Biogeographic and Biogeochemical Drivers of Microbial Community Assembly

John Lawrence Darcy
University of Colorado at Boulder, jack.l.darcy@gmail.com

Follow this and additional works at: https://scholar.colorado.edu/ebio_gradetds
Part of the Biogeochemistry Commons, Ecology and Evolutionary Biology Commons, and the Microbiology Commons

Recommended Citation
Darcy, John Lawrence, "Biogeographic and Biogeochemical Drivers of Microbial Community Assembly" (2017). Ecology & Evolutionary Biology Graduate Theses & Dissertations. 101.
https://scholar.colorado.edu/ebio_gradetds/101

This Dissertation is brought to you for free and open access by Ecology & Evolutionary Biology at CU Scholar. It has been accepted for inclusion in Ecology & Evolutionary Biology Graduate Theses & Dissertations by an authorized administrator of CU Scholar. For more information, please contact cuscholaradmin@colorado.edu.
BIOGEOGRAPHIC AND BIOGEOCHEMICAL DRIVERS OF
MICROBIAL COMMUNITY ASSEMBLY

by

JOHN LAWRENCE DARY

B.A., University of Colorado, Boulder, 2010

A thesis submitted to the
Faculty of the Graduate School of the
University of Colorado, Boulder in partial fulfillment
of the requirement for the degree of
Doctor of Philosophy

Department of Ecology and Evolutionary Biology

2017
This thesis entitled:
Biogeographic and Biogeochemical Drivers of Microbial Community Assembly
written by John Lawrence Darcy
has been approved for the Department of Ecology and Evolutionary Biology

________________________
Steve Schmidt

________________________
Nolan Kane

________________________
Christy McCain

________________________
Pat Kociolek

________________________
Cathy Lozupone

Date __________

The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline.
Abstract

The processes of community assembly shape all groups of coexisting organisms across all environments where life is found. Even for environments that are currently sterile, when they are colonized by life, community assembly processes will occur. The processes governing formation and diversification of microbial communities are vital to an understanding of how ecosystems develop in the human body, in emerging landscapes, and everywhere else. In this dissertation, I use community assembly theory to understand how microbial communities are shaped by geography, and also by the resources available to them. Using a microcosm-based nutrient addition experiment, I show that microbial communities in the plant-free, oligotrophic debris on top of the Middle Fork Toklat Glacier (Denali National Park and Preserve, Alaska) are strongly structured by the lack of available phosphorus, a result which differs significantly from ecological dogma that suggests early-successional primary producers are limited by nitrogen instead. I expand on my findings of P-limitation with an in situ nutrient addition experiment in soils exposed by the retreating Puca glacier (Cordillera Vilcanota, Peru), which showed that phosphorus limits primary producers (both microbes and plants), shaping the structure and function of microbial communities. The biogeochemical properties affecting microbial communities often have a geospatial component, and I found that atop the Toklat Glacier,
geographic space strongly shapes the interaction between biogeochemistry and microbial communities. But surprisingly, microbial community compositional data revealed that the top of the glacier is likely a hidden chronosequence, disguised as supraglacial debris. Chronosequences are natural experiments where space and time are conveniently conflated, and to better understand these time-series from a microbiological perspective, I developed a mathematical model that estimates the degree to which microbial communities form nepotistically. Using this model with time-series data from human microbiome studies, I found that in skin, feces and tongue, microbes are more likely to join those communities if a closely-related species is already present. Together, the chapters of this dissertation show how the environment, space, and time shape microbial communities on glaciers, near glaciers, on people, and in people.
Acknowledgements

I would like to thank my PhD committee members, Steve Schmidt, Diana Nemergut, Pat Kociolek, Christy McCain, Nolan Kane, and Cathy Lozupone, for taking the time to read my dissertation before my defense, and for all the guidance and help they provided. Thank you to my lab mates in the Schmidt lab and in the Nemergut lab, without whom my C.V. would be much shorter and my knowledge base much shallower. Also, I thank the C.U. EBIO department which gave me $1000 to spend on a drone and NIR camera, and the C.U. Graduate School which gave me $5000 for DNA sequencing. Free and open-source software has enabled much of my dissertation work, and as such I thank everyone who has contributed to R, R packages, and Linux. Finally, I thank Dr. Diana Nemergut for being my favorite committee member, valuable mentor, and good friend. Miss you; wish you were here.
4.3.1. Sample collection.................................................................58
4.3.2. DNA extraction and sequencing........................................58
4.3.3. Sequence data processing and bioinformatics.....................59
4.3.4. Sediment biogeochemical measurements..........................60
4.3.5. Statistical analyses.........................................................60

4.4. Results..................................................................................61
4.4.1. Bacterial biogeography....................................................61
4.4.2. Eukaryote biogeography..................................................61
4.4.3. Archaeal community.......................................................65
4.4.4. Spatial structuring of microbial communities....................67
4.4.5. Biogeochemical structuring of microbial communities........67

4.5. Discussion.............................................................................69

4.6. Acknowledgements.............................................................74

4.7. Data accessibility...............................................................74

Chapter 5: a phylogenetic model of community Assembly in the human microbiome (and in a glacial chronosequence, too)................................................75
5.1. Abstract..............................................................................75
5.2. Introduction.........................................................................76
5.3. Methods.............................................................................77
5.3.1. Statistical model..............................................................78
5.3.2. Simulations.....................................................................79
5.3.3. Parameter Estimation......................................................80
5.3.4. Hypothesis Testing..........................................................81
5.3.5. Analysis..........................................................................81
5.3.6. Code and data accessibility............................................84

5.4. Results.................................................................................84
5.4.1. Results from “Moving Pictures” data...............................84
5.4.2. Results from infant gut data............................................85
5.4.3. Results from the Toklat Glacier Chronosequence.............85

5.5. Discussion..........................................................................92
5.6. Conclusions......................................................................94
5.7. Acknowledgements..........................................................95

Chapter 6: conclusions.............................................................96
References..............................................................................100
Appendix.................................................................................113
List Of Tables

Table 2.1: Logistic growth model comparison.................................................................29
Table 4.1: Spatial correlations of abiotic variables.........................................................67

List Of Equations

Equation 2.1: Exponential decay.....................................................................................24
Equation 2.2: Logistic growth.........................................................................................24
Equation 5.1: Statistical model for phylogenetic community assembly.......................78
Equation 5.2: Equation to re-scale phylodiversity accumulation curves.....................81
## List Of Figures

Figure 1.1: Logistic growth model and equation.................................................................2
Figure 1.2: Satellite photograph of the Middle Fork Toklat Glacier.................................5
Figure 1.3: Photograph of the Middle Fork Toklat Glacier................................................6
Figure 1.4: Plant counts along the Toklat Glacier’s forefield chronosequence......................8
Figure 1.5: Conceptual view of microbial dispersal..............................................................12
Figure 1.6: Photograph of the Puca Glacier........................................................................16
Figure 2.1: Growth curves for Toklat Glacier microcosms..................................................28
Figure 2.2: Residual inorganic nitrogen concentrations of microcosms..............................30
Figure 2.3: Principle coordinates analysis of bacteria and eukaryotes from the microcosms....31
Figure 2.4: Rank-abundance plots of eukaryal OTUs by treatment....................................32
Figure 2.5: Alpha rarefaction for eukaryal communities.....................................................33
Figure 3.1: Results of microcosm studies showing P-limitation of early successional soils......47
Figure 3.2: Near-infrared enhanced photographs of field plots and mNDVI comparison........49
Figure 3.3: P-limitation as shown by stem counts and percent-cover..................................50
Figure 3.4: Shifts in cyanobacterial communities from nutrient addition, and nutrient limitation of microbial phototrophs........................................................................52
Figure 4.1: Topographic map of sampling sites across the Middle Fork Toklat Glacier......56
Figure 4.2: Relative abundance of bacterial phyla across the supraglacial transect...............63
Figure 4.3: Relative abundance of eukaryote phyla across the supraglacial transect..............64
Figure 4.4: Semivariograms of bacterial and eukaryote communities..................................66
Figure 4.5: NMDS analysis of bacterial and eukaryote communities and measured biogeochemical parameters.................................................................68
Figure 5.1: Phylodiversity accumulation in the female left palm........................................86
Figure 5.2: Model fitting and hypothesis testing for the female left palm.............................87
Figure 5.3: Dispersion parameter (D) estimates for Caporaso et al. (2011) data sets.............88
Figure 5.4: Empirical phylodiversity accumulation in the infant gut microbiome...............89
Figure 5.5: Dispersion parameter (D) estimates in the infant gut, pre- and post-formula........90
Figure 5.6: Phylodiversity accumulation across the Toklat Glacier’s chronosequence..........91
Chapter 2, Supplemental Figure 1: Mathematical model converting FOV data to point-intercept estimates..................................................................................................................113
Chapter 4, Supplemental Figure 1: Semivariograms of bacterial and eukaryote communities with no sliding-window overlap.................................................................114
Chapter 4, Supplemental Figure 2: Spatial patterns in Cyanobacteria..............................115
Chapter 4, Supplemental Figure 3: Spatial patterns in Betaproteobacteria.........................116
Chapter 4, Supplemental Figure 4: Spatial Patterns in algae.............................................117
Chapter 4, Supplemental Figure 5: Spatial correlations of abiotic variables.....................118
1.1. What does “community assembly” mean?

Community assembly processes are ubiquitous in nature, just like microbes. Microbial community assembly occurs (and is occurring) on every non-sterile surface in the world, including soils, people, rocks, my desk, the ocean floor, mountain peaks, and everywhere else in between. Community assembly broadly means “the processes that determine the patterns of the number and composition of co-occurring species” (Chase 2003), and these processes begin with an environment containing few or no inhabitants (a “blank slate”), and continue as the organisms within that environment compete or emigrate or immigrate or die over time (Vellend 2010; Nemergut et al. 2013). Although most of the work I present in this dissertation is focused on arctic and alpine environments, I frequently use the example of an influenza vaccination to explain microbial community assembly. When a flu shot is administered, first a nurse prepares an area of skin to receive the shot by cleaning it with an ethanol swab. I explain that this process is removing microbes from the skin, and skin has a large number of microbial inhabitants. While this is not perfectly true, it is illustrative: what happens after the shot is over and done with? Part of the skin is barren, but nearby the un-cleaned skin still has microbes living on it, as does the patient's surrounding world. What processes determine which microbes colonize and occupy the new frontier? What mathematics describe the order in which species arrive and settle there?

The vaccination case is an example of ecological succession, a term which is often used synonymously with community assembly (Hodkinson et al. 2003; Fukami and Nakajima 2011), or as a larger macro-scale process within which community assembly occurs (Jumpponen 2003; Chang and HilleRisLambers 2016). While these terms are tightly linked and there is significant grey-area surrounding their use, in this dissertation I will use the term succession in its historical context.
Succession has been observed in systems ranging from glacial forefields (Chapin et al. 1994) to cheese (Ercolini et al. 2004) to the human gut (Koenig et al. 2011), and in all cases it describes the development of an ecosystem from an uncolonized state toward one at a “stable” size, where the community's biomass has reached an equilibrium with the energy available to it (MacArthur and Wilson 1967; Hubbell 2001).

Figure 1.1: Logistic growth model and equation

Ecological succession is a phenomenon characterized by shifts in community composition, but also by increase in biomass. That biomass reaches its maximum as a community reaches the carrying capacity (K) of its environment. K may be limited by any of the essential resources for life, but typically photosynthetic communities in terrestrial systems are limited by nitrogen (N), phosphorus (P), or both. The relative exponential growth rate (r), aggregated across a community, determines how quickly that community reaches K.
This stable state is known as the ecosystem's carrying capacity (K, Figure 1.1), and a community at carrying capacity will be composed of different organisms than it was in previous stages of succession (MacArthur and Wilson 1967). The processes that govern this change over time are community assembly processes, but the overall trend is ecological succession.

A simplistic but classic view of ecological succession is the development of plant communities, starting from barren soil, then grasses and small plants, then larger plants, and eventually becoming an “apex” community of giant trees. While reductive, this model demonstrates that succession is not only an increase in biomass, but the phenomenon is also characterized by a dramatic shift in community composition over time. This shift in community composition is caused by community assembly processes.

1.2. Community assembly for microbes

Even though it can't be seen, succession is happening in the bare dirt even before plants arrive, much as it is happening on a patient's skin after a flu shot. Because of the invisible nature of microbial community assembly, understanding how community assembly processes work for microbes and how these processes shape microbial succession requires different methods than were traditionally employed for macrobes (Martiny et al. 2006). In the present decade, microbial communities are most frequently (and thoroughly) characterized using high-throughput DNA sequencing technology, which is similar to traditional methods of species abundance measurement (e.g. plant counts, mark/recapture) in some respects, and different in others. In modern use, DNA sequencing technology can provide sample-by-site tables that look identical in format to those that have long been used by macrobial ecologists. However only relative abundance (not absolute abundance) is often possible for microbes with the most widely-used experimental designs. Furthermore, unlike macrobes which can be poked and prodded and visually observed, microbes are much more diverse, and as such, almost all microbes that are detected
using molecular methods have never been cultured before (Hugenholtz et al. 1998; DeSantis et al. 2006).

Despite these differences between modern molecular data and the old (but good!) field methods on which most ecological theory is based, community assembly processes for microbes are not significantly different for microbes than they are for macrobes. Vellend (2010) outlines four categories of community assembly processes, which are selection, dispersal, speciation, and drift. I co-authored a review of microbial community assembly processes with Nemergut et al. (2013), and we discussed how contemporary research into microbial community assembly fits into those same four fundamental processes. The same selection, dispersal, speciation, and drift that describes how a patch of mossy dirt becomes a forest, can be used to understand how an infant's gut becomes that of an adult, or perhaps more interestingly, how a patch of bare dirt becomes a patch of mossy dirt. Thus, it is not in question whether microbial communities assemble given the same processes that occur for macrobes. Instead, questions about microbial community assembly are similar to questions about macrobial community assembly, but these microbial questions are more abundant and more diverse, just like microbes.

1.3. Microbial community assembly processes in the cryosphere

Arctic and alpine environments are surprisingly useful locations in which to study microbial community assembly. Ecological succession has most famously been studied in glacial forefield chronosequences, where successional timelines longer than a human lifespan can be studied over hundreds of meters of space instead of decades to millennia of time (Matthews 1992; Walker et al. 2010). As a glacier retreats, uncolonized mineral soil is exposed to the world, and to life. This naïve substrate is the youngest part of a chronosequence; as the glacier retreats farther through space and time, newer substrate is exposed while older substrate ages.
Figure 1.2: Satellite photograph of the Middle Fork Toklat Glacier

The colored overlay shows how the glacier has been retreating since its position was first measured in 1954. In 2014 when I visited the site, the glacier’s terminus was over 1.5 kilometers south of the 1954 terminus. This allowed me to sample along the glacier’s chronosequence over the span of 60 years by walking only a short distance. This image was produced by the United States National Parks Service, and can be found on their website at: [https://www.nps.gov/articles/denali-glacier-monitoring.htm](https://www.nps.gov/articles/denali-glacier-monitoring.htm).
I took this picture during my expedition to the Toklat Glacier (Denali National Park and Preserve, Alaska, USA) in 2014, and it illustrates how barren and inhospitable the recently deglaciated landscape is. The glacier’s terminus is roughly 100 meters in front of Steve Schmidt (pictured). Also visible in this picture are the debris that cover the glacier. White patches on top of the glacier are not ice, but snow that sits above the supraglacial debris.
During this aging, communities develop through the process of ecological succession. Since the youngest communities are closest to the glacial terminus and the oldest communities are farthest away, a gradient of community ages can be studied at the same place and time. This convenient observational approach is called a space-for-time substitution (Matthews 1992).

One such natural experiment is the Middle Fork Toklat Glacier, which will be discussed in depth in this dissertation (Figure 1.2 and Figure 1.3). Glacial forefield chronosequences have been used to study the community assembly processes mediating plant succession (Vitousek 1994; Wardle et al. 2004; Walker et al. 2010), and the Toklat glacier is no exception, with a significant pattern of plant biomass increase with substrate age (Figure 1.4). But with the advent of high-throughput sequencing, these same natural experiments are being used to study microbes as well (Kaštovská et al. 2005; Castle et al. 2016; Nemergut et al. 2016). Experiments like these in the cryosphere have been used to look at microbial communities under the light of all four community assembly processes (Vellend 2010; Nemergut et al. 2013): selection, dispersal, speciation, and drift.

1.4. Selection

Selection describes the process by which fit species are more successful than less fit species (Bell 2008; Vellend 2010). This is similar to the use of the word “selection” in the term “natural selection”, although in terms of community assembly there is no evolutionary aspect to the process. Instead, selection can incorporate many different aspects of fitness. For example, perhaps the most notorious use of the word is in regard to $r$ vs. $K$ selection (MacArthur and Wilson 1967). This difference in “ecological strategy” refers to the parameters in the logistic equation shown in Figure 1.1, where $r$ is the relative exponential growth rate, and $K$ is the upper asymptote, also called the carrying capacity. $r$-strategists have high growth rates as a result of traits that promote rapid growth, and for microbes this includes large number of ribosomal genes (Klappenbach et al. 2000; Shrestha et al. 2007;
Yano et al. 2013). As a co-author with Nemergut et al. (2016), I looked at the predicted ribosomal copy numbers of bacteria living in the forefield of the Toklat Glacier. We found that bacteria with many ribosomal genes are more abundant during early succession, and less abundant during later successional stages. This is commensurate with a shift from $r$-selection to $K$-selection. This sort of shift was shown using culture-based methods by Sigler et al. (2002), although at the time it was uncertain whether the observed shift from fast-growing bacteria to slower-growing bacteria was due to culture media.

![Figure 1.4: Plant counts along the Toklat Glacier’s forefield chronosequence](image)

Near the terminus, there are no plants and the recently exposed substrate is rocky and inhospitable. But in this seemingly lifeless waste, microbial succession is invisibly taking place. Further from the terminus, plants begin to appear, and at sites that are roughly 50 years old there are more than 500 plants per square meter.
However, $r$ and $K$ strategies are not the only types of selection, and perhaps the most common type of selection is environmental. In any environment, species that are better adapted to environmental conditions will prosper over mal-adapted species. One commonly cited example for microbes is the use of ammonia. Low ammonia concentrations in soil necessitate high-affinity ammonia monooxigenase (AMO) enzymes, which are inefficient under high concentrations of ammonia, where low affinity AMO enzymes are better suited (Martens-Habbena et al. 2009). So depending on which traits (low-affinity or high-affinity) a species expresses, it will either do well or do poorly. Since succession is characterized not only by a net increase in biomass, but also by fundamental soil biogeochemical changes (Vitousek 2004), it is no surprise that these same ammonia oxidizing microbes show significant spatial patterns across glacial chronosequences (Brankatschk et al. 2011), as a result of selection.

But as with many natural systems, it is difficult to determine causality among covariates; even if one assumes that microbial community structure is a dependent variable, this may not be appropriate. For example, if the abundance of cyanobacteria within cryoconite holes is correlated with inorganic nitrogen (Stibal et al. 2006), it is difficult to know whether N-fixing cyanobacteria changed their environment, or whether the environment selected for the cyanobacteria. Fortunately, manipulative experiments offer the opportunity to know the directionality, and be sure that observed microbial community shifts are an effect of treatment. Toward this end, microcosm experiments can be an effective tool to control environmental conditions and measure the response of microbial communities (Schmidt et al. 2012; Darcy and Schmidt 2016).

In addition to abiotic factors like ammonia concentration or soil water content or UV flux or salinity or pH or photosynthetically available radiation, biotic factors can be agents of selection as well. The presence of a mutualist organism or of a symbiont is a strong determinant of many species' presence (Tilman 2004). Perhaps most famous in the microbial world are mycorrhizal fungi, which
need certain plants as hosts (Gange et al. 1993). Indeed, in glacier forefield chronosequences, plant-associated fungi are only found in abundance in older soils, and younger soils have different sorts of fungi altogether (Jumpponen 2003; Dong et al. 2016). If mycorrhizal fungi are absent, plants that utilize them will be less competitive and will be selected against. Likewise, if those plants are absent, mycorrhizal fungi will not have the niche they have evolved to use, and will be selected against as well.

1.5. Dispersal

But community assembly is not so simple that we would expect the mycorrhizal fungi to be selected for only because their host plant is present. Nor would we expect an acidophilic bacterial species to be abundant just because pH is low enough to be permissible for growth. These organisms can't be selected for (or against) if they aren't dispersed to the environment in question. Dispersal is the movement of organisms through space and time, and is especially relevant to the glacial forefield chronosequence example. When a glacier retreats, naïve substrate is exposed. And while that substrate may already contain microbes living within it, perhaps from on top of the melting glacier (Sattin et al. 2009; Rime et al. 2016) or from underneath it (Sharp et al. 1999; Foght et al. 2004; Boyd et al. 2014), it certainly does not contain the sum total of phylogenetic and functional diversity that will occupy that soil until the heat-death of the universe.

Microbes have many avenues of dispersal open to them, but studying the processes of microbial dispersal has been historically difficult. The vast metabolic diversity of bacteria caused Baas-Becking to famously state that “everything is everywhere; the environment selects” (de Wit and Bouvier 2006), a statement which interestingly incorporates two community assembly processes. He was stating that because he observed redundant functional diversity in many different environments, the small size of microbes must allow the same species to exist in all environments he analyzed. So, he concluded that selection (in his case, using selective media) determined microbial community composition, and
dispersal was irrelevant. In the 21st century we know that to be false, thanks to modern sequencing technology, but the diversity of microbes coupled with their small size makes the study of microbial dispersal extremely difficult. For example, measuring how well a given plant species is able to disperse has been done using dispersal curves (e.g. Gomez and Espadaler, 1998; Nathan et al., 2003), which are probabilistic functions generated by surveying the spatial distribution of seeds around a given plant. This is not feasible for microbes for a myriad of reasons, chiefly that they are too numerous to actually count, and to small to see, so they can't be tracked from a source to a destination.

Instead of observing dispersal directly via propagule tracking, the past dispersal of microorganisms is usually inferred from their present spatial distributions. This approach has been particularly useful for microbial biogeography due to the wealth of genetic sequence data available in public databases like GenBank and SILVA. Much in the same way that publicly available genetic data have improved microbial phylogenies (Nilsson et al. 2011), several recent studies of microbial biogeography in arctic and alpine environments have supplemented their analyses with sequences from public databases. My first ever scientific publication looked at the distribution of bacteria from the genus Polaromonas throughout the cryosphere, using 16S gene sequence data generated by myself and others to compare the similarity of bacteria found near each other to the similarity of bacteria found on opposite ends of the earth (Darcy et al. 2011). I found that Polaromonas phylotypes were highly similar even at global ranges, which suggests that these organisms are capable of being distributed worldwide.
In some cases, dispersal and establishment are successful (solid blue arrows). In other cases, dispersal is not successful and the microorganisms perish in transit or on arrival (dashed lines) or may be poorly adapted to the new environment (teal hexagons). Priority effects may also play an important role in microbial community assembly during succession. For example, the red ovals and yellow squares arrive first, gaining a competitive advantage as they monopolize resources. Later migrants (small green circles) may not be competitive due to their lower relative abundance and the sequestration of limiting nutrients by earlier colonists (e.g. the red ovals). Had the green circles arrived first instead of the red ovals, they may have been more successful and the community would have assembled differently. I made this figure, and it is reproduced with permission from Schmidt et al. (2014).
Metagenomic evidence can also be used to learn about microbial dispersal, albeit indirectly. In my paper on *Polaromonas*, I searched a glacier ice metagenome (Simon et al. 2009) for *Polaromonas* genomes. I found that a *Polaromonas* genome contained genes that allow for dormancy, which could allow the bacteria to be more easily transported via wind. Dormancy processes for bacteria generate even smaller cells that are less easily damaged by environmental stresses. These smaller, hardier cells are better transported through the air, as shown by mathematical models of global particle circulation (Wilkinson et al. 2012).

Contrary to notions of somewhat deterministic dispersal ability as mentioned above, dispersal is most often thought of as a stochastic community assembly process (Chase 2010; Wilkinson et al. 2012; Nemergut et al. 2013). If an organism has a strong ability to be dispersed, and we find evidence that this trait has enabled the organism to obtain a wide spatial distribution, this is not an overly stochastic process. However, among organisms with similar dispersal abilities, dispersal may be a dice-roll (Schmidt et al. 2014). Under the conceptual model of dispersal structuring microbial communities presented in Figure 1.5, stochasticity of dispersal, exacerbated by a low immigration rate, may lead to stochastic outcomes. A maladapted organism may gain an advantage simply because it arrived before any competition (Fukami and Nakajima 2011).

1.6. Drift

Unlike dispersal which may have varying degrees of stochasticity, or selection which is defined as a deterministic process, ecological drift is purely stochastic. Drift is the random change in organism abundances (Vellend 2010; Nemergut et al. 2013), and most commonly the term refers to random local extinction events (Chase 2010). Drift has been shown to be most powerful in communities with low population sizes (Chase 2010), which is an intuitive result since at low population sizes there will be less redundancy within species, so extinction is much more common. This also means that rare taxa are
more vulnerable to drift, even in large communities (Allison and Martiny 2008). But in general, microbial communities have very large population sizes, so drift is not thought to be as important of a community assembly process for microbes.

In experimental designs with replication, however, drift is an important consideration. For example, when creating microcosms from homogenized parent material, the presence or absence of rare taxa may vary among subsamples. But if the microcosms select for those rare taxa, drift becomes an extremely important community assembly process. In this way, drift may explain variation among communities in microcosms of the same treatment, even when the treatment is a highly significant effect (Darcy and Schmidt 2016). The previous example somewhat stretches the definition of ecological drift, since the “extinction” events were caused by sampling, rather than random natural processes. But experiments seeking to reproduce ecological drift have used the same sampling process described above as a proxy for drift (Chase 2010; Chase and Myers 2011).

1.7. Speciation

Speciation, sometimes referred to as diversification (Nemergut et al. 2013), is the process by which new species form. As with dispersal, the influence of speciation is not directly observed or experimentally tested, but instead the community assembly process observed indirectly by measuring its effects. Like the shadows on the walls of Plato’s cave, the effects of speciation can be observed and even tested. But the process itself takes place out of view, just like puppets and fire casting shadows. Speciation is an even more elusive process than dispersal, because the dimension of difficulty is time instead of space. Even for microbes with fast generation times and horizontal gene transfer (a process that arguably falls within “diversification”), directly observing speciation is no easy task.

Fortunately, the limitations and benefits of modern microbiology have conspired to make microbial speciation a tractable focus of study, even in the context of community assembly. Because
microbial communities are studied using DNA sequence data, creating phylogenies of microbes is relatively easy (Price et al. 2010; Nilsson et al. 2011; Schmidt and Darcy 2015). A phylogeny is a nested history of speciation, so speciation can be studied historically. One way speciation is incorporated into community assembly is through the concept of niche determinism, which predicts that selection acts similarly on more closely related species (Webb et al. 2002; Letcher and Chazdon 2012). In other words, species that share more evolutionary history are thought to be more ecologically similar. Indeed, studies of microbial genomes have found that core genome content, a proxy for ecological function, has a strong phylogenetic signal, supporting niche determinism.

1.8. Project Overview

Plant-free, early successional environments like recently deglaciated soils and supraglacial debris provide natural experiments where community assembly theory (reviewed above) can be applied to understand primary succession in natural systems. While plant communities in glacial forefield chronosequences have been extensively studied for decades (Stevens and Walker 1970), only recently has succession in the unvegetated soils recently exposed by glaciers attracted the interest of microbiologists and community ecologists (Kaštovská et al. 2005; Schmidt et al. 2008; Kim et al. 2017). Furthermore, the debris atop debris-covered glaciers represents a relatively un-studied frontier in the cryosphere, with many similarities to glacial chronosequences.

In a previous paper I published before I began work on this dissertation, I presented a phylogeographic analysis of bacteria of the genus *Polaromonas*, which are abundant in many glacial retreat forefields, on top of glaciers, in snow and ice, and are generally found in alpine and polar environments (Michaud et al. 2012b; Margesin et al. 2012; Franzetti et al. 2013). I sequenced the 16S rRNA gene of *Polaromonas* phylotypes from atop the Middle-Fork Toklat glacier (Figure 1.3), which is a debris-covered glacier in the Alaska range (Darcy et al. 2011). In that paper I focused on dispersal as
a community assembly process, and dispersal was again in question when I published another phylogeographic paper on certain algae in the Ulvophyceae, which are also abundant atop the Toklat glacier and near other glaciers as well (Schmidt and Darcy 2015).

Figure 1.6: Photograph of the Puca Glacier

Like the Toklat Glacier’s forefield chronosequence (Figure 1.3), the forefield of the Puca glacier (Peruvian Andes) is barren and seemingly devoid of non-robotic life. If it weren’t for the meltwater, the red color of the rocks would make this environment look martian. The name of the glacier is actually derived from that same color, as Puca means “red” in Quechua.
However, as discussed above, dispersal is an important first-step in community assembly, but selection is perhaps the most important and most tractable community assembly process. I followed-up my preliminary work on the Toklat glacier site with a full-factorial nitrogen (N) and phosphorus (P) addition experiment, in order to understand how nutrient limitation and selection determine microbial community composition and primary productivity in the debris atop the glacier (Chapter 2).

However, microcosm experiments suffer from the limitation that a microcosm can never perfectly re-create the natural environment in question. Unfortunately, the Toklat glacier site is in a national park, and manipulative experiments at that site are not legally or logistically feasible. My second field site is in front of the rapidly retreating Puca glacier in the Cordillera Vilcanota mountain range in the Peruvian Andes (Figure 1.6), where I conducted a similar experiment to the Toklat Glacier microcosms, but in situ and over the course of 5 years. This field experiment also had a corresponding microcosm experiment (Schmidt et al. 2012), but the field component enabled much more thorough measurements of microbial and plant community assembly (Chapter 3). This allowed me to test hypotheses generated in the Toklat Glacier microcosm experiment without microcosms, specifically the nutrient limitation of P which is surprising in early-successional systems.

I also investigated spatial patterns along a transect above the Toklat glacier, using an observational and non-manipulative experimental design, in order to see what effect geography and soil biogeochemistry have on the microbial communities living atop debris-covered glaciers (Chapter 4). This experiment yielded surprising insights into the biogeographic patterns in microbial communities living in the supraglacial debris, and the community assembly processes that structure those supraglacial microbes. My research suggests that the top of the Toklat glacier actually is a chronosequence, just like the glacial forefield left behind by the glacier's retreat, and the two systems are likely connected as well.

Glacial chronosequences are space-for-time substitutions (Stevens and Walker 1970; Walker et al.
2010), and are analyzed as though they are temporally sampled data sets. However, temporal studies can also be conducted by repeatedly sampling the same location over time. I developed a mathematical model for describing how newly observed microbes are added to microbial communities, in light of their shared evolutionary history (Chapter 5). In a stark contrast to the other work in this dissertation on glaciers, I applied my model to several data sets from the human microbiome (Caporaso et al. 2011; Koenig et al. 2011), and show that phylogenetic relationships among bacteria are very strong predictors of the order in which new bacteria are observed in these data sets. I also applied this model to data I collected from the Toklat glacier's forefield chronosequence, and I show that these temporal phylogenetic patterns in microbial community assembly are likely universal to microbial succession.
2.1. Abstract

Photosynthetic microbial communities are important to the functioning of early successional ecosystems, but we know very little about the factors that limit the growth of these communities, especially in remote glacial and periglacial environments. The goal of the present study was to gain insight into the degree to which nutrients limit the growth of photosynthetic microbes in sediments from the surface of the Toklat Glacier in central Alaska. Previous studies and historical observations indicated that this environment is dominated by unique soil algae, and that succession from a microbial to a plant-dominated system is very slow. We used a soil microcosm approach to determine if nitrogen (N) and/or phosphorus (P) additions would affect the development and final biomass of microbial phototroph communities in this system. We found that fertilization with P significantly increased the exponential growth rate ($r$), but P alone did not affect the final percent soil cover (K) by microbial phototrophs. Nitrogen alone had no effect on either $r$ or $K$, but the combination of P and N dramatically increased $K$, thus showing that algal growth rate in this system is likely P-limited, but total productivity may be co-limited by P and N. In addition, nutrient treatments differentially stimulated microbial groups resulting in significantly different microbial communities among treatments. Overall, these results give a preliminary indication of the factors that might limit the development and productivity of photosynthetic microbial communities in an extreme and remote glacial system.

2.2. Introduction

Very few studies have examined nutrient limitation of photosynthetic community development in plant-free periglacial and glacial environments (Schmidt et al. 2012). The development of
photosynthetic microbial communities is a critical early step in primary succession, contributing to soil formation that in turn is necessary for the establishment of macroscopic plant communities (Belnap and Lange 2001; Nemergut et al. 2007). Schmidt et al. (2012) observed that in the glacial forelands of the Pucará glacier in Perú, the development of photosynthetic crust communities was limited by phosphorus availability. However, this limitation may or may not be exclusive to that site or to the Peruvian Andes. Other environments in the cryosphere may impose different limitations on the development of microbial phototroph communities, especially since cold desert environments differ in important biogeochemical factors (Schmidt et al. 2011b).

One type of cold desert environment that has received very little study are debris-covered glaciers, which are very common and extensive in terms of geographic coverage, especially in active geological areas such as the Himalayas and Alaska Range (Nakawo et al. 2000; Scherler et al. 2011). This environment is characterized by a thin layer of mineral debris on top of glacial ice, which is deposited allochthonously from the glacier's surroundings. One such glacier is the Middle Fork Toklat Glacier in the Alaskan interior (Alaska Range) which is covered with oligotrophic mineral soils, is not colonized by plants (Schmidt and Darcy 2015), and is located in Denali National Park and Preserve, which has very low rates of nitrogen deposition (Fenn et al. 2003). Before the present study, no work had been done on microbial phototrophs and nutrient limitation in the extreme environment atop debris-covered glaciers. However, nutrient limitation has been observed in heterotrophic microbial communities in several early-succession cold desert biomes. P limitation was observed for heterotrophic microbes of periglacial soils in the Colorado Rocky Mountains (King et al. 2008) and Peruvian Andes (Schmidt et al. 2011a), while N limitation (after C limitation was alleviated) was observed in the Swiss Alps (Göransson et al. 2011). However, nutrient limitations of heterotrophic communities may not translate to their co-occurring phototroph communities since microbial phototrophs may have access to different pools of P than their heterotrophic counterparts (Cleveland
Although there has been much previous work on nutrient limitation and microbiology of cold-desert environments (King et al. 2008; Göransson et al. 2011; Schmidt et al. 2011b, 2012; Knelman et al. 2014), most of it has focused on recently deglaciated landscapes. However, except for the current study, work on supraglacial nutrient limitation has been limited to cryoconite holes (Stibal et al. 2009), which are substantially different from the soil-like surface of the Toklat Glacier. Cryoconite holes form when small amounts of debris are warmed by solar radiation and melt into the ice forming enclosed micro-communities. Although this process may happen in places atop the Toklat glacier, the supraglacial debris layer is over 20 centimeters thick, enough so that the ice beneath the debris cannot be seen. Microbiological studies of supraglacial debris are rare, with only two studies focusing on bacteria atop debris-covered glaciers (Darcy et al. 2011; Franzetti et al. 2013). Unsurprisingly, culture-independent studies of microscopic eukaryotes in supraglacial debris are even rarer yet, with the only currently published work being our previous study of unique algal clades on the Toklat Glacier (Schmidt and Darcy 2015).

Here, we use a microcosm experiment, biogeochemical analysis, and culture-independent molecular methods to better understand the extreme supraglacial environment, and the role nutrient limitation may play in limiting microbial growth on top of a debris-covered glacier. To measure nutrient limitation, we employed and improved upon recently developed microcosm-based experimental protocols for comparing the degree to which common limiting nutrients (N and P) influence the growth of diminutive photosynthetic communities (Schmidt et al. 2012). We have taken this approach a step farther by phylogenetically characterizing the microbial communities from these microcosms to better understand selection processes that occur under different nutrient limitation conditions, and how they relate to organisms that may be found in situ. Together, our results form a detailed glimpse of phototrophic life in this harsh environment, and provide new insights into how
different cryospheric bacteria and microbial eukaryotes may be affected by nutrient limitation.

2.3. Methods

2.3.1. Nutrient addition experiment

Microcosm plates were created using homogenized soil samples collected from atop the Toklat Glacier by Darcy et al. (2011). Homogenization was used to ensure that starting communities were similar among microcosms, and also representative of the organisms found in situ. A total of 20 microcosms were made, each consisting of 13 g of soil added to a 55 mm diameter Petri dish (Fisher Scientific 8-757-13A), sealed with Parafilm (Pechiney Plastic Packaging, Menasha WI, USA). Treatments consisted of a control (no nutrient addition), +N, +P, and +N+P. For each treatment, 75 μg of N (as NH₄NO₃), P (as KH₂PO₄), or both were added (in aqueous solution) per gram of soil. All soils were initially amended to 70% of water holding capacity as described elsewhere (Schmidt et al. 2011a, 2012). Water holding capacity averaged 29% of dry weight across microcosms, therefore aqueous concentration was approximately 370 μg of N and/or P per ml of water. Preliminary experiments indicated that these concentrations were high enough to overcome nutrient limitations in calcareous, early successional soils (Schmidt et al. 2011a, 2012). Microcosms were incubated at 21°C under 16 hours of light per 24 hours, conditions that approximately mimic the relatively mild summer conditions at the toe of the Toklat Glacier (unpublished data). The arrangement of the plates was randomized every three days to account for any variation in light and temperature. Microcosms were surveyed twice weekly for coverage of microbial phototrophs using the field of view (FOV) method described by Schmidt et al. (2012), for a total of 15 time points over 51 days. When FOV measurements began to saturate (plateau), a variation of the point-intercept method was used instead, where a reticule was placed on the microscope's objective. Instead of counting a 'hit' when a photosynthetic colony was within the field of view, a 'hit' was counted when the cross-hairs (a single point) of the reticule
intersected any photosynthetic organism or structure. This method was much more time consuming, but allowed for more accurate estimates of percent cover. Both types of measurement (FOV and point-intercept) were used in conjunction for several measurement periods before the FOV measurements began to reach saturation at day 34.

2.3.2. In-silico methodological validation

To correlate the two methods, an in-silico microcosm experiment was run (R code available upon request), which used both the FOV and point-intercept methods on randomly generated microcosm plates. To ensure a broad distribution of covers across generated microcosm plates, plates were generated with preset naïve covers ranging from 0-80% cover, calculated by the total area of all colonies divided by the total area of the plate. This naïve cover is inaccurate by design because of overlap between colonies, however it was only used to ensure a wide range of actual in silico covers (as estimated by percent cover) for correlative purposes. We also confirmed that the in-silico point-intercept method is highly accurate, by using Adobe Photoshop's (Adobe Inc., San Jose CA, USA) magic wand tool to calculate the ratio of green pixels to white pixels on graphical representations of the in-silico microcosms (Supplemental Figure 1, first panel). The trend between FOV and point-intercept data observed in the in-silico experiment was best modeled using a general 1-parameter exponential decay model (Equation 2.1) (Supplemental Figure 1, second panel), which was fit to the observed data using the Gauss-Newton algorithm in R 3.0.2 (Supplemental Figure 1, third panel). This model was then used to convert real FOV data from before day 34 of the microcosm experiment to percent cover estimates.
Equation 2.1: Exponential decay

\[ f(x) = 1 - e^{-Ax} \]

2.3.3. Growth curve modeling and statistical analysis

Time series of percent cover data for each of the 20 microcosms were modeled using the general logistic growth function (Equation 2.2) in R using the Gauss-Newton algorithm. The logistic growth function we used has been described elsewhere (e.g. Schmidt et al. 2011b) and models growth as a function of time (t, days), with three parameters: The upper asymptote (K, final percent cover), the exponential growth rate (r, days\(^{-1}\)), and the position of the inflection point (i, days). The point on the logistic curve where \( t = i \) has the steepest instantaneous growth rate (i.e. % cover gained per day). In this version of the logistic equation, the lower asymptote is fixed at zero. Fit parameter values for each nonlinear model were used in three 2-way ANOVAs with interaction to test for the effects of N and P addition on growth response, using Boolean independent variables for N and P.

Equation 2.2: Logistic growth

\[ f(t) = \frac{K}{1 + e^{-r(t-i)}} \]

2.3.4. Microcosm biogeochemical analysis

Microcosms were destructively sampled after the microcosm experiment to determine residual concentrations of nitrate, nitrite and ammonia. Five grams of soil from each sample were combined with 25 ml 0.5 M K\(_2\)SO\(_4\) and shaken at 150 rpm for 1 hour, then centrifuged for 1 hour at 4000 rpm. This same procedure was carried out on a “blank” sample containing no soil. Dissolved nitrate+nitrite
concentrations for each sample (including the blank) were analyzed using a Lachat QuickChem 8500 (Hach Ltd, Loveland CO, USA) and dissolved ammonia was analyzed colorimetrically using a BioTek Synergy 2 microplate reader (BioTek, Winooski VT, USA). Nitrogen (N) concentrations were back-calculated stoichiometrically and corrected for background N levels using the blank sample data. Corrected values were tested for a difference among treatments using Tukey's HSD test.

### 2.3.5. High-throughput 16S and 18S rDNA sequencing

After the microcosm experiment, genomic DNA was extracted from 3 replicate microcosms of each treatment (12 total) using the MoBio PowerSoil kit (Carlsbad, CA, USA). Each extraction was amplified in triplicate using the bacterial primer set 515F/806R (Caporaso et al. 2012) and the eukaryal primer set 1391f/EukBr (Amaral-Zettler et al. 2009). Each primer oligo included an Illumina flowcell adapter, and different GoLay barcodes were used for each of the 12 samples and 2 genes (16S and 18S), for a total of 24 barcodes. Both primer sets are from the Earth Microbiome Project standard protocols (accessible at [http://press.igsb.anl.gov/earthmicrobiome/emp-standard-protocols](http://press.igsb.anl.gov/earthmicrobiome/emp-standard-protocols)). Each set of triplicate reactions was pooled, and amplicon concentrations were assayed using Pico Green flurometry on a BioTek Synergy 2 microplate reader (BioTek, Winooski VT, USA). Amplicons were diluted to equimolar concentration, pooled, and sequenced on the Illumina MiSeq platform (Illumina, Inc.) using paired-end 2x150bp chemistry. A 30% phiX spike was added to the run to compensate for the otherwise limited amplicon variability (Caporaso et al. 2012).

### 2.3.6. Bacterial and eukaryal community analysis

Raw reads were demultiplexed and quality filtered using QIIME (Caporaso et al. 2010b). For bacterial reads, paired-end sequences were joined. However, this process did not work for eukaryal reads, so only the read corresponding to the 1391F primer was used. This read was selected instead of
its pair because a sequence closer to the center of the 18S gene overlaps more with the majority of sequences in the NCBI and SILVA databases, allowing for better taxonomic assignment. Both the bacterial and eukaryal data sets were separately clustered at 97% sequence similarity using UCLUST (Edgar 2010), and taxonomy was assigned using QIIME's parallel_assign_taxonomy_blast.py script and the SILVA Ref NR 99 database's taxonomic information. When this method was not sufficient for classification of an abundant OTU (>2% community composition) to the genus level because it returned “unclassified”, BLAST (Altschul et al. 1990) was used with the NCBI Nucleotide database to find a close match, cultured or otherwise. Using this taxonomic information, all mitochondrial and chloroplast OTUs were removed from the bacterial data set, and all bacterial OTUs were removed from the eukaryal data set. Both data sets were then rarefied to the number of sequences equal to their least populous sample. The bacterial data set was rarefied to 19,000 sequences per sample, and the eukaryal data set was rarefied to 5,000 sequences per sample. Weighted UniFrac (Lozupone and Knight 2005) was used to compute beta-diversity matrices for both data sets, which were tested for the influence of N and P using ADONIS from the R package vegan (Oksanen et al. 2016) with Boolean independent variables for N and P. Principal coordinate analysis was used to visualize these data. Alpha rarefaction was performed in QIIME using the rarefied eukaryal data set with both the species observed and Faith's (1992) phylodiversity metrics. Phylogenetic trees were constructed for each data set, since UniFrac and Faith's (1992) phylodiversity are both phylogenetic metrics, and require trees as input. Bacterial and eukaryal representative sequence sets were each aligned to reference using the SINA aligner (Pruesse et al. 2012), with the SILVA Ref NR 99 database version 119 as reference (available at http://www.arb-silva.de/download/arb-files/). FastTree (Price et al. 2010) was then used to build phylogenetic trees from the SINA alignments, using default settings.
2.3. Results

2.3.1. Microcosm growth experiment

The microcosm experiment revealed that growth rate of microbial phototrophs in the Toklat Glacier's supraglacial soils is limited by P, but that the final % cover in the microcosms was co-limited by P and N (Figure 2.1). Until day 34 of the microcosm experiment, +P and +N+P microcosms showed similar growth patterns, increasing in percent cover more rapidly (higher growth rate) than control or +N microcosms. However, after day 34, +N+P microcosms continued to grow, while growth in the +P microcosms plateaued. Thus, microbial phototrophs in +N+P microcosms attained total covers over four times higher than all other microcosms by the end of the experiment, and the other treatments and control plateaued with similar final percent cover values ($K$, Equation 2.2, Table 2.1). Therefore, both N and P significantly affected $K$, however the presence of a significant interaction term ($P_{N,P}$, Table 2.1).
Figure 2.1: Growth curves for Toklat Glacier microcosms

Each point is the mean of five replicate microcosms (error bars are standard error of the mean). Both the +N+P (green split rectangles) and the +P (orange triangles) treatments exhibited rapidly accelerating increases in percent cover. However, the +P treatment was quickly limited by a lack of N, as it plateaued at roughly 30 days. Both the control (blue circles) and the +N treatment (red squares) had a much slower acceleration of growth than the treatments that included P, and also had a much lower final percent coverage. The +N treatment had the lowest percent coverage, indicating that without added P, N inhibited growth of microbial phototrophs to some extent, however this pattern is not robust (Table 2.1). Data points from before day 34 were obtained via the FOV method (Schmidt et al. 2012) and were converted to percent cover estimates using our mathematical model (Supplemental Figure 1).
indicates that the effect of P or N is different when the other is present (as in the +N+P treatment). The other parameters in the logistic equation also responded to the addition of N and P, however only the ANOVA for $K$ produced a significant interaction term. The logistic inflection point, $i$, responded significantly to both N and P. However, the exponential growth rate, $r$, was higher in treatments containing P (+P, +N+P) but was not significantly affected by N (Table 2.1).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>“Control”</th>
<th>“+N”</th>
<th>“+P”</th>
<th>“+N+P”</th>
<th>$P_P$</th>
<th>$P_N$</th>
<th>$P_{NP}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K$ (% cover)</td>
<td>16.7 (6.17)</td>
<td>14.0 (3.15)</td>
<td>20.1 (5.67)</td>
<td>88.4 (11.1)</td>
<td>9.94E-9</td>
<td>6.10E-8</td>
<td>9.21E-9</td>
</tr>
<tr>
<td>$r$ (days$^{-1}$)</td>
<td>0.22 (0.62)</td>
<td>0.14 (0.22)</td>
<td>0.25 (0.06)</td>
<td>0.25 (0.07)</td>
<td>9.17E-3</td>
<td>0.129</td>
<td>0.126</td>
</tr>
<tr>
<td>$i$ (days)</td>
<td>36.7 (2.50)</td>
<td>43.2 (3.10)</td>
<td>28.2 (2.34)</td>
<td>39.1 (2.80)</td>
<td>5.37E-5</td>
<td>5.35E-6</td>
<td>0.099</td>
</tr>
<tr>
<td>$n$</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2.1: Logistic growth model comparison

This table shows the parameter estimates of logistic models fit to growth data from each microcosm, averaged by treatment. Numbers in parenthesis are standard deviations of $n$ replicate microcosms. Only 4 of the 5 +N+P microcosms were used in these calculations because one microcosm did not plateau, so the logistic model estimated over 100% cover. P-values are shown in the rightmost 3 columns for the 2-way ANOVAs run for each parameter. Independent variables in the three ANOVAs were Boolean: N (true or false) and P (true or false). The significant interaction term for $K$ indicates that asymptotic percent cover responded differently to P addition when N was added versus when N was not added (or vice-versa).

2.3.2. Residual nitrogen biogeochemistry

Commensurate with +N microcosms exhibiting the slowest growth, these microcosms also contained the highest residual N levels (Figure 2.3). +N microcosms contained concentrations of nitrate+nitrite roughly ten times higher than other treatments, but no significant differences were observed between other treatments ($P_{+N/Control}=0.027$, $P_{+N/+P}=0.025$, $P_{+N/+N+P}=0.026$, Tukey’s HSD). The mean ammonia concentration was also highest in the +N treatment (Figure 2.2). However, no statistically significant difference in ammonia concentration was detected among the treatments.
Figure 2.2: Residual inorganic nitrogen concentrations of microcosms

+N microcosms had very high levels of residual inorganic N at the end of the experiment, presumably due to an inability to metabolize excess N without sufficient P. However, there was no excess N detectable in the +N+P treatment, indicating that added P enables N use. Red dots indicate the mean.
Both bacterial and eukaryal communities significantly responded to N and P addition, and communities from the same treatment were more similar to each other than they were to communities from other treatments. In the bacterial plot (right panel), PC1 corresponds to N addition, since the two treatments that cluster on the left do not have added N and the two communities that cluster on the right do have added N. Similarly, PC2 corresponds to P addition, since the two communities on the bottom do not have added P and the two on the top do. The eukaryal community also segregated based on the treatment, but the interpretation of the principal coordinate axes is not so straightforward. Nevertheless, each treatment resulted in a unique microbial community of eukaryotes.

**Figure 2.3: Principle coordinates analysis of bacteria and eukaryotes from the microcosms**
In all treatments except the control (blue), *Bracteacoccus* dominated the eukaryal community. In the control treatment, a moss, *Dawsonia*, was the most abundant OTU. Algae closely related to the genus *Pseudendocloniopsis* have been previously detected in the same supraglacial debris that were used to make the microcosms discussed here (Schmidt and Darcy 2015), however they are much less proportionally represented in the microcosm communities than they were *in situ*.
Figure 2.5: Alpha rarefaction for eukaryal communities

Alpha rarefaction using the OTUs observed metric (also called “species observed”) showed that fewer OTUs were present in +N+P microcosms (top panel). Faith's (1992) phylodiversity (bottom panel) is a different alpha-diversity metric, which sums branch lengths of divergent OTUs, meaning that the calculated value is indicative of how many deeply divergent taxa are present at a given sampling depth. This metric reveals that P addition increased phylodiversity relative to the other three treatments. While OTUs observed do not saturate with increased resampling size, Faith's (1992) phylodiversity does, indicating that the lack of saturation in the first metric is due to incomplete sampling of OTUs that are phylogenetically similar to those already detected.
2.3.3. Bacterial community analysis

High-throughput sequencing of the microcosms for all three domains of life revealed that bacterial and eukaryal communities were significantly changed by treatment (Figure 2.3). Archaea were not detected at all, even though the bacterial primer set does amplify archael 16S rDNA. Archaeal sequences were detected in positive controls run in the same MiSeq lane using the same PCR reagents and the same thermocycler, indicating that their absence in the Toklat Glacier microcosm data set may be real (data not shown). ADONIS analysis of both data sets revealed significant effects of both N and P (for bacteria, $P_N=0.001$, $P_P=0.002$; for eukaryotes, $P_N=0.001$, $P_P=0.001$). Phylogenetic analysis of the microbial communities from each microcosm treatment revealed that photosynthetic bacteria were not highly abundant in any treatment, although photosynthetic eukaryotes dominated every microcosm's 18S rDNA library. Several bacterial OTUs were highly abundant across different treatments, such as an OTU which was abundant in the control and +P treatments, and was a close BLAST match to *Thiobacillus denitrificans*. Other abundant bacterial OTUs were related to *Lysobacter* (+P and +N+P), *Kaistobacter* (+N), an uncultured Acidobacterium (+P), *Fimbriimonas* (+N+P), and an uncultured Planctomycete (control). The only cyanobacteria within the top 20 most abundant OTUs were from the control. One was an uncultured basal cyanobacterium, and the other was a close BLAST match to *Crinalium epipsammum*.

2.3.4. Eukaryal community analysis

Eukaryal OTUs were mainly algal, and *Bracteacoccus* spp. were the dominant OTUs in the non-control microcosms (Figure 2.4). In the control microcosms, a moss in the Polytrichaceae was the dominant OTU, and it was also abundant (but not dominant) in the +N and +P treatments. Abundant algal OTUs were related to *Stichococcus*, *Characium*, and *Pseudendocloniopsis*, the latter two of which have already been shown to inhabit the Toklat Glacier site (Schmidt and Darcy 2014). Some
heterotrophic eukaryotes were abundant as well, including the rotifer *Philodina* and the amoeba *Glaeseria*. Richness (number of OTUs) was lower in +N+P microcosms than it was in microcosms from other treatments, and phylodiversity was higher in +P microcosms than it was in other treatments (Figure 2.5).

**2.4. Discussion**

Although we previously identified some of the organisms that live on the Toklat glacier (Darcy et al. 2011; Schmidt and Darcy 2015), and can even speculate as to how they form a simple, low-diversity community, phylogenetic data alone do not lend themselves to mechanistic explanations of which factors limit phototrophic growth in this unique environment. To this end, the present study showed that P limited the growth rate whereas P and N limited the final percent cover (Figure 2.1). Several other studies have examined nutrient limitations of microbial phototrophs in arctic and alpine environments, but none so far have studied debris-covered glaciers. Furthermore, there have been only three previous studies of the indigenous microbiota of supraglacial debris (Darcy et al. 2011; Franzetti et al. 2013; Schmidt and Darcy 2015). However, studies of similar environments indicate that P may limit growth. For example, analysis of cryoconite on the Svalbard Glacier indicated that P may be limiting (Stibal et al. 2009) and microcosm studies similar to those done here showed that P limited both the growth rate and percent cover of phototrophic communities near the retreating Puca glacier in the Peruvian Andes (Schmidt et al. 2012). An *in-situ* fertilization experiment at the same site showed that co-addition of N and P accelerated the succession of the microbial community leading to a significant increase in phototrophic microbes (Knelman et al. 2014).

Similar to the abovementioned studies of nutrient limitation in the cryosphere, our results show P as the principally limiting nutrient (Figure 2.1). P-addition significantly accelerated growth rate (Table 2.1), which is especially evident between days 20 through 34 of the microcosm experiment.
Had we stopped the microcosm experiment at day 34, we would have observed a clear pattern of P limitation even for percent cover. However at later time points when growth had leveled off, +N+P microcosms outpaced the other treatments by a large margin. The final percent cover estimates ($K$ values, Equation 2.2) overlapped to a large extent for all treatments except +N+P (Table 2.1), and the final cover for +N+P treatments was greater than the sum of control, +N and +P treatments. This non-additive result has been previously described as “Synergistic Co-limitation” (Allgeier et al. 2011). In synergistic co-limitation, the effect of the +N+P treatment is greater than the effects of +N added to +P, which is what we observed (Figure 2.1, Table 2.1). This result is similar to results from many aquatic systems, but differs from most terrestrial systems, including Arctic systems, where an antagonistic response is usually observed (Allgeier et al. 2011). But although we observed N/P co-limitation of final percent cover, it is clear that P-limitation occurred before N limitation, so we characterize the microcosms as principally P-limited.

The $K$ parameter in our logistic model represents percent cover values used in other full-factorial N and P addition studies (Elser et al. 2007; Harpole et al. 2011), but the growth rates we report ($r$, equation 2.2) have not been measured in many previous studies. Had we not collected time-series data on this experiment, we would only have observed the end effect, and concluded that the system is synergistically co-limited by N and P. However thanks to the resolution of our time series, we observed that growth rate of microbial phototrophs is P-limited. Furthermore, from the inflection point parameter ($i$, equation 2.2) we observed that +N microcosms took the longest to reach their peak instantaneous growth rate (i.e. change in % cover gained per day), but +P microcosms reached their peak instantaneous growth rate the fastest, over two weeks earlier than +N microcosms. The slow growth of +N microcosms is commensurate with the inorganic nitrogen data we collected, which clearly show that added N was not utilized unless P was also added (Figure 2.2). The +N+P microcosms received the same amount of ammonium nitrate as the +N microcosms, but presumably due to a lack of P, the +N
microcosms were not able to use the added N. Although they did not measure inorganic N concentration after their microcosm experiment, Schmidt et al. (2012) observed that N addition alone resulted in microcosms that grew less than the control, and at a slower growth rate than microcosms with added P.

Unlike the patterns of percent cover and residual inorganic N where a single treatment was highly different than the rest, the microbial communities of all four microcosm treatments were distinct from each other, and all were significantly affected by both N and P (Figure 2.3). This result suggests that the mechanism by which nutrient addition affected growth (Figure 2.1) was not simply an effect that caused more of the same organisms to grow, but rather that the treatments selected for the growth of different organisms (Figure 2.3). Even when total growth (in terms of percent cover) was not dramatically affected, as was the case between the control, +N, and +P microcosm treatments, different microbial communities developed indicating strongly deterministic selection (Nemergut et al. 2013) by each nutrient regime.

In addition, the bacterial communities of the microcosms were very diverse, with no OTU composing more than 4% of the 16S amplicon libraries from control microcosms, and no OTU composing more than 9% of any other microcosm. Among the most common bacteria that grew in the microcosms without added N were *Thiobacillus*, which have been detected before in other periglacial environments, such as beneath the Bench glacier (Skidmore et al. 2005), which is also in Alaska although it is roughly 342 km from the Toklat glacier. In treatments containing added P (+P and +N+P), an OTU assigned to the genus *Lysobacter* was highly abundant, which may be a denizen of the rhizosphere (Hayward et al. 2010). However, for such an inconspicuous genus it is difficult to generalize traits based on a ~250 bp 16S rDNA sequence. Even the ecological role of one cyanobacterial OTU (ML635J-21) detected within the microcosms is not easily understood. ML635J-21 was only detected in the control microcosms, where it was the most abundant OTU in one
microcosm, and is the most basal clade in the Cyanobacteria, has no cultured representatives, and may not even be phototrophic since many phylotypes come from aphotic environments (Soo et al. 2014). The only other cyanobacterium we detected was a close match to *Crinalium*. This cyanobacterium is definitely phototrophic, and the genus *Crinalium* has been detected in Antarctic glacial cryoconite (Broady and Kibblewhite 1991; Mueller and Pollard 2004; Porazinska et al. 2004).

Unlike the bacteria, the eukaryotes we sequenced were almost entirely phototrophic (Figure 2.4). In every microcosm except the controls, an OTU from the algal genus *Bracteacoccus* dominated. *Bracteacoccus* have been isolated from cryoconite holes, but also from supraglacial debris (Stibal et al. 2006). These algae were also observed by Kaštovská et al. (Kaštovská et al. 2005) in barren glacial soils, but not in vegetated soils nearby. Commensurate with our observations, Shukla et al. (Shukla et al. 2011) found that out of 6 algal isolates from recently deglaciated soil, only *Bracteacoccus* responded well to excess N. However, this N copiotrophy does not explain why *Bracteacoccus* still dominate the 18S libraries for +P microcosms, where no N was added. These algae were not the dominant OTU in the control microcosms, and we found a bryophyte OTU to be most abundant instead. The two different classification methods we used disagreed on the genus of this OTU, returning closely related genera *Dawsonia* (SINA) or *Notoligotrichum* (BLAST) both of which are in the family Polytrichaceae (Bell and Hyvönen 2012). Whatever the exact identity of this moss OTU, it was clearly selected against by any nutrient addition. Similarly, Schmidt et al. (2012) found that mosses were inhibited by all nutrient addition treatments and they speculated that these mosses could be adapted to oligotrophic conditions, and our observations support this hypothesis because of the dominance of putatively copiotrophic *Bracteacoccus* algae in nutrient addition microcosms.

Our phylogenetic comparisons also show that the excess growth of phototrophs in the +N+P treatment was likely due to the growth of a few dominant organisms, because the total number of OTUs detected was reduced by this treatment (Figure 2.5, top panel). Similar to the pattern observed with
final percent cover estimates, control, +N, and +P microcosms overlap in the number of OTUs observed during alpha rarefaction, while the +N+P microcosms had lower richness. However, when a metric such as Faith's Phylodiversity (Faith 1992) is used instead of the number of observed OTUs, a different pattern emerges in which +P microcosms contain significantly more phylodiversity than the other three treatments (Figure 2.5, bottom panel). Faith's phylodiversity is an alpha diversity metric that uses phylogenetic distance instead of OTU counts, so that closely related OTUs (which have low phylogenetic distance) are less important to the output than distantly related OTUs. This means that the +P microcosms contain more distantly related eukaryal OTUs than other microcosms, indicating that P allowed the growth of a broader diversity of organisms than did the other treatments.

Taken together, the results of our molecular, physiological, and biogeochemical studies form a preliminary picture of how low nutrient levels might limit life atop the Middle Fork Toklat Glacier, and perhaps other debris-covered glaciers. Our microcosm results suggest that the growth rate of microbial phototrophs on the Toklat Glacier is P-limited, but total growth (final percent cover) may be co-limited by N and P. Our results also suggest that N and P differentially stimulate members of the indigenous microbiome resulting in the formation of different microbial communities in response to each treatment. Future work will be focused on understanding the growth strategies of these individual taxa to better understand how they may respond to future inputs of nutrients either due to continued successional processes or anthropogenic inputs of nutrients.

2.5. Acknowledgements

We thank B.T. Todd and W. A. Schrepel for laboratory assistance and A.J. King, B.-L. Concienne and M. Mitter for collecting the soils used in this study. Funding was provided by NSF grants for studying microbial community assembly (DEB-1258160), and the LTER program (DEB-1027341) and the USAF Office of Scientific Research (FA9550-14-1-0006).
2.6. Data Accessibility

Sequence data and associated metadata from this study have been deposited in FigShare and are available with the DOI http://dx.doi.org/10.6084/m9.figshare.1427427.
Chapter 3: Phosphorus Availability Limits Both Visible And Microscopic Primary Producers During The Earliest Stages Of Primary Succession

by J.L. Darcy and S.K. Schmidt

3.1. Abstract

Current models of ecosystem development hold that low nitrogen availability limits the earliest stages of primary succession, but these models were developed from studies conducted in areas with temperate climates. Global warming is now causing rapid glacial retreat even in inland areas with cold dry climates, areas where ecological succession has not been adequately studied. Here we combine field and microcosm studies of both plant and microbial primary producers, and found that phosphorus, not nitrogen, is the nutrient most limiting to the earliest stages of primary succession along glacial chronosequences in the Central Andes and Central Alaska. These results overturn the universality of early-succession being N-limited, and will inform conservation and management of these fragile and threatened ecosystems.

3.2. Introduction

Current models of ecosystem development and biogeochemistry hold that low nitrogen availability limits the earliest stages of primary succession. We combined field and microcosm studies of both plants and microbial primary producers, and found that phosphorus, not nitrogen, is the nutrient most limiting to photosynthetic plants and microbes in recently deglaciated soils, and barren soils atop debris-covered glaciers. We support this conclusion using both traditional ecological techniques like measuring the percent cover of plants across our full-factorial N and P addition experiment in the Peruvian Andes, but also with modern techniques such as high-throughput DNA sequencing, digital image processing with mNDVI, and chlorophyll autofluorescence digital microscopy. A major paradigm of biogeochemical and ecological theory is that early successional ecosystems are primarily
nitrogen (N) limited whereas later successional ecosystems tend to become phosphorus (P) limited (Vitousek 2004; Selmants and Hart 2010; Menge et al. 2012). This hypothesis grew out of classic ecological studies and the observation that all of the elements required for plant growth, except N, are found in geologic substrates on which primary succession occurs, and that soils tend to weather from young, P-rich systems to older, P-poor systems (Stevens and Walker 1970; Walker and Syers 1976; Vitousek 1994). However, this paradigm was developed, and has mostly been tested in humid temperate and subtropical ecosystems (Walker and Syers 1976; Vitousek 2004) and to a more limited extent in semi-arid temperate ecosystems (Selmants and Hart 2010; Newman and Hart 2015). To date this theory has not been tested in polar or alpine environments where glacial and ice cap retreat due to global warming is opening up vast new landscapes to primary ecosystem succession. Polar and alpine environments are among the systems most at risk due to global warming (Ernakovich et al. 2014), and are also areas where climate may further limit the rate of ecosystem succession beyond limitations imposed by low nutrient availability. It has recently been shown that the pre-plant stages of ecosystem succession are protracted in such environments (Nemergut et al. 2007; Sattin et al. 2009; Schmidt et al. 2016), and in the most extreme polar and alpine ecosystems succession never progresses beyond the stage where microbes are the primary producers of the system (Zeglin et al. 2009). Therefore, it is important to test models of ecosystem development for both microbes and plants in polar and alpine ecosystems.

Here we show that early ecosystem development (for both plants and microbes) is primarily P limited in young, plant-free soils from two alpine sites. Primary productivity in soils from the retreating Puca glacier (Peruvian Andes) as well as soil atop the debris-covered Middle Fork Toklat Glacier (Alaska range) is primarily limited by P. We compare our findings of P-limitation at these sites using a microcosm approach, which showed P limitation from a primary productivity perspective and also from a biogeochemical perspective. However, since microcosms cannot mimic many aspects of these natural
systems, we also performed a 6-year in situ nutrient addition experiment at the Puca glacier site. This experiment also strongly showed P-limitation of primary productivity, for both plants and microbes, using traditional techniques like percent cover by plants and density of microbial phototrophs, and also modern techniques like mNDVI and microbial community composition.

3.3. Methods

Field plots were created in August of 2010 in soil at the terminus of the retreating Puca Glacier, in the Cordillera Vilcanota of Peru. These 16 permanent plots (1 m²) were marked using nails and string, with small aluminum numbered tags for future identification. Plot treatment identity was assigned randomly from the four treatments of our factorial N and P design such that each treatment identity (Control, +N, +P, and +N+P) was represented by four plots. All plots were completely devoid of visible plants or lichens at the time of nutrient addition. Nutrient addition was done using spray bottles as described elsewhere (Knelman et al. 2014). Soil samples were collected before treatment was added to plots, and kept frozen at -20C. This soil was used for the nutrient addition microcosm experiment by Schmidt et al. (Schmidt et al. 2012) which used the same N/P factorial design as the in situ field plots. Microcosm data from Schmidt et al. (Schmidt et al. 2012) and Darcy and Schmidt (Darcy and Schmidt 2016) were analyzed by first reducing the Toklat data set to only include those time points that were measured in the same way as Schmidt et al. (Schmidt et al. 2012). A discussion of the different ways these microcosm experiments were measured can be found in Darcy and Schmidt (Darcy and Schmidt 2016). Repeated-measures ANOVA was used to test the influence of N and P addition on percent cover.

Soil samples were collected from each of the field plots in November of 2012 in order to analyze the developing microbial communities in the soil. Soil samples were kept at -20C until genomic DNA was extracted using the MoBio PowerSoil kit (Carlsbad, CA, USA). Each extraction
was amplified in triplicate using the bacterial primer set 515F/806R (Caporaso et al. 2012), using a unique barcode for each of the 20 samples. Each set of triplicate reactions was pooled, and amplified DNA concentrations were assayed using Pico Green fluorimetry on a BioTek Synergy 2 microplate reader (BioTek, Winooski VT, USA). DNA was then diluted to equimolar concentration, pooled, and sequenced using an Illumina Miseq (Illumina, Inc) with 2x150bp chemistry. 16S rDNA data were demultiplexed and processed using QIIME software (Caporaso et al. 2010b). Sequence data were rarefied to 20,000 sequences per sample. Genomes of cyanobacteria most closely related to our most abundant cyanobacterial OTU were found in NCBI's genome database using nucleotide BLAST (Altschul et al. 1990).

Because 16S rDNA amplicon data only yield estimates of relative abundance, we used microscopy to estimate the density of microbial phototrophs within soil samples. One gram of frozen soil from each sample was placed in a 2 ml microcentrifuge tube, and 1 ml of deionized water was added to it. These tubes were incubated overnight to allow microbial phototrophs to re-activate. Tubes were vortexed briefly, then 10 ul of liquid was pipetted from the tube onto a microscope slide. A cover slip was added, and then a chlorophyll autofluorescence lamp was used to visualize microbial phototrophs containing chlorophyll. Images were captured using a Nikon D311 DSLR camera (Nikon, Tokyo, Japan) fitted to an AxioSkop 2 (Carl Zeiss AG, Oberkochen, Germany) light microscope using a 3-D printed Nikon F-mount adapter (available for download at https://www.thingiverse.com/thing:1722898). Ten photographs were taken of each slide from different fields of view, at 200X magnification. These photographs were analyzed in R 3.3.3 using custom software. Briefly, each image has red, green, and blue channels. Since chlorophyll autofluoresces red, only the red channel was used. When read into R, the red channel is represented as a matrix of numeric values, with the dimensions of the camera's image resolution (4000x3000 px). Numeric values over a threshold (determined using a negative control) were counted as “hits”, and the percent cover (on the
(slide) of microbial phototrophs was calculated as hits / total * 100.

Field plots were re-visited in 2015 and 2016 in order to take precise measurements of percent cover by plants. These measurements were taken during the wet season (February-April) so that plant cover could be recorded at its zenith. Percent cover was measured using the point-intercept method, which was done by laying marked strings over the plots and counting how many times the marks intersected plants. One hundred intersections were surveyed for each of the 16 plots, for both years. However, this method may miss small plants and microbial phototrophs, so we also used a specially-made camera that can capture near-infrared (NIR) light, in order to do mNDVI. The camera was originally a Hero 4+ (GoPro, San Mateo, CA, USA), but it was modified to capture NIR light by the company Agribotix (Boulder, CO). Photographs were taken of each site, making sure to capture all four corners of the site using the camera's rear screen preview. Adobe Photoshop (Adobe Inc, Carlsbad, CA) was used to make masks for each photograph, which are images where the plot space is colored white, and everything else captured in the photograph is black. The original photos and the masks were processed in R using custom software. The mNDVI equation was used on the original photograph, which is an equation identical to NDVI except the NIR channel is normalized using only one other channel. Orbital imaging platforms (on satellites) traditionally have separate NIR and visible light sensors, which is a viable solution for multi-spectral imaging because the two sensors are so far from the target that the two sensors produce an image of the same focal area. But on the ground, less than a meter from the target, the two-sensor solution is not viable (mathematically or economically). So we used mNDVI instead, and applied that function using the entire original photograph's NIR channel and blue channel. Pixels that were not within the plot area were discarded, using the mask. This process was done for each plot across both years. Percent-cover estimates using mNDVI and using the point-intercept method were tested with ANOVA.
3.4. Results and Discussion

Our initial microcosm experiments showed strong evidence of P-limitation of early-successional alpine environments, at both the Puca Glacier site (Figure 3.1, left)(Schmidt et al. 2012) and the Toklat Glacier site (Figure 3.1, right)(Darcy and Schmidt 2016). In both microcosm experiments, P addition resulted in higher percent cover by microbial phototrophs (Figure 3.1, A), and the effect of P addition was much larger than the effect of N addition (Figure 3.1, B). Although N still had a significant effect on primary productivity in both cases (Figure 3.1, B), the difference in effect size between N and P makes it clear that these microcosms were P-limited (Elser et al. 2007; Harpole et al. 2011). Residual N was only found in +N treatments of both experiments as well (Figure 3.1, C), indicating that P addition allowed for use of added N, but N added to the +N microcosms could not be used without P added (as in the +N+P microcosms).
Figure 3.1: Results of microcosm studies showing P-limitation of early successional soils

Microcosm experiments with soil from the Puca Glacier site (left) and the Toklat Glacier site (right) both used factorial additions of N and P, and both show P-limitation (Schmidt et al. 2012, Darcy and Schmidt 2016). The percent cover of microbial phototrophs over time is significantly higher in treatments with P added, and lower when P is not added (A). Repeated-measures ANOVA shows a much larger effect size of P than of N as well (B). Furthermore, when N is added in the absence of P, that N is left unused (C).
However, microcosm experiments are limited by the difficulty of replicating in-situ conditions, so we also conducted a full-factorial N and P addition experiment at the Puca Glacier site. This 6-year experiment began with completely barren soil at the terminus of the Puca Glacier, but P-limitation was evident within the second year in 2012. Within one year of nutrient addition, we observed small mosses and grasses in plots with P added, but the control plots and plots with N added were still barren.

Initially, we used stem counts within plots to track the progression of plant community succession, but this became intractable within the third year of the study because much of the plant biomass was in mosses, which can't be easily quantified this way. Furthermore, the difference in size of plants was apparent, thus it was inappropriate to treat the stem of a large plant with the same statistical weight as that of a small plant.

Instead, the plots show visually obvious signs of P-limitation (Figure 3.2), and our percent cover measurements show consistent patterns of plant primary productivity responding mainly to P across two years and two methods of percent cover estimation (Figure 3.3). Both point-intercept and mNDVI data show strong evidence of P-limitation across both years that percent-cover data were collected. +P and +N+P treatments had similarly high responses, while control and +N treatments had similarly low responses, which is a very clear signal of strict P limitation (Elser et al. 2007; Harpole et al. 2011). This result is reflected in the effect sizes of N and P: the presence or absence of added P explained 78% of variation in percent-cover (point-intercept) in 2015, and 79% in 2016 (P'15 & P'16 < 10-5, ANOVA), but N explained less than 4% for both years (P'15 & P'16 < 0.10). While N did have a statistically significant effect, the difference in effect sizes suggests that these soils are P limited instead of co-limited.
Figure 3.2: Near-infrared enhanced photographs of field plots and mNDVI comparison

In these false-color photographs, the visible light channels of the sensor have been averaged and rendered as a grayscale background, and the NIR channel has been overlayed in red. Control plots (A) did not contain any visible plants, but some pixels in the plot area still had mNDVI values above the threshold of 0.1 (B), possibly indicating detection of microbial phototrophs. However, plots with P added (C) contained many plants, which were detected with the NIR sensor and statistically differentiated from barren areas using mNDVI. The mNDVI distribution plot (middle) shows that the +P plot has many more pixels above the mNDVI threshold.
Figure 3.3: P-limitation as shown by stem counts and percent-cover

Stem counts showed P-limitation early on (A), but did not account for mosses, which were abundant in some plots. The point-intercept method (B, top) yielded almost identical results for 2015 (left) and 2016 (right), and ANOVA of each year showed an overwhelmingly large effect size of P addition (inset pie charts). mNDVI analysis (B, bottom) showed very similar results, although the effect size of P was lower. mNDVI also captured potential primary productivity in the control plots, possibly from microbial sources. The effect of N was not statistically significant in any of these four analyses.
Point-intercept and mNDVI data showed almost identical trends, but mNDVI detected active chlorophyll in control plots even though no plants were observed using percent cover. Microbial phototrophs are invisible to the naked eye, and were not counted as “hits” when we performed point-intercept experiments. However, the sensor we used for NIR photography does not distinguish between chlorophyll in plants and chlorophyll in cyanobacteria or algae. So it is likely that microbial phototrophs were detected by our sensor in otherwise barren soil. NDVI has been successfully used to detect cyanobacterial blooms in the ocean from orbit (Oyama et al. 2015) and to detect cyanobacteria in desert biological soil crusts (Karnieli et al. 1999; Burgheimer et al. 2006), so it is not surprising that our sensor may have detected cyanobacterial crusts as well.

Indeed, high-throughput DNA sequencing revealed that cyanobacteria comprised a large fraction of microbial communities in the plots (Figure 3.4, left). Control plots, which were otherwise barren, harbored microbial communities composed of over 12% cyanobacterial sequences on average. Cyanobacterial soil crusts have been shown to be abundant at the Puca Glacier site (Schmidt et al. 2009; Sattin et al. 2009) and in the youngest soils exposed by other retreating glaciers as well (Kaštovská et al. 2005; Duc et al. 2009). But similar to the plant primary productivity response to nutrient addition, the relative abundance of cyanobacteria also increased sharply when P was added. This shift was entirely caused by an increase in sequences from Nostocales (Figure 3.4, left), a group of cyanobacteria known for nitrogen fixation. In fact, the genomes of cyanobacteria most closely related to our most abundant Nostocales OTU all contained the nifH gene (Zehr and Turner 2001), and other genes involved in N-fixation as well (NCBI Genome references NC_019682=Calothrix sp. PCC7507, NC_019676=Nostoc sp. PCC 7107, NZ_KB235896=Anabaena sp. PCC 7108).

The sharp increase in relative abundance of putative nitrogen-fixing cyanobacteria when P is added is commensurate with P limitation of microbial primary producers in additional to plants. Excess P added to the system can be used by these cyanobacteria to produce their own N, which in itself is a
mechanism for P limitation since N is readily available to be fixed from the atmosphere. In this sense, the structure of the microbial community at this site may underlie the mechanism that causes P-limitation, but an underlying resource-ratio equilibrium likely still exists (Vitousek et al. 2002). But relative abundances of DNA sequence data are insufficient to support this hypothesis, because relative abundance is philosophically divorced from the actual biomass of the microorganisms in question. It could easily be the case that even though relative abundance of Nostocales was high in treatments with added P, there were orders of magnitude fewer microbes in those samples to begin with.

Figure 3.4: Shifts in cyanobacterial communities from nutrient addition, and nutrient limitation of microbial phototrophs

Both N and P addition changed cyanobacterial community structure (A), but P-addition caused a dramatic increase in the relative abundance of N-fixing Nostocales (A). We also quantified the density of microbial phototrophs in soil (B) using a microscopic percent cover technique (C), which showed strong P-limitation of microbial phototrophs.
To address this, we used a technique to compare the amount of photosynthesis in microbes that is analogous to the percent-cover techniques we used for plants. Our chlorophyll autofluorescence percent-cover estimates for microbes show a strikingly similar pattern to that of plants (Figure 3.4, right), showing that both plants and microbial phototrophs are P-limited at this site.

3.5. Conclusions

The work we present here clearly shows that early-successional photoautotroph communities can be strongly P-limited during the earliest stages of ecosystem succession following glacial retreat, both in terms of plants and microbes. Previous work at the Puca Glacier site has shown that bioavailable P is higher in older (vegetated) sites compared to younger (barren) sites, possibly due to microbial weathering processes that take time to process recalcitrant P (Schmidt et al. 2011a). Both this result and our current work support a model of nutrient limitation during primary succession where P-limitation occurs in new soils due to the relatively low bioavailability of P and the presence of N-fixers. P-limitation may then be ameliorated later in succession by biological weathering processes.

With support from other studies (Fitter and Parsons 1987; Schmidt et al. 2011a, 2016; Knelman et al. 2014), these results point to a new paradigm for primary succession in cold inland ecosystems compared to well-studied coastal and island landscapes. Despite high concentrations of P in the primary minerals on which succession occurs (Schmidt et al. 2011a), the availability of this P is highly restricted in climates where rock weathering is inhibited by cold temperatures and aridity. This results in the severe P limitation described here, which in turn explains why succession of plant and microbial primary producers is extremely slow in places like the High Andes and Central Alaska. More work is needed to determine if this new paradigm applies to vast inland areas such as the Tibetan Plateau where deglaciation due to global warming is occurring at a record pace.
Chapter 4: Spatial Autocorrelation Of Microbial Communities Atop A Debris-Covered Glacier Is Evidence Of A Supraglacial Chronosequence

By J.L. Darcy, A.J. King, E.M.S. Gendron, and S.K. Schmidt

4.1. Abstract

Although microbial communities from many glacial environments have been analyzed, microbes living in the debris atop debris-covered glaciers represent an understudied frontier in the cryosphere. The few previous molecular studies of microbes in supraglacial debris have either had limited phylogenetic resolution, limited spatial resolution (e.g. only one sample site on the glacier), or both. Here, we present the microbiome of a debris-covered glacier across all three domains of life, using a spatially-explicit sampling scheme to characterize the Middle Fork Toklat Glacier’s microbiome from its terminus to sites high on the glacier. Our results show that microbial communities differ across the supraglacial transect, but surprisingly these communities are strongly spatially autocorrelated, suggesting the presence of a supraglacial chronosequence. This pattern is dominated by phototrophic microbes (both bacteria and eukaryotes) which are less abundant near the terminus and more abundant higher on the glacier. We use these data to refute the hypothesis that the inhabitants of the glacier are randomly deposited atmospheric microbes, and to provide evidence that succession from a predominantly photosynthetic to a more heterotrophic community is occurring on the glacier.

4.2. Introduction

Glaciers are an important and popular system of study for ecologists, especially considering their importance in various fields of research, such as global warming (Oerlemans 1994; Kaser et al. 2004), ecological theory development (Chapin et al. 1994; Kaštovská et al. 2005; Nemergut et al. 2016), and resource management (Bury et al. 2011). While much research on the ecology of glacial
microbes has focused on glacial forefields (Kaštovská et al. 2005; Bradley et al. 2014; Castle et al. 2016; Nemergut et al. 2016), these environments are geographically and physically distinct from the environment on or in the glacier’s surface. While microbiological studies of glacial surfaces exist, almost all to-date have been glaciers with exposed ice surfaces (Stibal et al. 2012; Edwards et al. 2014; Boetius et al. 2015), rather than debris-covered glaciers. Debris-covered glaciers are common throughout the cryosphere, especially in the Alaska Range and the Himalayas. The debris atop these glaciers are added to a glacier's surface from its surroundings, and contain unique microbial communities that have only been described in a few studies (Darcy et al. 2011; Franzetti et al. 2013; Azzoni et al. 2015; Schmidt and Darcy 2015).

Preliminary work characterizing the indigenous microbes of debris-covered glaciers has focused on microbes that are abundant within debris samples. Bacteria from the genus Polaromonas have been found to be prevalent on debris-covered glaciers by multiple studies (Darcy et al. 2011; Franzetti et al. 2013), however their role in these environments is still mysterious. It is possible that they are dormant propagules landing in an otherwise low-biomass environment, and it is also possible that Polaromonas are somehow functioning in-situ. Other microbes such as the green alga Pseudendocloniopsis (Ulvophyceae) have been detected in abundance from debris-covered glacier samples (Schmidt and Darcy 2015) and also from microcosms made from glacial debris (Darcy and Schmidt 2016). From an even more taxon-specific perspective, both nematodes and rotifers have been surveyed on debris-covered glaciers (Azzoni et al. 2015).

However there is very little evidence addressing whether these organisms are functioning on debris-covered glaciers, or whether they are just inactive (dormant) atmospheric transients that are easily detectable in a low-biomass environment (Darcy et al. 2011). Thus, our null hypothesis is that microbial communities in supraglacial sediment are random assemblages of dormant propagules and therefore should show no correlation with spatial variation in environmental variables, such as nutrient
Figure 4.1: Topographic map of sampling sites across the Middle Fork Toklat Glacier

Sample sites (white dots) were located along a staggered transect, beginning at the glacier’s terminus (dashed black line) and ending slightly more than 600 meters to the south, corresponding to an elevation increase of 100 meters (contours represent 10-meter elevation differential). Inset, top: Map of Alaska, showing the location of Denali National Park and Preserve. Inset, bottom: Map of Denali National Park and Preserve, showing location of the Middle Fork Toklat Glacier within the park.
availability, pH, or other biogeochemical factors. Alternatively, microbial communities that are functionally active on the glacier should be shaped by, and correlate with, spatial patterns in these environmental variables, as is often the case in soil microbial communities, which can be strongly shaped by nutrient and water availability (King et al. 2010; Manzoni et al. 2012).

In glacial forefield chronosequences, these soil biogeochemical variables are often structured by substrate age (Schmidt et al. 2016), but this may or may not be the case atop debris-covered glaciers. Some authors have speculated that debris-covered glaciers are chronosequences, where recently deposited debris high on the glacier give way to older substrate at the terminus (Gobbi et al. 2011; Franzetti et al. 2013). If this is the case, this supraglacial chronosequence hypothesis makes two predictions about the microbial communities atop debris-covered glaciers. First, since chronosequences are space-for-time substitutions, we expect microbial communities atop the glacier to be highly spatially structured. Sites that are geographically proximate are also temporally proximate, and are therefore in similar successional stages. Thus, the microbial communities of sites close to each other should be more similar (in terms of beta-diversity) than geographically/temporally distant sites. Second, the nature of these spatially/temporally structured communities should reflect their age, especially in terms of the relative abundance of microbial phototrophs. Ecosystem succession begins with primary producers (although there may be exceptions (Bardgett et al. 2007), and as such we expect chronosequences to exhibit a pattern where phototrophic organisms are more proportionally abundant at young sites, and less so at older sites.

Here, we test the above hypotheses, and also present the most thorough picture so far of life atop a debris-covered glacier, the Middle Fork Toklat Glacier in Denali National Park and Preserve. Although previous sequencing studies of debris-covered glaciers were limited to bacteria or select taxa therein (Darcy et al. 2011; Franzetti et al. 2013) or limited by spatial resolution (Schmidt and Darcy 2015; Darcy and Schmidt 2016), here we present an in-depth analysis of life in the Middle Fork Toklat
Glacier's supraglacial debris, using both 16S and 18S high-throughput rDNA sequencing to look at all 3 domains of life.

4.3. Materials and Methods

4.3.1. Sample collection

Samples were collected from the surface of the Middle Fork Toklat Glacier in the summer of 2009, along a transect that started on top of the glacier near its terminus and ended approximately 600 m up-glacier (Figure 4.1). Sample sites near the terminus were high enough on the glacier and far enough away from recently exposed subglacial sediment to be sure of their supraglacial origin. Samples consisted of roughly 50 grams from the top 4 cm of sediment. During sampling, GPS coordinates and elevations for each sample point were recorded using a Garmin 60CSx GPS unit. Samples were frozen on ice in the field and were shipped to the University of Colorado, where they were kept at -80°C until DNA was extracted in 2010. Figure 4.1 shows the spatial origins of the 21 samples collected in this manner. A thorough description of the Middle Fork Toklat Glacier and pictures of the site can be found elsewhere (Schmidt and Darcy 2015; Darcy and Schmidt 2016; Schmidt et al. 2016).

4.3.2. DNA extraction and sequencing

DNA was extracted from each of the 21 samples using the MoBio PowerSoil DNA isolation kit (Carlsbad, CA, USA), and DNA was kept frozen at -20°C until 2014, when each extraction was amplified in triplicate using 515F/806R (Caporaso et al. 2012) for bacteria and archaea, and 1391F/EukBR (Amaral-Zettler et al. 2009) for eukaryotes. Samples were amplified with barcoded versions of these primers, which also contained Illumina flowcell adapters. Both primer sets can be accessed from the Earth Microbiome Project website (http://press.igsb.anl.gov/earthmicrobiome/emp-
standard-protocols). Triplicate reactions were pooled, and amplified DNA concentrations were assayed using Pico Green fluorimetry using a BioTek Synergy 2 microplate reader (BioTek, Winooski VT, USA). Reactions were diluted to equimolar concentration and pooled, then sequenced using an Illumina MiSeq (Illumina, inc.) with 2x150bp chemistry. A 30% phiX spike was added to the run, in order to increase read variability (Caporaso et al. 2012).

4.3.3. Sequence data processing and bioinformatics

Raw sequence reads were demultiplexed using QIIME (Caporaso et al. 2010b). 16S paired-end reads were joined, but this process did not work for 18S reads due to insufficient overlap, so only one end was used. Sequences were clustered at the 97% similarity level using UCLUST (Edgar 2010) and taxonomy was assigned with QIIME using the SILVA Ref NR 99 database's taxonomy for 18S sequences and the GreenGenes database's taxonomy (DeSantis et al. 2006) for 16S sequences. We used different databases because the GreenGenes taxonomy is the most commonly used taxonomy for 16S sequences and we wanted our results to be comparable to other publications. However, the GreenGenes database does not contain taxonomy for 18S sequences, so the SILVA database was used instead. All mitochondrial and chloroplast OTUs were removed from the 16S library, and archaeal sequences were separated. Bacterial and archaeal OTUs were removed from the 18S data set where present (possibly due to PCR primer promiscuity). The filtered data sets were each rarefied to a depth of 6000 sequences per sample, except for the archaeal data set which had 0 sequences per sample for most samples, and only 10 sequences for the most populous sample. Weighted UniFrac (Lozupone and Knight 2005) was used to calculate beta-diversity matrices for the 16S and 18S data sets. BLAST (Altschul et al. 1990) was used to compare sequence data to the NCBI nucleotide database and to find phylotypes similar to common OTUs from our data sets.
4.3.4. Sediment biogeochemical measurements

Soil water content was measured gravimetrically by determining weight loss of roughly 1 gram of soil after 48 hours at 60°C. Dissolved organic carbon (DOC), dissolved organic nitrogen (DON), NH$_4^+$ and NO$_x$ were extracted from ~5 gram soil samples by adding 25 ml of 0.5 M K$_2$SO$_4$ and shaking at 150 rpm for 1 hour. Samples were centrifuged for 1 hour at 4000 rpm. A blank sample that contained no soil was also processed. NO$_x$ concentration was analyzed for each extraction using a Lachat QuickChem 8500 (Hach Ltd, Loveland CO). NH$_4^+$ concentration was analyzed for each sample colorimetrically using a BioTek Synergy 2 microplate reader (BioTek, Winooski VT). DOC and DON were analyzed using a Shimadzu total organic carbon analyzer (TOC 5000) equipped with a total dissolved nitrogen (TDN) module (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA). DON was calculated as TDN-DIN. All concentrations were corrected using measurements from blanks.
Concentrations of C and N were calculated stoichiometrically using the original soil masses, and were corrected for background levels of C and N using the blank sample data.

4.3.5. Statistical analyses

Pair-wise geographic distances were calculated for sample locations (Figure 4.1) using the R package “Fields” (Nychka et al. 2016). Semivariograms (Bachmaier and Backes 2008) were then made in R by combining 16S and 18S beta-diversity matrices with their geographic distances. We used a sliding-window approach to geographic distance classes within the semivariograms in order to better visualize the spatial structuring of bacterial and eukaryote beta-diversity, with a window overlap of 2/3 and even sample size between bins; this means that all distance classes contain the same number of pairwise points, and each class shares 2/3 of its points with the class before it. Versions of these figures without the sliding-window approach are available as supplemental material (Supplementary Figure 1). Nonmetric Multidimensional Scaling (NMDS) was calculated from 16S and 18S beta-diversity
matrices using the R package “vegan” (Oksanen et al. 2016) to test how the biogeochemical parameters we measured were related to bacterial and eukaryote beta-diversity, and correlations between geographic distance and biogeochemical parameters were calculated in R.

4.4. Results

4.4.1. Bacterial biogeography

The most striking biogeographic pattern was that of cyanobacteria, which comprised over 13% of the bacterial communities at sites high on the glacier, but were almost absent near the terminus (Figure 4.2). The most common cyanobacteria were in the class Synechococcophycideae, and clustered into three main OTUs which were most similar to sequences in GenBank that originated from polar and alpine environments. Cyanobacteria in the Chamaesiphonaceae and Phormidiaceae were also present and showed similar spatial patterns, although they were less abundant than the Synechococcophycideae (Supplemental Figure 2).

Other bacteria showed patterns over the glacier as well, including the Acidobacteria (Figure 4.2) which were slightly more abundant near the terminus. Within the Acidobacteria, OTUs in the orders DS-18 and iii1-15 were responsible for this trend. Betaproteobacteria were generally less abundant near the terminus and more abundant higher on the glacier, with the genera Polaromonas, Methylibium and Methylotenera contributing greatly to this pattern, but the Thiobacillus exhibited the opposite pattern with higher abundances near the terminus (Supplemental Figure 3).

4.4.2. Eukaryote biogeography

Similar to the bacteria, phototrophic eukaryotes had much higher relative abundance at sites high on the glacier compared to sites near the terminus (Figure 4.3). This was the case for both Chlorophyta (green algae) and Ochrophyta (brown algae, mostly Chrysophyceae), which together made 61
up over 50% of 18S sequences high on the glacier, but less than 8% at the terminus. Within the Chlorophyta, the Ulvophyceae decreased along the transect (from top of glacier toward terminus) by the largest magnitude, but Trebouxiophyceae and Chlorophyceae decreased as well (Supplemental Figure 4). The Ulvophyceae were dominated (>99%) by just one OTU, which was in the genus *Pseudendocloniopsis*. 
Figure 4.2: Relative abundance of bacterial phyla across the supraglacial transect

In this plot, each polygon represents a different bacterial phylum. The vertical width of the polygon for any sample location (x-axis) represents the relative abundance of that phylum within the rarefied sample. Polygons are ordered vertically by their correlation coefficient with geographic distance. Cyanobacteria (bottom, red) were the most positively correlated with geographic distance, meaning that they were not abundant near the glacier’s terminus but were more abundant higher on the glacier. Acidobacteria showed the opposite trend, and were more abundant near the terminus and less abundant higher on the glacier.
Similar to the pattern observed for phototrophic cyanobacteria (Figure 4.2), phototrophic eukaryotes (Ochrophyta, Chlorophyta) were much less abundant near the glacier’s terminus than they were high on the glacier. Please see the Figure 4.caption of Figure 4.2 regarding the interpretation of this figure.
Heterotrophic eukaryotes did not show obvious spatial patterns, although in aggregate heterotrophs exhibited the opposite pattern of the phototrophs (above) in terms of relative abundance; although this pattern may be driven solely by the lack of phototrophs near the terminus. Chytridiomycetes were the most abundant fungal clade, and while they were generally more prevalent near the terminus, this pattern had high variance. Within the Chytridiomycetes, the Chytridiales were absent at the terminus but present higher on the glacier, although they never made up more than 5% of any site's community. Pezizomycotina were also abundant, and within them the Leotiomyces were more abundant near the terminus, but the Pezizomyces were more abundant higher on the glacier. Metazoa sequences were also abundant, but like the Fungi they did not appear to be spatially structured. The most common metazoa were either rotifers (Philodina) or hexapods (Collembola).

4.4.3. Archaeal community

Although we detected very few archaeal sequences, they were only observed in samples in the upper half of the transect (not near the terminus). Because we detected so few archaeal 16S sequences, this is not enough information to indicate a biogeographic signal in archaea; we only detected 22 archaeal 16S sequences which clustered into 8 OTUs, out of a total sampling depth of 449,630 sequences. Nevertheless, these few Archaeal phylotypes were mostly unclassified, but the remainder were in the Nitrososphaeraceae, Methanoregulaceae, and Methanobacteriaceae families. The scarcity of archaeal phylotypes was not due to solely to primer bias, because many similar archaea (also in the Nitrososphaeraceae) were successfully amplified and sequenced from other soil samples that were included on the same MiSeq run (data not shown).
Figure 4.4: Semivariograms of bacterial and eukaryote communities

These semivariograms show that for both bacterial communities and eukaryote communities, community composition is highly spatially autocorrelated across the entire transect. If these semivariograms were to plateau, it would indicate that at some distance the difference between communities becomes random. Instead, both semivariograms show a strong spatial signal across the entire transect, indicating that the phylogenetic difference between communities is related to the spatial distance between them, even at our largest scale (~600 meters, Figure 4.1).
4.4.4. Spatial structuring of microbial communities

Beta-diversity analysis revealed that both bacterial and eukaryote communities were strongly spatially structured (Figure 4.4). Our beta-diversity semivariograms did not plateau, which would have indicated a distance at which spatial structuring breaks down (King et al. 2010; Robeson et al. 2011). Said another way, a plateau would indicate that at some distance, the difference between communities becomes random instead of being structured by space. Since we did not observe this pattern in our semivariograms, the microbial communities we sampled across the Middle Fork Toklat Glacier are spatially structured across the entire transect.

Table 4.1: Spatial correlations of abiotic variables

Each variable is correlated with geographic distance from the glacier's terminus. P-values were corrected for multiple hypothesis testing using the false discovery rate (FDR) algorithm. Plots can be seen in Supplementary Figure 5.

<table>
<thead>
<tr>
<th>Variable</th>
<th>r-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO(_x)</td>
<td>-0.879686</td>
<td>4.47*10(^{-7})</td>
</tr>
<tr>
<td>NH(_x)</td>
<td>-0.438332</td>
<td>0.047</td>
</tr>
<tr>
<td>Soil H(_2)O</td>
<td>0.607676</td>
<td>0.005</td>
</tr>
<tr>
<td>DOC</td>
<td>0.511891</td>
<td>0.021</td>
</tr>
<tr>
<td>DON</td>
<td>0.653155</td>
<td>0.003</td>
</tr>
<tr>
<td>m.a.s.l.</td>
<td>0.987865</td>
<td>9.88*10(^{-10})</td>
</tr>
</tbody>
</table>

4.4.5. Biogeochemical structuring of microbial communities

NMDS analysis showed that soil water content, dissolved organic carbon (DOC), dissolved organic nitrogen (DON), and spatial distance all affected the bacterial community composition similarly, but inorganic nitrogen (NO\(_x\) and NH\(_x\)) was found to have an opposite relationship with bacterial beta diversity than other measured variables (Figure 4.5A). The same was true for eukaryote communities, but to an even greater extent. Both NO\(_x\) and NH\(_x\) concentrations were negatively correlated with geographic distance from the glacial terminus (Table 4.1, Supplementary Figure 5),
meaning that NO$_3^-$ concentration is generally high near the glacier’s terminus (dashed line, Figure 4.1), and is low higher up on the glacier. The other biogeochemical parameters we measured were positively correlated with distance from the glacial terminus (Table 4.1, Supplementary Figure 5), meaning that they were low at the terminus and higher on top of the glacier.

Figure 4.5: NMDS analysis of bacterial and eukaryote communities and measured biogeochemical parameters.

NMDS showed that inorganic nitrogen (NO$_3^-$ and NH$_4^+$) were orthogonally related to bacterial and eukaryote beta-diversity, as compared to dissolved organic carbon and nitrogen (DOC and DON). The vector arrows in these plots show how these variables relate to the beta-diversity of samples (black dots) in the NMDS ordination.
4.5. Discussion

Although many studies have examined microbial communities on glacier ice surfaces, relatively few studies have focused on microbes on debris-covered glaciers. Here we present the most comprehensive view of the debris-covered glacier microbiome to-date, across all three domains of life, and at a large enough spatial scale to capture community variation across the Middle Fork Toklat Glacier. We found that both bacterial (Figure 4.2) and eukaryote (Figure 4.3) communities were strongly structured by geographic distance (Figure 4.4), along the entire length of the exposed debris on top of the glacier (Figure 4.1). This finding is evidence against the hypothesis (Darcy et al. 2011) that organisms (e.g. *Polaromonas*) found on this glacier are uniformly distributed dormant propagules from the atmosphere that remain inactive atop the glacier, and are only detected because they landed in an otherwise low-biomass environment. If a uniformly distributed rain of dormant atmospheric propagules were the driving force structuring microbial community composition, there should be no spatial autocorrelation in beta-diversity (Figure 4.4).

Relative abundance of microbial phototrophs contributed greatly to the spatial structuring of microbial communities across the glacier. Cyanobacteria comprised a significant part of bacterial communities at sites high on the glacier, but were almost absent near the terminus (Figure 4.2). A similar pattern was observed for eukaryote phototrophs, but for eukaryotes, phototrophic OTUs made up a much larger proportion of 18S sequences high on the glacier (over half) than bacterial phototrophs did (Figure 4.3). These biogeographic patterns of microbial phototrophs are surprising, in part because the only other spatial study of debris-covered glacier bacteria did not detect significant phototroph (cyanobacteiral) abundance (Franzetti et al. 2013). However, the authors of that study only sequenced bacterial 16S, and it may have been that algal phototrophs were more abundant.

The strong spatial patterning of both 18S and 16S communities across the Middle Fork Toklat Glacier not only increases our understanding of the biogeographic structure of an extreme
environment, but also provides insight into the interactions between biogeography and biogeochemical functioning of this system. Especially relevant are the patterns of inorganic nitrogen availability observed in the present study, which show large increases in availability near the glacier terminus (Table 4.1, Supplemental Figure 5) coinciding with the observed decline in both 16S and 18S phototrophs (Figures 2 and 3). A likely cause for both the decline of phototrophs and the spike in inorganic N availability is that the glacier is undergoing severe disturbance in the form of break-up at the terminus, whereas the upper reaches of the glacier have a more uniform surface (AJK personal observation, 2009). Thus, it is likely that inorganic N is not structuring the microbial community as much as the microbial community is structuring the supply of inorganic N; that is, as disturbance disrupts the functioning of microbial phototrophs (e.g. by burying them), ending inputs of newly photosynthesized C which may result in the accumulation of inorganic N. Similar patterns of N availability linked to disturbance and disruption of microbial N demand have been observed in other ecosystems (Vitousek and Matson 1985; Vitousek 2004).

A further sign of disturbance at the toe of the glacier is the increase in relative abundance of *Thiobacillus* phylotypes. Thiobacilli thrive in environments where reduced sulfur compounds (e.g. pyrite) are exposed to the oxidative world, often due to mining or other disturbances (Male et al. 1997; Skidmore et al. 2005). Previous work on the valley floor near this glacier terminus also indicated a spike in the relative abundance thiobacilli near the glacier toe that corresponded to higher pyrite levels, compared to down-valley sites further from the glacier (Schmidt et al. 2016). Likewise, studies along the down-valley chronosequence going away from the Toklat Glacier also showed higher availability of N near the glacier terminus and a sharp decrease in inorganic N availability further from the glacier were phototrophs were again establishing on the glacial debris left behind by the retreat of the glacier. Thus, the patterns seen on top of the Middle Fork Toklat Glacier are a mirror image of the down valley chronosequence previously described at this site (Schmidt et al. 2016).
This mirroring of biotic and abiotic features between the glacial forefield chronosequence (Adema et al. 2007; Schmidt et al. 2016) and the supraglacial transect we describe here suggests that the surface of the Middle Fork Toklat Glacier is actually a chronosequence. Indeed, the debris atop many other glaciers have been shown to have a strong spatiotemporal distribution. Models of debris-covered glacier surface flow show debris are transported downhill by underlying ice (Konrad and Humphrey 2000), with debris accumulating high on the glacier and moving downward (Potter 1972). Supraglacial debris have even been aged using techniques like $^{14}$C dating and lichenometry; on the mendel glacier (Sierra Nevada Range) debris were young at the glacier’s cirque and older at downhill sites (Konrad and Clark 1998). Likewise, debris at higher sites on Søre Illåbreen and Hellstugubreen glaciers (Scandinavian Range) had no lichens, but lichen density increased down-glacier (Griffey 1978) indicating a substrate age gradient.

Across our supraglacial transect, we observed a strong biogeographic pattern for microbes on top of the glacier (Figures 4 and 5), and similar biogeographic patterns for plants and animals have been observed on the debris-covered Miage glacier (Caccianiga et al. 2011; Gobbi et al. 2011). This has led some ecologists to hypothesize that the spatiotemporal structuring of supraglacial debris leads to spatiotemporal structuring of ecological communities as well (Gobbi et al. 2011; Franzetti et al. 2013), just like in glacier forefield chronosequences. This hypothesis is supported by our observation of strong spatial autocorrelation of microbial communities (Figure 4.4), again, mirroring results from glacial forefield systems (Castle et al. 2016; Nemergut et al. 2016). Specifically, the strong spatial autocorrelation we observe (Figure 4.4) indicates that microbial community structure changes with geographic distance from the glacier’s terminus. This observation, coupled with the strong spatial pattern in microbial phototrophs, matches the predictions made by the supraglacial chronosequence hypothesis. However, it is not possible to disentangle the effects of substrate age from the effects of surface disturbance, since older sample sites near the terminus were more disturbed by glacial melting.
than newer sites higher on the glacier. Indeed, one of the major processes of debris-covered glacier formation is the collapse of lateral moraines (Nakawo et al. 1986; Konrad and Humphrey 2000), which spreads debris over the glacier’s surface. In this case, the glacier’s surface would be a chronosequence of disturbance (Boerner et al. 1998) instead of a simple substrate age chronosequence.

It is unlikely that the Middle Fork Toklat glacier is unique, as other debris-covered glacier surfaces have been shown to be chronosequence-like as well (Caccianiga et al. 2011; Gobbi et al. 2011), just not yet for microbes (Franzetti et al. 2013). Furthermore, the indigenous microbiota of the Middle Fork Toklat Glacier resemble those of other cold and dry environments, especially the cyanobacteria and algae. The most abundant cyanobacterial OTU in our sequence library was phylogenetically similar to cyanobacteria found on other glacial surfaces (Simon et al. 2009; Xiang et al. 2009; Segawa and Takeuchi 2010; Ni et al. 2014) and near glaciers (Nemergut et al. 2007; Michaud et al. 2012a). Cyanobacterial endemicity to cold desert environments has been demonstrated before using other cyanobacterial taxa (Bahl et al. 2011), so it is not surprising that this OTU from the Synechococcophycideae may be native to glacial environments. Certain algae from the Ulvophyceae are also commonly found in cold and dry environments, although the presence of these algae on the Middle Fork Toklat Glacier was already known (Schmidt and Darcy 2015).

Similar to the Ulvophyceae, previous research showed that Polaromonas bacteria were abundant atop the Middle Fork Toklat Glacier (Darcy et al. 2011) and on other glaciers and cryospheric locations as well (Rime et al. 2016; Ambrosini et al. 2016; Kim et al. 2017). With the spatial transect we present here (Figure 4.1), it is now apparent that these bacteria have a biogeographic pattern within the glacier. Polaromonas were abundant high atop the glacier, but were less abundant near the terminus. This pattern is similar to that of the cyanobacteria and algae we detected, suggesting that perhaps similar forces structure the relative abundance of both. If Polaromonas are undergoing selection across the transect, they are not random “propagule rain” appearing in otherwise low-biomass
environments (Darcy et al. 2011; Schmidt et al. 2014) but are instead active members of the glacier’s microbial community.

The existence of a spatially structured chronosequence of microbial communities on the surface of debris-covered glaciers may indicate that soil formation begins before the depositing of the debris at the melting terminus. The buildup of N and the weathering of P from the debris may allow for faster ecological succession after the melting of the glacier at the terminus in cases were glaciers are debris-covered. In contrast, debris-free glaciers may have less nutrient input from the melt-out because microbial activity is almost entirely restricted to cryoconite holes (Bagshaw et al. 2013, 2016). Also, some glacial recession chronosequences have high rates of ecological succession, and nutrient inputs from melt-out are thought to be an important driver (Sattin et al. 2009). Thus, active microbial communities on top of debris-covered glaciers likely influence the course of ecological succession in the foreland after glacial melt (Rime et al. 2016).

Cumulatively, our findings inform a broader understanding of life atop debris-covered glaciers, from a biogeochemical and biogeographic perspective. The strong spatial autocorrelation we observed for both bacteria and eukaryote communities is evidence against the hypothesis that supraglacial communities consist of randomly deposited, non-functioning transients. Instead, the spatial patterns in beta-diversity and in the relative abundances of microbial phototrophs suggests that the surface of the glacier is actually a chronosequence. This conclusion is further supported by our biogeochemical analysis, which revealed that supraglacial biogeochemistry mirrors that of the adjacent glacier forefield chronosequence. The biogeochemical and biogeographic patterns we observed atop the glacier also suggest that ecosystem succession in the glacier forefield gets its start on the glacier's surface. Our findings represent a large but early step toward understanding life on (and near) debris-covered glaciers.
4.6. Acknowledgements

We thank B.-L. Concienne, M. Mitter, M. Harris, m. Concienne, and J. Onorato for help in the field. This work was supported by NSF grants for studying microbial community assembly in disturbed and peri-glacial environments (DEB-1258160 and PLR-1443578) and a Discover Denali Research Fellowship to B.-L. Concienne.

4.7. Data accessibility

Sequence data and metadata from this study are available at FigShare:

https://figshare.com/s/07d6a70e199f99f612e9.
Chapter 5: A Phylogenetic Model Of Community Assembly In The Human Microbiome (and In A Glacial Chronosequence, Too)

By J.L. Darcy, A.D. Washburne, M.S. Robeson, T. Prest, S.K. Schmidt, and D.R. Nemergut

5.1. Abstract

Community assembly is ubiquitous in the human microbiome (and in other biomes as well), but the rules and mechanisms underlying assembly are difficult to parse. Fortunately, microbial communities offer a data-rich opportunity to test mathematical models of assembly, especially from a phylogenetic perspective. We developed a mathematical model that can be used to test hypotheses about how phylogenetic diversity is added to microbial communities over time. We applied our model to time-series, high-throughput sequencing data to estimate the extent to which previously unobserved species appear during community assembly, based on their phylogenetic similarity to species already present. We tested the model outcomes against a neutral model of community assembly, wherein previously unobserved species are added with no regard to the phylogenetic makeup of the existing community. For several recent time-series human-microbiome data sets, the model clearly demonstrates that species closely related to existing species in the community are more likely to be added to the community over time (i.e. underdispersion). Community assembly on the palms had stronger (i.e. more “nepotistic”) patterns of underdispersion than on the tongue or in feces. We also found that community assembly in an infant gut microbiome became less underdispersed (but still different than a neutral model) after the subject began consuming baby formula. Community assembly followed a very similar pattern in a glacial chronosequence microbiome data set as well. All data sets analyzed showed phylogenetic underdispersion, and more importantly, the magnitude of this underdispersion varied by sampling location on the human body. We also provide R code for our
model, which can be used in future studies that test phylogenetic patterns of community assembly against a neutral model, or compare phylogenetic patterns of community assembly between sites, treatments, or individuals.

5.2. Introduction

Every non-sterile surface in the world is in some stage of community assembly, from a forest of tropical trees to a constellation of microbes in a mammalian gut. The communities of organisms inhabiting these environments are dynamic through time, and studying patterns of assembly may shine light on general rules that govern their change. Understanding these rules may aid habitat restoration (Palmer et al. 1997; Temperton 2004), the management of nature reserves with disturbances (Richards et al. 1999; Bengtsson et al. 2000), and ecological theory of phylogenetic signatures in community assembly (O’Dwyer et al. 2012; Goberna et al. 2014). Patterns and rules of community assembly are particularly important in human systems, including the primary succession of microbes on a human host following birth (Koenig et al. 2011), secondary successions following disease, disturbances caused by host lifestyle or antibiotic use (Peterfreund et al. 2012; David et al. 2014; Kennedy et al. 2016), and the natural turnover of microbial communities over time (Caporaso et al. 2011).

However, community assembly is a complex mosaic of overlapping processes (Vellend 2010; Nemergut et al. 2013). We cannot observe community assembly processes directly (especially for microbes), but instead we can observe their effects as empirical patterns of community change (Gerhold et al. 2015). These patterns can be accessed via time-series studies, which seek to understand how a community of microorganisms changes over time (Caporaso et al. 2011; Koenig et al. 2011). Community and metacommunity dynamics can be understood from the theory of island biogeography in which the fundamental processes driving community change are arrivals, changes in abundance, and extirpation (MacArthur and Wilson 1967).
The arrival of species in a community signifies the arrival of new traits to a community, and thus provides a window into the assembly of ecosystem function. In particular, arriving species that have not previously arrived (first-time arrivals) constitute new genetic diversity being added to a community. Any organism of interest in a human microbiome data set, from the pathogenic to the probiotic, will at some point be a first-time arrival, and the order in which these organisms arrive in the community is determined by community assembly processes (Nemergut et al. 2013). Predicting which lineages of organisms can arrive and establish in a given environment can have far-reaching implications for ecosystem remediation and management, especially in microbial communities where the functional traits of many microbes are still largely unknown.

In this paper we investigate the phylogenetic signal of arrival order for microbial communities undergoing primary succession and fluctuations in community composition. Our analysis extends previous phylogenetic community ecology frameworks (e.g. (Webb 2000; Webb et al. 2002; Letcher et al. 2012)) by parameterizing the probability of a species arriving for the first time as a monotonic function of its phylogenetic distance to members of the community that have already arrived. Our general model of phylogenetically-mediated arrival ranges from the standard neutral model in which all lineages are added to the community with the same probability, to an underdispersed model where close relatives are more likely to be added to the community than distant relatives, and an opposite, over-dispersed model.

5.3. Methods

Our modeling approach determines to what extent the arrival order of new species into a community can be predicted by the phylogenetic distance of the arriving species compared to the set of all species that have already arrived. Thus, our methods section is broken into five parts. First, we discuss our statistical model of phylogenetically-mediated first-time arrival. Second, we describe our
simulations, where we use the statistical model to resample communities. Third, we describe the parameter estimation approach we use, which allows for comparison of the phylogenetic signal of first-time arrivals across data sets. Fourth, we describe our hypothesis testing, where we tested these patterns against the null hypothesis of no phylogenetic signal in first-time arrivals. Fifth, we apply our statistical model to real data sets and explain the bioinformatic and technical details of our analysis, and then in the sixth section we make our code available for others to replicate our analysis or perform similar analyses.

5.3.1. Statistical model

At any point in time, a local community is composed of many species, and other species are not present but are available to be added. Species not yet added (“species pool”) represent organisms present within the metacommunity but not the local community. Our model parameterizes the probability of species arriving in a local community for the first time, based on their phylogenetic distances from species that have already arrived.

In a species-neutral model of community assembly, each species $i$ in the species pool has the same probability of arrival at time $t$, irrespective of how different it is from species that are already present at time $t$. Thus, the neutral model for first-time arrivals is a random draw without replacement of species from the species pool. We extend the species-neutral model by modeling the probability $p_{i}$ of species being observed for the first time at time $t$ as

\[ p_{i,t} = \frac{d_{i,t}^{D}}{\sum d_{i,t}^{D}} \]

*Equation 5.1: Statistical model for phylogenetic community assembly*
where $d_{it}$ is the phylogenetic distance from species $i$ to its closest relative that has already been observed prior to time point $t$, and $D$ is a dispersion parameter.

When $D = 0$, our model functions as a neutral model; all species have the same probability of arriving in the community for the first time, since $p_{it}$ is the same for every species. When $D < 0$, $p_{it}$ decreases with $d_{it}$, meaning that species from the species pool have higher probabilities of arriving when they are more closely related to species that have already been observed in the local community (underdispersion; phylogenetically constrained). When $D > 0$, the opposite is true (overdispersion; phylogenetically divergent). Our hypothesis testing and parameter estimation focus on the dispersion parameter, $D$.

5.3.2. Simulations

Our analysis of a data set relies on re-constructing that data set via simulation of our statistical model, allowing for hypothesis testing and parameter estimation. Using the empirical data as a starting point, we simulate many surrogate data sets with $D$ values ranging from $D<0$ (underdispersed) to $D=0$ (neutral) to $D>0$ (overdispersed), in order to determine whether the empirical datasets differ from a null hypothesis of neutral arrival, and to estimate the empirical phylogenetic dispersion of first-time arrival order.

We start each surrogate data set with the same species present in the first sample of its corresponding empirical data set. Then, surrogate data sets are constructed forward in time by randomly drawing $M_t$ new arrivals from the species pool, where the probabilities of species arriving at any given time is given by Equation 5.1, and $M_t$ is the number of new arrivals in the empirical dataset from times $t-1$ to $t$. The species pool is updated to exclude those species drawn at previous time points, and the newly sampled species are recorded. This process is repeated for all time points. Surrogate data sets are produced for many different $D$ values, ranging from underdispersed to overdispersed models.
In the analyses we present here, we performed 500 simulations (as described above) for each data set analyzed.

5.3.3. Parameter Estimation

Our main goal is to estimate the empirical dispersion parameter $D$ (Equation 5.1), which quantifies the degree to which first-time arrivals are phylogenetically underdispersed ($D>0$), neutral ($D=0$), or overdispersed ($D>0$). To this end, we use Faith's phylodiversity (Faith 1992) to compare each of the 500 surrogate data sets (described above) to the empirical data set. Phylodiversity is the sum of branch-lengths on a phylogenetic tree for a set of species, so phylodiversity of a set of highly related species is low (i.e. phylogenetically constrained) because there are no long branch lengths in the tree, but phylodiversity is higher (phylogenetically divergent) for a set of more distantly related species (Faith 1992). If $D\neq 0$, then species are preferentially added if they have relatively low ($D<0$) or relatively high ($D>0$) phylogenetic distance to the resident community ($d_{it}$, Equation 5.1), yielding accumulations of total phylodiversity that are relatively slow ($D<0$) or relatively fast ($D>0$) compared to the neutral model (Figure 5.1). In other words, at any time point $t$, the phylogenetic diversity of species which have already been observed is $PD_t$, and the extent to which $PD_t$ accelerates or decelerates over a sampling effort depends on $D$. Because of this, we can estimate $D$ by comparing the empirical phylodiversity curve to our surrogate phylodiversity curves, which have known $D$ values (Figure 5.1).

For the comparison of an empirical phylodiversity accumulation curve to curves for corresponding surrogate data sets, we re-scale the absolute phylodiversity accumulation curves, $PD_t$, to measure the relative phylodiversity accumulation. In particular, we used relative phylodiversity accumulation curves,
Equation 5.2: Equation to re-scale phylodiversity accumulation curves

\[ Y_t = \frac{PD_t - PD_0}{PD_{t_{\text{max}}} - PD_0} \]

where \( PD_t \) is the accumulated phylodiversity at time point \( t \), \( PD_0 \) is the phylodiversity at the initial time point, and \( PD_{t_{\text{max}}} \) is the phylodiversity at the final time point. We calculate the KS distance (Massey 1951) from each scaled surrogate phylodiversity accumulation \( Y_t^D \) to the empirical phylodiversity accumulation \( Y_t^{\text{emp}} \), and minimize this value to obtain an estimate of \( D \) for the empirical data set (Figure 5.2). Minimization was performed using the R package SiZeR (Sonderegger 2012), which fit a segmented regression (“broken stick”) model to our KS distances, producing an estimate for the empirical \( D \), as well as 95% confidence intervals for that estimate. Segmented regression was used because plots of KS distances between \( Y_t^D \) and \( Y_t^{\text{emp}} \) appeared linear (Figure 5.2).

5.3.4. Hypothesis Testing

We test against the null hypothesis \( D=0 \) by comparing the estimate for the empirical \( D \) value and its 95% confidence interval to 0. If 0 is not within the 95% confidence interval, we reject the null hypothesis. Evidence of either overdispersion (\( D>0 \)) or underdispersion (\( D<0 \)) allows us to reject the null hypothesis. An example of an empirical \( D \) estimate (black line), 95% confidence intervals for that estimate (dashed lines) and the null hypothesis (red line) can be seen in Figure 5.2.

5.3.5. Analysis

16S rDNA sequencing data from Koenig et al. (Koenig et al. 2011) were downloaded from the NCBI Short Read Archive (SRA) website (http://www.ncbi.nlm.nih.gov/sra) along with their metadata.
These data are a time-series of fecal microbiota from an infant subject, over the first 500 days of life. QIIME (Caporaso et al. 2010b) was used to trim primer regions from these data. Our approach depends on closed reference OTU picking against a chimera-checked reference database (DeSantis et al. 2006). Any query sequence that does not match the reference database, has sequencing errors, or is chimeric, will be discarded by this process. However, using the closed reference approach means that we need to account for any disparities when matching short amplicon reads to a reference database that has been pre-clustered across the full length of the gene under study (e.g. 16S rRNA). That is, over the amplicon region in question, a query sequence can be equally similar to multiple representative sequences within a reference database pre-clustered at 97% over the full length of the gene. Alternatively, sequences can be up to 94% dissimilar from one another yet still be merged into one reference OTU. In either case the result is potentially unstable OTU assignments (Westcott and Schloss 2015). We circumvent this issue by using the UTAX protocol of usearch (v8.1.1861)(Edgar 2010), meaning we extracted the specific 16S rDNA amplicon region of interest from the GreenGenes reference database (DeSantis et al. 2006). Then all of these extracted regions were dereplicated to 100% sequence identity and then clustered via swarm2 (Mahé et al. 2015). Exhaustive searches via uclust were used to pick the best matching closed reference swarm OTUs. This enabled us to make robust use of the chimera-checked reference phylogeny provided with the GreenGenes reference database (DeSantis et al. 2006) for use in calculating phylogenetic diversity, and pairwise phylogenetic distance between OTUs (branch-length distance). Chloroplast, mitochondrial, and archaeal OTUs were identified using the GreenGenes taxonomy, and were discarded.

The resulting OTU table was rarefied to 1000 sequences per sample, and samples with fewer sequences were excluded. The last five time points were excluded as well because they were sampled at a much lower temporal resolution. This left 52 time points spread over the first 469 days of the infant subject's life. The OTU table was then split into two OTU tables, one for time points before the infant
started using baby formula, and one for those after. The “pre-formula” OTU table contained ages 4
days through 146, and the “post-formula” OTU table contained ages 161 days through 469 days. Each
OTU table was used to run our model as described above using 500 $D$ values (Equation 5.1), ranging
from underdispersed ($D=-1$) to overdispersed ($D=0.5$), using OTUs in lieu of species. The 500
resulting phylodiversity accumulation curves were compared to the empirical curve using the KS-
statistic (Massey 1951) as described above. Minimization of the resulting KS-statistic values was used
to determine which value $D$ best fit the data using the R package SiZer (Sonderegger 2012), which fit a
segmented regression (“broken stick”) model to the KS distances. 1000 bootstrap replicates of the
segmented regression generated 95% confidence intervals for the $D$ parameter estimates.

Sequence data from Caporaso et al. (Caporaso et al. 2011) were downloaded from the MG-
RAST database (http://metagenomics.anl.gov/) along with associated metadata, and as above. These
data are time-series data from one male subject and one female subject, over a period of several
hundred days, across multiple sample sites (feces, both palms, tongue). Time points were excluded
which did not have sequence data for each of the 8 environments (male and female by L hand, R hand,
mouth, feces), and rarefied to 5000 sequences per sample. This left 107 time-points, ranging from day 1
to day 185. Analysis for each environment within the data set was carried out as described above. These
data were also processed using the Unoise3 OTU-picking strategy (Edgar 2016). Since this is a de-novo
OTU clustering approach, a phylogenetic tree was made to accompany the data using PyNast
(Caporaso et al. 2010a) to align sequences and FastTree (Price et al. 2010) to build a phylogenetic tree.

Sequence data from the Toklat Glacier Chronosequence dataset from Nemergut et al. (Nemergut
et al. 2016) were obtained from the authors along with associated metadata. Minimal processing was
required because these data had already been filtered to exclude mitochondria and chloroplast
sequences, and OTUs had already been picked against the GreenGenes reference database (DeSantis et
al. 2006). Since the chronosequence is a space-for-time substitution (Walker et al. 2010), we used
geographic distance of samples from the glacier's terminus in lieu of time. Samples were excluded that had significant spatial gaps between pairs, leaving a data set of 25 samples that were fairly evenly spaced. Otherwise analysis was performed as above.

5.3.6. Code and data accessibility

R code and data to replicate our analysis, or to perform a similar analysis on other data, are available at (redacted until publication – email me).

5.4. Results

Figure 5.1 shows model simulations demonstrating how varying $D$ affects the model output, and compares it to an actual data set (Caporaso et al. 2011). Running our model (Equation 5.1) at a low $D$ value produced curves where phylodiversity accumulated slowly, compared to curves where $D$ is closer to 0 (i.e. underdispersion of first-time arrival order). The opposite is true as well, and with a high $D$ value, phylodiversity is added more quickly than the neutral model (i.e. overdispersion of first-time arrival order). These results show that the $D$ parameter in our model does in fact correspond to over- and underdispersion relative to the neutral model.

5.4.1. Results from “Moving Pictures” data

All eight data sets from Caporaso et al. (Caporaso et al. 2011) (feces, left hand, right hand, and tongue for one male and one female subject) showed phylogenetic underdispersion of first-time arrival order, since their $D$ estimates were negative (Figure 5.3) and their 95% confidence intervals did not overlap with 0 (neutral model). This means that when an OTU was observed for the first time in one of these communities, it was more likely to be phylogenetically similar to an OTU that had previously arrived in that community. For both the male and female subject, $D$ estimates were lowest in the palms,
and 95% confidence intervals of the palm $D$ estimates did not overlap with those of tongue or feces communities. In the female subject, the $D$ estimate for the feces was much lower than it was in the male subject, and was closer to that of the palm communities, but the 95% confidence intervals still did not overlap. Similar results were obtained when the Unoise pipeline (Edgar 2016) was used, except fecal environments had similar $D$ estimates to skin environments, while tongue $D$ estimates were much lower as above. All eight environments were still significantly underdispersed when the Unoise data were used.

5.4.2. Results from infant gut data

The phylodiversity accumulation pattern in the infant gut microbiome (Koenig et al. 2011) showed a sharp increase in phylodiversity after day 161 (Figure 5.4), the same date that the infant began consuming baby formula. This suggests that baby formula changed the phylogenetic colonization patterns of the developing infant gut. We analyzed this data set as two separate time-series, one before formula use and one after, and both had negative $D$ estimates with 95% confidence intervals that did not overlap with 0 (neutral model). The pre-formula time-series was more underdispersed than the post-formula time-series, and the 95% confidence intervals for the $D$ parameter estimates did not overlap with each other, indicating a change in the dispersion of arrival order post-disturbance (Figure 5.5). Because the post-formula $D$ estimate is higher than the pre-formula $D$ estimate, arrivals were more overdispersed after the subject began consuming formula.

5.4.3. Results from the Toklat Glacier Chronosequence

Phylodiversity accumulation in the Toklat Glacier's forefield chronosequence showed patterns highly similar to those observed in the human microbiome datasets (Figure 5.6). As with the others, this data set showed significant underdispersion as compared to the neutral model (Figure 5.6).
Figure 5.1: Phylodiversity accumulation in the female left palm

The dashed line is the empirical phylodiversity accumulation curve, meaning that each time point's phylodiversity value is the cumulative sum of all branch lengths observed up to that time point (Faith 1992). This curve has been scaled to the 0-1 scale using Equation 5.2. The colored lines are 500 surrogate (simulated) phylodiversity curves with different $D$ values (Equation 5.1). These lines are color coded by their $D$ value (see key at right). The empirical model (dashed) is below the neutral model (purple), signifying underdispersion in the order of first-time arrivals.
Figure 5.2: Model fitting and hypothesis testing for the female left palm

Each surrogate (simulated) curve from Figure 5.1 is compared to the empirical curve using the KS statistic, with a lower KS statistic indicating a closer fit. Each dot on this Figure 5. is colored according to the surrogate curve’s $D$ value (Equation 5.1), and corresponds to its line color on Figure 5.1. The black solid vertical line is the estimate for the best-fit model, and the vertical dashed lines are the 95% confidence intervals for that estimate. The red solid vertical line is placed at $D=0$, which is the neutral model (null hypothesis). Note that the confidence intervals of the estimate do not overlap with the neutral model.
All environments analyzed from the Caporaso et al. (2011) data set exhibited underdispersion. For both the female (left) and male (right) subjects, the hands had lower $D$ estimates than the tongue or feces (note the negative Y axis). This pattern was robust in both cases, but the 95% confidence intervals of the female feces and left palm were close, but did not overlap.
Figure 5.4: Empirical phylodiversity accumulation in the infant gut microbiome.

Phylodiversity increases sharply after day 161 of the infant’s life, then plateaus. This timing coincides with the day the subject began consuming baby formula. As the curve saturates, new OTUs added to the community were phylogenetically redundant, adding little to the community’s cumulative phylodiversity because close relatives had already been observed in the community. Non-redundant OTUs join the community after the subject began consuming baby formula. The curve is scaled using Equation 5.2. The times of sampling points are shown as vertical blue lines below the X-axis.
Figure 5.5: Dispersion parameter (D) estimates in the infant gut, pre- and post-formula

Formula use began on day 161, thus the first 160 days of the subject's life were analyzed separately. Community assembly was underdispersed both pre- and post-formula, but moreso pre-formula.
Figure 5.6: Phylodiversity accumulation across the Toklat Glacier’s chronosequence

The other data sets analyzed here are strictly temporally sampled, but this data set is a glacial chronosequence and its interpretation as a time-series is slightly more convoluted. As a glacier retreats, it exposes barren soil beneath it. This soil ages over time, and a time-series of soil ages can be collected by sampling different ages of soil exposed by the glacier (Walker et al. 2010). We include this very different data set and its analysis to show that our model can be used for microbiological data sets outside the human microbiome. Glacial chronosequences are very well studied systems, and the selective forces at play structuring microbial communities over time are well understood. From the spatio/temporal autocorrelation that glacial chronosequences often exhibit (Hodkinson et al. 2003), it is expected that our model shows phylogenetic underdispersion in such a data set.
5.5. Discussion

The world is a mosaic of communities undergoing community assembly following different magnitudes and types of disturbance. If there are patterns or general rules for how diversity accumulates during community assembly or succession, and which taxa have higher probabilities of arriving, these rules can guide habitat restoration projects, help us better design probiotics for colonization, and better exploit disturbance as a tool for managing microbial systems related to human health and disease. We found that assembly during primary succession of the infant gut and during turnover of the microbial communities on the adult palms, tongue, and gut, follows a predictable pattern: OTUs are more likely to arrive for the first time if a close relative has already arrived.

This “nepotistic” pattern in arrivals suggests that traits are driving community assembly in these human environments. In our model, each arrival is a new branch of a phylogenetic tree (alpha diversity accumulation), and these branches are indicators of trait differences in OTUs. Although the phylogenetic tree we use here is constructed with 16S rDNA sequences, such phylogenies have been shown to track genomic differences in bacteria (Zaneveld et al. 2010; Langille et al. 2013). This suggests that the phylodiversity accumulation we model here is also trait accumulation. Indeed, in previous frameworks of phylogenetic over/underdispersion (Webb 2000; Webb et al. 2002; Letcher et al. 2012), non-neutral assembly has been interpreted to mean that traits are under ecological selection (sensu Nemergut et al. (Nemergut et al. 2013)). If traits are not driving community assembly (neutral model; (Hubbell 2001)) or if the traits driving microbial community assembly are largely horizontally transferred between taxa independent of their relatedness (as estimated by a 16S rDNA phylogeny), we would expect no phylogenetic signature in first-time arrivals. Instead, we observed very a strong phylogenetic signal in arrival order for all data sets we analyzed.

However, even if non-neutral assembly is trait-based, selection on traits may not occur within the host environment. An alternative explanation for the underdispersion we observed is that selection
is external to the host environment (i.e. selection occurs within the neighboring species pool from which emigration occurs), causing change in the community entering the host to already be underdispersed. Similarly, phylogenetic dispersion has been unable to distinguish between selection and differences in migration rates (Emerson and Gillespie 2008), so a pre-underdispersed community entering the host is a plausible mechanism for phylogenetic underdispersion of arrivals. But selection of microbial communities within the host has been shown by multiple studies (Peterfreund et al. 2012; David et al. 2014; Kennedy et al. 2016), so we think that trait-based selection within the host is a more likely scenario. Furthermore, trait-based selection has been observed by numerous studies in glacial chronosequences (Hodkinson et al. 2003; Duc et al. 2009; Walker et al. 2010), so our observation of significant phylogenetic underdispersion in the Toklat Glacier chronosequence data set fits this pattern.

The burst of newly observed phylogenetic diversity in the infant gut microbiome after the subject began consuming baby formula (Figure 5.4) indicates that this particular disturbance resulted in a re-assembly of a community that was approaching island-biogeographic equilibrium (MacArthur and Wilson 1967; Jean et al. 2016). The pre-formula community had reached a state where phylogenetic diversity was not being added (Figure 5.4), but post-formula, there was a significant jump in novel phylogenetic diversity arriving in the community. After a few months post-formula, the community again moved toward a state where novel diversity was no longer being added. Our model shows that both of these time series showed underdispersion (Figure 5.5) as did each of the 8 data sets from Caporaso et al. (Caporaso et al. 2011) (Figure 5.3), which lends support to the hypothesis that microbial systems, surveyed through 16S rDNA sequencing, have underdispersed phylodiversity accumulation over time.

If our hypothesis is true, our findings have major implications for the management and restoration of human-associated microbial systems (Shooner et al. 2015), in particular for probiotic development and remediation of pathological microbial communities. Without any other information,
our finding of consistent phylogenetic under-dispersion in arrivals suggests that probiotics for sustained colonization of the human gut should be close relatives to the microbes already present. Indeed, recent research has shown that for fecal transplants, donor strains are able to integrate into the recipient's gut community when a conspecific strain is already present, but novel donor strains are unlikely to successfully integrate into the recipient (Stecher et al. 2010; Li et al. 2016). Different body sites - as we saw with the skin – may have qualitatively similar patterns of underdispersion, yet quantitatively different magnitudes of this effect. Thus the efficacy of an engineered probiotic based on similarity to organisms already present in the community for which it was engineered may largely depend on the body site for which it's intended.

Microbial communities provide a unique opportunity to study community assembly in primary and secondary succession. In addition to standard cross-sectional studies of communities of different ages or successional stages, the short timescales of microbial community dynamics allows longitudinal studies of community assembly over manageable time frames (David et al. 2014; Kennedy et al. 2016). Microbial communities allow large sample sizes, longitudinal studies, and experimental manipulations that enable us to identify general rules and statistical patterns of community assembly. The model presented here makes use of such data, and to facilitate further discovery both in the human microbiome and in other environments, we have made our R code available at FigShare: (Redacted until publication – email me).

5.6. Conclusions

We developed a mathematical model that describes the order in which species arrive during community assembly, and applied it to several human microbiome data sets and also a glacial chronosequence microbiome data set. For each data set, we used our model to estimate the degree to which arrival order was phylogenetically overdispersed, neutral, or underdispersed. All data sets
showed underdispersion that did not overlap with the neutral model. More importantly, the magnitude of underdispersion varied by sampling site on the human body; palm communities had more underdispersed arrival order than tongue or feces communities. We also found that the microbial community of a developing infant gut had less underdispersed arrival order after the infant began consuming baby formula. These findings suggest that different environments have quantitatively different magnitudes of phylogenetic underdispersion. This may have implications for probiotic development, since the success of a probiotic treatment may depend on the presence of a conspecific strain and on the intended body site.

5.7. Acknowledgements

The authors thank J.P. O'Dwyer, P. Sommers, E.M. Gendron, J.S. Clark, and S. Sauce for many helpful discussions.
Chapter 6: Conclusions

Communities of organisms don't spontaneously appear in previously vacant environments. Instead there are patterns and processes that govern which species can be successful in a given environment, which species can successfully reach that environment, which species will randomly disappear, and which new species will develop or have historically developed. The earliest stages of ecosystem development are particularly important to study under the light of community assembly processes, because these early time points set the stage for all future ecological succession, including that of macrobes like plants and animals. In this dissertation, I've shown how biogeochemical and biogeographic factors conspire together to shape microbial communities in early successional environments, through these same community assembly processes.

Most of the analyses I presented here focus on selection as the force shaping microbial communities, and also shaping the functional relationship between microbial communities and their environment. For example, in terms of nutrient limitation, different nutrient addition treatments select for different communities. Those different communities are selected because of their ability to function under the treatment conditions, and that function is differential among treatments because of an interaction between the community and the biogeochemical differential among treatments. This framework is reflected in both the experimental design and subsequent data analysis on supraglacial soils from the Middle Fork Toklat Glacier in Alaska's Denali National Park and Preserve (Chapter 2). In that experiment, I used factorial N and P addition to soil microcosms to test the nutrient limitation of microbial phototrophs in the glacier's supraglacial debris. In barren sites like the surface of the Toklat Glacier, all primary productivity is microbial. Thus, the biogeochemical factors limiting that primary productivity are extremely important to the future of that environment, both in terms of primary
productivity and community composition. I found that both N and P had a significant effect on microbial community structure, and on the primary productivity in that system. But the large effect of P, especially in the growth rate of microbial phototrophs, was not expected in early-successional environments, like the supraglacial debris of the Toklat Glacier. The community shifts I observed for both bacteria and eukaryotes in that experiment showed the power of interaction between stoichiometric nutrient limitation and the corresponding microbial community shifts.

The Toklat Glacier N and P limitation experiment was done with microcosms, so I also carried out an in situ nutrient addition experiment at the terminus of the retreating Puca Glacier in Peru's Cordillera Vilcanota (Chapter 3). Similar to the Toklat site, the barren soil exposed by the Puca Glacier's retreat is in the earliest stages of ecosystem succession. This experiment was conducted over the first 6 years of succession, and primary productivity was measured using a variety of traditional ecological methods and modern high-tech methods. I employed the point-intercept method to measure percent cover of microbial phototrophs, which has been used by ecologists for over a century. But I also used multi-spectral imaging so that a computer could calculate percent cover instead. On the smaller, microbial scale I used chlorophyll autofluorescence to quantify the extent to which microbial phototrophs are nutrient limited by N or P. My results showed that both plants and microbes are strongly limited by P at this site, mirroring the strong effect of P on top of the Toklat Glacier. High-throughput 16S rDNA sequencing revealed that the structure and function of microbial communities also changed as a result of nutrient addition treatments, with N-fixing cyanobacteria flourishing when P-limitation was alleviated. My thorough measurement of P-limitation at the Puca Glacier site, combined with the results from the Toklat Glacier microcosm experiment, are evidence that primary succession in alpine environments may not occur under the dogmatic notion of N-limitation during primary succession.

The biogeochemical parameters underlying selection in these cryospheric sites also have a
strong biogeographic component, as illustrated by my study of microbial community diversity in a transect of sites across the top of the Toklat Glacier (Chapter 4). In this chapter I tested against the neutral hypothesis that communities of microbes across the top of the glacier were randomly distributed; a hypothesis inheriting from the stochastic nature of dispersal as a community assembly process. Instead, I found that microbial communities in the supraglacial debris were highly structured by geographic space. This biogeographic pattern in microbial community composition was present in both bacteria and microbial eukaryotes, and in both cases, the biogeographic signal was strongest in microbial phototrophs. Together with my spatial analysis of biogeochemical variables, these results suggested, suprisingly, that the surface of the Toklat Glacier is a hidden chronosequence. In this sense, the selection of microbial communities atop the glacier has a temporal as well as biogeographic component – sites near the glacier's terminus are likely older versions of sites higher up on the glacier, which explains the strong spatial pattern and the increase in the relative abundance of microbial phototrophs along the transect.

In addition to the historical legacy of succession that is likely recorded in the microbial communities atop the Toklat Glacier, selection of species within a community has an evolutionary legacy of fitness differences that is described in the phylogeny of member species. A phylogeny is a history of speciation, and speciation is a difficult community assembly process to observe directly because of the large time scales involved in evolution, even for microbes. Instead of observing speciation driving microbial community assembly experimentally, I have integrated speciation into the chapters of this dissertation indirectly through phylogenetic metrics. But I also explicitly used the relationships among species to model microbial community assembly (Chapter 5). The model I developed can be used to estimate the extent to which microbial community assembly is nepotistic ("phylogenetically underdispersed") – meaning that over time, species are more likely to join a community if a closely-related species is already there. Using an early-successional time-series data set
from an infant gut, I show that the community's recruitment of species is highly nepotistic, but this pattern is changed slightly when the biochemical nutrient regime of that environment is altered by the infant beginning to consume milk. I also applied this model to time-series data from adult human-microbiomes, showing that different environments have different degrees of preference for closely-related species. But humans are not the only environments in which time significantly structures microbial communities. In fact, that happens in all environments, everywhere. But perhaps the most famous studies of ecological time-series are glacial forefield chronosequences, like the one left behind by the retreat of the Toklat Glacier. With my model, I show that the accumulation of phylogenetic diversity across this glacial-retreat chronosequence follows the same nepotistic patterns seen in the human microbiome time-series studies.

The research I have presented here provides a thorough look at the biogeochemical and biogeographic drivers of microbial community assembly, especially in the most important, early stages of ecological succession. I've shown how nutrient limitation in early-successional alpine environments may not proceed as previously suspected, and shown how microbial community assembly is inextricably linked to this result (Chapters 2 and 3). I have also shown how geographic space and soil biogeochemistry can be linked together in suprising ways, even in barren, extreme environments that have previously thought of as dumping grounds for random transient microbes (Chapter 4). Finally, I have shown how the shared evolutionary history of species can be used to understand the patterns of change in communities over time, in early-successional sites in humans, and early-successional sites in alpine environments.
References


Franzetti A, Tatangelo V, Gandolfi I, et al (2013) Bacterial community structure on two alpine debris-
covered glaciers and biogeography of Polaromonas phylotypes. ISME J 7:1483–92. doi: 10.1038/ismej.2013.48


Iahs Publ 255–266.


Matthews JA (1992) The ecology of recently deglaciated terrain: A geoeological approach to glacier
forelands and primary succession.


Nilsson HR, Ryberg M, Sjökvist E, Abarenkov K (2011) Rethinking taxon sampling in the light of


Sattin SR, Cleveland CC, Hood E, et al (2009) Functional shifts in unvegetated, perhumid, recently-


Chapter 2, Supplemental Figure 1: Mathematical model converting FOV data to point-intercept estimates

Data were initially collected using the FOV method (Schmidt et al. 2012), however FOV results began to saturate at day 38. To remedy this, we also collected point-intercept data for the rest of the experiment. This figure shows the model we created to convert the FOV data from before day 38 to percent cover estimates like those from the point-intercept method. The first panel of the figure shows an in silico microcosm plate with green photosynthetic colonies growing on it. For this plate, the point-intercept method returns a value of 22.5% cover. The second panel shows the relationship between in silico point-intercept values and their corresponding in silico FOV values. This relationship was used to inform our modeling effort, and fit an exponential decay function very well (Equation 1, shown on figure). For the in silico data, the function's coefficient $A=14.981$. The third panel shows the observed data for the period of the experiment when both point-intercept and FOV data were collected. These data fit the exponential decay model very tightly, and even had a similar parameter estimate to the in silico data of $A=13.798$. Note that the X and Y axes of these two panels are identically scaled.
Chapter 4, Supplemental Figure 1: Semivariograms of bacterial and eukaryote communities with no sliding-window overlap

These semivariograms are identical to those shown in the main chapter, except without a sliding window overlap. This means that each distance class (“bin”) in the plot was made from a unique and even set of pairwise comparisons.
Chapter 4, Supplemental Figure 2: Spatial patterns in Cyanobacteria
Chapter 4, Supplemental Figure 3: Spatial patterns in Betaproteobacteria
Chapter 4, Supplemental Figure 4: Spatial Patterns in algae
Chapter 4, Supplemental Figure 5: Spatial correlations of abiotic variables.