GS FLX Titanium pyrosequencing of the 16S rRNA gene amplicons was completed by the Duke Institute for Genome Sciences & Policy (Duke University, North Carolina).

Pyrosequence and statistical analysis

Using QIIME, sequences were limited to those of a sequence length of 200 to 400 base pairs, a maximum of 5 homopolymers, a minimum quality score of 25, and a maximum of ambiguous bases/primer mismatches of 0; reverse primers were removed, and all samples were then denoised using flowgram clustering in QIIME (Reeder and Knight, 2010). Chloroplast sequences were removed. OTUs were selected at a 97% identity level by clustering based on representative sequences via UCLUST (Edgar, 2010). The Ribosomal Database Classifier (Qiong Wang et al., 2007), a naïve Bayesian classifier, was employed to assign taxonomic identification to OTUs. After sequence alignments based on the NAST algorithm (DeSantis et al., 2006), a phylogeny was constructed with the FastTree algorithm (Price et al., 2009). OTU tables were rarified to the lowest number of sequences in a sample: 407 for community dissimilarity analyses of fertilization plots. Reference transects of advancing age included 6, 5, and 3 sequenced replicate samples, respectively, and were rarefied to 71 to include all of these samples. For comparison of reference samples and fertilization plots this workflow was repeated. In order to examine differences among bacterial communities, pairwise distance matrices based on weighted UniFrac, a phylogenetic distance metric, were generated for entire communities and

the cyanobacterial subset of communities in fertilization plots (Lozupone *et al.*, 2006, 2007). The Principal Coordinate Analysis (PCoA) ordinations were constructed based on OTU tables and weighted UniFrac distance matrices for overall communities. The QIIME-generated OTU tables were used to evaluate the relative abundance of all taxa.

Primer v6 software (Clarke and Gorley, 2006) was used to perform permutational ANOVAs (PERMANOVA) to compare phylogenetic distances among bacterial communities. PERMANOVA tests were used on both UniFrac beta diversity matrices of the entire communities and cyanobacterial portions of communities. PERMANOVA analysis was also employed to assess differences among treatment-affected communities and successional reference communities. For all comparisons with reference communities, data were rarefied to the lowest sampling depth among both fertilization plot and reference plot samples.

R software (R Development Core Team, 2013) was used for further statistical analysis. The PERMDISP procedure (with permutational P-values) from the R vegan package to test homogeneity of group dispersions (variances) was also employed via QIIME in order to test for differences in community phylogenetic dispersion (UniFrac) in fertilized samples and reference successional communities (Oksanen *et al.*, 2013; Caporaso *et al.*, 2010). As well, the pgirmess package in R was used to evaluate comparisons among reference chronosequence soil and relative abundance data via the Kruskal Wallis test. To assess treatment vs. temporal effects underlying shifts in overall phylogenetic community composition, a Tukey's HSD post-hoc test was used to compare UniFrac distances of paired pre- and post-treatment plots with paired control plots from both years. Additionally, to assess the relative abundances of bacterial taxa, I compared the differences in paired pre- to post-treatment taxon relative abundances for each treatment with that of paired control plots via Tukey HSD post-hoc tests. To examine the

relationship between treatment-related community shifts from my fertilization experiment and reference communities across advancing stages of soil development, I examined the relationship between weighted UniFrac phylogenetic dissimilarity between +NP communities and reference communities across time via a Spearman correlation Mantel test. The Mantel test tests the null hypothesis that there is no correlation between +NP and reference community dissimilarity and chronosequence age rank.

All relative abundance data and environmental variables were evaluated for normality. Taxon relative abundances and fertilizer plot NO₃⁻/NO₂⁻ were square root transformed to achieve a normal distribution prior to statistical analysis. All other edaphic factors were natural log transformed. ANOVAs, Tukey HSD, and Kruskal Wallis post-hoc tests were used to assess differences in pH, %C, P, N pools and soil moisture in fertilization plots and reference chronosequence soils. Percent N was below the detection limit in a majority of samples and thus removed from statistical evaluations.

Sequences and metadata have been deposited in FigShare and are available with the DOIs: 10.6084/m9.figshare.1050042 (metadata) and 10.6084/m9.figshare.1048992 (sequences).

Results and Discussion

Together, my analyses demonstrate that a single +NP application caused the bacterial community structure of the 3-year-old barren soils to converge with the structure of 85-year-old vegetated soils after only one year. First, paired pre- and post-treatment plot community differences (weighted UniFrac distance) were assessed among all plot categories using an ANOVA. A post-hoc Tukey's HSD test of this before and after phylogenetic community change

for all plot categories reveals no significant differences in community change for treatment plots vs. control plots (Tukey's HSD; P>0.05), except for +NP plots (Tukey's HSD; P= 0.037), thus demonstrating a treatment effect. A PCoA ordination (Figure 2-1) further revealed a successional trend in community composition across the reference chronosequence, with post-treatment +NP communities clustering with the oldest reference communities.



Figure 2-1. Principal Coordinates Analysis (PCoA) ordination plot of bacterial communities from the field fertilization experiment and bacterial communities from the successional chronosequence. Only the +NP treatment communities are shown because the +N and +P treatments did not result in significant community shifts. PCoA visually represents differences among community composition as the distance between points. Triangles represent communities from the natural chronosequence: red = 5 years old; orange = 20 years old; blue = 85 years old. Circles represent communities from the fertilization experiment: black = pre-treatment control; grey = post-treatment control; purple = pretreatment +NP; Pink = post-treatment +NP. My
analysis revealed significant community shifts over the reference chronosequence (triangles) as well as a significant response to +NP fertilization (circles). As well, the PCoA analysis demonstrates that the +NP communities (pink circles) group with the oldest soils from the chronosequence (blue triangles). doi:10.1371/journal.pone.0102609.g001

A PERMANOVA analysis demonstrated that there were no significant differences among pre-treatment communities (Table 2-1). However, communities in post-treatment +NP plots were significantly different from both pre- and post-treatment controls, including the paired pretreatment +NP plots (PERMANOVA, P<0.05, Table 2-1). When +NP communities were compared to reference communities across the natural chronosequence, a Mantel test of pairwise average UniFrac (Lozupone *et al.*, 2006, 2007) distances between +NP plots and reference samples revealed significant patterns of decreasing dissimilarity: +NP communities were most similar to the 85 year old successional soils (Figure 2-2, ρ_M = -0.3465367 P= 0.01498501).

Table 2-1. Results of PERMANOVA analysis. Post-treatment +NP phylogenetic community structure was significantly different from controls and from all communities from the reference chronosequence with the exeption of communities in the oldest soils (P<0.05).

Permutational MANOVA (PERMANOV)	A) contrast P-values			
Sample vs. Sample	post-treatment control	post-treatment +N	post-treatment +P	post-treatment +NP
pre-treatment control	0.415	0.066	0.031	0.026
post-treatment control		0.422	0.072	0.036
pre-treatment +NP-paired	0.114	0.085	0.024	0.032
post-treatment +NP plots	0.036	0.023	0.18	
succession timepoint 1	0.124	0.003	0.005	0.006
succession timepoint 2	0.105	0.009	0.018	0.022
succession timepoint 3	0.152	0.055	0.179	0.162
Significant P-values (P<0.05) bolded.				

Controls showed no differences from any contrasts (P>0.05). Significant P-values (P<0.05) are bolded. doi:10.1371/journal.pone.0102609.t001



Figure 2-2. Relationship between +NP treatment-affected communities and reference communities. A box plot shows the average weighted UniFrac distance between +NP-treated communities and reference communities with increasing successional time. A Mantel test demonstrates that +NP communities show decreasing dissimiliarty as compared to the reference communities over advancing stages of succession ($\rho_M = -0.35 P = 0.01$). doi:10.1371/journal.pone.0102609.g002

The PERMANOVA analysis also showed that +NP communities were significantly different than communities of all successional stages except those of the oldest transect (85 years old) (Table 2-1). In contrast to controls, such trends suggest that fertilization drives shifts in

community composition away from early successional stages and results in convergence with communities of older soils. Likewise, the phylogenetic dispersion (Oksanen et al., 2013; Caporaso et al., 2010) of +NP communities was significantly different from all reference communities except those in the 85 year old soils (Table 2-2). I note that my PERMANOVA analysis is not corrected for multiple comparisons due to the low statistical power of my study, but the general results of this analysis regarding treatment effect and convergence relating to +NP plots is nonetheless corroborated by my other statistical analyses of treatment effect (ANOVA/Tukey's HSD of pre- and post-treatment community shifts) and convergence (Mantel test of +NP community distance compared to successional reference samples).

Table 2-2. Permutational analysis of dispersion (PERMDISP) among post-treatment +NP plots and post-treatment controls

Homogeneity of Dispersion (PERMDISP) P-values							
Sample vs. Sample	post-treatment +NP	post-treatment control					
succession timepoint 1	0.022	0.508					
succession timepoint 2	0.042	0.588					
succession timepoint 3	0.555	0.997					
Significant P-values (P<0.05) bolded.							

Controls showed no difference in community dispersion from communities of any of the reference succession transects (P>0.05). Significant P-values (P<0.05) are bolded. doi:10.1371/journal.pone.0102609.t002

My results suggest that nutrient colimitation is an important control on microbial primary succession in this system. Because of low statistical power, it is difficult to discern whether this colimitation is simultaneous, meaning that both nutrients need to be present for a community

response, or independent, meaning that each nutrient in isolation may elicit some response (Harpole *et al.*, 2011). However, there is some evidence that single nutrient additions may cause a smaller response than when both nutrients are abundant. For example, my results show that post-treatment +P communities are not significantly different from post treatment +NP communities (Table 2-1). Both +N and +P plots show patterns of convergence similar to +NP plots in comparison with ongoing natural succession; by contrast, control plots do not display convergence (Table 2-1). Thus, +N and +P communities may represent intermediate states between control and +NP plots, but I was not able to statistically demonstrate an underlying treatment effect.

While my study is unique as I established and resampled nutrient addition plots in a remote glacial forefield, the rapidly changing nature of the Puca Glacier landscape and criteria for setting up plots on a stable and relatively homogenous landscape limited replication and necessitated rarefaction of sequencing depth to include all available samples. As such, I acknowledge the need to be circumspect in drawing conclusions as such factors curbed the statistical power of my study and the ability to detect smaller magnitude treatment effects in +N and +P additions, for example. However, I note that the patterns shown here are robust to even lower rarefaction depths (55-70), thus it is likely that observed patterns are real. Nonetheless, my research shows the greatest, and only statistically significant treatment effect on microbial communities under +NP additions, suggesting the effect of both nutrients in tandem is important in succession.

Interestingly, standing nutrient pool analysis lends some insight into particular dynamics that may underlie nutrient colimitation in this autotrophic chronosequence. For example, +P and +NP soils both show significant increases in ammonium pools in comparison with control plot

soils (Table 2-3), which is consistent with a body of research that demonstrates P limitation is a strong control of N-fixation (Vitousek, 1999; Vitousek *et al.*, 2010), and may be particularly strong in this autotrophic chronosequence that features cyanobacterial N-fixers (Nemergut *et al.*, 2007). Likewise, +N plots show a significant increase in bioavailable-P from control plots (Table 2-3), a pattern supported by research that shows N availability may limit the production of phosphatase enzymes (Olander and Vitousek, 2000; Vitousek *et al.*, 2010; Y.-P. Wang *et al.*, 2007). Thus, these particular biochemical pathways lead to a coupling of nutrient cycles, which appears to be reflected in a colimitation to successional processes.

Table 2-3. Edaphic property means and standard deviations for post-treatment fertilization j	plots
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Mean (SD) of Edaphic Properties and	Tukey HSD Comparisons for	r post-treatment plots (P<0.05)	

Factor	Control	N	Р	NP
рН	8.97 (0.05) ^A	8.99 (0.15) ^A	8.98 (0.09) ^A	8.97 (0.06) ^A
% Soil Moisture	0.76 (0.82) ^A	0.34 (0.26) ^A	0.23 (0.09) ^A	0.42 (0.44) ^A
% Carbon	0.057 (0.016) ^A	0.040 (0.006) ^A	0.071 (0.030) ^A	0.068 (0.017) ^A
Bioavailable-P (µg P /g soil)	0.47 (0.051) ^A	6.11 (0.026) ^B	69.88 (24.98) ^c	82.88 (23.99) ^c
Extractable NH_4^+ (µg N /g soil)	0.43 (0.06) ^A	0.81 (0.19) ^{AB}	1.04 (0.57) ^B	0.96 (0.16) ^B
Extractable NO_3^{-}/NO_2^{-} (µg N /g soil)	0.13 (0.12) ^A	1.07 (0.67) ^B	0.16 (0.06) ^A	1.39 (1.00) ^B

Letters denote differences in Tukey HSD contrasts (P<0.05)

Despite the multitude of well documented changes across successional gradients including shifts in pH, C pools, plant cover and biotic historical factors, nutrient addition alone not only caused changes in early successional community structure, but induced convergence with late successional soil communities of the natural chronosequence (Figures 2-1 and 2-2 and Tables 2-1 and 2-2). For example, unlike patterns in measured N and P nutrient pools, strong changes in %C, another known filter on microbial communities, were observed across the natural chronosequence but not in +NP plots (Tables 2-3 and 2-4). In other ecosystems, the effects of fertilization on microbial community structure have also been attributed to changes in plant productivity or community structure (Ramirez *et al.*, 2010). However, it is important to note that while the +NP fertilization caused sparse vegetation (<15 cm tall) to colonize after one year at my site, soils were collected at least 75 cm from these small plants. Altogether, my results suggest that the effects of the +NP fertilization on microbial community succession were direct and not mediated through changes in other aspects of the abiotic environment or through the effects of plants on soil communities.

Table 2-4. Edaphic property/cyanobacterial relative abundance means and standard deviations for reference plots.

Property		Time Point	
	5 years old	20 years old	85 years old
рН	7.37 (0.24) ^A	7.52 (0.12) ^A	7.01 (0.255) ^A
% Soil Moisture	6.92 (9.53) ^A	0.95 (0.21) ^A	1.67 (1.66) ^A
% Carbon*	0.53 (0.13) ^A	1.43 (0.72) ^A	16.82 (3.08) ^B
Bioavailable-P (μg P /g soil)	1.12 (1.04) ^A	3.00 (0.62) ^A	4.74 (1.42) ^B
Extractable NH_4^+ (µg N /g soil)	0.26 (0.21) ^{AB}	0.21 (0.24) ^A	2.42 (1.94) ^B
Extractable NO_3 / NO_2 (µg N /g soil)	0.39 (0.17) ^A	1.81 (0.89) ^A	6.19 (1.62) ^B
Cyanobacteria Relative Abundance (% total community)	29.04 (6.79) ^A	36.45 (6.89) ^A	56.18 (25.75) ^A

Mean (SD) of Edaphic Properties and Cyanobacterial Relative Abundance for Reference Chronosequence Plots (P<0.05)

Letters denote differences in Kruskal-Wallis multiple comparisons (P<0.05) *All data is from 2011 chronosequence samples, with exception of %C from 2010 chronosequence samples

My field-based fertilization experiment helps to extend existing ecological theory regarding the role of nutrient limitations in succession (Vitousek and Farrington, 1997; Crews *et al.*, 1995; Walker and Syers, 1976) to microbial communities present in the earliest primary successional soils, which are important for biogeochemical cycling, physical soil development, and plant colonization (Schmidt *et al.*, 2008; Nemergut *et al.*, 2007; Knelman *et al.*, 2012).

While it is widely acknowledged that microbes can alter soil fertility and nutrient cycling processes, and that changes in soil nutrient pools and microbial communities occur over primary succession (Zumsteg *et al.*, 2012; Edwards *et al.*, 2006; Nemergut *et al.*, 2007; Schmidt *et al.*, 2008), to what extent nutrients directly structure soil microbial communities is not clear. My fertilization experiment allowed us to decouple the effects of changes in microbial communities on nutrient cycles and to directly demonstrate the influence of nutrient pools on microbial succession. Correlative studies are less powerful because they cannot isolate the impact of individual factors amidst the multiplicity of soil properties that change with succession, and because measured soil properties may be decoupled from microbial community composition in time.

Despite the high fertilization rate I used, the nutrient addition treatment did not push communities to an alternative or novel state, but simply accelerated succession, rapidly producing a community that was structurally most similar to the community in the 85 year old soils in the chronosequence (Figures 2-1 and 2-2 and Tables 2-1 and 2-2). Thus, my data highlight the stability of soil microbial communities (Griffiths and Philippot, 2013). Few studies have explicitly evaluated nutrients in the context of longer-term successional reference plant communities to understand how nutrients may either drive succession or shape alternative stable states in communities. However, in a study of salt marsh vegetation, Van Wijnen and Bakker (Van Wijnen and Bakker, 1999) observed that fertilization of young marsh communities resulted in plant communities that resembled those of older, unfertilized marshes. These results further suggest that nutrient-related mechanisms for succession may be generalizable between plant and microbial communities.

The relative abundance of cyanobacteria significantly increased in the +NP plots and the

phylogenetic structure of the cyanobacterial communities in post-treatment +NP plots was

significantly different from the paired pre-treatment +NP and pre-/post-treatment control plots

(PERMANOVA, P<0.05, Table 2-5).

Table 2-5. Bacterial taxa relative abundances in pre and post-treatment plots.

Таха	Pre-treatment	-	Post-treatmer	nt	
		Control	N	Р	NP
Acidobacteria	6.05 (2.59)	6.70 (0.81)	3.13 (0.55)	2.64 (1.41)	2.33 (1.09)
Actinobacteria	10.96 (4.39)	6.51(4.66)	9.71 (4.44)	2.21 (1.51)	1.97 (1.12)
Bacteroidetes	12.35 (5.07)	10.20(0.95)	10.32 (2.83)	10.50 (3.96)	5.96 (1.34)
Cyanobacteria	37.00 (7.37)	37.70 (13.25)	40.48 (7.13)	56.51 (12.98)	62.90* (7.97)
α-Proteobacteria	9.52 (3.16)	14.25 (4.06)	13.51 (2.58)	11.92 (4.16)	10.69 (2.73)
β-Proteobacteria	4.94 (1.55)	2.95 (0.40)	5.41 (1.75)	2.27 (0.97)	3.07 (0.62)

Mean (SD) Relative Abundances of Major Taxa (>5% of community) at Puca Glacier site

Tukey HSD test of changes in paired pre/post treatment samples versus changes in paired pre/post control samples. *P=.0335

Although not significant, cyanobacterial relative abundance doubled between the oldest and youngest stages of the reference chronosequence and past work at this site has documented similar successional changes in cyanobacterial community structure (Table 2-4) (Nemergut *et al.*, 2007; Schmidt *et al.*, 2008). Consistent with these results, a laboratory experiment evaluating microbial autotrophs from this site demonstrated that P additions resulted in significant increases of the growth rate of photoautotrophic crusts (Schmidt *et al.*, 2012). Both N fixation rates and the relative abundance of N-fixing cyanobacteria show successional trends at this site as well (Schmidt *et al.*, 2008), suggesting that N availability may also limit microbial growth and activity. The current study adds to this work and demonstrates that both N and P together are important colimiting controls over community successional processes in this system (Tables 2-1 and 2-2). The increase in the relative abundance of cyanobacteria in the +NP plots may reflect their ecological advantage in this low C environment. In a laboratory study, Drakare (Drakare, 2002) observed that P additions enhanced cyanobacterial populations, but only in an environment where low C concentrations constrained heterotrophic growth. Incubation studies of early successional soils that found increases in heterotrophic activity in response to both N and C (but not to N alone) are also consistent with this interpretation (Yoshitake *et al.*, 2007; Göransson *et al.*, 2011). These results indicate that C often limits the response of the heterotrophic community to nutrient additions, whereas cyanobacteria can readily take advantage of such nutrients to fuel photosynthesis. By extension, I argue that the observed effects of N and P additions on microbial community succession are likely to apply only to autotrophic successional sequences, and that heterotrophic succession (sensu Fierer *et. al* (2010)) may be controlled by a different suite of resources, including C availability.

Conclusion

Microbes are fundamental to soil physical and chemical development and underlie ecosystem function, thus understanding the factors that drive soil microbial community succession is key to predicting and managing ecosystem development. Particularly in low nutrient environments, microbial activity has major effects on soil, plant community, and ecosystem development (Schmidt *et al.*, 2008; Nemergut *et al.*, 2007; Borin *et al.*, 2010; Van Der Heijden *et al.*, 2008). Likewise, low nutrient environments may feature more prominent nutrient colimitations (Harpole *et al.*, 2011). As such, the results of this study have important implications for understanding nutrient controls on ecosystem development and relevant models for microbial succession. Furthermore, while early successional microbial communities may

vary strongly in both composition and in terms of the specifics of resource availability (e.g., heterotrophic vs. autotrophic), my study provides evidence that nutrient colimitation may provide a generalizable mechanism for microbial community succession in such autotrophic successional sequences. My data also support recent evidence for the stability of soil microbial communities, as fertilization simply accelerated succession and did not push communities into a novel state. Overall, the details of microbial nutrient limitations presented herein are essential to understanding the factors that structure early successional microbial communities, the profound contributions they make to soil development, and the ecosystem processes they mediate.

CHAPTER 3

CHANGES IN THE COUPLING OF RESOURCE ENVIRONMENT AND ECOSYSTEM FUNCTION ACROSS ADVANCING STAGES OF SECONDARY SUCCESSION

Abstract

In post-disturbance ecosystem development, the role of environmental conditions as a control on ecosystem function is fundamental to understanding ecological succession and may strongly inform management and predictions of biogeochemical cycling. While past work has shown that environmental, community, and functional aspects of ecosystems show patterns, at times predictably, across succession in general, little work has sought to evaluate how the nature of the relationships among these factors may vary across secondary succession. In particular, there is a need to better understand connections between soil resource environments and ecosystem function. Understanding when and where these connections are amplified or muted will inform a more mechanistic understanding of how communities and ecosystems assemble to provide ecosystem services. Most past studies examining the connection between environment, microbial communities, and enzyme activity have assumed a strong role of ecological selection in structuring such relationships. However, recent work has demonstrated that more stochastic assembly processes are central to succession as well. Other factors relating to details and distribution of microbial traits and physiologies that vary across succession could also mitigate or strengthen the influence of environment on microbial community structure and function at different times in succession.

In this study, I explicitly evaluated the coupling of environment, microbial communities, and related ecosystem function (enzyme activity) across post-burn secondary succession that has

previously been shown to undergo a relative shift in the importance of stochastic and deterministic assembly processes as well as changes in microbial community composition with advancing succession. I found that with advancing succession, as well as in reference forest soils compared to successional soils, the relationship between environmental similarity and similarity in enzyme activity became stronger. Early successional samples showed a decoupling of resource environment and enzyme activity. Existing theory predicts this increased coupling of environment with ecosystem process across succession should correspond with observed shifts in stochastic to deterministic assembly processes, though other factors, such as the prominence of spore forming organisms and dormancy, could also lead to observed changes in the coupling of environment and function. This work also shows that under environmental selection, edaphic properties that correlate with enzyme activity shift across succession in relatively short time, reflecting shifts in nutrient limitation and environmental perturbation from flooding. Finally, we also find that microbial life history strategy, in terms of rRNA operon copy number, varies across succession in generalizable ways and corresponds to environment and enzyme activity, thus providing another perspective on connections between the environment and function.

Introduction

While past studies have documented well defined patterns in microbial community structure across space and time (Nemergut *et al.*, 2011; Hanson *et al.*, 2012; Bahl *et al.*, 2011; Martiny *et al.*, 2011) only recently have studies focused on generalizable processes that may underlie such observations. Successional systems, rich in empirical ecological research and theory, provide an opportunity to examine processes and patterns that relate community assembly and the development of ecosystem function. Past studies in successional systems have

demonstrated the role of abiotic environmental filters as controls on microbial succession (Cline and Zak, 2015; Freedman and Zak, 2015; Dini-Andreote *et al.*, 2015; Zumsteg *et al.*, 2012) while others have suggested the importance of stochastic processes in structuring communities (Zhou *et al.*, 2014; Ferrenberg *et al.*, 2013; Dini-Andreote *et al.*, 2015; Zhang *et al.*, 2015; Fukami *et al.*, 2010a).

Less attention, however, has been paid to the relationship between environmental factors and ecosystem processes across succession. Nonetheless, given its pertinence to managing and predicting ecosystem function, questions of when the environment may predictably control microbial mediated ecosystem function have recently gained attention (Graham et al., 2014). When environmental filters dominantly shape microbial community structure they may also drive patterns in ecosystem function. However, environmental parameters may also be decoupled from microbial mediated ecosystem function for a variety of reasons, including physical barriers for microbes in accessing substrates (Schimel and Schaeffer, 2012), stochastic assembly processes (Nemergut et al., 2013), functional redundancy, and phenotypic plasticity/dormancy (Shade et al., 2012). Because these factors also have been shown to vary across succession (Ferrenberg et al., 2013; Fukami et al., 2010b; Lennon and Jones, 2011; Yin et al., 2000), the relationship between environment and function may show predictable, dynamic patterns across succession rather than as a single or persistent pattern. Thus, I predict that not only will changes be observed in the particular environmental factors that may correlate with ecosystem function, but also that the relative strength of environment-function relationships in themselves may fluctuate across succession with shifts in assembly processes and microbial community attributes.

At the Four Mile Canyon outside of Boulder, Colorado, Ferrenberg et al. (2013) showed that shifts in microbial community structure across secondary succession after a severe wildfire were driven by changes in the relative importance of stochastic and deterministic processes of microbial community assembly that varied across succession after disturbance. As well, this work showed the presence of spore-forming taxa in early succession versus later succession, indicating differences in microbial community traits across succession. And yet, such attributes of this system may not only implicate the structure of bacterial communities, as evaluated in this previous work, but also the relationship between environment and community function. Here, I examine post-burn, secondary succession soils from two time points of succession (1 and 4 months after burn) of Ferrenberg et al. (2013), as well two additional time points at 29 and 33 months post-burn. I assess microbial enzyme activities relating to carbon (C) and nitrogen (N) cycling of soils across all these time points of secondary succession in order to evaluate aspects of microbial mediated ecosystem function important to biogeochemical cycles. I analyze the connections between edaphic properties and ecosystem function (enzyme activity) across succession as well as between the post-burn successional soils and a reference forest. Past work has evaluated general shifts in enzyme activity (Holden *et al.*, 2012) and broad scale environmental controls on enzyme potential across secondary succession (Cline and Zak, 2015), but this work contributes to an understanding of how the fundamental relationship between environment and ecosystem function may change across succession as well as how edaphic controls on enzyme activity may vary in different stages of succession.

I hypothesize that in parallel with the increasing correspondence between environmental parameters and bacterial community composition found previously at this site with advancing succession (Ferrenberg *et al.*, 2013), increasingly prominent connections between environment

and enzyme activity will be observed as well across succession. In later successional soil communities that may show stronger coupling of environment and function, I also hypothesize that the prevailing edaphic controls will shift over time to reflect nutrient limitation, a biologically driven limiting step to decomposition (Sinsabaugh et al., 2009; Gartner et al., 2012). Therefore, in later succession, I expect correlations between abiotic factors and enzymes may indicate shifting biological limitations on the cycling of nutrients, based in nutrient limitations. Given the dynamism of the successional soils versus reference forest soils, I also hypothesize that while relationships between environment and enzyme activity may vary according to patterns of nutrient limitation across samples (Sinsabaugh and Moorhead, 1994), reference communities will show more consistent patterns of nutrient controls on enzyme activity whereas successional soils will show more variable correspondence between edaphic properties and enzyme activity. Given that successional communities show more dynamic shifts in microbial communities and edaphic properties over time versus reference communities (Cline and Zak, 2015; Ferrenberg et al., 2013), these soils may show a lessor connection between environment and enzyme activity by a prevailing environmental factor.

Generating insights of when and where environment, microbial community, and function are congruent or not may be obfuscated by the vast phylogenetic and taxonomic diversity of microorganisms. Because of this, microbial community data may provide relatively little explanatory power in predicting ecosystem function beyond environment (Graham *et al.*, 2014). In light of functional redundancy and high diversity of microbes (Wohl *et al.*, 2004), advancing a generalizable understanding of the connection between environment, microbial community structure, and function may require a more functional, trait-based perspective (Burke *et al.*, 2011; Nemergut *et al.*, 2014). Here, I expand questions of the relationship between environment and

ecosystem function to examine connections with microbial communities from a trait-based perspective. I assess the connections between environment, the microbial community, and enzyme activity from a trait-based perspective by examining average rRNA operon copy number of communities across reference and post-burn successional soils. rRNA operon copy number has been shown to correspond with r- and K- selected strategies of microbial growth (Klappenbach *et al.*, 2000) that are known to vary across microbial succession (Sigler and Zeyer, 2004; Sigler *et al.*, 2002; Shrestha *et al.*, 2007). As this trait may both respond to environment and constrain microbial function, rRNA operon copy number may hold strong ecological implications for the assembly and function of microbial communities across succession.

Methods

Site and Soil Properties

Samples at 29 (June 2013) and 33 months (October 2013) post-burn were collected from the Four Mile Canyon, outside of Boulder, CO, and processed according to the site description and collection methods enumerated in Ferrenberg et al. (2013). Samples of 33-month soils also experienced major rains and flooding between September 9 and 16th, 2013, with rainfall exceeding 400mm in the area during this time (the great Colorado flood 2013). Unique from the 1 and 4 month soils, 29 and 33 month soils were revegetated with understory herbaceous plants by seeding. 10 replicates for both burn and forested reference soils were collected at each time point. After sieving soils through a 2mm mesh, a subsample of soils was stored in a -70 degree C freezer for molecular analysis, and samples for soil chemistry and enzyme assays were refrigerated at 4 degrees C. Soil moisture, pH, total dissolved nitrogen (TDN), extractable organic carbon (NPOC), and ammonium (NH4⁺) were evaluated. A subsample of each soil was dried at 100°C for 48 hours to determine gravimetric soil moisture. Dried soils of all samples were ground and 50mg were packed into tin capsules for %C and %N analysis using a Thermo Finnigan EA 1112 Series Flash Elemental Analyzer; (Thermo Fisher Scientific, Inc., Waltham, Massachusetts, USA) (Matejovic, 1997). Immediately following collection, 8g of soil were extracted for 1 hour in 40mL of .5M K2SO4 and filtered with Whatman no.1 paper (Whatman Incorporated, Florham Park, NJ, USA). Extract filtrate was frozen until analysis. To evaluate microbial biomass, paired samples were chloroform-fumigated for 72-hours as per standard methods (Brookes et al., 1985; Jenkinson and Powlson, 1976) and then extracted in of .5M K2SO4 as described above. Microbial biomass-C as reported was adjusted for extraction efficiency based on the literature correction value of 0.45 (Beck et al., 1997; Jenkinson and Ladd, 1981; Vance *et al.*, 1987). Extracts were analyzed for NH4⁺ on a BioTek Synergy 2 Multidetection Microplate Reader (BioTek, Winooski, VT, USA) and TDN/NPOC were measured on a Shimadzu TOC-V CSN Total Organic Carbon Analyzer (Shimadzu TOCvcpn, Kyoto, Japan). TDN, NPOC, NH4⁺, and microbial biomass-C were completed for all 4 and 29 month soils. pH was measured with a ratio of 2 dry soil: 4mL water for all samples. Soils slurries were shaken at 250rpm for one hour, allowed to equilibrate, and then measured on a pH meter.

DNA extraction, PCR, and sequencing

DNA was extracted using MoBio's PowerSoil DNA Isolation Kit (MO BIO Laboratories, 186 Carlsbad, CA), according the manufacturer's protocol and final DNA was eluted in TE buffer. PCR was performed on all samples and negative controls using 515F/806R primers. The forward primer was comprised of the 5' Illumina adaptor, a forward primer pad, forward primer linker, and forward 515 primer. The reverse primer was comprised of the reverse complement of the 3' adaptor, golay barcode, reverse primer pad, reverse primer linker, and the reverse 806 primer. PCR was run with a reaction mixture of 25.0 µL including 8.6 µL PCR Grade H2O, 12.5 μL FideliTaq Master Mix, 1.0 μL Primer 515 192 F (10μM), 1.0 μL Primer 806R (10μM), 0.11 µL MgCl2 (25mM), and 2.79 µL template DNA (all samples normalized to 3.583ng/µL). Reaction mixtures were amplified in triplicate for each sample with an initial denaturation at 94°C for 2 minutes and then 25 cycles of 94°C for 45 seconds, 50°C for 60 seconds, and 68°C for 45 seconds. A final extension of 5 minutes at 68°C concluded all reactions. To eliminate primer dimer contamination, barcoded PCR product was purified using a QIAquick Gel Extraction Kit, according to the manufacturer's protocol. DNA purity and quality was determined on a NanoDrop800 and samples DNA concentration was determined using the PicoGreen method on a microplate reader according to the manufacturer's protocol. Samples, including negative controls, were pooled and purified using the UltraClean PCR Clean-up Kit, according to the manufacturer's protocol. This final multiplexed DNA was sequenced at the University of Colorado (BioFrontiers Institute, Boulder, CO) on an Illumina MiSeq with the MiSeq Reagent Kit v2, 300 cycles.

Enzyme analysis

A subset of samples from Ferrenberg et al (2013) were randomly selected for enzyme analysis that included 8 replicates of reference forested soils (4 months) and 7 replicates of burn soils for each time point (1 and 4 months) in the original study. All replicates from 29 and 33 month reference and burn soils were also included. Enzyme activities for β -1,4-glucosidase (BG), and β -1,4-N-acetylglucosaminidase (NAG) were evaluated in order to assess microbial investment in C and N acquisition and connections with edaphic properties. The activity of these enzymes are indicative of microbial (both bacterial and fungal) investment in the acquisition of C and N, and the limiting nature of these nutrients (Sinsabaugh et al., 2008). Enzyme activity was measured via fluorometric microplate methods (Sinsabaugh et al., 2002; Weintraub et al., 2012). The methods of Weintraub et al. (2012) were used based on a 96-well assay plate method with 1M sodium acetate buffer titrated to a pH of 7.0, and 4-methylumbelliferone standards. ~1 g of refrigerated soil was used from each sample. Each sample was run with 16 analytical replicates, quench corrections, standards, and negative controls. Fluorescence was measured using a microplate reader (Thermo Labsystems, Franklin, MA, USA) at 365 nm excitation and 460 nm emission to calculate nmol activity h⁻¹ g soil⁻¹.

Statistical and sequence analysis

Sequence and edaphic data that corresponded to the Ferrenberg et al. (2013) soils analyzed for enzyme potential in this study were used for all downstream analyses, along with data for all sampled replicates from the 29 and 33 month time points. The pgirmess package in the R statistical environment (R Development Core Team, 2013) was used to evaluate changes in edaphic properties within reference and burn soils across the various time points using Kruskal-Wallis contrasts. Differences in enzyme activity were also evaluated as such. I examined bacterial community structure and its relationship to edaphic properties and enzyme activity through the UPARSE and QIIME software packages according to Knelman et al. (2015). Sequences for both the original and new datasets were separately quality filtered, chimera checked, picked for OTUs at a 97% identity level, and assembled into OTU tables by using UPARSE (Edgar, 2013). OTU tables were rarefied to 1,000 and 13,000 sequences (the minimum sequencing depth for a given sample in each dataset) for each sample in the original and new datasets respectively. The construction of dissimilarity matrices based on the Bray-Curtis and weighted UniFrac metrics were completed in QIIME (Caporaso et al., 2010; Lozupone et al., 2006). Further, the two sequence datasets were jointly closed reference OTU picked using the uclust algorithm (Edgar, 2010) and a 97% identity threshold against the Greengenes gg 13 5 otus file in QIIME to create a single OTU table, which was then rarefied to 200 sequences per sample, the minimum sequencing depth across samples. A Bray-Curtis dissimilarity matrix was then constructed. PRIMER E (Clarke and Gorley, 2006) was used to examine correlations between community dissimilarity matrices, edaphic properties, and enzyme activity. Edaphic properties and enzyme activity were made into distance matrices based on Euclidean distance and Mantel-like RELATE tests were used to test correlations between edaphic properties, community dissimilarity, and enzyme activity for all samples that included these measurements. Environmental factors of percent C, percent N, pH, C:N ratio, and percent moisture were tested against BG and NAG activity. Analysis across all reference and burn samples used the closed reference OTU Bray-Curtis dissimilarity matrices, while within time point analyses of post-burn succession drew on the de novo OTU picking and related UniFrac

and Bray-Curtis distance matrices. PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Langille et al., 2013), was used to estimate rRNA operon copy number and calculate the community aggregate trait values for all samples. First, OTU table counts were normalized (standardized) for copy number and the community aggregated trait value (weighted mean) was calculated based on the product of the estimated operon copy number and the relative abundance for each OTU, summed for the entire sample to give a single weighted mean value for the trait. This workflow was then employed for samples from disparate environments from two additional studies. I examined salt marsh sediment (Dini-Andreote et al., 2015) and a glacial forefield soils from the Toklat Glacier (Darcy et al., unpublished). Changes in weighted mean value of the trait were evaluated with Kruskal-Wallis contrasts as well as a linear regression model on log-transformed data for the Toklat Glacier. Kendall rank correlations were calculated between weighted mean trait value and edaphic factors/enzyme activity across all time points for reference and burn samples as well within each burn time point. Kendall rank correlations were also calculated for biomass normalized enzyme activity versus copy number across burn and reference soils in 4 and 29 month time points, for which biomass data was available.

Results and Discussion

Overall, analysis to evaluate correlations among edaphic and enzyme activity of samples revealed that edaphic properties more strongly correlated to enzyme activity in reference forest soils than in the post-burn soils (Figure 3-1). The dynamic changes in successional microbial communities may mute correlations between a prevailing environmental factor and community function. In contrast, I hypothesized that reference forest communities would show a stronger correspondence between environment and enzyme activity due to more stable environmental selection. Consistent with previous work that observed reduced community dissimilarity and less variability in microbial community composition in reference communities as compared to successional soils (Ferrenberg *et al.*, 2013), here we find similar patterns in the correlation between environment and enzyme activity, where stronger relationships are noted for reference communities.



Figure 3-1 Statistically significant relationships among edaphic properties and enzyme activity, for both burn and reference soils. Circle size corresponds to effect size, the Rho value for correlation. Rho value and P value from RELATE tests are reported.

In reference soils, for example, BG significantly correlated with %N, but not in post-burn soils. Both reference and successional soils correlate with %C, but at a Rho of .267 vs. .173, respectively (Figure 3-1). As such, this work suggests that resource environment-function relationships indeed may vary between a post-disturbance and developed ecosystem landscape, representing an endpoint in ecosystem development. Over time, more environmentally stable reference forest communities may yield more consistent associations between environment and microbial communities and their function, while the dynamism associated with secondary succession may obfuscate such relationships. Past work has also demonstrated that that genes associated with the ability to degrade organic substrates varied across secondary succession in coordination with variation in pH and soil organic matter (Cline and Zak, 2015). Interestingly, pH and %C are found to significantly correlate with BG activity across both reference and successional soils here, and these edaphic properties are known to be dominant factors in structuring microbial communities in general (Fierer and Jackson, 2006).

I more directly examined the relationship among environment and enzyme activity within each stage of the post-burn landscape, first focusing on the initial two time points where shifts in the relative importance of stochastic and deterministic assembly have been documented (Ferrenberg *et al.*, 2013). Here, I saw shifts in the coupling of edaphic properties with enzyme activity. In the first time point, which corresponded with relatively stochastic assembly, I found that similarity in enzyme activity showed no correlation with similarity in the environment (Table 3-1). Then, in the second time point, 4-month soil communities that show relatively more deterministic assembly, I observed very strong connections between edaphic properties and

enzyme activity (Rho of >0.8 between percent C and N with BG activity) (Table 3-1). No correlations were found between edaphic properties and community composition (for either Bray-Curtis or Unifrac) at either of these time points. In contrast, in reference soils for each of these time points, environmental correlates were observed with community composition. One month reference soil communities (Bray-Curtis) correlated with moisture (rho=.524, P=0.025) and 4 month soil communities (UniFrac) correlated with %N (rho=.378, P=0.041), %C (rho=.467, P=.027), and the C:N ratio (rho=.451, P=0.019), however, connections between microbial community structure and enzyme activity were only observed in 4 month reference soils for Bray-Curtis dissimilarity and BG activity (rho=.411, P=0.041) and UniFrac and BG activity (rho=.388, 0.02). Combined with the fact that past work has demonstrated the increasing role of determinism over these two time points in the assembly of microbial communities, such patterns suggest the possibility that relationships between edaphic properties, communities, and enzyme activity may modulate based on relative changes in stochastic to deterministic assembly (Knelman and Nemergut, 2014; Nemergut et al., 2013). Indeed, the coupling between environmental similarity and similarity in environmental process would be expected to increase with more deterministic assembly, a pattern that is consistent with assembly processes observed in these soils (Nemergut *et al.*, 2013). Interestingly, I propound that this mechanism and the shifts in relationship between environment and function may be generalizable across other successional systems, as shifts in the importance of stochastic to deterministic assembly processes have been observed as a general property of microbial succession (Ferrenberg *et al.*, 2013; Dini-Andreote *et al.*, 2015). Other factors could also underlie this pattern of increasing coupling between environment and function over advancing succession. For example, dormant, spore-forming organisms observed in the earliest stages of succession in these samples could

attenuate the relationship between environment, microbial community structure and its associated function (Lennon and Jones, 2011; Shade *et al.*, 2012). Further, as it is known that C chemistry can change substantially after fires (Certini, 2005; Knelman *et al.*, 2015; Knicker *et al.*, 2013), and the physical access to substrates may impact the production of enzymes rather than any biological constraint, thus obfuscating the relationship between environment and microbial mediated function. Regardless of the mechanism, this work shows that in looking at individual stages of succession, the relationship between environment and function shifts in its strength with advancing succession. Here, we observed a decoupling of environment and function early on with an increased coupling between the two in later succession.

Table 3-1. Significant correlations across post-burn succession between environmental factors and enzyme activity/community composition.

Significant correlations as per RELATE tests wit	thin each stage	e of post-burn	secondary su	ccession
Rho/P-value for:	Stage post-fire			
Enzyme-Environment	1 month	4 months	29 months	33 months
BG vs. %N	NS	.823 / 0.005	NS	NS
BG vs. %C	NS	.825 / 0.004	.364 / 0.047	NS
BG vs. CN	NS	NS	.776 / 0.001	NS
NAG vs. %N	NS	NS	NS	.544 / 0.016
NAG vs. %C	NS	NS	.425 / 0.031	.484 / .026
NAG vs. CN	NS	NS	.504 / 0.032	NS
Community composition-Environment				
UniFrac vs. % moisture	NS	NS	NS	.488 / 0.004
UniFrac vs. % N	NS	NS	NS	.423 / 0.043
Bray-Curtis vs. % moisture	NS	NS	NS	.439 / 0.005

When assessing the extended dataset, the 4 and 29-month time points show correlations between edaphic properties and BG activity, while later, 29 and 33 month time points, show correlations with edaphic properties and NAG activity. Shifts in nutrient limitation may contribute to when particular functional responses correspond with environmental parameters under strong environmental selection (Table 3-1). Such patterns are consistent with general patterns in nutrient limitation across succession where C is a primary limitation, with N limiting earlier in succession and P being limiting in later succession, under deterministic assembly (Vitousek and Farrington, 1997; Vitousek *et al.*, 1993; Fierer *et al.*, 2010). Further, the presence of plants in 29 and 33 month soils may also strongly structure microbial enzyme activity (Cline and Zak, 2015) and accentuate N limitations leading to strong correlations between environment and NAG activity, for example.

In this way, under strong environmental filtering of microbial communities, biological/physiological controls on function, such as resource demand determined by fundamental cellular processes, may contribute to responses across succession (Knelman *et al.*, 2014). In considering the relative importance of physical or biological limiting steps to decomposition processes (Schimel and Schaeffer, 2012), this work suggests that later succession may be more dominantly governed by biological controls, which could provide another mechanism by which connections between environment and microbial community function would be strengthened across secondary succession. Further, changes in carbon chemistry after burns, advancing succession, and the colonization by plants could affect the role of physical or biological rate limiting steps on enzyme production and decomposition (Schimel and Schaeffer, 2012; Knelman *et al.*, 2015).

Major flooding that occurred between 29 and 33 months provided the opportunity to assess the impact of an additional environmental perturbation on fire-disturbed soils. It appears the flooding may explain certain shifts in the relative importance of structuring environmental factors in these samples. For example, only burned soils were susceptible to significant, lasting changes in soil moisture (Table 3-2), and only in these 33-month soils did soil moisture emerge as a correlate with bacterial community composition (Table 3-1). In this case, fires that may actually destroy microbial communities and open up habitat patches (Bárcenas-Moreno *et al.*, 2011; Bárcenas-Moreno and Bååth, 2009) have been shown to result in increases in the relative importance of stochastic assembly (Ferrenberg *et al.*, 2013), whereas other disturbances such as floods which do not necessarily reset the microbial community but strongly change environmental conditions such as moisture (Table 3-2), may result in an increased role of deterministic processes in microbial community assembly, and therein connections between edaphic factors related to that disturbance and community composition.

Table 3-2. Edaphic properties across all time points for reference and post-burn successional samples.

Edaphic pro	perties mean a	ind standard devia	ation across b	urn and refer	ence soils							
Category	рН	% moisture	% N	% C	C:N	NH4 (mg/kg soil)	NPOC (mg/g soil)	TDN (mg/g soil)	Biomass-C (mg C/g soil)	BG (nmol activity/h/g soil)	NAG (nmol activity/h/g soil)	BG:NAG ratio
Burn												
1 month	7.21 (0.30) ^{AB}	9.93 (4.55) ^{AC}	.14 (0.04)	2.43 (0.95)	17.07 (2.71)	N/A	N/A	N/A	N/A	53.94 (12.02) ^A	78.53 (31.46) ^{AB}	0.74 (0.23)^
4 months	8.08 (0.41) ^A	8.55 (2.36) ^{ABC}	0.11 (0.04)	2.33 (1.07)	20.76 (1.67)	48.94 (14.95) ^A	0.30 (0.16)	0.06 (0.02)A	0.14 (0.13)	76.38 (27.67) ^{AC}	107.54 (43.07) ^A	0.75 (0.18) ^A
29 months	7.00 (0.28) ^B	2.16 (0.80) ^B	.12 (0.03)	2.48 (0.66)	19.60 (2.31)	2.30 (1.33) ^B	0.31 (0.30)	0.02 (0.02)B	0.31 (0.19)	127.00 (27.57) ^B	44.41 (14.61) ⁸	3.01 (0.71) ^B
33 months	7.40 (0.33) ^{AB}	18.08 (2.47) ^c	.11 (0.02)	2.29 (0.56)	20.41 (1.79)	N/A	N/A	N/A	N/A	115.23 (40.82) ^{BC}	52.72 (13.64) ^{AB}	2.26 (0.83) ^B
Reference												
1 month	6.44 (0.67) ^{AC}	19.74 (10.17) ^A	.27 (0.08)	6.21 (2.01)	23.07 (2.70) ^{AB}	N/A	N/A	N/A	N/A	N/A	N/A	N/A
4 months	7.08 (0.46) ^B	6.35 (3.50) ^B	0.24 (0.17)	6.59 (5.08)	25.99 (2.66) ^A	2.39 (1.40)	0.26 (0.18)	0.02 (0.01)	.55 (0.61)	147.83 (54.97) ^A	136.29 (63.12)	1.17 (0.38)
29 months	6.63 (0.34) ^{BC}	8.40 (6.12) ^{BC}	.24 (0.08)	5.20 (2.19)	21.67 (3.42) ^B	1.24 (0.60)	0.09 (0.04)	0.01 (0.004)	0.096 (0.02)	254.41 (86.14) ^B	251.77 (116.38)	1.13 (0.38)
33 months	6.29 (0.41) ^c	14.58 (5.05) ^{AC}	.25 (0.16)	5.47 (3.48)	21.67 (2.39) ^B	N/A	N/A	N/A	N/A	278.12 (132.57) ^B	264.42 (140.26)	1.16 (0.38)
Letters den	oto significant (differences as ner	Kruckal-Walli	s contrasts /D	2<0.05)							

While this work, shows changes in the importance and details of environment-ecosystem function relationships over time, connections with microbial phylogenetic/taxonomic community composition are not clear. Because of the vast diversity contained in microbial communities and the potential for functional redundancy, past studies have shown that trait-based approaches in place of phylogenetic or taxonomic ones may hold great value in elucidating processes and patterns relating to microbial community assembly (Burke *et al.*, 2011; Fox, 2012). Here, I also examined microbial communities related to reference and post-burn secondary succession in terms of rRNA operon copy number, a microbial trait that has been shown to underlie fundamentally distinct r and K selected life history strategies of microbes (Klappenbach *et al.*, 2000; Lauro *et al.*, 2009; Stevenson and Schmidt, 2004), which have been noted as characteristics central to microbial successional dynamics (Sigler *et al.*, 2002; Sigler and Zeyer, 2004; Shrestha *et al.*, 2007). In this way, I sought to examine how microbial communities, from a trait-based perspective, may connect with environment and function across succession.

Decreases were observed in average copy number across secondary succession of post burn soils (Figure 3-2). Likewise significant differences between reference forest and post-burn communities, with post-burn soil communities showing higher copy number, were found (Figure 3-2). This trait, underlying ecological strategies of microbes, may therein prove central to the assembly and function of communities across succession in post-burn soil. Interestingly, selection for high versus low copy number relating to different stages of succession and burn versus reference soils is represented in correlations with edaphic properties (Table 3-3). For example, across all samples, operon number shows the strongest (positive) correlation with pH (tau=0.476, P<0.0001) as well as significant negative correlations with %C, %N, and C:N ratio (Table 3-3), thus reflecting decreases in copy number with environmental properties of advancing succession.

Table 3-3. Correlations between rRNA copy number, environmental factors, and enzyme activity.

Significant rl	RNA oper	on copy number correlations with edaphic properties and enzyme activty
Factor	tau	P-value
рН	0.476	< 0.0001
% C	-0.336	< 0.0001
% N	-0.31	0.0001
C:N ratio	-0.237	0.0037
BG	-0.519	< 0.0001
NAG	-0.348	< 0.0001

Communities with aggregate community trait values of low copy number show the potential for far higher levels of enzyme activity than with those of a high copy number (Figure 3-2), though there is great variability in this trait, suggesting the other functional aspects of microbial communities may also be associated with the ecological life history characteristics corresponding to high and low copy number. This pattern in correlation between copy number and enzymes remains true for biomass normalized BG activity (tau=-.30, P=0.011) and marginally so for NAG activity (tau= -.23, P=0.057). Patterns of high enzyme activity with later successional, K-selected soil communities have been observed in the past (Pancholy and Rice, 1973; Tscherko et al., 2004; Sigler et al., 2002) as such communities may invest more in decomposition of litter for resource acquisition whereas inorganic nutrient availability in the soil is much more abundant to fast growing r-selected organisms in early secondary succession that is largely absent vegetation (Table 3-2, Table 3-3). Indeed, my research demonstrates a pattern of high enzyme activity with later succession, low rRNA operon copy number aggregate community trait values (Table 3-3, Figure 3-2). Interestingly, shared resources or function, such as extracellular enzymes, have been strongly implicated in microbial community evolutionary

dynamics (Morris *et al.*, 2012) because certain microbes may produce the resource while others may gain an advantage in simply using the resource as a cheater, and thus future work may more closely examine the connection between production of this shared resource pool and life history strategy. In total, the correspondence of this trait with both environment and function suggests that it may be a valuable trait related to life history strategy that may clarify connections among environmental factors, microbial communities, and ecosystem function in succession.



Figure 3-2 A: Plot of community aggregate trait value for rRNA operon copy number across soils from post-fire succession and reference communities at all time points. Letters denote significant differences as per Kruskal-Wallis contrasts (P < 0.05) B: Plot of relationships between the weighted mean copy number (aggregate trait value) and BG activity. C: Plot of relationships between the weighted mean copy number (aggregate trait value) and NAG activity.

The overall pattern of decreasing rRNA operon copy number observed between burn and reference forests as well as with advancing post-fire secondary succession, may provide a generalizable trait based approach by which communities are assembled across succession (Figure 3-2). In particular, rRNA operon copy number has in the past been strongly tied to microbial physiology and life history strategy (Shrestha et al., 2007; Stevenson and Schmidt, 2004; Klappenbach et al., 2000), suggesting its potential as a trait of interest that may constrain both the ecology and evolution of bacteria, and vary predictably across succession. To examine the generalizability of this successional pattern in copy number, I repeated my analysis on two additional, disparate successional environments: marsh sediments and a glacial forefield. These data demonstrated similar patterns in declines in aggregate community copy number over succession, with significant differences in community aggregate copy number between early and late communities (Figure 3-3). Thus, my work supports the notion that rRNA operon copy number may act as a trait that fundamentally underlies life history strategy of microorganisms to yield communities associated with early and late successional environments across a variety of systems.



Figure 3-3 Weighted mean rRNA copy number (community aggregate trait value) across succession of post-burn soils, marsh sediments, and glacial forefield soils. Letters denote significant differences as per Kruskal-Wallis contrasts (P<0.05). A significant linear model is shown for the glacier dataset (multiple R^2 =0.4949, P<0.0001).

While my research has focused on bacteria, fungi are often highly sensitive to heating, strong increases in pH, and loss of plant biomass; thus, their role in post-fire soil ecology may be at least temporarily muted (Bárcenas-Moreno *et al.*, 2011; Bárcenas-Moreno and Bååth, 2009; Ponder Jr. *et al.*, 2009). Still, I also acknowledge that fungi can play an important role in postfire landscapes (Dooley and Treseder, 2012; Holden *et al.*, 2012; Treseder *et al.*, 2004). Although the particular changes in bacterial communities may not mechanistically explain changes in enzyme activity under a scenario where fungi are contributing strongly to this function (and may explain the lack of consistent patterns in environment-microbial relationships but patterns of environment-function relationships), this work importantly points out the changing relationship between environment and enzyme activity across secondary successional soils, which is not impacted by the bacterial-restricted community analysis.

Conclusion

In total, this work provides evidence that the relationship between environmental factors and function changes over succession with early communities exhibiting a decoupling of edaphic factors (relating to resources) and ecosystem processes, while later communities show a more prominent coupling of these factors. My work specifically draws on a post-fire secondary successional system in which research has demonstrated that across secondary succession soils there is a shift from relatively stochastic to deterministic assembly, which would lead to expected increases in coupling between environmental similarity and similarity in ecosystem function (Nemergut *et al.*, 2013). Other possibilities for mechanisms underlying this shift in the strength of environment-function relationships may be at work as well. For example, the relative importance of physical or biological controls on decomposition as well as spore-formation,

which varies across these successional soils, may contribute to the relative coupling of environment and function. A variety of other factors such as phenotypic plasticity and functional redundancy could also influence environment-function relationships, and these aspects of microbial succession should be evaluated in future work to better understand the how the relative importance of environmental properties in ecosystem function may shift across succession. Further, my work also notes that rRNA operon copy number as a community aggregated trait may in fact prove important in the assembly and succession of microbial communities and the linkage between environment, microbial community, and function. My work demonstrates patterns of decreasing copy number with advancing succession both in terms of this particular study system, as well as across other, disparate environments. Interestingly, though further research is needed, low copy number organisms, which are characteristic of later succession, correspond with higher levels of enzyme activity per unit of biomass. Importantly, this study provides evidence that the relative importance and dominant controls of environmental selection may shift over succession with implication for environment-ecosystem function relationships. Here, early successional soils show a decoupling of environment and function whereas later successional soils show a coupling that is variable based on possible changes in nutrient limitation and/or environmental perturbation. While this work describes shifts in the coupling of environment and function across succession, future work should more closely examine the possible mechanisms that I have considered underlying these patterns, such as the role of stochastic and deterministic assembly, functional redundancy, microbial traits and phenotypic plasticity, and physical vs. biological controls on process.

CHAPTER 4

THE ROLE OF PLANT-MICROBE INTERACTIONS IN BACTERIAL COMMUNITY STRCTURE AND MICROBIAL FUNCTION DURING POST-FIRE SECONDARY SUCCESSION

Adapted from: Knelman, Joseph E., et al. "Fire severity shapes plant colonization effects on bacterial community structure, microbial biomass, and soil enzyme activity in secondary succession of a burned forest." *Soil Biology and Biochemistry* (2015).

Abstract

The increasing frequency and severity of wildfires has led to growing attention to the effects of fire disturbance on soil microbial communities and biogeochemical cycling. While many studies have examined fire impacts on plant communities, and a growing body of research is detailing the effects of fire on soil microbial communities, little attention has been paid to the interaction between plant recolonization and shifts in soil properties and microbial community structure and function. In this study, I examined the effect of a common post-fire colonizer plant species, Corydalis aurea, on soil chemistry, microbial biomass, soil enzyme activity and bacterial community structure one year after a major forest wildfire in Colorado, USA, in severely burned and lightly burned soils. Consistent with past research, I find significant differences in soil edaphic and biotic properties between severe and light burn soils. Further, my work suggests an important interaction between fire severity and plant effects by demonstrating that the recolonization of soils by Corydalis aurea plants only has a significant effect on soil microbial communities and biogeochemistry in severely burned soils, resulting in increases in percent nitrogen, extractable organic carbon, microbial biomass, β -glucosidase enzyme activity and shifts in bacterial community diversity. Overall, my study demonstrates that plant recolonization has a strong effect on soil edaphic, microbial, and biogeochemical properties, but
only in severely burned soils with more extreme abiotic soil environments. This work supports the increased role of plant colonization in succession of harsh environments and adds insights to a nascent understanding of when and where plant influence on belowground communities may act as a prominent feature of ecosystem dynamics in early succession.

Introduction

As patterns of fire severity and frequency shift amidst climate change, understanding how fires may influence ecosystem structure and function is of growing importance (Nibovet *et al.*, 2011; Reichstein et al., 2013). In particular, the western U.S. and Rocky Mountain regions are expected to face more frequent and severe fires (Miller *et al.*, 2009; Rocca *et al.*, 2014; Westerling, 2006). The details of ecological succession, including revegetation, can strongly influence ecosystem structure and function after such fire disturbance (Scheiner and Willig, 2011). Revegetation is a vital process for recovery of ecosystem function due to both direct and indirect effects on soil physical, chemical, and biological properties. While a vast body of research has described ecological consequences of fire to aboveground (Bond et al., 2005; DeBano et al., 1998) and belowground communities (Dooley and Treseder, 2012; Ferrenberg et al., 2013; Pourreza et al., 2014), a dearth of research has addressed the effect of colonizer plants on belowground soil microbial communities after fire disturbance (Hart et al., 2003; López-Poma and Bautista, 2014), or explicitly evaluated such interactions in the field. Nonetheless, interactions between soil bacteria and plants may themselves be drivers of ecosystem succession (Jangid et al., 2013; Knelman et al., 2012; Reynolds et al., 2003) and overall microbial communities (bacteria and fungi) are central to fundamental ecosystem soil processes including

carbon (C) flux, nutrient (nitrogen (N) and phosphorous (P)) cycling, and soil fertility (Schmidt *et al.*, 2014; Van Der Heijden *et al.*, 2008).

Extensive research has also established that fire severity strongly influences the degree to which soil physical, chemical, and biological properties are altered. For example, the severity of a fire can alter the magnitude of chemical and biological changes experienced by soils, such as changes in C, microbial biomass, enzyme activity, ammonium, and/or pH (Certini, 2005; Neary et al., 1999). As well, fire severity can also strongly influence carbon chemistry (Certini et al., 2011; Knicker et al., 2013) and microbial community structure/activity (Hamman et al., 2007; Weber et al., 2014), and because fire severity affects a multitude of soil properties so strongly, it may also modulate the relative importance of plant-microbe interactions. While research continues to uncover aspects of when and where changes in plant communities may affect microbial community structure and function, existing theory suggests that links between microbes and plants may be strongest amidst harsh abiotic conditions (Van Der Heijden et al., 2008). In this way, fire severity may prove important in understanding plant-microbe interactions in secondary succession. In particular, since early successional heterotrophic microbial communities often face C and nutrient constraints both after fire-disturbance and in general (Fierer et al., 2010; Treseder et al., 2004; Zak et al., 1990), I expect plant colonization to alter microbial community structure and function via alterations in soil edaphic properties. Given existing research and theory that suggests the importance of plant-microbe interactions varies across different abiotic conditions, I hypothesize that fire intensity will modulate plant effects, in which soils experiencing severe burns will exhibit a greater response to plant colonization than soils experiencing light burn.

Thus, in order to generate a better understanding of plant-microbe interactions following fire disturbances of varying severity, I examined patterns of soil chemistry, bacterial communities, microbial biomass, and overall extracellular enzyme activity of C, N, and P-acquiring enzymes, in four soil categories: severe burn unvegetated soils, severe burn revegetated soils, light burn unvegetated soils, and light burn revegetated soils from approximately one year after a major wildfire. I examined bacteria to assess a higher resolution picture of soil biotic responses to plant colonization during secondary succession revegetation processes, but I also assayed changes to total microbial biomass and enzyme activity – metrics that also integrate over the fungal community which may play a prominent role in post fire soil dynamics (Gartner *et al.*, 2012; Holden *et al.*, 2012; Treseder *et al.*, 2004). Here, I investigated how plant recolonization after fire may vary in its impact on soil biogeochemistry under conditions of varying fire severity.

Methods

Site description and sampling

I sampled soils from the High Park fire burns outside of Ft. Collins, CO, in July 2013. Soils were collected at the Buckhorn Camp property on the Colorado Front Range, which sits at ~2377 meters above sea level. Samples were collected approximately one year after the fire, which occurred in June 2012 and is the 3rd largest fire in Colorado's recorded history to date based on area burned. The site included both severely and lightly burn areas within a continuous area of forest dominated by Ponderosa Pine *(Pinus ponderosa)* on similar slope and aspect, and

with similar tree cover (in the area of latitude: 40.59N; longitude: 105.32W). General characteristics of these Front Range ponderosa pine forests are described by Veblen et al (2000). Severe burn was defined as areas with no soil litter layer and trees that were fully scorched to the crown (Figure 4-1). Light burn areas had a litter layer of 0.5-3.5 cm and partially scorched trees (55-286 cm scorch height) (Figure 4-1). All trees were dead in the severe burn areas while live and dead trees were mixed in light burn areas.



Figure 4-1 Light and severe burn sample sites. (Top) Light burn sample sites showed scorch marks at heights partially up trees. A pine need litter layer remained on the forest floor. (Bottom) Severe burn sample sites included completely charred trees with no litter layer on the forest floor.

Eight replicate revegetated and unvegetated soil samples were collected from both severe and light burn areas at least 5 meters apart across transects spanning 50 meters in both light and severe burn landscapes. Revegetated soil samples were collected under *Corydalis aurea* plants, a native plant that is a common member of communities after fire disturbance and was dominant in both light and severe burn areas. Vegetated soil samples were taken from under *Corydalis aurea* plants that were free of any other vegetation within a radius of at least 32 cm from the sampled soil. Unvegetated samples were free of vegetation within a 1.5 m radius. Soils were collected using a 5 cm diameter coring device to 5 cm depth and, for light burn soils, pine litter was removed prior to sampling. Thus, all samples included the top 5 cm of soil. Soils were immediately transported to labs at the University of Colorado at Boulder, passed through 2 mm mesh size sieves, and subsampled to be stored at 4° C for soil chemical and enzyme analysis. A subsample was stored at -70° C for molecular analysis.

Soil Properties

Soils were dried at 100° C for 48 hours to determine gravimetric soil moisture, and soil pH was determined on fresh soils using a ratio of 2 g soil to 4 mL DI H₂O. Thirty milligrams of dried, ground soils were packed in tin capsules and then run on a Thermo Finnigan EA 1112 Series Flash Elemental Analyzer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) to determine % C and % N of samples (Matejovic, 1997). Within a day of collection, ~8 g fresh soil were extracted in 40 mL 0.5M K₂SO₄ to determine NH₄⁺ and NO₃⁻/NO₂⁻ extractable N, total extractable non-purgeable organic carbon (extractable carbon excluding carbonates) (NPOC),

and total dissolved nitrogen (TDN). Microbial biomass C was ascertained through a paired set of extractions on 72-hour chloroform-fumigated samples as per standard methods (Brookes *et al.*, 1985). Microbial biomass-C as reported was adjusted for extraction efficiency based on the literature correction value of 0.45 (Beck *et al.*, 1997). All extractions included shaking for 1hour and filtering with Whatman no.1 paper (Whatman Incorporated, Florham Park, NJ, USA). Extracts were frozen until chemical analysis. NH₄⁺ was measured on a BioTek Synergy 2 Multidetection Microplate Reader (BioTek, Winooski, VT, USA) and NO₃⁻/NO₂⁻ were measured on a Lachat QuikChem 8500 Flow Injection Analyzer (Lachat Instruments, Loveland, CO, USA) from pre-fumigation extracts. NPOC in pre and post fumigation soils were measured on a Shimadzu TOC-V CSN Total Organic Carbon Analyzer (Shimadzu TOCvcpn, Kyoto, Japan).

To determine changes in carbon chemistry via humification, fluorescence spectroscopy was employed (Fellman *et al.*, 2010). For each sample, 5g fresh soil were extracted in nanopure H₂O, shaken for 1 hr at 250 rpm and filtered through combusted (4 hours at 450°C) Whatman GF/F filters (Whatman Incorporated, Florham Park, NJ, USA) into combusted amber viles. First, UV–vis analysis was performed using an Agilent 8453 spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). For fluorescence analysis, a 1:10 dilution of extract:water was completed so that UV absorbance at 254 nm fell between 0.1 and 0.2 cm⁻¹. These diluted samples were then used for fluorescence analysis on a Fluoromax-3 spectrofluorometer (Horiba Jobin Yvon, Kyoto, Japan). A three dimensional excitation-emission matrix (EEM) was collected for each sample at excitation wavelengths of 245-450 in increments of 10 nm, and at emission wavelengths of 300-600 nm in increments of 2 nm (Cory *et al.*, 2010). EEMs were then corrected for instrument biases and processed according to Gabor et al. (2014) using MATLAB software (The MathWorks, Inc., 2014). In order to examine the degree to which organic mater was humified in each sample, the humification index (HIX) was calculated from collected EEMs (Ohno, 2002; Zsolnay *et al.*, 1999).

Enzyme analysis

Enzyme activities for β -1,4-glucosidase (BG), β -1,4-N-acetylglucosaminidase (NAG), and acid phosphatase (aP) were determined; these enzymes are commonly used to assess the investment of the overall microbial community (fungi and bacteria) in the acquisition of the limiting elements C, N, and P (Sinsabaugh *et al.*, 2008). Enzyme activity was measured via fluorometric microplate methods (Sinsabaugh *et al.*, 2002; Weintraub *et al.*, 2012). Enzyme analyses were completed using a 96-well assay plate method using ~1 g of refrigerated soil, 1M sodium acetate buffer titrated to a pH of 7.3, and 4-methylumbelliferone standards, with the protocol detailed by Weintraub *et al.* (2012). The setup included 16 analytical replicates, quench corrections, standards, and negative controls for each sample. Fluorescence was measured using a microplate reader (Thermo Labsystems, Franklin, MA, USA) at 365 nm excitation and 460 nm emission to determine nmol activity h⁻¹ g soil⁻¹.

DNA extractions and Illumina sequencing

DNA was extracted using MoBio's PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA), according the manufacturer's protocol. DNA samples were eluted in TE buffer. Samples were amplified with barcoded PCR using 515F/806R primers. The forward primer included the 5' Illumina adaptor, a forward primer pad, and forward primer linker followed by the forward 515 primer. The reverse primer contained the reverse complement of the 3' adaptor, golay barcode, reverse primer pad, reverse primer linker, and the reverse 806 primer. PCR was run with a reaction mixture of 25.0 µL including 8.6 µL PCR Grade H₂O, 12.5 µL FideliTaq Master Mix, 1.0 µL Primer 515F (10µM), 1.0 µL Primer 806R (10µM), 0.11 µL MgCl₂ (25mM) in order to quench EDTA, and 2.79 μ L template DNA (all samples normalized to 3.583ng/ μ L). Negative controls were included. Samples were denatured at 94°C for 2 minutes and then amplified in 25 cycles at 94°C for 45 seconds, 50°C for 60 seconds, and 68°C for 45 seconds. A final extension was included of 5 minutes at 68°C. All PCR products were run in triplicate and then combined into single samples. To eliminate primer dimer contamination, barcoded PCR product was purified using the QIAquick Gel Extraction Kit, according to the manufacturer's protocol. Samples were multiplexed according to DNA concentration as per quantification using the PicoGreen method on a microplate reader according to the manufacturer's protocol. Pooled DNA purity and quality was determined on a NanoDrop800. Final pooled DNA was purified using the UltraClean PCR Clean-up Kit, according to the manufacturer's protocol. The final multiplexed DNA sample, including negative controls, was sequenced at CU Boulder (BioFrontiers Institute, Boulder, CO) on an Illumina MiSeq with the MiSeq Reagent Kit v2, 300 cycles.

Statistical and sequence analysis

Using the R statistical environment (R Development Core Team, 2013), Analysis of Variance (ANOVA) tests and Tukey's honestly significant difference (HSD) post-hoc tests were employed to evaluate differences in all soil parameters and taxon relative abundances across the four categories: light and severe burn and vegetated and unvegetated. All enzymes, biomass, moisture, pH, TDN, NH₄⁺, NO₂³⁻,NPOC , and taxon relative abundances were natural log transformed to achieve normal distribution as checked via a Shapiro-Wilk test before ANOVA and Tukey's HSD post hock tests. Pearson's product-moment correlation coefficients were calculated for relationships among specific enzyme activities.

I assessed bacterial community composition and diversity using a combination of UPARSE and QIIME software packages for quality checking/OTU picking at a 97% identity level and community level analysis (Caporaso *et al.*, 2010; Edgar, 2013). Quality filtering, chimera checking, OTU picking, and constructing an OTU table was performed in UPARSE according to default parameters and workflow on joined, paired end reads. QIIME was employed for downstream community analysis. Sequencing yielded a total of 781,359 sequences for 32 samples with a median sequence length of 253 base pairs. After quality filtering, 669,405 sequences remained in total and 5,662 OTUs across all samples resulted from UPARSE OTU clustering. All samples were rarefied to an even 11,700 sequences, and rarefaction plots were constructed based on the Chao1 estimator (Figure 4-2). All samples showed Good's coverage of over 95% as calculated in QIIME.



Number of sequences sampled

Figure 4-2 Rarefaction curves for all samples. Rarefaction curves based on Chao1 estimator of alpha diversity for all samples: BUN= burn unvegetated; BPARS= burn revegetated; LBUN= light burn unvegetated; LBPARS= light burn revegetated. All samples show or approach asymptotic phase and demonstrate a Good's coverage estimator of over 95%.

In QIIME, sequences were aligned and a tree was built using the Randomized accelerated maximum likelihood (RAxML v7.3.0) method for tree building (Stamatakis, 2006). This tree was used for downstream dissimilarity analysis, though results with the FastTree tree building method (Price *et al.*, 2009) as described in Knelman et al. (2014) showed the same significant

patterns in downstream analyses. Alpha diversity metrics were calculated in QIIME using the gini index, Shannon, and PD whole tree methods. Community dissimilarity matrices were constructed using the unweighted UniFrac method (Lozupone et al., 2006). Permutational MANOVAs (PERMANOVA) were performed in PRIMER E on the UniFrac beta diversity matrix and Mantel-like RELATE tests were used to examine correlation between this dissimilarity matrix and edaphic properties for severe burn soils (Clarke and Gorley, 2006). I assessed the relationship between all measured edaphic properties and BG activity in severe burn soils using the adonis function in the vegan package in R (Oksanen et al., 2013). This analysis showed if factors explained significant variation in BG activity across samples, and then demonstrated how much of that described variation was non-independent of revegetation as a factor in the model. Based on the findings of relationships between BG activity and particular edaphic factors, structural equation modeling in R (lavaan package) was used to more specifically determine how vegetation effects impacted BG activity in severe burn soils out of a variety of edaphic factors that showed relationships with BG activity (Grace, 2006; Rosseel, 2012). An a-priori model, based on my variance partitioning and documented ecological relationships, included both direct effects of vegetation on BG activity as well indirect effects via soil N, C, pH, moisture, and biomass. All variables were natural log transformed. This model, significantly different from the data, was respecified through the elimination of insignificant paths, resulting in an overall model that did not significantly differ from the data (P>0.05) and individual pathways, which were in themselves significant (P<0.05) (Grace, 2006).

Data availability

Sequences and mapping file/metadata have been made available via FigShare with the DOIs 10.6084/m9.figshare.1295229 and 10.6084/m9.figshare.1400496, respectively.

Results and Discussion

Soil edaphic properties showed strong signatures of fire severity. Measures of C chemistry and quantity showed significant differences in light and severe burn unvegetated soils, with severe burn soils containing higher relative levels of humics and significantly lower percent carbon. Nitrogen pools also showed significant differences with burn intensity as evidenced by lower percent N and TDN in severe burn vs. light burn unvegetated soils: however, the inorganic nitrogen content (NO_3^- and NH_4^+) did not differ between burn intensities (Table 4-1). Finally, severe burn soils showed significantly higher pH and soil moisture content than light burn soils.

Table 4-1. Soil properties of unvegetated/vegetated and severe/light burn samples

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Category	severe burn	severe burn	light burn	light burn		
	unvegetated	revegetated	unvegetated	revegetated		
TDN (mg N/kg soil)	1.36 (2.36) ^A	6.18 (3.34) ^{AB}	24.40 (20.15) ^B	13.67 (13.03) ^B		
NH4+ (mg/kg soil)	27.49 (14.38) ^A	10.75 (4.20) ^B	27.92 (19.97) ^A	14.93 (7.67) ^A		
NO2-/NO3-(mg/kg soil)	21.48 (17.43) ^A	14.21 (7.87) ^A	11.23 (14.98) ^{AB}	3.68 (2.69) ^B		
Humification Index	5.03 (1.44) ^{AD}	6.16 (0.61) ^A	3.47 (0.81) ^{BC}	4.19 (0.76) ^{CD}		
NPOC (mgC/kg soil)	153.35 (74.03) ^A	$280.95\ (89.60)^{\rm B}$	157.97 (50.00) ^A	195.04 (62.34) ^{AB}		
Percent C	2.96 (0.94) ^A	5.50 (1.59) ^{AB}	6.15 (2.40) ^B	8.03 (2.30) ^B		
Percent N	0.15 (0.05) ^A	0.26 (0.05) ^B	0.27 (0.07) ^B	0.32 (0.08) ^B		
C:N ratio	19.35 (2.64) ^A	20.99 (2.91) ^{AB}	22.42 (3.34) ^{AB}	24.69 (3.78) ^B		
pН	6.41 (0.47) ^A	6.86 (0.37) ^A	5.62 (0.16) ^B	5.70 (0.23) ^B		
Moisture (%)	2.41 (0.69) ^A	1.36 (0.39) ^B	1.02 (0.33) ^{BC}	0.75 (0.24) ^C		

Edaphic properties means (SD) across soil categories

letters denote significant differences as per Tukey HSD tests (P<0.05)

In severely burned soils, *Corydalis aurea* acted to alter chemical properties of soils, whereas in light burn soils, *Corydalis aurea* had no statistically significant effects on soil edaphic properties (Table 4-1). Plant colonization in severe burn soils corresponded with significant increases in NPOC and percent N, and significant decreases in percent moisture in contrast with severe burn unvegetated soils (Table 4-1). Ammonium and nitrate showed declines under revegetation of both fire severities relative to unvegetated soil, but differences were not significant (Table 4-1).

I constructed a principal coordinates ordination, which illustrated phylogenetic dissimilarity among soil communities (Figure 4-3). PERMANOVA analysis demonstrated that overall phylogenetic bacterial community composition was significantly different between unvegetated soils of severe and light burn (Table 4-2).



Figure 4-3 This Principal Coordinates Analysis (PCoA) shows microbial community dissimilarity (UniFrac) among samples from the 4 different soil types: Purple = severe burn; Gold = light burn; Circles = unvegetated; Triangles= vegetated.

Table 4-2. Differences among communities of different soil categories as per PERMANOVA analysis.

contrasts	P-value
severe unveg vs. severe reveg	0.007
severe unveg vs. light unveg	0.001
severe reveg vs. light reveg	0.001
light unveg vs. light reveg	0.897

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PERMANDVA	contrasts of	UniFrac	distance	among soil	categories
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In part, this difference was evidenced in significant shifts in dominant taxa between soil communities of severe and light burn soils. For example, the relative abundance of Firmicutes in severe burn soils was significantly higher than in light burn soils, whereas Alphaproteobacteria was significantly lower in severe burn soils (Table 4-3). Verrucomicrobia also showed a significant decline in severe burn unvegetated soils. Microbial biomass was also significantly different between light and severe burn unvegetated soils, showing significant decreases with increasing fire severity (Table 4-3). No significant differences were found among categories in terms of alpha diversity. Plant recolonization only significantly increased overall microbial biomass in severe burn soils (Table 4-3). PERMANOVA analysis similarly demonstrated that plant recolonization only affected bacterial phylogenetic community structure in severe burn soils (PERMANOVA P<0.05, Table 4-2, Figure 4-3).

Table 4-3. Microbial properties (biomass and taxa relative abundances) associated with different soil categories.

Total microbial biomass C and bacterial community composition means (SD) across soil categories					
Catagoria	severe burn	severe burn	light burn	light burn	
Category	unvegetated	revegetated	unvegetated	revegetated	
Microbial biomass C (mg C/kg soil)	180.91 (46.18) ^A	375.83 (104.36) ^B	547.49 (315.39) ^B	480.15 (196.86) ^B	
Acidobacteria (%)	6.83 (2.65) ^{AB}	6.14 (1.71) ^A	11.23 (5.37) ^B	9.98 (2.96) ^{AB}	
Actinobacteria (%)	28.55 (4.29) ^{AD}	31.20 (3.52) ^A	20.85 (5.19) ^C	20.55 (6.84) ^{CD}	
Bacteroidetes (%)	11.05 (3.12)	10.35 (2.24)	8.63 (4.74)	9.35 (2.91)	
Firmicutes (%)	4.63 (3.52) ^A	2.93 (1.04) ^A	1.32 (1.22) ^B	0.85 (0.78) ^B	
Alphaproteobacteria (%)	15.42 (1.38) ^A	15.14 (1.88) ^A	21.17 (3.73) ^B	22.70 (2.18) ^B	
Betaproteobacteria (%)	16.41 (3.23) ^{AB}	18.24 (3.03) ^A	11.50 (6.90) ^B	10.59 (3.92) ^B	
Verrucomicrobia (%)	4.30 (3.23) ^B	4.65 (2.70) ^{AB}	8.65 (3.80) ^A	8.25 (3.10) ^A	

letters denote significant differences as per Tukey HSD tests (P<0.05)

Exoenzyme activity showed significant differences between severe and light burn unvegetated soils, demonstrating effects of fire severity on microbial mediated enzyme activity related to C, N, and P cycling (Figure 4-4). BG, NAG, and aP enzyme activity were significantly lower in severe burn soils than light burn soils (Figure 4-4). Meanwhile the BG:NAG ratio was significantly higher in severe burn soils than light burn soils (Figure 4-4). When enzyme activity was evaluated as specific enzyme activity per unit biomass C, NAG showed the same patterns across soil types as absolute activity, being significantly higher in light burn soils than severe burn soils (Tukey HSD, P<0.05). BG and AP specific enzyme activity showed no significant differences in either comparisons of light and severe burn vegetated soils or light and severe burn unvegetated soils. Specific enzyme activities significantly correlated to varying degrees (P<0.05, BG-NAG: r= 0.83; BG-aP: r= 0.67; NAG-aP: r= 0.52). Total BG enzyme activity was significantly greater in plant-colonized soils than unvegetated ones in severe burn soils (Tukey's HSD P<0.05); no other soil enzymes showed significant differences (Figure 4-4).



Figure 4-4 Exoenzyme activity (nmol activity h^{-1} g soil⁻¹) across different soil conditions. Letters denote significant differences (Tukey's HSD, P<0.05).

In severe burn soils, plant recolonization showed significant effects on edaphic properties, bacterial community structure, and enzyme activity. Variance partitioning demonstrated that a variety of edaphic factors showed significant relationships with BG activity, but that the described variation was largely non-independent from revegetation, indicating the importance of indirect revegetation effects on BG activity (Table 4-4). Structural Equation Modeling, informed by the variance partitioning analysis, showed that vegetation indirectly drives changes in BG activity, primarily by impacting overall microbial biomass and TDN, which in turn drive variation in BG activity (R^2 = .834) (Figure 4-5). This overall model fit the data (Chi-squared test, P=0.102; Comparative Fit Index= 0.956) and all individual standardized path coefficients were significant (P<0.05) (Figure 4-5). In total, plant colonization described significant variation in BG activity via its impact on edaphic properties.

Variance in BG activity as explained by significant edaphic factors of severe burn soils (adonis)				
factor	R ²	P value	percent of explained variation attributable to plant recolonization	
biomass	0.617	0.001	78.93	
TDN	0.512	0.002	83.2	
perC	0.562	0.001	80.96	
perN	0.587	0.001	85.52	
NPOC	0.337	0.008	95.85	
moisture	0.446	0.001	92.6	

Table 4-4.	Variance	partitioning	results fi	rom adonis	analysis
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Figure 4-5 This diagram of the Structural Equation Model for plant effects on BG exoenzyme activity shows that plant recolonization indirectly impacts BG activity via its influence on microbial biomass and TDN, for example. Standardized path coefficients are all significant (P<0.05). Arrows are weighted in relation to path coefficients. Chi-squared P=0.102 and n=16.

My study shows fire severity is a dominant factor in explaining the effects of fire on edaphic and microbial properties of soil. In agreement with past research, I show both decreases in C pools and shifts in C chemistry, humification, with increasing fire severity (Almendros and González-Vila, 1990; Certini, 2005; Neary *et al.*, 1999; Neff *et al.*, 2005) (Table 4-1). Indeed, severe burns may lead to decreases in soil organic nitrogen (Neary *et al.*, 1999; Neff *et al.*, 2005; Treseder *et al.*, 2004), consistent with my results that show significant declines in TDN and percent N in severe vs. light burn soils (Table 4-1). In acidic soils, pH is also known to increase after fires, with more severe burns contributing more strongly to alkalization of soils as documented in my research (Certini, 2005) (Table 4-1).

I show that burn severity also impacts bacterial community structure and overall microbial biomass. Severe burn soils showed a significant reduction in overall microbial biomass and a significant change in the phylogenetic composition of bacterial communities in comparison with lightly burned soils, an effect which has been noted in previous work (Hamman *et al.*, 2007; Pourreza *et al.*, 2014; Weber *et al.*, 2014). Indeed, post-fire changes to the soil environment, especially accentuated under severe burns, may create a unique environment that can influence microbial community assembly via both strong environmental filters and/or the opening of habitat to colonizer dispersal, which can therein influence ecosystem function as well (Ferrenberg *et al.*, 2013; Knelman and Nemergut, 2014; Nemergut *et al.*, 2013).

Although no changes were observed in alpha-diversity, changes in relative abundances of major microbial taxa are coherent with ecological strategies of associated microbes. For example, Firmicutes, with the capacity to form endospores and possibly withstand fire have been noted to burgeon after fire disturbances (Ferrenberg *et al.*, 2013; Smith *et al.*, 2008) (Table 4-3). Thus, in more severe fires, these organisms may have a comparative advantage, having withstood the severe burns, in immediately establishing in soils post disturbance. I also note significant decreases in Alphaproteobacteria relative abundance with increasing burn severity, which is consistent with research demonstrating greater Alphaproteobacteria abundances in higher nutrient soils (Cederlund *et al.*, 2014; Knelman *et al.*, 2012) (Table 4-3).

Importantly, my study is unique in exploring the effects of secondary plant succession via colonization by Corydalis aurea, under the conditions of different burn severities, in regulating ecosystem function. Consistent with my hypotheses, I demonstrate an interaction between burn severity and revegetation, in which re-establishment of plant communities has a significant impact on soil edaphic and microbial properties in severe burn soils but not in light burn soils. In severely burned soils, plant colonization significantly increased extractable organic C and percent N, thereby having the effect of amending the soil nutrient status relative to unvegetated soils in severe burns. Litter and exudate inputs from newly established plants may alter carbon and nitrogen status of soils and feedback on microbial biomass and community structure (Berg and Smalla, 2009; Van Der Heijden et al., 2008). Although plant colonization correlated with significant shifts in microbial communities in severe burn soils (Figure 4-3, Table 4-2), I was not able to attribute these observed overall community changes to shifts in measured edaphic properties, as Mantel-like RELATE tests showed no correlations among bacterial dissimilarity and edaphic factors. It is possible, that communities may be responding to unmeasured direct or indirect plant effects, such as more nuanced aspects of carbon chemistry (Meier and Bowman, 2008).

Plant-colonization only affected BG enzyme activity in severe burn soils. My variance partitioning identified relationships between biomass, TDN, percent C, percent N, NPOC, and moisture with BG activity, that were, however, non independent of plant effects. I then used SEM to generate a more mechanistic hypothesis of plant colonization driving shifts in BG activity via indirect effects on soil edaphic properties. In testing the indirect effect of plant recolonization via changes of these various edaphic properties, I found that plant driven changes in biomass and TDN fit the data to significantly describe variation in BG activity. As such my

results indicate a coupling between C and N cycles and suggest that plant organic inputs, in general, may play a role in altering microbial mediated biogeochemistry in early secondary successional soils. Indeed, past research has shown that microbes may invest in C acquisition enzymes in response to the availability of resources, such as via plant inputs of carbon and nitrogen (Sinsabaugh et al., 2008; Sinsabaugh and Moorhead, 1994), and my work extends this understanding to demonstrate a response in terms of microbial community function and bacterial structure to plant colonization in severe but not light burn soils. Specific enzyme activity confirms that investment in NAG per unit biomass C is significantly higher in light burn vs. severe burn soils, and correlations among specific enzyme activities per unit biomass showed that activity of all enzymes increased together. However, BG:NAG ratios are also significantly higher in severe burn soils versus light burn unvegetated soils, indicating a change in the relative investment in C vs, N acquisition. As such, it appears that microbial communities in severe burn soils may be more limited by C while microbial communities in light burn soils may be more limited by N. These differences in BG:NAG ratios have been used as an indication of the relative allocation of resources toward C or N acquisition, demonstrating an overall shift in resource allocation strategy toward C acquisition in severe burn soils, not purely an change in enzyme potential as a function of microbial biomass (Gartner et al., 2012; Sinsabaugh et al., 2009, 2008) (Figure 4-4).

Finally, while my research has focused on bacterial community structure, an important subset of the microbial community as a whole, for my higher resolution community analysis, my assessment of overall microbial biomass and soil enzyme activity may be driven by fungal components of the microbial community outside of the observed bacterial patterns, since fungi can play important roles in post fire landscapes, secondary succession, and biogeochemistry

more generally (Holden *et al.*, 2012; Reynolds *et al.*, 2003; Treseder *et al.*, 2004). Additionally, past research has demonstrated strong responses of mycorrhizal fungi and soil invertebrates to fire, which also may strongly impact secondary succession in soils after fire due to there importance in ecosystem dynamics (Dooley and Treseder, 2012; Certini, 2005). In total, while biomass and enzyme activity measured in this study may suggest important changes in fungal communities with integral feedbacks on ecosystem development, further research is needed to better appreciate the relative contribution of bacterial and fungal responses to biomass and enzyme activity reported here.

Conclusion

As fires become more prevalent across the American West and the globe, it is vital to better constrain how affected ecosystems – and the important functions associated with them – will respond to such disturbance and recover. Beyond well known differences in the effect of fire severity, my work fills a gap in our understanding of post fire-disturbance ecosystem succession, demonstrating that the effects of plant colonization during secondary succession revegetation depend on fire severity, with the greatest impact on soil edaphic factors, bacterial community structure, overall microbial biomass, and soil enzyme activity in higher severity burns. Plant recolonization effects on BG enzyme potential is of interest, given the central role of exoenzymes in decomposition and nutrient cycling, and suggests the importance of understanding plant effects on broader microbial community function associated with compositional changes as well (Uroz et al., 2010). My work demonstrates that indirect soil effects of plant colonization drive increases in BG activity in severe burn soils. Further, my study indicates that plant colonization during revegetation may have stronger effects on

belowground communities in harsher, more nutrient poor soil environments after severe burns, for example, building on a body of literature that is beginning to assess where and when aboveground communities will have effects on belowground microbial community structure and function in a rapidly changing world.

CHAPTER 5

CONCLUSION

Building knowledge of generalizable patterns and processes that underlie the assembly of microbial communities through succession will prove central to predicting and managing the functional outcomes of microbial systems, with implications from human to environmental health in a changing world. Increasingly perspectives in microbial succession are being considered beyond natural soil ecosystems where successional theory has traditionally been developed. The human gut (Koenig *et al.*, 2011; Peterfreund *et al.*, 2012), food production (Coppola *et al.*, 2000; Petersen *et al.*, 2002), waste water treatment (Yan *et al.*, 2008; Pholchan *et al.*, 2013), and agriculture (Ishii *et al.*, 2000; Chaparro *et al.*, 2014) present but a few environmental health. Indeed, insights generated from this research that sit at the intersection of community and ecosystems.

From this work, I demonstrate that N and P in combination play a strong role in controlling largely autotrophic microbial communities in primary succession. We find that nutrient addition not only results in phylogenetic community change, but drives succession within a single year in a way that is consistent with over 80 years of succession. These soils have no corresponding change in pH or soil carbon, for example, which have been known to structure microbial communities and change, showing that N and P addition alone largely control microbial succession. Thus, this research demonstrates the central role that nutrients can play as a control on microbial community succession and extends existing theory regarding nutrient

controls on succession from plant-centered studies into the microbial realm. Further, my work demonstrates nutrient controls on microbial mediated ecosystem function -- enzyme activity -- in succession of the Four Mile Canyon as well as High Park Fire post-burn soils. In both these studies, C and N are shown to correspond with enzyme activity, again demonstrating that nutrients play a dominant structuring role in microbial community succession both at the community and functional level. While nutrients are strong abiotic filters in succession, my work further explores how the relationship between environment and ecosystem function may change across succession, showing that the strength of such relationships increases with advancing succession. The more nuanced view of connections between environment and function that this work takes also demonstrates that the specific controls on function may shift during succession at relatively short time scales given change in biological constraints, such as nutrient limitation, or environmental disturbance, such as flooding. While the mechanism may be varied, my work importantly points out that the correspondence between environmental similarity and similarity in ecosystem function varies across succession and may relate to assembly process, microbial traits, or other community attributes such as functional redundancy that may vary in a generalizable manner across succession. In considering how microbial life history strategy may connect environment and function, I show that rRNA copy number varies predictably across succession of a variety of systems, and that this variation corresponds with both environmental parameters, including nutrients, as well as functional outcomes, enzyme activity. Thus, a trait-based approach provides the possibility of synthesizing microbial community assembly with environmental, community, and functional understandings of succession. While this work highlights the role of abiotic factors in driving microbial community succession, my work also elucidates the strong ecological selection related to plant-

microbe interactions in early succession, demonstrating the importance of biotic interactions in microbial succession. This study shows the prominent selective effect that plants may have in driving microbial community assembly across succession upon colonization of early successional soils. Further, this work demonstrates that the relative importance of plant-microbe interactions as a selective filter on microbial succession depends on the abiotic context, in this case in terms of fire severity. In total, my work highlights both abiotic and biotic controls on succession and clarifies patterns and processes that underlie microbial succession. As well, this work suggests that patterns in microbial community characteristics in terms of assembly process or traits, for example, across succession may also influence the strength of environment-function relationships, and further work should examine the generalizability of this observation.

Since deep into human history, civilization has relied heavily on disturbance and succession to maintain ecosystem services, and future advances in our understanding of microbial succession is particularly important given global climate change, where the increasing prevalence of disturbance and successional responses of ecosystems will have strong feedbacks on global biogeochemistry and the environments that sustain us. While academic research on ecological succession has been ongoing for well over a century, our continued efforts in understanding the patterns and processes of microbial succession are as relevant today as ever.

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APPENDIX

PLANT ASSOCIATED ROOT MICROBIAL COMMUNITIES IN EARLY PRIMARY SUCCESSION

Abstract

In low nutrient, early successional environments, plant-associated microbes may play an accentuated role in plant ecology. Details of the structure and assembly of such bacterial communities remain unexplored in these ecosystems; past research has largely focused on agricultural systems and/or culturable subsets of the bacterial community. Here I examine patterns in the root-associated bacterial communities of colonizer plants in early ecosystem succession that may therein impact the trajectory of further ecosystem development. In the Mendenhall Glacier forefield near Juneau, AK, USA, I used 454 pyrosequencing of the 16S rRNA gene to reveal patterns in bacterial community structure from roots of alder (Alnus sinuata) and spruce (Picea sitchensis) seedlings and unvegetated bulk soils within a single transect. Root-associated bacterial communities were phylogenetically distinct from bulk soil communities and also unique to plant species type. Roots show significant increases in Actinobacteria and γ -Proteobacteria as well as decreases in β -Proteobacteria and Acidobacteria compared to bulk soils. To better understand the presence of bacteria across the root-soil continuum (specialists or generalists) I identified particular OTUs that were common across a majority of both root types (widespread), and their patterns of occurrence in soil and root habitats using an indicator species value index. The patterns observed in beta diversity and taxon relative abundances suggest the importance of strong environmental selection and plant signatures on bacterial communities; however, the presence of widespread generalists indicate that alternate

processes – such as dispersal, competition, or ecological drift – may also prove central to the assembly of root communities.

Introduction

Feedbacks between plants and soil microbial communities are integral to plant performance, plant community ecology, and ecosystem development (Wardle, 2004; Bardgett *et al.*, 2005; Kardol *et al.*, 2007; Van Der Heijden *et al.*, 2008). Plants influence microbial community composition and function largely through rhizodeposition and the unique chemical and physical attributes of the root compartment (Grayston *et al.*, 1998; Hartmann *et al.*, 2008). In turn, root-associated microbial communities can play immense roles in plant performance. Plant growth-promoting rhizobia (PGPR), for example, can impact host plants through wideranging effects including the acquisition of nutrients, production of phytohormones, and provisions of pathogen control/stress resistance (Dobbelaere *et al.*, 2003; Vessey, 2003; Compant *et al.*, 2010).

Research on plant-microbe interactions in both agricultural and non-managed systems has focused on rhizosphere soils, typically defined as root adhering soil particles. Nonetheless, the bacteria that inhabit the rhizoplane and endorhizosphere compartments of plants, hereafter referred to as root-associated bacteria, are more proximate to plants and may strongly influence plant performance (Compant *et al.*, 2010; Lodewyckx *et al.*, 2002; Beattie, 2006; Hardoim *et al.*, 2008). Past research on root-associated bacteria has mostly centered on bacterial communities in managed/agricultural systems (Germida *et al.*, 1998; Marilley and Aragno, 1999; Chelius *et al.*, 2001; Wieland *et al.*, 2001; Kim *et al.*, 2006). Additionally, much of this work has focused on

culturable microbes (Kuklinsky-Sobral *et al.*, 2004; Puente *et al.*, 2004; Han *et al.*, 2009; Becerra-Castro *et al.*, 2010; Venieraki *et al.*, 2010). While research efforts have identified the importance of PGPR in agriculture and for bioremediation, root-associated bacteria can impact plant community ecology and ultimately ecosystem structure and function. Only a few studies, however, have begun to explore such bacterial communities (Nunan *et al.*, 2005; Van Der Heijden *et al.*, 2006; Oh *et al.*, 2012; Dean *et al.*, 2015).

In particular, ecological theory suggests that microbial communities are of primary importance for plant performance in low nutrient environments (Reynolds *et al.*, 2003; Van Der Heijden *et al.*, 2008). In this context, root-associated microbial communities may play a vital role in the ecology of the first vascular plant colonists in early succession, impacting overall ecosystem development. Thus, understanding how plants influence microbial community structure and function will help to explain the ecology of plant communities and overall ecosystem development.

In studies of developing ecosystems, past research has demonstrated plant effects on microbial community structure and function, typically at the rhizosphere scale or broader (Knelman *et al.*, 2015; Bardgett and Walker, 2004; Tscherko *et al.*, 2005; Edwards *et al.*, 2006; Miniaci *et al.*, 2007; Knelman *et al.*, 2012). However, there is practically no understanding of the composition and patterns of root-associated microbial communities at the rhizoplane and endorhizosphere scale in these environments. As such, I aimed to examine the composition and phylogenetic structure of root associated bacterial communities that occupy the physical root environment in early succession of the Mendenhall Glacier forefield. Such patterns in bacterial community structure can reflect processes underlying community assembly of root-associated bacteria. In particular, the root environment, with its unique physical and chemical

characteristics, may result in strong species sorting (Compant *et al.*, 2010). Nonetheless, studies have noted that bacteria can persist as generalists in habitat preference across different compartments and selective environments of the root-soil continuum (Germida *et al.*, 1998; Lilley *et al.*, 1996), suggesting the role of other processes beyond selection. Local dispersal as well as competition may impact the assembly of these generalist microbes that persist across both the soil and root habitats and are less constrained by environmental selection (Compant *et al.*, 2010; Benizri *et al.*, 2001; Rosenblueth and Martínez-Romero, 2006).

In total, I sought to characterize overall phylogenetic and taxonomic differences in root associated bacterial communities in comparison with background microbial communities of the bulk soil and understand how these patterns might reflect processes structuring the community. Additionally, I screened sequence data for particular OTUs that were "widespread" across roots (found in a majority of samples of both root types). I then analyzed their occurrence in soil samples using an indicator species index to test their specialist/generalist patterns across the rootsoil continuum. Given the potentially heightened importance of plant-microbe interactions in the early stages of ecosystem development studied here, I hypothesized the presence of a significant plant species signature on root-associated bacterial communities.

Methods

Study site and sampling

Sampling took place at the Mendenhall Glacier forefield, outside of Juneau, Alaska, October 2009. Details of the site and soils -- which have resulted from an ongoing deglaciation event of this high latitude, low elevation glacier -- have been previously described (Knelman *et al.*, 2012; Sattin *et al.*, 2009). I sampled a single transect of soils that have been exposed for \sim 6 years for the roots of both spruce and alder seedlings, as well as bulk, unvegetated soil (0-5cm). Seven replicates of each sample type analyzed in this study were collected from seedlings that were \sim .3-.7m tall and interspace soils that were free from vegetation including lichens and biological crusts. Roots and soils for DNA analysis were immediately placed on ice and soils sieved through a 4 mm mesh in Juneau. Samples were transported on ice to Boulder, CO, USA. Samples for DNA analysis were stored at -80°C.

DNA Extractions for 454 pyrosequencing

Roots were rinsed in a series of 3 washes of sterile water to remove root adhering soil particles, frozen in liquid nitrogen, and finely ground. The DNeasy Plant Mini Kit (QIAGEN, Valencia, CA) was used according to the manufacturer's protocols to extract DNA from the 14 root samples. Mo Bio PowerSoil[™] DNA Isolation kits were used as per the manufacturer's instructions for DNA extractions of the bulk soil samples (Mo Bio Laboratories, Inc., Carlsbad, CA). PCR-amplified bacterial 16S rRNA genes from the genomic DNA of the root and soil samples were generated using a universal bacterial 27F and 338R primer set as described by Hamady et al. (2008), and reaction conditions followed those described by Fierer et al. (2008). Primers included a 2 bp adapter, the 454 Roche Titanium A/B primer, and a unique, 12 base pair error-correcting golay barcode for pyrosequencing as detailed by Knelman et al. (2012). 454 Life Sciences GS FLX Titanium pyrosequencing of the 16S rRNA gene amplicons was completed by the Environmental Genomics Core Facility (Engencore) at the University of South Carolina. Resulting sequences were quality-checked and denoised using the Quantitative Insights into Microbial Ecology software package (QIIME) (Caporaso *et al.*, 2010; Reeder and Knight, 2010). Chloroplast and mitochondrial sequences were removed.

Pyrosequence and statistical analysis

Using QIIME, sequences were limited to those of a sequence length of 200 to 400 base pairs, a maximum of 5 homopolymers, a minimum quality score of 25, and a maximum of ambiguous bases/primer mismatches of 0; all samples were then denoised using flowgram clustering in QIIME. OTUs were selected at a 97% identity level by clustering based on representative sequences via UCLUST (Edgar, 2010). The Basic Local Alignment Search Tool (BLAST) method (Altschul et al., 1990) with the SILVA (Pruesse et al., 2007) database was used to assign taxonomic identification to OTUs. After sequence alignments based on the NAST algorithm (DeSantis et al., 2006), a phylogeny was constructed with the FastTree algorithm (Price et al., 2009). In order to examine differences among bacterial communities, a pairwise distance matrix based on unweighted UniFrac, a phylogenetic distance metric, was generated (Lozupone et al., 2006, 2007). The Principal Coordinate Analysis (PCoA) ordination was constructed with the UniFrac distance matrix. The QIIME-generated OTU table was used to evaluate relative abundance of all taxa. QIIME was also used to construct rarefaction curves of sampling in each replicate and filter OTUs to those present in over half of replicates in each of the plant species root samples to discern widespread OTUs associated with plants. OTUs that were present in a majority of both root type samples were considered "widespread." Among these widespread taxa, ratios of specialist: generalist OTUs were determined by assessing

generalist to specialist habitat occurrence via Dufrêne-Legendre Indicator Species Analysis (Dufrêne and Legendre, 1997). I assessed indicator species based on presence/absence of widespread OTUs in replicate samples of root and soil habitats. This analysis yields a value index that is calculated based on the distribution of an OTU across the two groups and the percent of samples out of a single group in which the OTU is present; a maximum indicator value, for example, occurs when an OTU is found in all of the samples of one group and none of the samples of another group. Statistical significance is tested based a randomization procedure that produces a null distribution of OTU frequencies across samples. For contrasts in beta diversity that involved all samples (UniFrac analysis), samples were rarified to the lowest sampling depth. Given the large differences in alpha diversity in bulk soil samples relative to root samples, the differential sequencing efforts resulted in comparable fractions of overall diversity recovered from both environment types (Figure A-1), albeit not exhaustive of total community diversity in either. Thus, the entire data set was used for analysis of relative abundance and widespread taxa, where analyses did not depend on absolute sequence counts. Primer v6 software (KR Clarke and Gorley, 2006) was used to perform Analysis of Similarity (ANOSIM) to compare phylogenetic distances among bacterial communities (UniFrac). R software (R Development Core Team, 2013), was used to test one-way analysis of variance (ANOVA) with Tukey's Honestly Significant Difference post hoc tests and unpaired t-tests to evaluate differences among bacterial taxa relative abundances across the three sampled environments and roots versus soils. The "labdsv" package in R was used for indicator species analysis (Roberts, 2007).



Figure A-1 Chao1 diversity estimator rarefaction curves of both bulk soil (top 7 curves) and root samples show that initial slopes of bulk soil curves are far greater than roots, whereas deeper sampling depths in bulk soils results in rarefaction curves more parallel to those of root samples. Rarefaction curves indicate that deeper sampling of bulk soil communities captures more comparable alpha diversity to that of the limited sampling of root communities.

Results and Discussion

I obtained a total of 4,349 sequences for roots and 19,935 sequences for bulk soils after quality checks, denoising, and the removal of chloroplast and mitochondrial sequences. I recovered an average of 2,848 reads per sample with a standard error of 130 in bulk soils. Because of the abundance of plant organelle 16S rRNA genes amplified, the number of sequences per root sample was much more variable: I obtained an average of 311 sequences per sample with a standard error of 70. Root bacterial communities of the two plant types were significantly different from bulk soil communities as well as each other (spruce roots vs. unvegetated, P=0.001; alder roots vs. unvegetated, P=0.001; spruce roots vs. alder roots P=0.017, ANOSIM analysis; Figure A-2).



Figure A-2 Principal Coordinates Analysis of Unweighted UniFrac distances among bacterial communities. Roots show greater similarity in community composition to one and another than to bulk soil. ANOSIM contrasts between all categories show significant differences for both weighted and unweighted UniFrac distances (P<0.05). Red= Bulk Soils, Blue=Spruce, Green=Alder.

Major bacterial phyla (or within Proteobacteria, sub-phyla) making up on average greater than 5% of community composition included Acidobacteria, Actinobacteria, Bacteroidetes, α -Proteobacteria, β -Proteobacteria, and γ -Proteobacteria (Table A-1). I observed changes in the relative abundances of these taxa between bulk soil and roots of the two plant species (One-way ANOVA with Tukey's Honestly Significant Difference a posteriori, Table A-1); broad scale shifts in dominant taxa were also observed between the root and soil environments (unpaired t-tests, Table A-1, Figure A-3).

Table A-1. Relative abundances of major bacterial taxa across environments.

 Bacterial Taxa 	Root Associated		Soil	
	Spruce	Alder	Bulk Soil	Roots vs. Bulk Soil
Acidobacteria	$5.65\pm0.90^{\rm A}$	$3.24\pm0.72^{\rm A}$	$10.67 \pm 1.32^{\mathrm{B}}$	t = -4.2322, P = 0.002198
Actinobacteria	$14.39\pm1.18^{\mathrm{AB}}$	$18.49 \pm 2.03^{\rm A}$	$8.33 \pm 1.16^{\rm B}$	t = 4.4302, P = 0.000308
Bacteroidetes	13.44 ± 1.29	16.11 ± 2.58	12.8 ± 0.71	NS
α -Proteobacteria	25.6 ± 3.28	23.65 ± 1.57	20.61 ± 0.94	NS
β-Proteobacteria	$15.57\pm1.38^{\mathrm{A}}$	$12.71 \pm 1.13^{\mathrm{A}}$	$21.55 \pm 1.14^{\mathrm{B}}$	t = -5.0136, P= 0.00019
γ-Proteobacteria	8.07 ± 1.15	8.61 ± 1.57	4.86 ± 0.78	t = 2.693, P= 0.01449

Relative Abundances of Bacteria Taxa >5% of total community (% of total community)

Letters denote significant differences in Tukey's HSD pairwise comparisons (P<0



Figure A-3 Significant shifts in dominant taxa between root-associated and bulk soil bacterial communities. Shifts in relative abundances of major bacterial taxa between root associated and bulk soil bacterial communities. Taxa displayed above are significantly different between Roots and Bulk Soil; Bulk Soil vs. Roots t-test, P< 0.05. Relative abundances with standard errors reported in Table A-1.

Together, both types of roots had significantly greater relative abundances of Actinobacteria and γ -Proteobacteria, and lower relative abundances of β -Proteobacteria and Acidobacteria when compared to bulk soils (P<0.05) (Table A-1). By filtering OTUs to only those that were present in a majority of root sample replicates, I identified OTUs that were widespread across roots even if not significantly enriched in relative abundance as compared to bulk soil (Table A-2). Taxa that were present in a majority of replicates from both root samples were compared across soil samples as well via indicator species analysis. Clusters of Actinobacteria, Rhizobiales, Burkholderiales, and Chitinophaga - Flavobacterium - Flexibacter proved consistent bacterial members across roots of both plant species (Table A-2). Indicator species analysis revealed statistically significant (P<0.05) occurrence patterns of OTUs that were specific in their occurrence to the root habitat and others that were generalists (Supplementary Table A-1). Based on this analysis, specialist:generalist OTUs (Table A-2) illustrated the habitat preference of widespread root OTUs. Burkholderiales and Rhizobiales were found to be the most generalist in habitat preference, spanning both roots and soil samples.

Table A-2. Widespread taxa across roots and habitat occurrence

Taxonomic	# of OTUs in	Average % of root communities	specialist:
Identification	cluster	Composed by OTUs in cluster	generalist*
Rhizobiales	11	11.40 ± 1.23	3:8
Chitinophaga- Flavobacterium- Flexibacter	5	6.12 ± 0.90	2:3
Burkholderiales	5	7.82 ± 0.90	1:4
Actinobacteria	5	8 51 ± 1 14	2.3

Widespread taxa across roots (present in majority of both Alder and Spruce roots) and habitat occurrence

*Specialists include OTUs that indicate root environments as per Indicator Species Analysis (P<0.05)

My analysis clearly shows that roots of early colonizer plants are selective environments for unique bacterial communities that are phylogenetically distinct from bulk soils and also unique based on plant species. (Figure A-2, Figure A-3, Table A-1). This overall finding is consistent with past research that indicates the bacterial communities of root environments display a strong signature of plant selective effects even in comparison with rhizosphere soils (Wieland *et al.*, 2001; Hartmann *et al.*, 2008). On one hand, the root surface and interior are selective environments, resulting in distinct occupants of the root habitat, as noted in my present study that shows phylogenetic differences in the communities of bulk soil and root samples (Figure A-2) (Sessitsch *et al.*, 2002). This is similar to research in macro-ecology that suggests that niche-based forces are important in structuring communities at successional transitions (Ellner and Fussmann, 2003). However, my indicator species analysis that reveals specialists and generalists in habitat occurrence points toward the concept of a root-soil continuum and community assembly processes that might extend beyond environmental selection of particular root or soil habitats. Many bacteria originate from the larger surroundings and may straddle habitats, living in distinguished environments of both root compartments and the soil (Lilley *et al.*, 1996; Rosenblueth and Martínez-Romero, 2006), such as the generalist taxa that I found across soil and root samples (Table A-2).

Interestingly, Lindstöm and Langenheder (2012) note that generalist versus specialist lifestyles are affected by different assembly processes and may be a useful dichotomy in understanding microbial community assembly. In general, microbial communities of plant influenced soils have been shown to follow alternative assembly processes than interspace soils (Hovatter et al., 2010). Indeed, amidst a multiplicity of factors likely affecting the assembly of root-associated communities, the prevalence of widespread generalists observed in my study (Table A-2) suggest that other factors may be at work in addition to the presence of environmental filters that was observed. For generalists that seem capable of persisting across different environments, small-scale dispersal capabilities from the soil to root environment or species-species interactions may be important in structuring these bacterial communities, for example. The role of active motility in bacterial root colonization has been previously recognized (Mishagi, I. J. et al., 1992; De Weger et al., 1987), and the prevalence of generalists found across habitats of the root-soil continuum in my study supports the possibility that factors outside of environmental filters, such as dispersal via motility, may pay a notable role in community assembly at the root scale. Indeed, in an analysis of genes unique to bacterial plant symbionts within the α –Proteobacteria the flagellar protein FliG was shared as a unique, common gene, likely underpinning the symbiotic lifestyle (Pini et al., 2011). Ecological drift may also play a

role in structuring the generalist constituents of root communities that are not so strongly determined by species sorting.

Furthermore, the analysis of specific OTUs that were widespread across root samples show the prominence of known plant symbionts. Interestingly, two of the clusters were identified as Burkholderiales and Rhizobiales, bacterial orders that contain well known plant symbionts and potential nitrogen fixing organisms that have been widely cited in the literature as important components of root-associated communities (Table A-2). For example, within the Rhizobiales, a single OTU present in every single root sample (Table A-2), was identified as Bradyrhizobium japonicum, which is not only well known as a symbiotic nitrogen fixer, but also has been characterized to enhance plant performance of non-legumes as a plant growthpromoting rhizobacterium (Antoun *et al.*, 1998). In particular this OTU was found across all soil samples as well, clearly displaying generalist patterns across environments (Table A-3). The relative abundance of this group of widespread Rhizobiales OTUs accounted for, on average, over 10% of the overall root-associated communities (Table A-2). Table A-3. Results of indicator species analysis for widespread OTUs

Taxonomic Identificati OTU ID)	indval	P-value	Frequency (samples present)
Actinobacteria	6436	0.7	0.004	17
Actinobacteria	2390	0.5952381	0.074	11
Actinobacteria	1811	0.7142857	0.004	10
Actinobacteria	8108	0.5748299	0.258	17
Actinobacteria	3994	0.5952381	0.069	11
Rhizobiales (Bradyrhiz	6541	0.5	1	21
Rhizobiales	7320	0.4450549	0.354	11
Rhizobiales	9045	0.4897959	0.363	15
Rhizobiales	11236	0.5833333	0.173	17
Rhizobiales	378	0.6428571	0.016	9
Rhizobiales	7090	0.5833333	0.17	17
Rhizobiales	5174	0.56	0.298	18
Rhizobiales	10671	0.5833333	0.17	17
Rhizobiales	2953	0.6648352	0.011	12
Rhizobiales	3934	0.9285714	0.001	13
Rhizobiales	2542	0.5384615	0.547	19
Chitinophaga - Flavoba	6557	0.525974	0.077	10
Chitinophaga - Flavoba	8729	0.4675325	0.583	17
Chitinophaga - Flavoba	3022	0.6428571	0.021	9
Chitinophaga - Flavoba	801	0.4115646	1	16
Chitinophaga - Flavoba	3803	0.7142857	0.005	10
Burkholderiales	3340	0.5185185	1	20
Burkholderiales	9386	0.7142857	0.004	10
Burkholderiales	1635	0.4571429	0.158	9
Burkholderiales	7176	0.56	0.316	18
Burkholderiales	11351	0.56	0.329	18

Dufrêne-Legendre Indicator Species Analysis Results

Rhizobiales, which shows a large proportion of generalists out of the widespread taxa identified in my research (Table A-2), has been previously described as displaying a generalist character. For example, Haichar et al. (2008) found Rhizobiales was able to utilize a variety of carbon sources and occurred across both roots and rhizosphere soils of all four plant species in consideration. In a global analysis across major Earth soil types, members of this order were

found to be widespread (Nemergut *et al.*, 2011). Both the prominence of certain Rhizobiales with potential PGPR in the root environment (e.g. Bradyrhizobium japonicum), as well as their generalist ecological strategy in associating across different species of plants (Table A-2), reinforce the prospective utility in employing particular clusters within this order for the purpose of enhancing plant growth, such as in agriculture and bioremediation, where persistence in the soil (generalist) may be an important attribute of effective inoculum. In the realms of agriculture and biotechnology, understanding the ecology of particular bacteria and how they are shaped by processes of community assembly will allow for better manipulations of their abundances and function in relation to innovation and implementation in these fields. Ecological lessons from early successional systems may be especially transferable to agriculture, where annual plant characteristics, low diversity, and disturbance, common aspects in modern agricultural management, are largely analogous to early successional habitats (Wood, 1998).

At a broad scale, my study reveals significant relative increases in Actinobacteria and γ -Proteobacteria, and reductions in β -Proteobacteria and Acidobacteria in root samples relative to bulk soils; no significant shifts in dominance at the phyla level were noted in Bacteroidetes or α -Proteobacteria (Table A-1). Many other studies also show the prevalence of Actinobacteria, α -Proteobacteria, β -Proteobacteria, and γ -Proteobacteria in root-associated communities (Marilley and Aragno, 1999; Chelius *et al.*, 2001; Han *et al.*, 2009; Becerra-Castro *et al.*, 2010; Andreote *et al.*, 2009); however, coherence of patterns between studies may be obfuscated by the biases of experimental technique or the effect of different plant species observed. In a study based on 16S rRNA gene clone libraries directly from samples, Marilley and Aragno (1999) also found increases of γ -Proteobacteria and decreases in Acidobacteria in plant roots as compared to rhizosphere soil of perennial ryegrass and white clover. My study further delineates the ecology

Acidobacteria, which are generally oligotrophic (Fierer et al., 2007), and may be ecologically illsuited for the carbon-rich plant rhizoplane and endorhizosphere environment. Past studies across scales from vegetated soils to the rhizoplane and endorhizosphere likewise show sharp declines in the abundances of Acidobacteria in plant influenced - carbon enriched - environments (Marilley and Aragno, 1999; Chelius et al., 2001; Knelman et al., 2012). It is important to note that while β-Proteobacteria are reduced in the root environment they remain a dominant component of the communities (Table A-1, Figure A-3). Past research in soils of early successional environments, including the Mendenhall Glacier forefield, have noted the dominance of β-Proteobacteria (Knelman et al., 2012b; Sattin et al., 2009; Nemergut et al., 2007), which perhaps is competitively superior or may be highly effective dispersers in the newly exposed soils of these ecosystems, though still relevant to root-associated communities. It is also important to point out that although relative shifts in α -Proteobacteria between the root and soil environments were not significant, this taxon is highly dominant in both environments as well (Table A-1). While there are increases in actinobacterial relative abundances in both Alder and Spruce roots, Tukey's HSD contrasts only show a significant increase in Alder roots versus bulk soil. This may reflect a root environment more conducive to Actinobacteria, as it is well known that Frankia nodulate Alder in a nitrogen fixing symbiosis, and Alder may support other Actinobacteria as well (Ghodhbane-Gtari et al., 2009).

Conclusion

As microbial communities are critical for the establishment of plants and plant community ecology, understanding the patterns behind the assembly, composition, and structure of the microbes most intimately connected with the plant root environment is of great importance. The aim of my study was to understand the dominant bacterial taxa that comprise the root associated communities of plants in early ecosystem development, where microbial feedbacks may act as a particularly strong driver of plant community dynamics in these nutrientlimited environments (Van Der Heijden *et al.*, 2008). Although sampling was not exhaustive of the microbial community diversity, my study sought to understand dynamics of dominant and widespread taxa, which were captured by my methods. By employing pyrosequencing at the study sampling depth I were able to ascertain major phylogenetic patterns central to microbial community dynamics that other profiling and culture techniques do not allow.

Although future research may begin to outline the functional role and potential impact of specific bacteria in the root-associated bacterial community of these ecosystems, the fact that PGPR are only truly effective through their interactions with other microbes in the community highlights the importance of understanding communities of bacteria as a baseline for future work rather than solely characterizing individual strains (Kim *et al.*, 2011; Lau and Lennon, 2012). Indeed, my research takes a community approach and demonstrates phylogenetically differentiated root-associated bacterial communities from those of the bulk soil, revealing the shifts in dominant bacterial taxa driving these patterns. Clusters of specific bacterial OTUs that were widespread across root samples identified in this study also suggest the presence of known plant symbionts involved in nitrogen fixation and other plant growth promoting mechanisms in the root-associated bacterial community. The patterns I discern in root-associated bacterial community. However, in this study I recognize the presence of generalist taxa that are prominent across the variable environments of the root-soil continuum. For widespread habitat

generalist organisms, which I note may be ecologically important to plants, processes such as dispersal at small spatial scales or competitive interactions may gain relative importance amidst the background of environmental filters in community assembly. In total, I delineate the patterns in community structure and major players in root associated bacterial communities of a natural ecosystem in the early stages of development, where such communities may have major impacts on ecosystem structure and function.