MICROBIAL BIODIVERSITY OF THE ATMOSPHERE

by

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A DISSERTATION

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DISSERTATION ABSTRACT

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Microorganisms are critical to the functioning of terrestrial and aquatic ecosystems and may also play a role in the functioning of the atmosphere. However, little is known about the diversity and function of microorganisms in the atmosphere. To investigate the forces driving the assembly of bacterial microbial communities in the atmosphere, I measured temporal variation in bacterial diversity and composition over diurnal and inter-day time scales. Results suggest that bacterial communities in the atmosphere markedly vary over diurnal time scales and are likely structured by inputs from both local terrestrial and long-distance sources. To assess the potential functions of bacteria and fungi in the atmosphere, I characterized total and potentially active communities using both RNA- and DNA- based data. Results suggest there are metabolically active microorganisms in the atmosphere that may affect atmospheric functions including precipitation development and carbon cycling.

This dissertation includes previously published and unpublished co-authored material.

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For Amie – my inspiration, my champion, my sister.

For Steven – with all my love. Always and forever.

TABLE OF CONTENTS

LIST OF FIGURES

Figure Page

LIST OF TABLES

CHAPTER I
INTRODUCTION

Biological diversity patterns in space and time and the processes underlying these patterns are central to ecology and evolutionary biology. Despite the extraordinary abundance, ubiquity, and diversity of microorganisms, the study of microbial biodiversity has lagged behind that of macroorganisms. Furthermore, most of what is known about microbial biodiversity and biogeography is limited to terrestrial and aquatic environments. But microorganisms are ubiquitous and diverse in the atmosphere as well (Després et al., 2012).

Little is known about the biology of the atmosphere relative to aquatic and terrestrial habitats due to technical and conceptual limitations. Technical limitations have significantly hindered the study of the air. Low densities of airborne microorganisms can make even sensitive molecular analyses difficult because of the small amount of biological material present in the air. Due to recent advances in molecular techniques and DNA sequencing capabilities, the technical limitations for study airborne microorganisms have been largely overcome. Conceptual limitations continue to impede the advancement of our understanding of life in the atmosphere. Most of what is known about airborne microorganisms is based on the longstanding assumption that the atmosphere is a conduit for the dispersal of microbes rather than a dynamic habitat where microorganisms actively metabolize and reproduce. Viewing the air as a microbial habitat has the potential to radically expand the scope of biodiversity and biogeography research (Womack, Bohannan, and Green, 2010).

These technical and conceptual limitations have resulted in two fundamental gaps in our understanding of atmospheric microbial ecology. First, although airborne communities are known to be diverse and dynamic in space and time (e.g. Brodie et al., 2007; Maron et al., 2006; Bowers et al., 2012), patterns in this variation and the forces underlying them remain relatively unexplored. Second, the majority of aerobiological research has focused on the role of the atmosphere in the dispersal of terrestrial and aquatic organisms rather than on the possibility that the atmosphere is a microbial habitat.

Thus, very little is known about the potential function of metabolically active microbial communities in the atmosphere.

For my dissertation research, I studied the diversity and potential function of bacterial and fungal communities in the atmosphere. In Chapter II, I review what is known about the function and diversity of microbial communities in the atmosphere and identify gaps in our knowledge of microbial communities in the atmosphere, which I attempted to address in my dissertation research. I conducted a time series study to measure bacterial community diversity and composition over multiple time scales in order to better understand the drivers of bacterial community assembly in the atmosphere. To study the metabolically active portion of microbial communities in the atmosphere, I developed a novel bioaerosol collection method. This allowed me use both RNA- and DNA-based analyses to study fungal communities in the atmosphere over the Amazon rainforest and bacterial communities in the atmosphere at a high elevation research station. The use of RNA-based methods allowed me to study the potentially metabolically active portion of microbial communities in the atmosphere and make inferences about their potential functions.

This dissertation includes previously published and unpublished co-authored material. Chapter II was previously published in *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* with Brendan J. M. Bohannan and Jessica L. Green as co-authors. Chapter III was previously published in *Biogeosciences Discussions* with Paulo E. Artaxo, F. Yoko Ishida, Rebecca C. Mueller, Scott R. Saleska, Kenia T. Wiedemann, Brendan J. M. Bohannan, and Jessica L. Green as co-authors. Chapter V was prepared for submission to *Frontiers in Microbiology* with Brendan J. M. Bohannan, Daniel A. Jaffe, David A. Levin, and Jessica L. Green as co-authors.

CHAPTER II
BIODIVERISTY AND BIOGEOGRAPHY OF THE ATMOSPHERE

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Introduction

As humans, we have an intimate relationship with the air around us. This relationship is by and large unconscious; we breathe in without thinking, move through the eddies and tides of air often without notice. This largely unconscious relationship has led to a delayed appreciation of the air as a biological entity. But air is as alive as soil or water. Not only does it host large macroscopic organisms such as a soaring hawk or a drifting wildflower seed, but it also hosts a wide variety of microorganisms. Hundreds of thousands of individual microbial cells can exist in a cubic meter of air (Burrows et al., 2009b), representing perhaps hundreds of unique taxa (Brodie et al., 2007; Fierer et al., 2008; Bowers et al., 2009). The ecology of these organisms – their diversity, distribution and interactions – is poorly understood. Given our intimate relationship with air, this lack of knowledge comes at a great cost. The life of the air, especially the microbial life, is in constant interaction with human life, both directly as a source of pathogenic and beneficial microbes (Kellogg & Griffin 2006) and indirectly through biological effects on atmospheric processes (Deguillaume et al., 2008). The atmosphere – the layers of air surrounding the Earth – has been described as 'one of the last frontiers of biological exploration on Earth' (Rothschild & Mancinelli 2001).

In this paper, we summarize our current state of knowledge of the ecology of the atmosphere, with an emphasis on the atmosphere's biogeography. Biogeography is the study of patterns in the distribution of life and the processes that underlie these patterns (Lomolino et al., 2006). The air has long been recognized as an important conduit for the movement of organisms from one geographical location to another, and thus is important for the biogeography of land and water. However, it is commonly assumed that the atmosphere is not a habitat in its own right but merely a conveyance for terrestrial and

aquatic life. We review evidence that challenges this assumption and suggests the existence of metabolically active and actively reproducing organisms in the atmosphere. We argue that the atmosphere has a biogeography of its own. Our discussion will focus on microorganisms, the numerically dominant forms of life in the atmosphere.

A Brief History of Aerobiology

Aerobiology has captivated scientists for centuries. Antoni van Leeuwenhoek – commonly known as the founder of microbiology – was one of the first to ask whether the air could be a habitat for microorganisms (Gregory 1971), observing that 'there may be living creatures in the air, which are so small as to escape our sight' (van Leeuwenhoek 1941). Charles Darwin collected airborne dust on the HMS Beagle; this dust was found to contain 17 'different organic forms' of microorganisms (Darwin 1846). Microorganisms have recently been isolated from these samples, demonstrating the ability of some airborne microbes to remain viable after long periods of time (Gorbushina et al., 2007). Darwin's contemporary Louis Pasteur, one of the first to systemically study airborne microorganisms, showed that there are viable bacteria and molds in the air, and that the densities of these organisms vary from location to location (Pasteur 1861). When flight in fixed-wing aircraft became possible in the early 1900s, interest in aerobiology took wing as well. Phytopathlogist Fred C. Meier was perhaps the most enthusiastic proponent of studying microbes at high altitudes. Meier was adept at creating sampling devices and recruiting others to participate in his studies. Charles and Anne Lindbergh collected fungal spore samples for Meier using his 'sky-hook' device during a flight over the Arctic from North Haven, Maine to Copenhagen, Denmark (Meier & Lindbergh 1935). Amelia Earhart took Meier's collection device with her during her attempt to circumnavigate the globe. According to Meier, Earhart's collections, had she not perished during her voyage, would have been an 'invaluable' sample set that spanned the circumference of the globe over massive bodies of water where little sampling had been previously conducted (Montague 1937).

Despite this long and rich history of study, we know very little about the biology of the atmosphere relative to aquatic and terrestrial habitats. Technical limitations have hindered the study of the air. Low densities of microorganisms in the air can make even

sensitive molecular analysis difficult because of the small amount of biological material present in the air. Additionally, the lack of standardization in air collection and sample-processing methods complicate comparisons across studies (Kuske 2006; Peccia & Hernandez 2006). Owing to this lack of methodological standardization, it is unclear whether large differences in density estimates among studies can be attributed to biological variation (reviewed in Peccia & Hernandez 2006; Burrows et al., 2009b).

Conceptual limitations also continue to impede the advancement of our understanding of life in the atmosphere. Most of what is known about airborne microorganisms is based on the assumption that the atmosphere is a conduit for the dispersal of microbes rather than a dynamic habitat where microorganisms actively metabolize and reproduce. Characterizing the role of biological processes in the atmosphere has enormous implications for furthering our understanding in a number of disciplines, from atmospheric chemistry and meteorology to biodiversity and biogeography.

An Atmospheric Habitat for Microorganisms

In the atmosphere, microorganisms may belong to one of three groups—those that are not metabolically active, those that are metabolically active but rarely reproduce and those that are both metabolically active and actively reproducing. Microbes can form inactive propagules (e.g. spores) that disseminate through the atmosphere; however, for these organisms, the atmosphere would not be a 'habitat' in the conventional sense. We suggest that microbes that remain metabolically active in the atmosphere but rarely reproduce are organisms for which the atmosphere serves only as an accidental dispersal mechanism. The last group – both metabolically active and reproducing – can be thought of as 'residents' of the atmosphere. We argue below that, despite past assumptions, residents of the atmosphere are likely to exist, and that the atmosphere can act as a habitat for microbial life. We rely on four sources of information to make these arguments: that large portions of the atmosphere have environmental characteristics consistent with other microbial habitats; that biogeochemical cycling (probably mediated by microbes) occurs in the atmosphere; that at least some microbes found in the atmosphere are metabolically

active; and that residence times of microbes in the atmosphere are long enough that actively reproducing residents could exist.

The atmosphere is not the most extreme microbial habitat

By several measures (pH, temperature, ultraviolet (UV) radiation, resource and water availability), the atmosphere appears to be less extreme than many other microbial habitats. The pH of clouds and rainwater ranges from 3 to 7 (Warneck 1988), a narrower range than that found in many microbial habitats. Microbes have adapted to a much wider range of pH conditions that occur in air, from highly acidic conditions near pH 0 (Schleper et al., 1995) to extremely alkaline conditions up to pH 11 (Jones et al., 1998). Temperature can vary widely throughout the atmosphere, but includes ranges that are suitable for microbial life. In the lower atmosphere (up to 20 km above the Earth's surface), average temperatures decrease with altitude and range from an average of 158C (at sea level) to 2568C (at 20km) (NOAA NASA US Air Force 1976). Many microorganisms are capable of growth at temperatures near and below 08C (Morita 1975), with some communities reported to be metabolically active at temperatures as low as 2188C (Rothschild & Mancinelli 2001).

As with temperature, UV radiation, including DNA-damaging UVB, increases with altitude (Blumthaler et al., 1992). Increased UV radiation at higher altitudes does not necessarily mean that airborne microorganisms are exposed to more UV radiation than their terrestrial counterparts, especially those terrestrial organisms that live at high elevations. Microorganisms in the atmosphere may have a variety of methods for protection from UV radiation in addition to the suite of DNA-repair mechanisms found in all microorganisms (Witkin 1976). It has been suggested that airborne microbes may mitigate levels of UV exposure by being embedded within larger particles with UV-attenuating properties, such as dust, pollen or water droplets (Lighthart 1997; Pearce et al., 2009). Pigments may also protect microbes from UV; the occurrence of pigmented microorganisms in the atmosphere has been correlated with the presence of high levels of solar radiation (Tong & Lighthart 1997). These protective mechanisms are especially important for the survival of organisms at the upper level of the stratosphere, where

levels of mutagenic UVB and UVC are not attenuated by the ozone layer (Smith et al., 1992).

Resource availability in the atmosphere is not necessarily lower than that of many terrestrial or aquatic environments. In clouds and rainwater, concentrations of nutrients (e.g. sulfate and nitrate) reach levels typical of oligotrophic lakes (Pearce et al., 2009). Numerous potential carbon sources are found in both clouds and the atmosphere, including carboxylic acids and alcohols (at concentrations up to 1 mg l21; Pearce et al., 2009) as well as a variety of hydrocarbons (at concentrations up to 4 ng l21; Warneck 1988). In addition to available resources for supporting heterotrophic metabolisms, the air provides a suitable habitat for phototrophs. Pigmented microorganisms found in the atmosphere could be using pigments for photosynthesis. Gene sequences from putative photoautotrophs have been amplified from air samples (Brodie et al., 2007), although to our knowledge, no photoautotrophs have been isolated from the atmosphere.

Microbes in air are metabolically active

Direct in situ evidence of microbial metabolic activity in the atmosphere is rare and limited primarily to approaches that require culturing of microbes in the laboratory. For example, bacteria aerosolized in the laboratory have been shown to be capable of metabolizing glucose (Dimmick et al., 1975) and dividing (Dimmick et al., 1979), suggesting that aerosolization is not a barrier to metabolic activity and reproduction. Sattler et al., (2001) showed that microorganisms incubated in cloud water at 08 have generation times of 3.6 – 19.5 days and take up labeled substrates at rates typical of bacteria in lake water. Microorganisms isolated from cloud water degrade organic acids when cultured in artificial cloud water at 58C and 178C (Vaïtilingom et al., 2010).

These approaches have significant limitations. The environmental conditions microbes are exposed to in clouds (e.g. temperatures of -15°C in super-cooled droplets) cannot be easily reproduced in the laboratory. Culturing aerosolized microbes in the laboratory is likely to impose a bias and may not be representative of the airborne community (Pace 1997). A few studies have avoided these biases by using culture-independent methods for detecting metabolic activity. For example, Hill et al., (2007) observed that 76 per cent of cells in cloud water reduced the dye CTC (5-cyano-2,3ditolyl

tetrazolium), suggesting that this proportion was metabolically active. Measurements of ATP concentrations in cloud water approximate what would be expected for metabolically active cells at the cell density at which they are found in clouds (Amato et al., 2007b), suggesting that microbes can be metabolically active in the atmosphere.

Biogeochemical cycling may occur in the atmosphere

If metabolically active microbes are present in the atmosphere, they should leave chemical 'footprints' of their metabolisms. For example, microbes are intimately involved in biogeochemical transformations, and evidence for such transformations in the atmosphere would support the hypothesis of a resident microbiota. Nitrogen cycling in clouds (including mineralization and nitrification) has been demonstrated (Hill et al., 2007), suggesting the presence of metabolically active microbes. There is some evidence for carbon cycling in clouds, although it is not as clear-cut as the case for nitrogen. For example, bacteria have been isolated from clouds that are able to use organic compounds commonly found in cloud water, including acetate, formate, succinate, L-lactate, formaldehyde and methanol as carbon sources (Amato et al., 2007a; Vaïtilingom et al., 2010). Bacterial end products of these metabolic reactions are also commonly found in cloud water (Amato et al., 2007a), suggesting that these microbes are actively transforming these compounds in clouds. Microbial degradation of organic compounds in the atmosphere may not be limited to cloud aerosols. Bacteria have been collected outside of clouds that can degrade a variety of dicarboxylic acids, producing end products that can be further transformed in the atmosphere (Ariya et al., 2002).

Microbes likely go through multiple generations of growth in the atmosphere

The studies described above support the idea that the atmosphere is an environment capable of supporting resident (i.e. metabolically active and actively reproducing) microbial communities. The environmental stressors imposed by an aerial habitat are not unique, and there are multiple examples of microorganisms that have adapted to live under conditions more extreme than those found in the atmosphere. If environmental conditions are not likely to prevent the presence of resident microbes in the atmosphere (at least in the lower atmosphere), what else might prevent their

presence? It has been suggested (Sattler et al., 2001; Burrows et al., 2009b) that residence time may be the largest limiting factor for resident microbial communities in the atmosphere.

Residence times of microbes probably vary as a function of the size of the particles they are associated with, and as a function of air temperature and relative humidity, among other factors (Williams et al., 2002; Burrows et al., 2009b; Pearce et al., 2009). There have been no direct estimates of microbial residence times in the atmosphere. Currently, the best estimates of residence times are derived from mathematical models of particle transport (Williams et al., 2002; Burrows et al., 2009a). The most recent estimates of residence times for bacteria-sized particles range from 2.2 to 188.1 days (Burrows et al., 2009a). The shorter estimates assume efficient removal of bacteria by rain, ice and snow; the longer estimates do not assume this. Is this sufficient time for a resident microbiota to develop (i.e. to complete one or more generations)? Microbes have been shown to have generation times as short as 20 min, under ideal conditions, but under the conditions present in the atmosphere (cold and nutrient-poor), microbial generation times are likely to be substantially longer. As discussed above, Sattler et al., (2001) measured generation times of microbes in cloud water, and reported generation times of 3.6 – 19.5 days, similar to the generation times of microbes in cold, oligotrophic Arctic lakes (Panzenboeck et al., 2000). These rough estimates of residence and generation times suggest that at least some microbes could be undergoing more than 50 generations of growth while in the atmosphere.

What We Know About Air Biogeography

Viewing the air as a microbial habitat has the potential to radically expand the scope of biodiversity and biogeography research. Biogeography has historically focused on understanding biological variation across the surface of the Earth, and has thus been primarily limited to the study of aquatic and terrestrial ecosystems. Understanding biological variation in aerial ecosystems opens the possibility for a truly unified view of biogeography, one that links biodiversity across each component of the biosphere: the lithosphere, hydrosphere and atmosphere. A preliminary picture of microbial life in aerial ecosystems is just beginning to emerge.

Density patterns for airborne microbes

The vast majority of aerobiology studies report patterns in the density (i.e. concentration) of microorganisms (reviewed in Burrows et al., 2009b). Although the quantification of total, community-level abundance has a rich history in microbiology (Whitman et al., 1998), plant and animal surveys rarely report patterns in community-level abundance. This difference may reflect the reality that researchers commonly document what is most tractable to measure.

Aerobiologists have historically measured the density of culturable microorganisms, reporting the number of colony-forming units per volume of air sampled (CFU m23). Culture-based studies suggest that, as in terrestrial and aquatic systems, microbial densities vary with space, time and environmental conditions in the air. For example, the density of culturable microbes has been shown to decrease with increasing altitude (Fulton 1966), and numerous studies have documented seasonal and diurnal temporal variation in the density of culturable microorganisms in the atmosphere (Bovallius et al., 1978; Lindemann & Upper 1985; Lighthart & Shaffer 1995; Tong & Lighthart 1999, 2000; Fang et al., 2007). Culture techniques, however, reveal only a fraction of microbial life. More recent studies use epifluorescent microscopy and report the total count of microbial cells per volume of air sampled (cells m23). There is some evidence that total cell density counts from microscopy parallel culture-based counts (Tong & Lighthart 1999, 2000); however, few studies have enumerated airborne microbial densities using both approaches, making comparative inferences problematic. The density of microorganisms in the atmosphere has also been estimated using particle transport models (Burrows et al., 2009a). Modeling approaches suggest that atmospheric cell density varies spatially, and that patterns in airborne cell density can occur on a global scale (Figure 1).

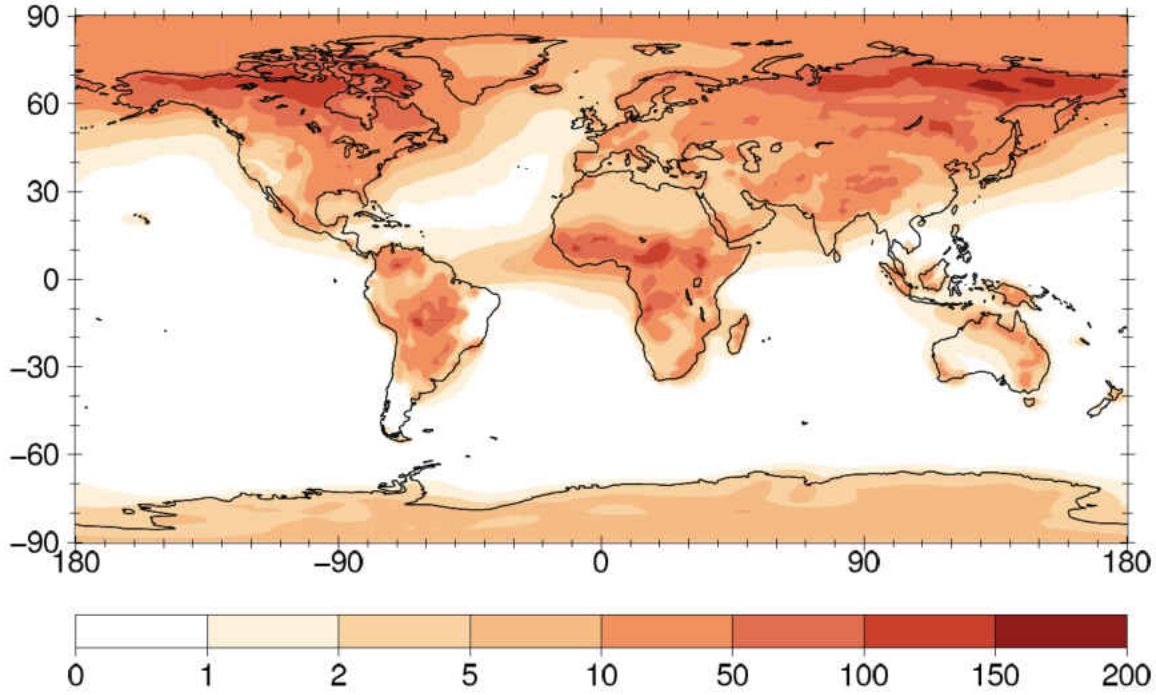

Figure 1. Simulated concentration (10^3 m $^{-3}$) of 1 μm bacteria in near-surface air based on an adjusted general circulation model (Burrows et al., 2009).

Patterns of species distribution in the atmosphere

Although the majority of aerobiology has focused on community-level abundance patterns, culture-based research has provided a foundation for exploring taxa-level patterns. The study of taxa-level distributional patterns, such as a species' geographical range, is central to biogeography. Culture-based work has begun to address fundamental questions about the upper boundary of microbial geographical ranges in the atmosphere. Isolated cultures of the common mold, *Penicillium notatum*, have been collected at an altitude of 77 km, and the bacteria *Micrococcus albus* and *Mycobacterium luteum* at an altitude of 70 km (Imshenetsky et al., 1978; Figure 2). Culture-based studies have also been used to understand the link between atmospheric environmental conditions and the occurrence of particular microbial species. For example, the occurrence of *Micrococcus* has been shown to correlate with the concentration of airborne particulate matter (Mancinelli & Shulls 1978); this might explain why airborne *Micrococcus* species are commonly dominant in urban environments (Fang et al., 2007). Finally, culture-based studies can help identify ubiquitous species that are likely to have large geographical

range sizes. Spore-forming organisms, such as *Bacillus* species and other Gram-positives, tend to dominate culture-dependent surveys of airborne microbial diversity and thus may have large geographical ranges (Mancinelli & Shulls 1978; Lighthart 1997; Fang et al., 2007).

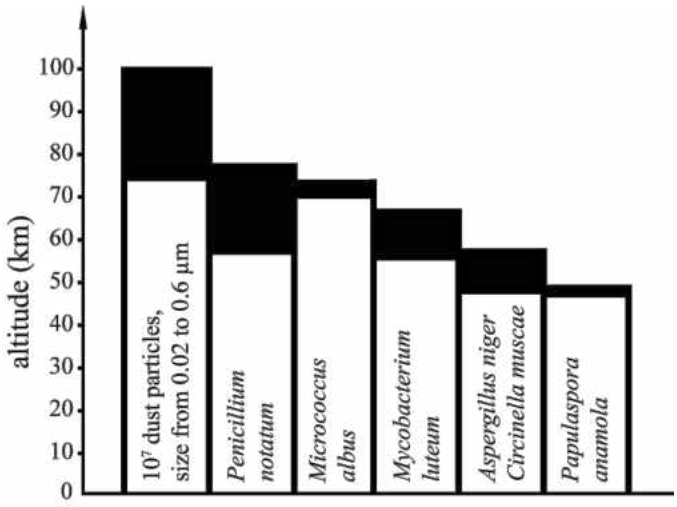

Figure 2. Isolation of microbes from the atmosphere. Shaded portions of the columns correspond to the altitude from which the organisms were sampled by meteorological rocket and isolated in the laboratory. The first column depicts the altitude at which dust particles were sampled and detected (adapted from Imshenetsky et al., 1978).

Airborne microbial community composition

Species do not exist in isolation; they occur together in complex ecological communities. To understand the mechanisms that shape biological variation on Earth, biogeographers study patterns in the composition and diversity of ecological communities in space and time. The development of environmental molecular biology has led to an explosion of investigations on the biodiversity and biogeography of microbial communities in terrestrial and aquatic environments (Green & Bohannan 2006). However, we currently know very little about microbial diversity in the atmosphere. Most studies demonstrating spatio-temporal variability of airborne microbial communities have been limited to culture-dependent methods. Recently, culture-independent molecular approaches have begun to be applied to airborne microbial communities. In contrast to culture-based studies, these molecular-based studies have revealed that airborne

microbial assemblages can be as diverse as those in terrestrial environments, including soils (Maron et al., 2005; Brodie et al., 2007).

The first applications of molecular techniques in aerobiology typically analyzed a single environmental sample (Hughes et al., 2004). More recently, investigators have begun to explore the biogeography of the atmosphere, by comparing microbial communities across multiple samples. Most comparative studies have focused on temporal variation in community structure at the same spatial location, with results ranging from pronounced differences in the daily (Fierer et al., 2008) and seasonal (Fröhlich-Nowoisky et al., 2009) cycles of airborne microbial community structure, to relatively static community structure across time (Bowers et al., 2009; Pearce et al., 2010).

There has been little research on the spatial variation of microbial communities in the atmosphere. Because microbial community composition can shift dramatically over short time scales (Fierer et al., 2008), comparative analyses between spatial locations require statistically controlling for time. Brodie et al., (2007) published the most definitive evidence for spatial variation of airborne microbial communities. In two Texas cities, these investigators pooled air filters in a manner that resulted in a random air sample per city, collected on a weekly basis for 17 weeks. In any given week, the data showed significant differences in community composition between cities; however, temporal and meteorological influences proved to be a greater factor in explaining variability of aerosol bacterial composition. Future research that explores spatial variability in microbial diversity, while accounting for temporal variability, will significantly expand our understanding of atmospheric biogeography.

Comparative Biogeography of Air, Water and Land

The studies reviewed above indicate that microorganisms vary in abundance, distribution and diversity in the atmosphere. Yet, the air remains the least understood environment from a biogeographic perspective. Patterns in the variation of microorganisms in the atmosphere have not been well documented, nor have the processes that underlie these patterns been identified. What might these patterns and processes look like in the atmosphere? Here, we consider defining attributes of land,

water and air environments, and how these attributes may contribute to similar and different biogeography patterns across these domains. Building on a rich history of research in terrestrial and aquatic systems, we explore two patterns that are likely to play an important role in shaping the emerging field of air biogeography: environmental diversity gradients and the existence of biogeographic regions. Ultimately, a more unified understanding of the biosphere will entail comparing and contrasting these patterns across the lithosphere, hydrosphere and atmosphere.

An ocean of air

The vast majority of biogeographic studies to date have focused on terrestrial environments. However, there is increasing interest in the biogeography of marine environments (e.g. DeLong 2009; Tittensor et al., 2010), and marine biogeography may be the best model for what a biogeography of the atmosphere might look like. Landscape-scale analyses of terrestrial environments have often been reduced to two spatial dimensions (with soil depth ignored), simplifying both the measurement of biogeographic patterns and the development of theory to explain these patterns. But marine environments, much like the atmosphere, are unavoidably three-dimensional. A given terrestrial environment (a particular forest, for example) is relatively long-lived and stationary; a given marine environment (e.g. a particular mass of ocean water) can be ephemeral and under constant motion, much like the atmosphere. We suggest that the major environmental gradients in marine environments (light/UV, temperature, nutrients etc.) vary in space and time at rates and scales more similar to the atmosphere than those of terrestrial systems. Given our assumption that atmospheric biogeography may be most similar to marine biogeography, we primarily focus our discussion below on the biogeographic patterns and processes shown to be important in marine systems.

Environmental diversity gradients in the atmosphere

Environmental gradients – geographical gradients in the abiotic and biotic environment – have been used for centuries as a tool to understand the ecological and evolutionary forces that shape biological diversity. Environmental gradients have inspired some of the earliest hypotheses about the origin and spread of life on Earth (Linnaeus

1781). Since Linnaeus, hundreds of studies of community structure along gradients of elevation, latitude and depth have contributed to the foundations of modern ecology and biogeography (Lomolino et al., 2006).

Despite the wealth of plant and animal environmental gradient research, there have been relatively few studies of microbial diversity gradients. The resounding message from recent microbial depth gradient research is that, as with macro-organisms, the structure and composition of microbial communities are significantly influenced by environmental variability. For example, in the ocean, temperature, pressure, light and nutrients vary from sea level to the sea floor. Recent culture-independent studies clearly demonstrate that this environmental variation influences the vertical distribution of oceanic microbial diversity, for example, patterns of taxonomic richness, RNA/DNA ratios, gene copy number and metabolic pathways (DeLong et al., 2006; Johnson et al., 2006; Wilms et al., 2006; De Corte et al., 2008; Brown et al., 2009; Treusch et al., 2009). In the atmosphere, temperature, pressure and moisture vary from sea level to the outermost layer of the atmosphere. Given the strong evidence for shifts in community structure along similar types of environmental gradients, it is parsimonious to assume that microbial biodiversity changes in predictable ways with altitude in aerial systems.

Another widely studied environmental diversity pattern is the increase in numbers of animal and plant species as one travels from the poles towards the tropics. This latitudinal diversity gradient has been recognized for centuries (Mittelbach et al., 2007), and in recent years, this pattern has received heightened attention in the microbial biogeography literature. Although the generality of latitudinal diversity gradients remains equivocal, both molecular-based studies (Fuhrman et al., 2008) and biodiversity models (Barton et al., 2010) have revealed a decrease in species richness with latitude for marine microbes. The most parsimonious explanation for an aerial latitudinal diversity gradient is that the diversity of the atmosphere reflects the diversity of terrestrial and marine systems (i.e. aerial communities are a random sample of metacommunities on the surface of the Earth). However, the possibility exists that the atmosphere has a unique latitudinal diversity pattern. Numerous mechanisms have been proposed to explain latitudinal diversity gradients that may be relevant in the atmosphere, including gradients in energy,

temperature and moisture. To our knowledge, there have been no published studies on a latitudinal diversity gradient for microorganisms in the atmosphere.

Biogeographic regions in the atmosphere

One of the most striking biogeographic patterns at a global scale is the existence of biogeographic regions. The globe is divided into six unique biogeographic regions, areas of the Earth's land surface that contain unique plants and animals (Wallace 1876; Lomolino et al., 2006). These unique biotas are hypothesized to exist because of vicariance, the evolutionary separation of species owing to historic barriers to dispersal. More recently, attempts have been made to define marine biogeographic regions (Lomolino et al., 2006). This is more challenging for several reasons: marine systems have fewer dispersal barriers, are more dynamic in space and time, have a more complicated geological history and are more obviously three dimensional in nature. Nonetheless, there are large-scale differences in marine biotas, even among pelagic organisms. These patterns are believed to be driven by environmental barriers (e.g. warm tropical oceans act as barriers to cold-adapted organisms) and differences in the biogeography of the underlying benthos and/or adjacent coastal regions, differences believed to reflect tectonic and oceanographic history.

Could there be biogeographic regions in the air? The short answer is that we do not know. No studies have attempted to ask whether there are large-scale patterns in the distribution of airborne microorganisms. However, there are reasons to believe that such patterns are possible. There are large-scale patterns in the distribution of masses of air that could conceivably drive large-scale patterns in air organisms. At the largest scale, differential heating of air at the tropics and poles combines with the Earth's rotation to produce six 'cells' of air blanketing the globe (Figure 3). Mixing of air is more frequent within cells than between them, resulting in barriers to air movement, and the potential for vicariance. These mixing barriers often coincide with strong differences in temperature between adjacent cells; thus, environmental barriers could augment physical barriers to dispersal. The major cells of air are relatively stable geographically, and rest on areas of the Earth's surface that are often biogeographically distinct. Thus the input of organisms to each cell could be distinct, and reflect tectonic and/or oceanographic

history, much like the influence of benthic or coastal biogeography on marine pelagic distributions. Together, this suggests that large-scale patterns in the distribution of air organisms are possible. We suggest that a logical starting place for such studies is to ask whether airborne communities are more similar within the six major air cells than among them.

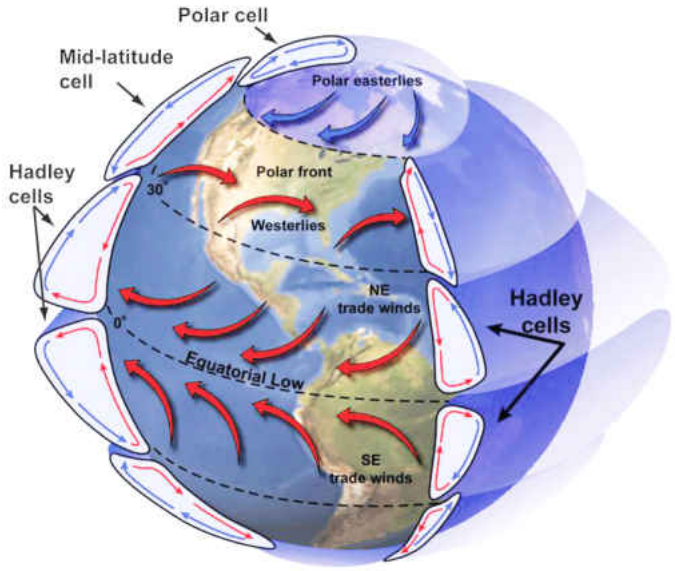

Figure 3. The six major air cells of the Earth's atmosphere (source: NASA).

These major cells of air are restricted latitudinally (i.e. they occur within particular bands of latitude; Figure 3). If these cells do represent biogeographic regions, differences in microbial community structure among these cells could reinforce latitudinal patterns in airborne microbial communities (if such patterns exist; see above). There is also the potential for biogeographic patterns within these major cells. Individual air masses (volumes of air with particular environmental characteristics) continually form, move and disperse within the major air cells. If these masses are sufficiently long-lived to allow for multiple generations of microbial growth, they could harbor unique resident microbial communities, analogous to patterns in the distribution of plant and animal communities in biogeographic 'provinces' within regions. However, to date, no study has attempted to determine whether microbial communities are more similar within particular air masses than among them.

Conclusion

Despite the potential importance of understanding the distribution of life in the air, there are major gaps in our current understanding of the air's biogeography. These gaps include a lack of accurate and comprehensive estimates of many important attributes of life in the air such as estimates of microbial densities and residence times, the proportion of organisms that are metabolically active, generation times of airborne organisms and the structure of airborne microbial communities. The use of new technology and standardization of techniques across studies will allow for a more complete understanding of the distribution of life in the atmosphere.

Most importantly, to move our understanding of life in the air forward, air biologists must learn to think like biogeographers. This includes designing studies that allow the disentangling of spatial and temporal effects on the distribution of life in the air, as well as using our knowledge of atmospheric dynamics to develop testable hypotheses regarding the biogeography of air. We feel that it would be especially fruitful to ask whether airborne communities are more similar within air cells and/or air masses than among them. Finally, studies of the biogeography of land and sea suggest that there are a number of biogeographic patterns that may be universal (Green & Bohannan 2006; Martiny et al., 2006). These include: (i) the distance– decay relationship (how similarity in community composition varies with the spatial, temporal or environmental distance that separates them), (ii) the taxa – area (or taxa – volume) relationship (how taxa richness increases with spatial scale), and (iii) latitudinal diversity patterns. These patterns are a promising starting point for developing a biogeography of the air.

The study of the biogeography of the air is in its infancy, but it has the potential to greatly alter how we think of the distribution of life on Earth. Given the important role the air plays in the dispersal of surface organisms, a more detailed understanding of the distribution of life in the atmosphere will allow us to better understand the distribution of life throughout the globe. It will also allow us to determine whether there are common patterns (and ultimately processes) underlying the distribution of life in the lithosphere, hydrosphere and atmosphere, bringing biologists a step closer to a comprehensive understanding of the distribution of all life.

Bridge to Chapter III

In Chapter II, I reviewed the history of aerobiology and the current state of knowledge about microbes in the atmosphere and I discussed the technical and conceptual limitations that have hindered their study. I presented evidence supporting the idea that the atmosphere could be a habitat for microorganisms and reviewed that is known about spatial and temporal diversity patterns in the atmosphere. I identified major gaps in our understanding of the microbial ecology of the atmosphere and suggested that overcoming these gaps will require studies that allow for the disentangling spatial and temporal effects on the diversity and composition of microbial life in the atmosphere. I advocated for the use of culture-independent DNA sequencing technology to explore the diversity and function of airborne microorganisms *in situ*. In Chapters III - V, I present my research on temporal patterns and potential function of microbial communities in the atmosphere, which was motivated by the information in Chapter II.

CHAPTER III
CHARACTERIZATION OF ACTIVE AND TOTAL FUNGAL COMMUNITIES IN THE ATMOSPHERE OVER THE AMAZON RAINFOREST

This paper was published in *Biogeosciences Discussions* and is in review at *Biogeosciences*. I conceived the experiments, processed the samples, analyzed the data, and wrote the paper. Assistance in experimental design, sampling, data analysis, and writing the manuscript was provided by my co-authors: Paulo E. Artaxo, F. Yoko Ishida, Rebecca C. Mueller, Scott R. Saleska, Kenia T. Wiedemann, Brendan J. M. Bohannan, and Jessica L. Green.

1. Introduction

Fungi are critical to the functioning of terrestrial ecosystems and may also play an important role in the functioning of the atmosphere. Fungi are abundant and ubiquitous in the atmosphere, with an estimated global land surface emission rate of 50 Tg/year for fungal spores alone. Fungal bioaerosols are not only abundant but also affect physical and chemical processes in the atmosphere. Fungal spores and cellular fragments affect precipitation by acting as ice and cloud condensation nuclei (Després et al., 2012; Morris et al., 2013; Pouleur et al., 1992; Richard et al., 1996), and metabolically active fungi sampled from the atmosphere are capable of transforming compounds known to play a major role in atmospheric chemistry, including carboxylic acids (Ariya, 2002; Côté et al., 2008; Vaïtilingom et al., 2013), formaldehyde, and hydrogen peroxide (Vaïtilingom et al., 2013)

The *in situ* function of airborne fungi will depend on the physiological state of fungal cells. Metabolically active vegetative cells have the potential to transform atmospheric compounds and ultimately alter atmospheric chemistry, whereas dormant spores do not. The ice nucleation efficiency of fungal cells also likely depends on their physiological state; vegetative cells derived from potentially active fungi are more efficient ice nucleators than spores. Vegetative forms of *Fusarium* (a filamentous fungi) as well as several lichen fungi have been shown to nucleate ice at temperatures as warm as -1°C (Després et al., 2012) (Figure 14), and ice nucleation by hyphae has been

observed at -2.5°C (Pouleur et al., 1992). In contrast, dormant spores – particularly those with surface hydrophobins – are generally poor ice nucleators. For example, ice nucleation of rust (*Puccinia*) spores requires temperatures lower than -10°C (Morris et al., 2013), and *Penicillium* spores nucleate ice at temperatures of approximately -22°C (Iannone et al., 2011).

Despite its importance, we know relatively little about the physiological state of fungal cells in the atmosphere. Specifically, we know little about the taxonomic composition of metabolically active airborne fungi and how this compares to the composition of the total fungal community. One way to survey the total and active communities is to measure community composition from rDNA (i.e. rRNA genes) and rRNA in ribosomes. Sequencing rDNA provides information about the total community, which includes both active and dormant individuals, whereas rRNA sequences provides information about the potentially active community, because ribosomes are more abundant in active cells than dormant cells (Prosser, 2002). This approach has been applied to study active fungal communities in soils and on decaying plant material (Baldrian et al., 2012; Barnard et al., 2013, 2014; Rajala et al., 2011) but has not been applied to fungal communities in the atmosphere.

Information about the taxonomic composition of airborne fungi that are present in different physiological states could be used to advance atmospheric science in multiple ways. For example, such data could be used to improve estimates of the ice nucleating capacity of fungal bioaerosols. Recent estimates of the ice nucleating capacity of fungal bioaerosols based on culture-based approaches – the abundance of colony forming units (CFUs) – have led some scientists to conclude that atmospheric fungi have a low ice nucleation efficiency (Iannone et al., 2011). However, these culture-based data may be misleading, as the vast majority of fungi require identification using culture-independent approaches (Borneman and Hartin, 2000). Culture-independent identification of active fungal taxa sampled from the atmosphere could be used to direct selective culturing of potentially important fungi to test their ice nucleation efficiencies and their metabolic capabilities in the laboratory.

In this study, we used culture-independent approaches to measure the composition of total and active atmospheric fungal communities *in situ* and a mass-balance model to

aid in the interpretation of our results. Our study system is the atmosphere above the Amazon rainforest canopy. We chose this system because fungal bioaerosols make up a substantial proportion of aerosol particulate matter over the Amazon (Elbert et al., 2007; Heald and Spracklen, 2009) and are estimated to be a dominant force responsible for cloud formation over the Amazon (Pöschl et al., 2010). We used a combined approach of DNA and RNA sequencing to address the following questions: 1) What is the composition of total airborne fungal communities? 2) What is the composition of active airborne fungal communities? 3) What likely drives differences in the composition of the total and active airborne fungal communities? 4) Is the diversity and structure of fungal communities in the atmosphere similar to that found in terrestrial environments?

2. Methods

2.1. Sample collection

Sampling was conducted on the ZF2 K34 flux tower (S -2.60907, W -60.20917, 67 m a.s.l.) in the Reserva Biologica do Cueiras in central Amazonia, about 60 km NNW of Manaus, Brazil. The site is operated by the Instituto Nacional de Pesquisas da Amazonia (INPA) under the Large Scale Biosphere-Atmosphere Experiment in Amazonia (LBA) program (Martin et al., 2010). Tower height is approximately 54 m. Surrounding vegetation is undisturbed, mature, terra firme rainforest, with a leaf area index of 5–6 and an average canopy height of 30 m. Samples were collected at the end of dry season over four days, December 8-11, 2010, from a height of 48m above the forest floor. Aerosol samples were collected using SKC BioSamplers (BioSampler SKC Inc.). Samplers were filled with 20 mL of a water-based preservation solution (LifeGuard Soil Preservation Solution, MO BIO Laboratories, Inc) to prevent DNase and RNase activity and maintain cells in stasis to allow accurate community profiling of the total and active fungal community. Twelve impingers were operated at 12.5 L/min from approximately 9:00 am – 4:00 pm each day. At the end of each day, the sampling liquid from all impingers was pooled and stored at -20C.

2.2. Nucleic acid isolation and cDNA synthesis

Samples were transported on ice to the University of Oregon where the liquid sample from each day was separated into two aliquots, one to be used for DNA extraction and the other for RNA extraction. The divided samples were filtered through 0.22 µm cellulose nitrate filters. DNA was extracted from filters using the MO BIO PowerWater DNA Isolation Kit according to the manufacturer's instructions with a 100 µl elution volume. RNA was extracted from filters using the MO BIO PowerWater RNA Isolation Kit with the following modifications. The DNase steps included in the kit were omitted. RNA was eluted in 50 µl. The extracted RNA was treated with DNase I (RNase-free) (Fermentas International, Inc) according to the manufacturer's instructions. DNase reactions were cleaned (Zymo Research Clean and Concentrate-5) and eluted into 50 µl. cDNA was synthesized from the total RNA extract using the SuperScript II First-Strand Synthesis System (Invitrogen, Life Technologies Corporation) with random hexamers. All RNA was converted into cDNA in six synthesis reactions and one reverse transcriptase negative control reaction.

2.3. Library preparation and sequencing

To increase the concentration of cDNA to levels required for sequencing, we used multiple displacement amplification (GenomiPhi V2, GE Healthcare) according to the protocol described in Gilbert *et al.,* (2010) including second-stand synthesis, amplification, and de-branching of amplification products. The fully de-branched products were sheared by sonication (24 cycles, 30 seconds each) using the Bioruptor sonication system (Diagenode). cDNA fragments were end-repaired (End-It DNA End-Repair Kit, Epicentre Biotechnologies), cleaned and concentrated (Zymo Research Clean and Concentrate-5) and eluted in 40 µl. A-overhangs were added to the end-repaired fragments using Klenow exo(-) (Epicentre Biotechnologies) in a 50 µl reaction. Reaction products were cleaned and concentrated (Zymo Research Clean and Concentrate-5). Standard paired-end, barcoded Illumina adaptors (Table 3) were ligated to the fragments using T4 ligase (Fermantas). Reaction products were cleaned and concentrated (Zymo Research Clean and Concentrate-5) and eluted in 12 µl. To enrich fragments with ligated adaptors, PCR amplification was performed using primers containing the flowcell

adaptor and complementary to the Illumina sequencing primer (Table 3). PCR reactions were performed using Phusion DNA polymerase (New England Biolabs) with 12 µl template, 10 µl 5x HF buffer, 1 µl 10 mM dNTPs, 2 µl 10 mM primer mix, 0,5 µl enzyme and 25.5 µl water for a final reaction volume of 50 µl. PCR cycling conditions were as follows: 30 seconds denaturation at 98°C followed by 18 cycles of 98°C for 30 seconds, 65°C for 30 seconds and 72°C for 30 seconds following by a final extensions at 72°C for 5 minutes. PCR products were size fractionated by gel electrophoresis (2.5%, low-melt agarose). Products in the range of 150-500 bp were excised, and DNA from the excised gel pieces was extracted (QiagenMinElute Gel Extraction) and eluted into 20 µl. DNA was quantitated using a Qubit 2.0 Fluorometer (Invitrogen, Life Technologies Corporation) and combined in equal molar concentrations. Shotgun metatranscriptome libraries were sequenced (150 base pairs, paired-end) on the Illumina HiSeq 2000 (Illumina, Inc.) platform at the University of Oregon Genomics Core Facility. LSU rDNA amplicons were sequenced (250 base pairs, paired-end) on the Illumina MiSeq platform at the Dana-Farber Cancer Institute Molecular Biology Core.

The D1-D2 region of the large subunit (LSU) rRNA gene was targeted using PCR with the primers LR0R (5'-ACCCGCTGAACTTAAGC-3') and LR3 (5'-CCGTGTTTCAAGACGGG-3') (http://sites.biology.duke.edu/fungi/mycolab/primers.htm). LSU amplicon libraries were prepared using a two-stage PCR procedure as described in (Kembel and Mueller, 2014) using unique combinatorial barcodes (Gloor et al., 2010) to identify samples (Table 4).

2.4. Sequence pre-processing

2.4.1. Metatranscriptome

Overlapping paired end reads were aligned and joined using fastq-join (https://code.google.com/p/ea-utils/wiki/FastqJoin). Joined reads and non-overlapping single-end reads were trimmed and filtered using PrinSeq (Schmieder and Edwards, 2011). Sequences <75 bp, > 2% Ns, and/or mean quality score <20 were removed. Sequence artifacts defined as exact duplicates with >5,000 sequences were removed. Sequences in the Dec. 10 sample were primarily artifacts, so this metatranscriptome sample was excluded from further analysis. Putative rRNAs in the remaining sequences

were identified using SortMeRNA (Kopylova et al., 2012) with the non-redundant version of the following databases: rfam 5.8S (version 11.0) (Burge et al., 2013); Unite (November 2011 version) (Kõljalg et al., 2013), and Silva 18S and Silva 28S (Release 115) (Quast et al., 2013). Of 5,165,185 quality-filtered reads, 1,915,994 with an average length of 137.5 bp were identified as putative rRNAs (Table 5).

2.4.2. LSU amplicons

Forward and reverse barcodes were combined to make a 12 bp barcode on the forward read. Only forward reads derived from the LR3 region were used for analysis. This region has been shown to have high species-level resolution even with short read lengths (Liu et al., 2012).

2.4.3. Multi-environment sequences

LSU sequences from four soil studies (Barnard et al., 2013; Kerekes et al., 2013; Penton et al., 2013, 2014) and one phyllosphere study (Kembel and Mueller, 2014) were compared to air samples collected for this study (Table 6). Raw sequence data and associated metadata were downloaded from publically available databases. 12 bp barcodes were added to all sequences to identify each sample in downstream analysis.

2.5. LSU amplicon and metatranscriptome sequence processing

All sequences were processed in QIIME version 1.7 (Caporaso et al., 2010). Briefly, libraries were individually demultiplexed and filtered for quality. Sequences with an average quality score less than 20, shorter than 150 bp and with greater than 2 primer mismatches were discarded. The same parameters were used across all samples except the metatranscriptome rRNAs were a size cut off of greater than 75 bp was used. Sequences from Kembel and Mueller (2014) and Penton *et al.,* (2014) were randomly subsampled to 25% and 60% respectively. Sequences were clustered into operational taxonomic units (OTUs) at 97% sequence similarity using closed reference BLAST (Altschul et al., 1990) against the Ribosomal Database Project Fungal LSU training set 1 (Cole et al., 2014). Taxonomy was assigned to each OTU was that of the most similar representative in the RDP database.

Following sequence processing and quality filtering, a total of 55,414 amplicon and 1,915,994 metatranscriptome LSU sequences generated for this study and 1,577,458 LSU sequences from soil and phyllosphere studies were retained (Table 6). For analyses using only samples from this study, the data were rarefied to 5,300 sequences per sample. For analyses that compare samples in this study to samples from other studies, the data were rarefied to 500 sequences per sample.

2.6. Statistical analyses and data availability

All statistical analyses were conducted in R (R Core Team, 2014) primarily using the vegan (Oksanen et al., 2013) package for ecological statistics and the ggplot2 (Wickham, 2009) package for visualizations. Sequence files and metadata have been deposited in Figshare (http://dx.doi.org/10.6084/m9.figshare.1335851). Data from other studies used for cross environment analyses are available using the databases and identifiers referenced in the respective manuscripts.

2.7. Mass-balance model

We use a global, well-mixed, one-box material-balance model to predict the relative abundances of fungal cells measured as gene copies sampled in the active and total portions of atmospheric bioaerosols. Model description and details are available in Appendix A.

3. Results and Discussion

3.1. Basidiomycota dominate total airborne fungal communities

Measurements of airborne fungi using culture-based methods such as quantifying spore and colony-forming unit counts have been conducted for centuries (Després et al., 2012). In comparison, there have been few culture-independent studies of the fungal composition of atmospheric samples (e.g. Boreson et al., 2004; Bowers et al., 2013; Fierer et al., 2008; Fröhlich-Nowoisky et al., 2009, 2012; Pashley et al., 2012; Yamamoto et al., 2012). Using a culture-independent approach, we found the composition of total airborne fungal communities primarily included taxa belonging to the phyla Ascomycota and Basidiomycota (Figure 4). This result is similar to what is observed in environments

on the Earth's surface (James et al., 2006) and what has been reported in other studies of fungi in the atmosphere (Bowers et al., 2013; Fröhlich-Nowoisky et al., 2009, 2012; Yamamoto et al., 2012).

Basidiomycota dominated the total airborne community in our air samples (mean relative abundance = 90.2±6.9%) (Figure 4). Within the phylum Basidiomycota, Agaricomycetes were the most abundant class in our samples. Agaricomycetes have been previously detected in air samples (Fröhlich-Nowoisky et al., 2012; Woo et al., 2013; Yamamoto et al., 2012) and are common in tropical soils (Tedersoo et al., 2014) and leaf surfaces (Kembel and Mueller, 2014). Within the Agaricomycetes, the most abundant order was the Polyporales (mean = 55.7±2.3%). Polyporales have been detected in culture-independent studies of urban aerosols (Yamamoto et al., 2012) and culturable representatives have been isolated from cloud water (Amato et al., 2007). Given that these are largely saprotrophic (i.e. wood-decay) fungi (Binder et al., 2013; Larsson et al., 2007), it is parsimonious to assume there is a significant local source of Polyporales on the forest floor.

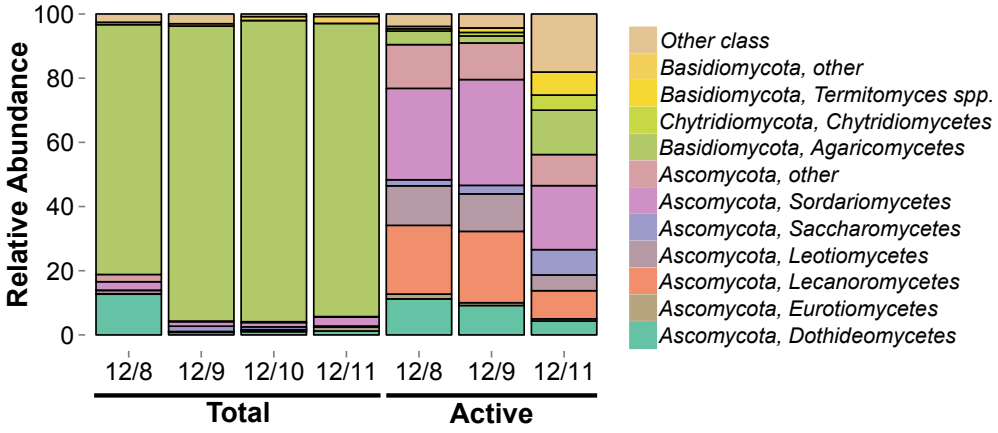

Figure 4. Basidiomycota dominate the total fungal community.

The presence of Agaricomycetes may have implications for atmospheric processes. Ice nucleation efficiency within the Agaricomycetes is variable, with some taxa capable of nucleating ice at temperatures as warm as -17°C (Haga et al., 2014) (Figure 14). These temperatures are warmer than what has been measured for *Penicillium* spores (Iannone et al., 2011) although not as warm as what has been measured for other

spore types (Morris et al., 2013), hyphal fragments (Pouleur et al., 1992), and lichen fungi (Després et al., 2012). Despite the low ice nucleation efficiency of some taxa in this group, given the high abundance of Agaricomycetes over the forest canopy, this group could still have a significant impact on cloud formation and precipitation in the tropics.

3.2. Ascomycota dominate active airborne fungal communities

The composition of total and active fungal communities over the Amazon rainforest canopy significantly differed (ADONIS, $R^2 = 0.342$, $p = 0.029$). The active community in the atmosphere over the forest canopy was dominated by Ascomycota (mean relative abundance = 80.4±20%) (Figure 4). Basidiomycota comprised a smaller fraction of the sampled genes (mean = 7.3±6.8%) with the remainder of identified sequences belonging to the phyla Chytridiomycota and Glomeromycota. This result makes sense in light of the natural histories of many of the Ascomycota, which have single-celled or filamentous vegetative growth forms that are small enough to become aerosolized, while many of the Basidiomycota are too large to be easily aerosolized, other than in the form of metabolically inactive spores.

The most abundant classes of Ascomycota detected were Sordariomycetes (mean = 27.1±6.6%), and Lecanoromycetes (mean = 17.5±7.6%). Sordariomycetes have been detected in air samples (Fröhlich-Nowoisky et al., 2009, 2012; Yamamoto et al., 2012) and have been shown to be abundant on tropical tree leaves (Kembel and Mueller, 2014) and tropical soils (Peay et al., 2013). In most ecosystems, Sordariomycetes are endophytes, pathogens, and saprotophs (Zhang et al., 2007). Xylariales, which includes both endophytes and plant pathogens (Zhang et al., 2007), was the most abundant order within the Sordariomycetes in our samples.

Lecanoromycetes were the second most abundant class of Ascomycota detected over the rainforest canopy. This group has been detected in other culture-independent studies of fungi in the atmosphere (Fröhlich-Nowoisky et al., 2012; Yamamoto et al., 2012). The Lecanoromycetes contain 90% of the lichen-associated fungi (Miadlikowska et al., 2007). Lichens are a symbiosis between a fungus and a photosynthetic partner such as eukaryotic algae or cyanobacteria. Lichens are known to be hardy and may be particularly well-adapted for long distance transport and metabolic activity in the

atmosphere. Lichens are often the dominant life forms in environments that have conditions similar to those found in the atmosphere, including low water (Kranner et al., 2008) and nutrient availability, wide temperature variations, and high UV irradiance (e.g. Solhaug, Gauslaa, Nybakken, & Bilger, 2003) (Onofri et al., 2004).

Another notable trait of lichens is their efficient ice nucleation capacity. Although there have been no investigations specifically on the most abundant lichen species detected in this study, *Physcia stellaris* (mean = 8.3±3.8%) and *Rinodina milvina* (mean = 4.8±3.4%), there have been multiple studies of the ice nucleation efficiency of many other lichen fungi species. Ice nucleation activity of lichens has been measured at temperatures warmer than -8°C, including 13 of 15 taxa tested by Henderson-Begg and colleagues (Henderson-Begg et al., 2009) and 9 of 15 taxa tested by Kieft (Kieft, 1988). These studies have demonstrated that lichens are among the most efficient biological ice nucleators. Therefore, their presence in the atmosphere may have a significant impact on cloud formation and precipitation. This ice nucleation capacity may also enable lichens to control the extent of their dispersal through the atmosphere. It is possible that lichens achieve this by nucleating ice formation, which leads to precipitation and ultimately deposition. This phenomenon has been shown to occur in some phytopathogenic bacteria (Morris et al., 2008, 2010) and potentially fungi as well (Morris et al., 2013).

3.3. Dominance of Basidiomycota in total communities and Ascomycota in active communities is consistent with mass-balance predictions

Our mass balance model (Appendix A) predicted Basidiomycota would dominate the total community because they produce orders of magnitude more spores and have smaller aerodynamic diameters compared to Ascomycota. Consistent with this prediction, the total airborne community was dominated by Basidiomycota in our air samples (mean relative abundance = 90.2±6.9%) (Figure 4). There have been some empirical studies reporting the opposite pattern, with a higher relative abundance of Ascomycota compared to Basidiomycota (Bowers et al., 2013; Fierer et al., 2008; Pashley et al., 2012). There has been one study focused on airborne fungal communities in the Amazon Basin (Fröhlich-Nowoisky et al., 2012). Although the site of this study was the atmosphere

above a rural pasture (versus a tropical rainforest, as in our study) these investigators also found that Basidiomycota dominate airborne fungal communities

Our mass-balance model explains the differences in composition between the total and active air communities. However, some of the differences we observed may be partially attributable to the use of different approaches in characterizing the total and active communities. In this study, the total community was characterized by PCR-based amplification and sequencing of LSU genes, whereas the active community was characterized through random sequencing of all the RNA present in the samples. Shotgun metatranscriptome sequencing and PCR-based community characterization approaches each have their own biases (Hong et al., 2009; Morgan et al., 2010). Our data suggest that the selection of LSU primers led to biased results. For example, the high relative abundance of lichen fungi in the active community was unexpected because this group was not present in the total community and has only been detected in low abundance in other PCR-based studies of fungi in the atmosphere (Fröhlich-Nowoisky et al., 2012). We tested the LR0R-LR3 primer pair using the SILVA TestPrime tool (Klindworth et al., 2013) and found coverage of the Lecanoromycetes with this primer pair was 71.4%. Importantly, the order Teloschistales, which contains the most abundant species in the active community, would not be detected with this primer pair. However, the general pattern that Ascomycota were much less abundant than Basidiomycota in the total community is not likely due to primer bias as overage of the phylum Ascomycota by the LR0R-LR3 primer pair is 85.5% according to TestPrime. Our findings underscore the value of using a combination of PCR-based and shotgun-based sequencing approaches, particularly in environments that are understudied and where little is known about microbiome structure and function.

3.4. Fungal air communities above the forest canopy are most similar in composition to tropical phyllosphere and soil communities

We compared total and active fungal air communities to communities from tropical, temperate, and tundra soils and from the surfaces of tropical tree leaves. Community composition significantly differed across environment types (ADONIS, R^2 = 0.167, p = 0.001), and fungal communities in the atmosphere were compositionally

distinct from communities in other environments (Figure 5). Ascomycota was the most abundant phylum across all soil and phyllosphere samples (soil mean relative abundance = 78.4±14.9%, phyllosphere = 90.9±4.9%) followed by Basidiomycota (soil mean relative abundance = 19.0±14.9%, phyllosphere = 7.4±4.5%) (Figure 6). We expected communities to be distinct across habitat types because environmental conditions may differ across the habitat types and select for different communities. However, in the atmosphere, dispersal and mixing of fungi from multiple habitat types may be driving the observed community composition differences instead of environmental selection.

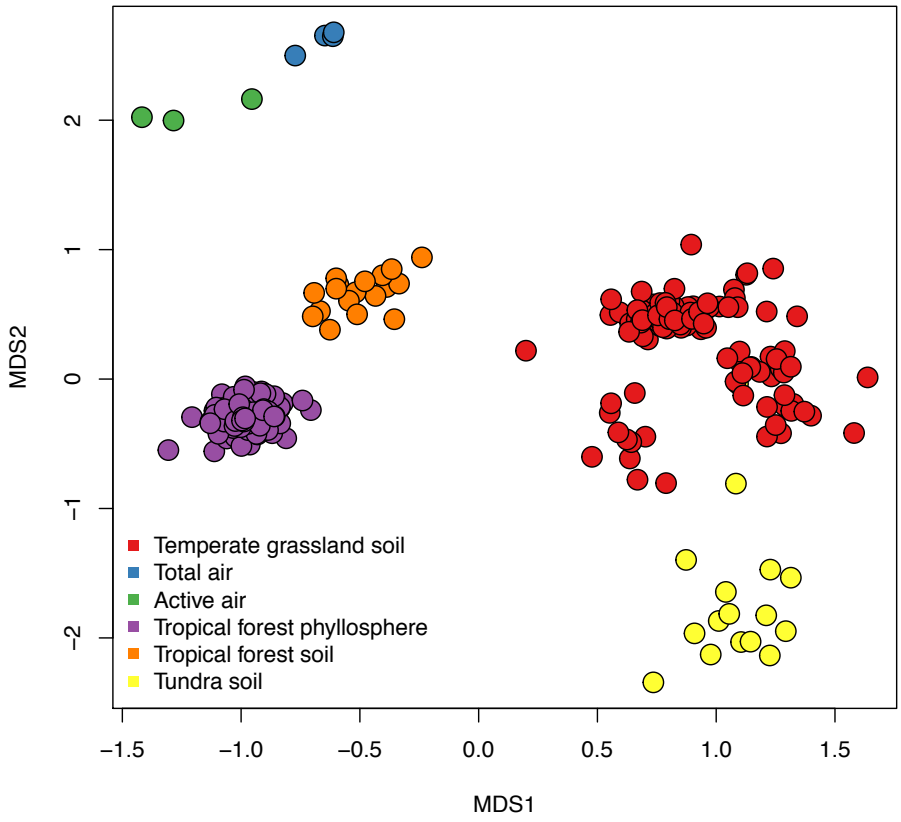

Figure 5. The compositions of the total and active fungal communities are distinct. Sørensen similarities are depicted, ordinated via NMDS.

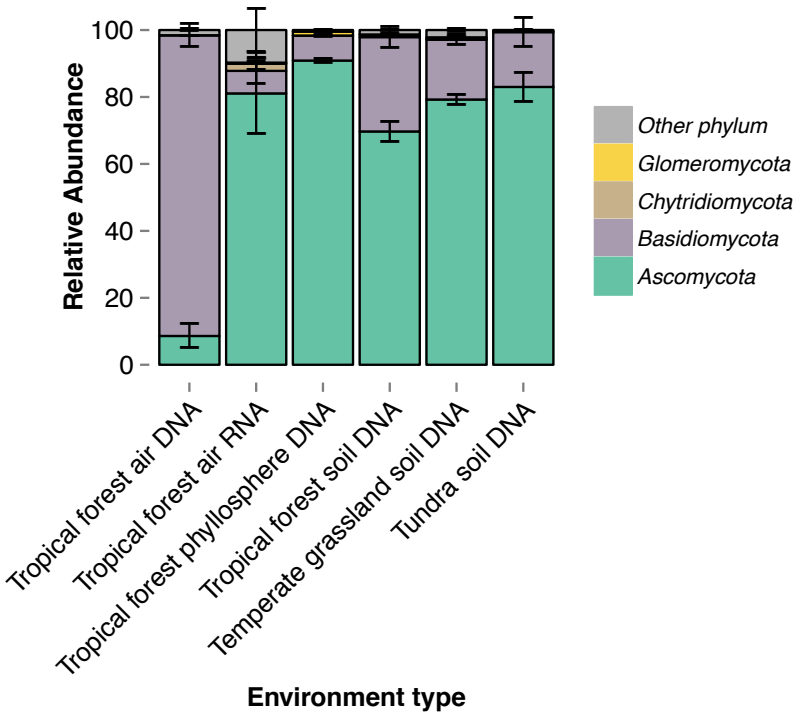

Figure 6. The active atmospheric fungal community over the Amazon rainforest is most similar to fungal communities found in tropical soils and on plant leaves.

The diversity of fungal communities in the atmosphere is within the range of diversities reported for terrestrial environments, including those of tropical leaf surfaces, tropical soils, temperate grassland soils, and tundra soils. Overall taxonomic richness, defined as the number of OTUs, significantly varied among environment types (ANOVA, $F(5,237) = 66.89$, $p < 0.001$) (Figure 15). Tukey's HSD post-hoc comparisons indicated that the richness of air communities, both total and active, was greater than tundra soil communities and did not significantly differ from temperate grassland soil communities. In general, air communities were less diverse than tropical forest phyllosphere and soil communities with the exception of tropical forest soils and active air communities, which did not significantly differ. Similar patterns have been observed in soil communities where taxonomic richness in arctic soils was significantly lower than soils from temperate and tropical ecosystems (Fierer et al., 2012).

Total air communities were most similar to tropical phyllosphere communities (mean Sørensen similarity = 0.015±0.009; statistic) and active air communities were most

similar to tropical soil communities (mean Sørensen similarity = 0.010±0.007) (Figure 16). These results suggest that inputs of fungi into the atmosphere over the canopy are derived from local, as opposed to long-distance, sources. This suggestion makes sense since fungal spores and hyphae are relatively large aerosol particles with short residence times in the atmosphere, limiting opportunities for long-distance dispersal. While these results are suggestive, detailed information is lacking regarding the potential influence of terrestrial source environments and their role in structuring airborne fungal communities.

4. Conclusion

Fungi in the atmosphere play an important role in atmospheric processes including precipitation development through ice nucleation. This is of particular significance in the atmosphere over the Amazon rainforest canopy where fungi constitute a large fraction of the total aerosol content (Elbert et al., 2007; Heald and Spracklen, 2009) and precipitation is aerosol-limited (Pöschl et al., 2010). Our study represents the first culture-independent survey of fungal communities over the Amazon rainforest canopy. It is also the first to measure metabolically active microbial communities in the atmosphere using an RNA-based approach. Using this RNA-based approach, we found evidence for the presence of potentially active fungi in the atmosphere, including lichen fungi. While an understanding of the structure of fungal communities in the atmosphere is beginning to emerge, studies on the function of these communities have lagged behind. We suggest that future research focus on understanding the functional capacity of airborne microbes with traits particularly well-suited for survival and metabolic activity in extreme environments. As with any environment, understanding both the structure and function of microbial communities in the atmosphere is needed to assess their potential impact on ecosystem processes such as water and carbon cycling. This study opens the door for future investigations of the diversity and function of fungal communities in the atmosphere.

5. Bridge to Chapter IV

In Chapter III, I used RNA- and DNA- based analyses to study active and total fungal communities in the atmosphere over the Amazon rainforest and their relationships

to potential source environments. I found that lichen fungi were abundant in the active community. This finding has implications for atmospheric function in the Amazon because lichen fungi are efficient ice nucleators and precipitation in the Amazon is dependent on nucleation by aerosols. I found the composition of the active community more closely resembled local source environments including tropical soils and leaf surfaces than did the composition of the total community. This suggests that composition the active fungal community was likely influenced by local sources. In Chapter IV, I build upon this finding with a study of the temporal dynamics of the diversity and composition of bacterial communities in the atmosphere and their relationships to potential sources at a high elevation research station.

CHAPTER IV
TEMPORAL VARAITION IN BACTERIAL DIVERSITY OF THE ATMOSPHERE

1. Introduction

Studies of temporal variation are valuable for understanding what drives the assembly of biological communities and for predicting how these communities may change in the future (Shade et al., 2013). In the atmosphere, bacterial communities vary over multiple time scales including seasonal, inter-day, and diurnal (reviewed in Després et al., 2012). Time series analyses have provided key information about the influence of terrestrial and aquatic sources in structuring atmospheric bacterial communities. However, it is not known over which time scales they are most variable, and how this variation may be related to changes in inputs from source environments or to environmental conditions.

Most studies of bacterial dynamics in the atmosphere have focused on changes in the concentration of cells rather than their taxonomic composition. This is largely because most studies have used culture-based methods, which miss the majority microbial diversity (Rappé and Giovannoni, 2003). Culture-independent methods have been applied to bacterial communities in the atmosphere only in recent years. Variation in the concentration of cells in the atmosphere has been measured over seasonal and diurnal time scales (reviewed in Despres et al., 2012). Seasonally, the highest concentrations typically occur in summer and fall (Despres et al., 2012). Potential mechanisms that contribute to high densities of cells in the atmosphere include the presence of a strong source from deciduous leaf surfaces, anthropogenic activity such as tilling and crop harvesting (Lighthart 1984), and seasonal changes in meteorological conditions including temperature and precipitation (Burrows et al., 2009). Diurnally, airborne cell concentrations tend to rise during the morning as leaf and soil surfaces dry and as wind speed in the boundary layer increases, and then they decrease in the afternoon hours (Jones and Harrison, 2004). These temporal changes in concentration are due, in part, to changes in source inputs. Moving beyond the study of cell concentrations in the atmosphere, to that of bacterial community composition, may tell us about the relative

importance of different source inputs and the conditions that favor upward flux from those sources.

Our understanding of bacterial community dynamics in the atmosphere remains in its infancy. Culture-independent analyses have demonstrated significant seasonal variation in overall community composition and in the relative abundances of specific taxonomic groups (e.g. Bowers et al., 2013; Bowers et al., 2011; Maron et al., 2006). This variation across seasons has been explained by changes in weather and atmospheric conditions (Maron et al., 2006), human activity (such as the seasonal application of fertilizer to agricultural fields (Fröhlich-Nowoisky et al., 2014), and changes in sources such as the emergence and senescence of deciduous tree leaves (Bowers et al., 2013). Less is know about inter-day variability in atmospheric community composition, and the few studies that exist report conflicting results. For example, some studies have documented patterns of high variability in community composition from day to day (Bertolini et al., 2013; Fierer et al., 2008; Smith et al., 2013), whereas others have reported the opposite pattern (Bowers et al., 2009). When observed, variation across days has been attributed to variation in human activity in urban environments (e.g. grass mowing (Fierer et al., 2008)) and meteorological changes (e.g. amount of precipitation (Maron et al., 2006)). Significant day to day variation in community composition has also been observed due to inputs from long-distance sources caused by dust storm events, which launch dust-associated bacteria into the upper levels of the atmosphere, where they can then travel thousands of miles before returning to Earth's surface (Smith et al., 2013). To our knowledge, no studies have used DNA sequencing to investigate diurnal variation in community composition in the atmosphere. One study by Maron et al. (2006) used a DNA fingerprinting technique to investigate temporal variability in composition over diurnal time scales. However, relative to sequencing approaches, fingerprinting approaches do not provide the same resolution of taxonomic information, and they are difficult to standardize which makes comparison across studies challenging.

To address this gap, we collected bioaerosol samples diurnally over multiple days at Mt. Bachelor Observatory (MBO), a mountaintop high elevation research station. MBO is an ideal site for studying temporal dynamics in airborne microbial communities, particularly in relation to sources of airborne bacteria. MBO has been used as a model

system to look at long distance sources of bacteria because the summit of Mt. Bachelor (elevation 2.8 km above sea level) regularly encounters air masses from the free troposphere with no recent contamination from the boundary layer or local emissions, making it possible to collect cells which have been aloft in the atmosphere for over one week (Smith et al., 2013; Weiss-Penzias et al., 2006). MBO is also ideal for analyzing local sources of bacteria, due to diurnal dynamics at the summit. Air from the boundary layer, which has interacted with the earth's surface, predominates at MBO during the day, and air from higher elevations, which has not recently interacted with the surface, predominates at MBO during the night. Thus during the day, solar radiation heats air in the boundary layer, and air surrounding the mountain rises over the summit via convection. At night as solar heating and convection taper off, cooler air masses, sometimes from the troposphere, drop down and push the heated boundary layer down the mountain. Combined, this means that bacterial communities in the atmosphere at the summit may vary diurnally and across days as they are derived from different layers of the atmosphere with different properties and histories. Specifically, atmospheric bacteria sampled at night are more likely to have dispersed over long distances compared to bacteria sampled during the day, which likely come from local sources. In this manuscript, we asked the following questions: 1) How does airborne bacterial diversity and composition vary over diurnally and across days? 2) How does temporal variability in bacterial community diversity and composition correlate with changes in the atmospheric environment and air mass sources?

2. Methods

2.1. Sample collection

Sampling was conducted over five days August 12-16, 2013 at the Mt. Bachelor Observatory (MBO) (43.98° N, 121.7 °W), a mountaintop research station, 30 km WSW of Bend, OR. MBO is located at the summit of Mt. Bachelor, an inactive volcano, at 2763 m above sea level. Aerosols were collected on to 47mm diameter cellulose nitrate filters with 0.2 μm pore size (Nalgene Analytical Test Filter Funnels, Thermo Fisher Scientific). Filters were placed approximately 1 m above ground level. Each sterile filter and housing unit was individually wrapped and sealed until use. Three filters were run in parallel with

each filter powered by two vacuum pumps (Welch model 2524) connected in series. Flow rates were approximately 12 L/min. Sampling was conducted continuously from 12:00 pm August 13, 2013, until 12:00 pm August 17, 2013. Filters were changed every fours hours over five days resulting in 30 total samples. After each sampling interval, lids were placed on filter housing, and stored at -20°C.

2.2. Environmental data and back trajectories

Meteorological and atmospheric chemistry data including atmospheric pressure, temperature, water vapor, wind speed and direction, UV irradiance, total gaseous mercury, CO, and ozone were collected as part of normal operations at MBO (see Weiss-Penzias et al., 2006 for details). Sub-micron scattering (450 nm, 550 nm, and 700 nm), PM2.5, and PM10 data were also collected, however these measurements were not used in our analyses due to an instrument failure which resulted in the loss of 24 hours of data. The four-hour means corresponding to the bioaerosol sampling intervals were used for statistical analysis. Kinematic back trajectories were calculated using HYbrid Single-Particle Lagrangian Integrated Trajectory (HYSPLIT) model version 4 (Draxler and Rolph, 2003) using global meteorological data from the Global Data Assimilation System archive.

2.3. Nucleic acid isolation and library preparation

Samples were transported on dry ice to the University of Oregon and stored at -80°C. Two types of controls, with and without blank filters, were processed identically to samples including DNA extraction, barcoding, and sequencing. Each of the three filters per time point were processed and sequenced separately. Sequences for each time point were combined for downstream analysis. DNA was extracted from filters using the MO BIO PowerWater DNA Isolation Kit according to the manufacturer's instructions with the following modifications (MO BIO Laboratories, Inc.). DNA was eluted in 100 µl elution buffer (QIAGEN). Due to the presence of inhibitors, DNA was diluted 1:8 for PCR. 16S genes (rDNA) were amplified using bacterial/archaeal primers 515 forward (5'-GTGTGCCAGCMGCCGCGGTAA-3') and 806 reverse (5'-GGACTACHVGGG TWTCTAAT-3') (Caporaso et al., 2012). The reverse primer also contained a 12 base-

pair barcode at the 5' end in order to assign sequences to samples. PCR reactions were performed in triplicate on each DNA and cDNA sample. Each 25 µl reaction contained 0.25 µl Phusion Hot Start II DNA polymerase (Life Technologies Corporation) 5 µl HF buffer, 0.5 µl 10 mM dNTPs (New England Biolabs, Inc.) 0.5 µl forward primer (10 µM), and 0.5 µl reverse primer (10 µM), 13.25 µl H_2O, and 5 µl template. PCR cycling conditions were as follows: initial denaturation at 98°C for 90 seconds followed by 35 cycles 98°C for 20 seconds, 52°C for 30 seconds and 72°C for 30 seconds following by a final extension at 72°C for 10 minutes. Triplicate reactions were pooled, cleaned using the MinElute 96 UF PCR Purification Kit (QIAGEN), and eluted in 20 µl elution buffer. The cleaned PCR products were quantitated using a Qubit 2.0 Fluorometer (Invitrogen, Life Technologies Corporation) and combined in equal molar concentrations. The pooled library was concentrated (Zymo Research Clean and Concentrate-5, Zymo Research) and eluted into 50 µl elution buffer. PCR products were size fractionated by gel electrophoresis (2%, low-melt agarose). Products in the range of 250-350 bp were excised, and DNA from the excised gel was extracted (Qiagen MinElute Gel Extraction, QIAGEN) and eluted into 30 µl elution buffer. The eluate was cleaned a final time (Zymo Research Clean and Concentrate-5, Zymo Research) and eluted into 30 µl elution buffer. The final library was quantitated and diluted from 59.42 nM to 10 nM. The amplicon library was sequenced (250 base pairs, paired-end) on the Illumina MiSeq platform at the Dana-Farber Cancer Institute Molecular Biology Core.

2.4. Sequence processing

Sequences were processed using QIIME version 1.8 (Caporaso et al., 2010) and the UPARSE pipeline (USEARCH version 7.0.1090) (Edgar, 2013). Briefly, libraries were demultiplexed in QIIME using the split_libraries_fastq.py without quality filtering or trimming. Sequences in fastq output from QIIME were quality filtered and trimmed using the fastq_filter USEARCH script. Sequences were trimmed to 296 bp. Sequences with a maximum expected error (fastq_maxee) >0.5 were removed. Sequences for which there was no identical match were removed using the derep_fulllength and sortbysize USEARCH scripts. Sequences were clustered into operational taxonomic units (OTUs) at 97% sequence similarity using the UCLUST algorithm (Edgar, 2010).

Representative sequences from each OTU were screened for chimeras using the uchime_ref script against the ChimeraSlayer reference database (Broad Microbiome Utilities (http://microbiomeutil.sourceforge.net/). Headers for representative sequences were reformatted and sequences were numbered sequentially using the fasta_number.py script. Original sequences were mapped to OTUs using the usearch_global script. The resulting OTU map was converted to a tab-delimited OTU table using a modified version of the uc2otutab.py script. The tab-delimited OTU table was converted to BIOM (McDonald et al., 2012) format. Taxonomy was assigned to representative sequences using the RDP Naïve Bayesian Classifier (Wang et al., 2007) against the Greengenes database (version 13_5, http://greengenes.secondgenome.com/downloads/database/13_5) in QIIME.

OTUs for which relative abundance in control samples was significantly correlated with their relative abundance in air samples were removed using the filter_otus_from_otu_table.py script in QIIME. A total of 89 of 1,060 OTUs were removed. Additionally, all OTUs identified as belonging to the class "Chloroplast" and family "Mitochondria" were removed using the filter_taxa_from_otu_table.py script.

2.5. Statistical analyses

All statistical analyses were conducted in R (R Core Team, 2014) using the vegan package (Oksanen et al., 2013) for ecological statistics and the lubridate package (Grolemund and Wickham, 2011) for parsing date-time data. The JTK_Cycle algorithm (Hughes, Hogenesch, and Kornacker, 2010) implemented in R was used to identify OTUs with significant periodicity in their relative abundances.

3. Results

3.1. Sequence statistics

A total of 5,163,477 sequences were generated. Following sequence processing and quality filtering, a total of 1,174,199 sequences remained, which were binned into 991 OTUs. The mean number of sequences and OTUs per sample was 19,136 ± 7,708.60. Each sample was subsampled to 13,000 sequences, after which 962 OTUs remained.

There were 38 OTUs shared across all samples, and 557 that were unique to one sample. The average number of unique OTUs per sample was 18.567 ± 13.599.

3.2. Environmental characteristics

There were two general patterns of transport histories of the air masses that arrived at the summit during sampling. On the dates 8/12-8/14 air masses moved over the Pacific Ocean and on to land from the north and west. On 8/13 and 8/14 the air masses at that reached the summit of Mt. Bachelor at 2:00 pm travelled at low elevations and likely had significant interaction with the Earth's surface (Figure 7).

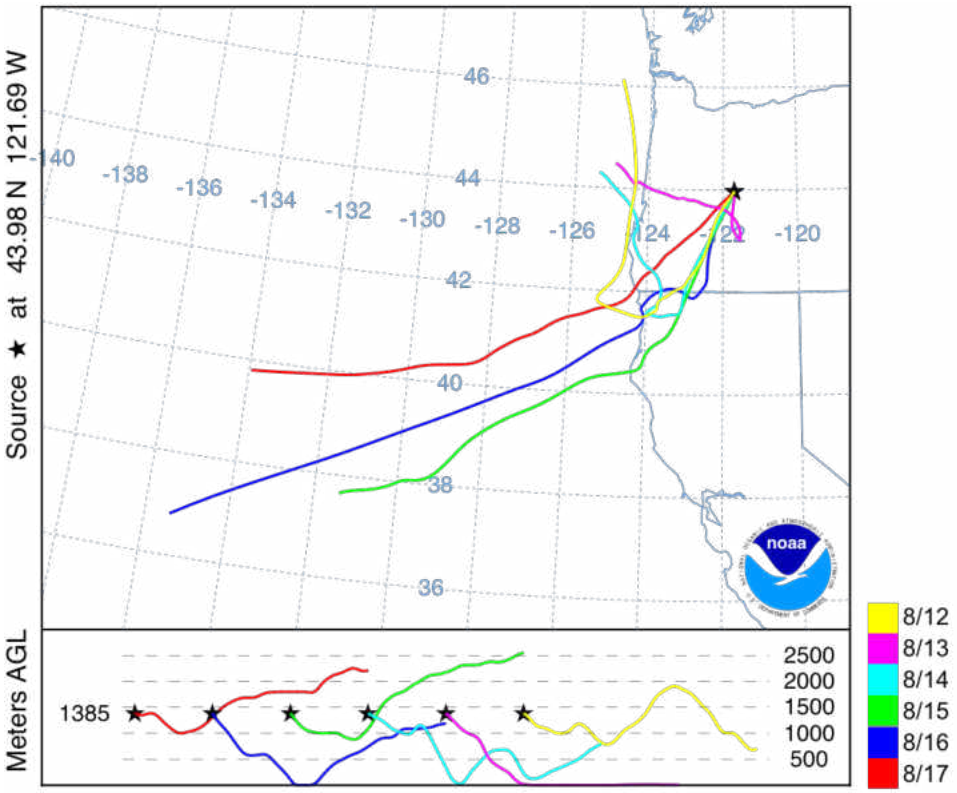

Figure 7. NOAA HYSPLIT back trajectories of air masses arriving at the Mt. Bachelor summit at 2:00 pm each day of sampling. Each trajectory represents the transport history of air masses 72 hour before reaching the summit of Mt. Bachelor.

Conditions at the summit over these days were generally characterized by high ozone, low/increasing CO, and low relative humidity (Figure 8). These conditions are consistent with what is observed when air from the free troposphere interacts with the summit. Air

masses that arrived at MBO during the last half of the sampling period traveled longer distances in the three days before reaching the summit than did air masses during the first three days of sampling. These faster traveling air masses moved on to land from the south and west. On 8/15 and 8/17 the air masses reaching the summit traveled at elevations higher than the summit and thus did not likely interact much with the surface (Figure 7). Conditions at the summit on 8/15-8/17 were generally characterized by decreasing ozone, high/decreasing CO, and high relative humidity (Figure 8). High and increasing CO was likely due to the transport of smoke from wildfires burning in northern California and southern Oregon during sampling.

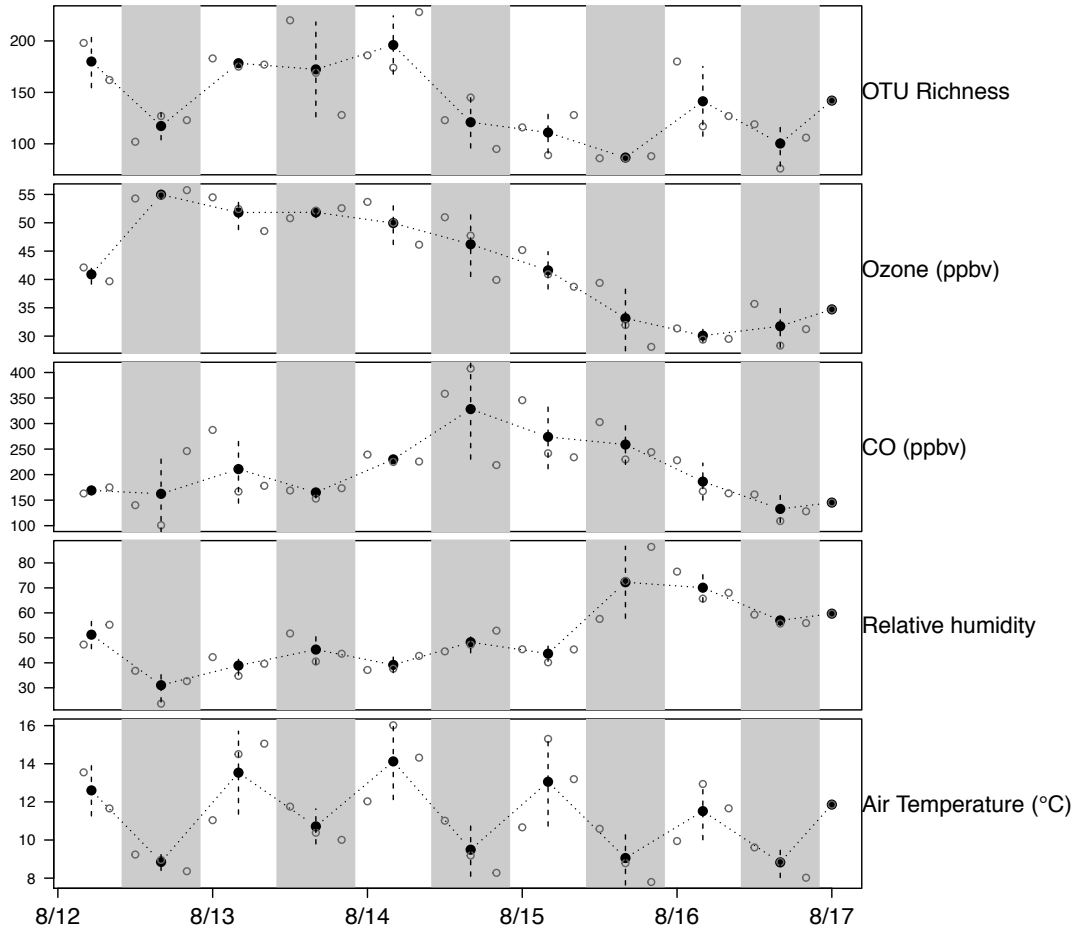

Figure 8. Significant predictors of community richness

3.3. Temporal variation in composition

Communities significantly varied in day (8:00 am – 8:00 pm) versus night (8:00 pm – 8:00 am) samples (ADONIS, Canberra distances, $F = 1.5346$, $p < 0.001$). Communities did not significantly differ by day of the week. In ordination space, several environmental conditions were significantly correlated with variability in community composition. Environmental conditions that vary diurnally including air temperature ($R^2 = 0.4187$, $p = 0.0004$) and UV ($R^2 = 0.2947$, $p = 0.008$) were significantly correlated with changes in composition. As can be seen in Figure 9, the environmental vectors are in the same direction as spread of day versus night samples and so were associated with variation between day and night samples. Other environmental variables significantly associated with community composition included ozone ($R^2 = 0.2231$, $p = 0.0315$), wind speed ($R^2 = 0.3586$, $p = 0.0021$), and wind direction ($R^2 = 0.3866$, $p = 0.008$) varied across days. In Figure 9, vectors for these variables are orthogonal to the axis showing diurnal variation.

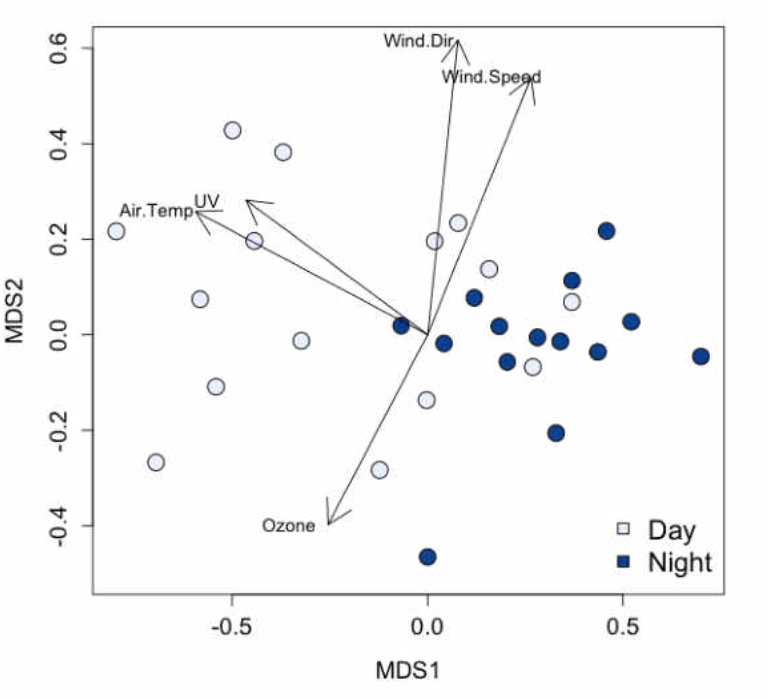

Figure 9. Ordination of Canberra distances with environmental vectors

Five OTUs were found to have significant diurnal variation in relative using the JTK_CYCLE algorithm (Hughes, Hogenesch, and Kornacker, 2010) (Figure 10). Two OTUs were members of the phylum Chloroflexi. One Chloroflexi OTU (class C0119) exhibited somewhat irregular periodicity but generally peaked in relative abundance in the early evening. The second Chloroflexi OTU (order WCHB1.50) generally peaked in relative abundance around midday. Two Solirubrobacterales OTUs reached their maximum relative abundance during the midday hours. The relative abundance of one Rhizobiales OTU also varied diurnally and had highest relative abundance at night.

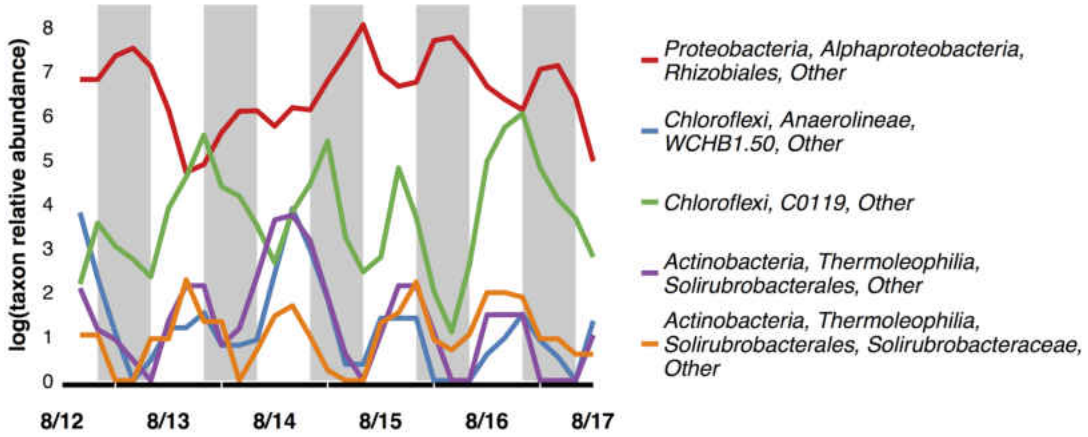

Figure 10. Taxa with significant diurnal periodicity in relative abundance. Grey bars indicate night samples (8:00 pm – 8:00 am).

3.4. Temporal variation in diversity

Richness significantly varied across days ($F = 3.349$, $p = 0.0196$) and was significantly greater in day versus night samples ($F = 8.26$, $p = 0.00765$). Variation in community richness was significantly associated with environmental factors that vary across days (ozone, CO, relative humidity) and within days (air temperature) (Figure 9 and Table 1). The best model explained an estimated 49% of the total variance in richness. Significant predictors of community richness based on stepwise model selection included variables associated with wildfire activity (high CO) and influence of free troposphere air (low relative humidity and high ozone). Richness was also significantly predicted by air temperature.

Table 1. Parameter estimates for linear model predicting community richness

	Estimate	SE	t-value	p-value
Ozone (ppbv)	4.87664	1.27552	3.823	0.000779
Carbon monoxide (ppbv)	-0.13415	0.08181	-1.64	0.113571
Temperature (°C)	10.42569	2.55455	4.081	0.000402
Relative humidity	2.49157	0.86974	2.865	0.008337

4. Discussion

In this paper, we described the temporal dynamics of bacterial community diversity and composition at a high elevation site over diurnal and inter-day time scales. Information about how communities vary through time can increase understanding of bacterial community assembly and transport patterns in the atmosphere. This is important because the transport and composition of bacteria through the atmosphere has consequences for microbial biogeography and ecosystem function at the Earth's surface (e.g. through the introduction of pathogens from the atmosphere). There is a paucity of information about how atmospheric bacterial communities vary diurnally and across days, and the few previous studies of variation in community composition across days have yielded conflicting results. We found strong signatures of diurnal variation in composition and richness, and in contrast to most other studies of variation across days, we found a form of stability across days, with communities that did not significantly vary in composition but did vary in richness.

4.1. Communities were most variable over diurnal time scales

In the atmosphere at the summit of Mt. Bachelor, variation in bacterial communities over diurnal time scales was more pronounced than variation across days. Both composition and richness varied diurnally, whereas only richness varied across days. Diurnal variation in richness could be explained by diurnal periodicity in cell concentrations in the atmosphere. While we did not quantify cell concentrations in this study, strong diurnal periodicity in the concentration of atmospheric bioaerosols has been observed in multiple studies across many environments (Després et al., 2012; Tong and Lighthart, 1999; Lighthart and Shaffer, 1995), with higher cell concentrations during the

day versus night. If cell concentrations were higher at MBO during the day relative to during the night, this larger pool of individuals could result in higher taxonomic richness. Research in terrestrial and aquatic systems has shown that taxa richness increases with increasing numbers of individuals per sample (Bunge and Fitzpatrick, 1993).

In addition to richness, the overall composition of bacterial communities in the atmosphere varied diurnally. Specifically, five taxa exhibited significant diurnal periodicity in their relative abundances. There was only one OTU, from the order Rhizobiales, which decreased in relative abundance during the day and increased in relative abundance at night. This could be because this OTU is at a near constant *absolute* abundance in the atmosphere at MBO and at night when there is less influence from local sources, the relative abundance of the Rhizobiales OTU increased relative to the abundance of other OTUs. Alternatively, the Rhizobiales OTU may have been abundant in higher atmospheric elevations, and then move down to the summit sample location at night. Two OTUs that also exhibited significant diurnal periodicity in their relative abundances were members of the order Solirubrobacteriales, a group of Gram-positive bacteria within the phylum Actinobacteria. Most Solirubrobacteriales species are soil-associated (Albuquerque and da Costa, 2014). They have been detected in outdoor air samples (Brodie et al., 2007; Kembel et al., 2012) and in dust (Favet et al., 2013). Two of the OTUs with diurnal periodicity in their relative abundances were members of the phylum Chloroflexi. Chloroflexi are anoxygenic photoheterotrophs, and many are also thermophilic. Members of the phylum have been previously detected in the atmosphere (Brodie et al., 2007; Kembel et al., 2012) as well as other types of atmospheric samples including rainwater (Cho and Jang, 2014) and dust (Barberán et al., 2014).

Interestingly, four of the five OTUs with diurnal periodicity are members of the Solirubrobacteriales and Chloroflexi, which are frequently found in rock varnish (Esposito et al., 2015) Rock varnish is a thin layer composed of metal (Fe and Mn) hydroxides and clay minerals that develop on rock surfaces across the globe. Assemblages of bacteria are found in rock varnish are distinct from those found on the surfaces of non-varnished rock (Esposito et al., 2015). Conditions in rock varnishes select for anoxygenic phototrophs such as Chloroflexi, and members of this group are often dominant members of the rock-associated community (Esposito et al., 2015). The summit

of Mt. Bachelor is primarily covered in volcanic rock including basalt and basaltic andesite with little vegetation, so it is parsimonious to assume that these taxa inhabit rock surfaces on the summit of Mt. Bachelor. The two Solirubrobacteriales and two Chloroflexi OTUs that increased in abundance during the day over the course of sampling may have lifted into the atmosphere from local rock surfaces by convection.

4.2. Community composition was stable across days but richness varied

In contrast to diurnal time scales, bacterial community composition in the atmosphere at the summit of Mt. Bachelor did not vary across days. Most other studies have found significant variation across days (Bertolini et al., 2013; Fierer et al., 2008; Smith et al., 2012) (although see Bowers et al., 2009 for an exception). Studies that found significant variation in composition across days attributed this pattern to human activities such as grass mowing in nearby fields (Fierer et al., 2008). It is possible that during the time of sampling at MBO, the composition of communities in the atmosphere was stable from day to day because there were no significant local disturbances or perturbations that were a source of variation in the number and types of bacteria that become airborne. These results also suggest that inputs of bacteria from long-distance sources were less important relative to local sources in structuring the composition of the communities. If long-distance sources were more important, variation across days would be more pronounced than diurnal variation because large masses take days to move through a location. However, the sampling period would need to be sufficiently long to observe the movement of multiple air masses through a single location. Instead we observed significant diurnal variation presumably due to the upward flux of bacteria into the atmosphere from local sources, which has a strong diurnal pattern.

4.3. Community variation was associated with atmospheric conditions and air mass history

Across all samples, variation in bacterial composition was correlated with environmental conditions with strong diurnal patterns including UV and temperature as well conditions that varied across days including wind speed/direction and ozone. Previous work has shown that atmospheric cell concentrations are positively correlated

with air temperature (Bovallius et al., 1978; Harrison et al., 2005). It is possible that temperature variation at MBO was linked to the observed variation in community composition in our study, because changes in the concentration of cells is likely related to changes in the relative inputs from different sources. For example, there were five taxa with relative abundances that varied diurnally. Taxa that increased during the day may have originated in local sources (i.e. rock surfaces at the summit) and increased in abundance as thermal convection increased during the day. Taxa that increased overnight may have consistent absolute abundances in the atmosphere. Their relative abundance may have increased at night as the abundance of taxa from local sources decreased (due to decreases in thermal convection).

The richness of bacterial communities in the atmosphere at MBO varied diurnally and across days and was significantly correlated with three environmental variables that varied across days including CO, ozone, and relative humidity as well as temperature, which varied diurnally. Across days, richness was greatest when ozone was high and relative humidity was low. The occurrence of free tropospheric air at MBO is characterized high ozone and low relative humidity (Weiss-Penzias et al., 2006), so it is possible that the increased richness could be due to inputs of bacteria from long distances sources in free troposphere air. In addition, richness was low when CO levels were high. High levels of CO were likely due to the influence of wildfire smoke, which impacted the summit of Mt. Bachelor during sampling. This suggests airborne bacterial communities associated with wildfire may be less diverse than air masses not impacted by wildfire. To summarize, richness was highest when air masses were more influenced by free troposphere air (high ozone, low RH) and less influenced by wildfires (low CO), and during the day when thermal convection increases upward flux of microbes from surfaces into the atmosphere (Lighthart and Shaffer 1995). This suggests that richness is influenced by both local sources (thermal convection during the day lofted microbes up from local soils, rocks) and long distance sources (free troposphere air).

5. Bridge to Chapter V

In Chapter IV, I presented a study of diurnal and inter-day temporal variation in the composition and diversity of bacterial communities at Mt. Bachelor Observatory. I

found communities in the atmosphere vary over multiple times scales and the mechanisms underlying that variation differ depending on time scale. Specifically, I showed that variation in community composition within days was more pronounced than variation across days, and this variation is likely driven by diurnal variation in upward fluxes of bacteria from local sources including rock surfaces. While composition did not vary across days, I found significant variation in richness across days and suggested that changes in richness could be due to the arrival of distinct air masses at the site. Thus, changes in richness across days could be driven by the influence of long distance dispersal of bacteria in the atmosphere. In this chapter, I described the temporal dynamics of bacterial community diversity and composition. In Chapter V, I expand on these results and explore the potential functions of bacteria in the atmosphere Mt. Bachelor Observatory by comparing the total and potentially active bacterial communities.

CHAPTER V
MOLECULAR EVIDENCE FOR ACTIVE BACTERIAL COMMUNITIES IN THE ATMOSPHERE

This paper was prepared for submission to *Frontiers in Microbiology*. I conceived the experiments, conducted the experiments, analyzed the data, and wrote the paper. Assistance with experimental design, data analysis and writing the manuscript was provided by my co-authors: Brendan J. M. Bohannan, Daniel A. Jaffe, David A. Levin, and Jessica L. Green.

1. Introduction

Studies of microorganisms in terrestrial, aquatic, and host-associated environments have demonstrated that surveying the metabolically active community is key to characterizing the functions of microbial communities (e.g. Haglund et al., 2003; del Giorgio and Scarborough, 1995; del Giorgio, Prairie, and Bird, 1997; Lillis, Doyle, and Clipson, 2009; Peris-Bondia et al., 2011; Baldrian et al., 2012). Yet, little is known about the metabolic activity of microbial communities in the atmosphere, an environment that is intimately connected to all biomes spanning the globe. This lack of knowledge is due to both conceptual and technical limitations. Conceptually, the atmosphere has historically been regarded as a dispersal vector for dead, dormant, or inactive cells instead of as a habitat with actively reproducing microbial life (Womack, Bohannan, and Green, 2010). Technically, low densities of cells have made it difficult to study the activity of airborne microorganisms *in situ* (Behzad, Gojobori, and Mineta, 2015). However, there is evidence to suggest that airborne microorganisms may be metabolically active. Culture-based analyses of bacteria isolated from clouds have shown that bacteria can transform atmospheric compounds including carbon, nitrogen, and oxidative species (Vaïtilingom et al., 2011; Vaïtilingom et al., 2013; Amato et al., 2007; Hill et al., 2007). Research has also demonstrated that aerosolized cultured bacterial cells increase their ribosome production, and thus their protein synthesis potential, when supplied carbon substrates in the lab (Krumins et al., 2014). The next step is to apply

culture-independent methods on atmospheric samples, targeting active cells, in order to learn about their potential functions.

One culture-independent approach for surveying the composition of active microbial communities is through the analysis of rRNA in ribosomes (Schippers and Neretin, 2006; Lennon and Jones, 2011; see review by Blazewicz et al., 2013). The vast majority of culture-independent microbiology research relies on rDNA (i.e. rRNA gene) sequence data, which provides information about the total community (including both active and dormant individuals). In contrast, rRNA sequences provide information about the potentially active community, because ribosomes are more abundant in active than dormant cells (Kerkhof and Kemp, 1999; Fegatella et al., 1998). Studies that have combined both rDNA and rRNA data have led to a wide range of ecological insights including how microbial communities respond to environmental change (Barnard, Osborne, and Firestone, 2013), which taxa contribute to key biogeochemical processes (Schostag et al., 2015), and what mechanisms shape microbial community assembly (Zhang et al., 2014). An emergent theme from comparative rDNA and rRNA analyses is that the active and total community can be fundamentally different from one another, in both structure and composition. For example, in many environmental systems it is the rare members of the total community that are dominant in the active community (Zhang et al., 2014; Baldrian et al., 2012; Hugoni et al., 2013; Jones and Lennon, 2010; Wilhelm et al., 2014; Campbell et al., 2011). This suggests that through using rDNA data alone, we may be underestimating the functional importance of taxa that are members of the "rare biosphere" (Jones and Lennon, 2010; Campbell et al., 2011) in the total community.

In this paper, we applied a culture-independent approach to survey the composition of potentially active microbial communities in the atmosphere. We focused our study on bacteria because they are abundant in the atmosphere with concentrations ranging from 10^4-10^5 cells/m^3 (Burrows et al., 2009) and, due to their small size, can have atmospheric residence times that are long enough for growth and reproduction to occur (see Womack, Bohannan, and Green, 2010). We applied comparative 16S rDNA and rRNA sequence analyses to characterize the structure and composition of the active and total bacterial community in air sampled from a high elevation research station location at the summit of Mt. Bachelor, Oregon. Mt. Bachelor is an ideal site for studying the

potential activity of airborne microbial communities. Due to the geography of the mountain and the surrounding topography, the summit (elevation 2.8 km above sea level) regularly encounters air masses from the free troposphere, making it possible to collect cells, which have been aloft in the atmosphere for over one week (Weiss-Penzias et al., 2006). By collecting cells that have been in the atmosphere for extended periods of time, the active community should more closely reflect the activity of cells in the atmosphere and not activity in potential local source environments like water or soil. We asked the following questions: 1) What is the diversity and composition of the active and total airborne bacterial community, and how do they compare? 2) Which bacteria in the atmosphere are potentially metabolically active?

2. Methods

2.1. Sample collection

Sampling was conducted over five days August 12-16, 2013 at the Mt. Bachelor Observatory (MBO) (43.98°N, 121.7°W), a mountaintop research station, 30 km WSW of Bend, OR. MBO is located at the summit of Mt. Bachelor, an inactive volcano, at 2763 m above sea level. Aerosol samples were collected using SKC BioSamplers (BioSampler SKC Inc.). Samplers were filled with 20 mL of a water-based preservation solution (LifeGuard Soil Preservation Solution, MO BIO Laboratories, Inc) to prevent DNase and RNase activity and maintain cells in stasis to allow accurate community profiling of the RNA and DNA communities. Twenty-four impingers were operated at 12.5 L/min from approximately 8:00 am – 4:00 pm each day. At the end of each day, the sampling liquid from all impingers was pooled and stored at -20°C.

2.2. Nucleic acid isolation and cDNA synthesis

Samples were transported on dry ice to the University of Oregon where the liquid was thawed and filtered through 0.22 μm cellulose nitrate filters (Nalgene Analytical Test Filter Funnels, Thermo Fisher Scientific). Field blanks were generated by filtering unused LifeGuard Solution through new, sterile filters. RNA and DNA were co-extracted from filters using the MO BIO PowerWater RNA Isolation Kit according to the manufacturer's instructions with the following modifications (MO BIO Laboratories, Inc.). The initial

DNase step was omitted. RNA and DNA were eluted in 100 µl elution buffer (QIAGEN) and then divided in half. One ~50 µl aliquot was treated with 1 µl DNase (DNase I, RNase-free, Thermo Fischer Scientific, Inc.) and the other was treated with 2 µl RNase (RNase A, DNase and protease-free, Thermo Fischer Scientific, Inc.). Both reactions were incubated at 37° for 30 minutes. Reactions were cleaned using the Qiagen MinElute Enzymatic Reaction Cleanup Kit (QIAGEN). DNA was eluted in 100 µl and RNA was eluted in 50 µl elution buffer.

cDNA was synthesized from the total RNA extract using the SuperScript II First-Strand Synthesis System (Invitrogen, Life Technologies Corporation) with random hexamers. All RNA was converted into cDNA in seven synthesis reactions and one reverse transcriptase negative control reaction. The seven cDNA reactions for each sample were pooled, cleaned using Qiagen MinElute Enzymatic Reaction Cleanup Kit (QIAGEN), and eluted in 50 µl elution buffer. Blanks were processed identically to samples including nucleic acid extraction, cDNA synthesis, barcoding, and sequencing.

2.3. Library preparation and sequencing

16S genes (rDNA) and transcripts (rRNA) were amplified using bacterial/archaeal primers 515 forward (5'-GTGTGCCAGCMGCCGCGGTAA-3') and 806 reverse (5'-GGACTACHVGGGTWTCTAAT-3') (Caporaso et al., 2012). The reverse primer also contained a 12 base-pair barcode at the 5' end in order to assign sequences to samples. PCR reactions were performed in triplicate on each DNA and cDNA sample. Each 25 µl reaction contained 0.25 µl Phusion Hot Start II DNA polymerase (Life Technologies Corporation) 5 µl HF buffer, 0.5 µl 10 mM dNTPs (New England Biolabs, Inc.) 0.5 µl forward primer (10 µM), and 0.5 µl reverse primer (10 µM), 13.25 µl H_2O, and 5 µl template. PCR cycling conditions were as follows: initial denaturation at 98°C for 90 seconds followed by 35 cycles 98°C for 20 seconds, 52°C for 30 seconds and 72°C for 30 seconds following by a final extension at 72°C for 10 minutes. Triplicate reactions were pooled, cleaned using the MinElute 96 UF PCR Purification Kit (QIAGEN), and eluted in 20 µl elution buffer. The cleaned PCR products were quantitated using a Qubit 2.0 Fluorometer (Invitrogen, Life Technologies Corporation) and combined in equal molar concentrations. The pooled library was concentrated (Zymo Research Clean and

Concentrate-5, Zymo Research) and eluted into 50 µl elution buffer. PCR products were size fractionated by gel electrophoresis (2.%, low-melt agarose). Products in the range of 250-350 bp were excised, and DNA from the excised gel was extracted (Qiagen MinElute Gel Extraction, QIAGEN) and eluted into 30 µl elution buffer. The eluate was cleaned a final time (Zymo Research Clean and Concentrate-5, Zymo Research) and eluted into 30 µl elution buffer. The final library was quantitated and diluted from 59.42 nM to 10 nM. The amplicon library was sequenced (250 base pairs, paired-end) on the Illumina MiSeq platform at the Dana-Farber Cancer Institute Molecular Biology Core.

2.4. Sequence processing

Sequences were processed using QIIME version 1.8 (Caporaso et al., 2010) and the UPARSE pipeline (USEARCH version 7.0.1090) (Edgar, 2013). Briefly, libraries were demultiplexed in QIIME using the split_libraries_fastq.py without quality filtering or trimming. Sequences in fastq output from QIIME were quality filtered and trimmed using the fastq_filter USEARCH script. Sequences were trimmed to 296 bp. Sequences with a maximum expected error (fastq_maxee) >0.5 were removed. Sequences for which there was no identical match were removed using the derep_fulllength and sortbysize USEARCH scripts. Sequences were clustered into operational taxonomic units (OTUs) at 97% sequence similarity using the UCLUST algorithm (Edgar, 2010). Representative sequences from each OTU were screened for chimeras using the uchime_ref script against the ChimeraSlayer reference database (Broad Microbiome Utilities (http://microbiomeutil.sourceforge.net/) version microbiomeutil-r20110519). Headers for representative sequences were reformatted and sequences were numbered sequentially using the fasta_number.py script. Original sequences were mapped to OTUs using the usearch_global script. The resulting OTU map was converted to a tab-delimited OTU table using a modified version of the uc2otutab.py script. The tab-delimited OTU table was converted to BIOM (McDonald et al., 2012) format. Taxonomy was assigned to representative sequences using the RDP Naïve Bayesian Classifier (Wang et al., 2007) against the Greengenes database (version 13_5, http://greengenes.secondgenome.com/downloads/database/13_5) in QIIME.

OTUs for which relative abundance in control samples was significantly correlated with their relative abundance in air samples were removed using the filter_otus_from_otu_table.py script in QIIME. Of the 90 OTUs that were removed, 26 could not be taxonomically classified. Additionally, all OTUs identified as belonging to the class "Chloroplast" were removed using the filter_taxa_from_otu_table.py script.

2.5. Statistical analyses

All statistical analyses were conducted in R (R Core Team, 2014) primarily using the packages vegan (Oksanen et al., 2013), BiodiversityR (Kindt and Coe, 2005) for ecological statistics and the ggplot2 (Wickham, 2009) package for visualizations. All analyses of beta-diversity were based on Canberra distances. DESeq2 (Love, Huber, and Anders, 2014) implemented in QIIME was used to analyze differential abundance of OTUs between the RNA and DNA communities.

3. Results

3.1. Sequence statistics

A total of 153,088 16S rRNA and rDNA sequences were generated from four aerosol samples. Following sequence processing and quality filtering, a total of 55,414 sequences remained, which were binned into 1,144 OTUs. The mean number of sequences and OTUs per sample was 19,136 ± 7,708.60 and 293.88 ± 39.21, respectively. Each sample was subsampled to 7,400 sequences, after which 1,076 OTUs remained. There were 23 OTUs shared across all samples, and 606 that were unique to one sample. The average number of unique OTUs per sample was 75.75 ± 50.26. There were 767 OTUs across all DNA samples, 652 OTUs across all RNA samples, and 343 OTUs were shared between RNA and DNA samples.

3.2. Composition and structure of active and total communities

Community composition of the RNA and DNA communities significantly differed (ADONIS, $F(1,6) = 1.22$, $p = 0.027$) at the level of OTUs and at the order level (Figure 11). The RNA and DNA communities were dominated by the orders Actinomycetales, RB41, Saprospirales, Cytophagales, and Rhodospirillales. The relative

abundances of the following orders significantly differed between RNA and DNA, RB41 ($t(3) = 4$, $p = 0.04$), Saprospirales ($t(3) = 6$, $p = 0.01$), Rhodospirillales ($t(3) = -7$, $p = 0.006$), Sphingomonadales ($t(3) = 10$, $p = 0.002$). Across all OTUs, relative abundance in RNA community were correlated with relative abundance in DNA community (Kendall rank correlation coefficient, tau = -0.078, p = 5e-04). The structure (richness, diversity, patterns of abundance) of the RNA and DNA communities also differed. Shannon diversity ($t(3) = 10$, $p = 0.002$) and evenness were significantly greater in the DNA community ($t(3) = 10$, $p = 0.002$) (Figure 12). Richness did not significantly differ ($t(3) = -0.5$, $p = 0.7$).

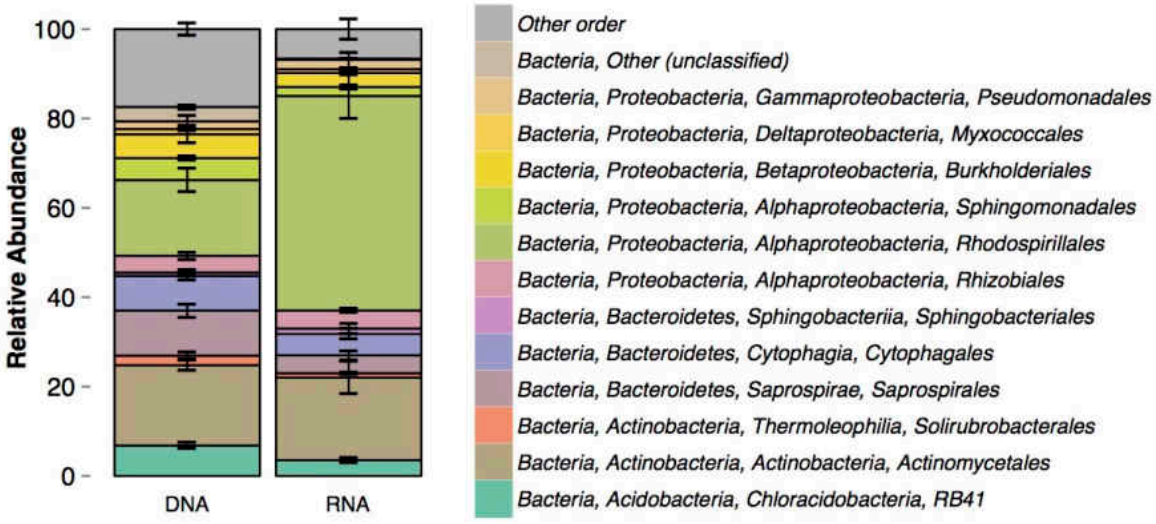

Figure 11. Taxonomic composition of RNA and DNA communities

3.3. Potentially active taxa

59 OTUs were identified as differentially abundant (DESeq2, adjusted $p < 0.05$) between the RNA and DNA communities including 12 OTUs that were more abundant in the DNA community and 47 that were more abundant in the RNA community. To identify potentially active taxa, we focused our analysis on OTUs that were shared between the RNA and DNA communities and that were overrepresented in the RNA community (DESeq2, $p < 0.01$). Using these criteria, 17 OTUs were identified as potentially metabolically active (Table 2).

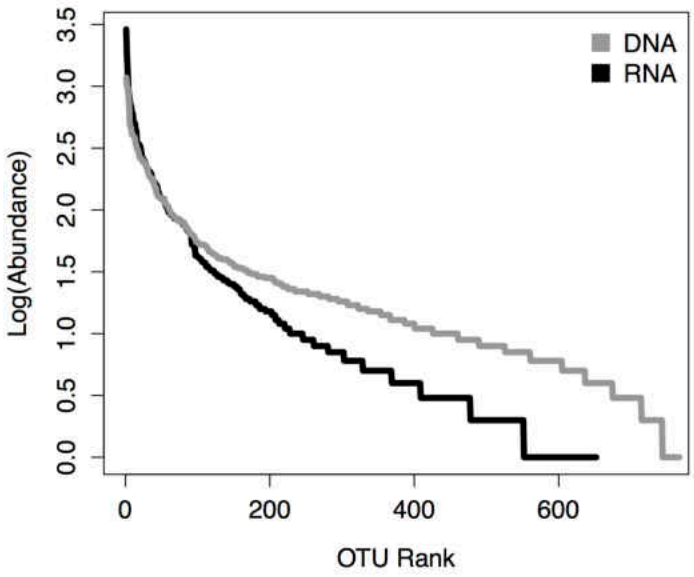

Figure 12. Rank abundance in RNA and DNA communities.

To compare the potential activity of these 17 OTUs to the rest of shared OTUs in the community, we plotted the RNA:DNA ratios of all shared OTUs against their rank in the DNA community. RNA:DNA ratios are frequently used as an index of bacterial activity (e.g. Campbell et al., 2011; Zhang et al., 2014; Baldrian et al., 2012) because the number of ribosome per cells is correlated with growth rates in cultured bacterial isolates (Fegatella et al., 1998; Kerkhof and Kemp, 1999; but see Blazewicz et al., 2013). The taxa with highest potential activity were rare members of the DNA community (Figure 13). Overall, the higher the RNA:DNA ratio, the rarer the taxon was in the DNA community. Specifically, the RNA:DNA ratio was positively correlated with rank in DNA (Spearman correlation, $\rho = 0.421$, p-value = 3.604e-16).

Table 2. OTUs significantly overrepresented in RNA

$\log_2$ Fold change	**Adjusted p-value**	**RNA: DNA**	**Taxonomy**
6.09	<0.001	115.33	Actinobacteria, Actinobacteria, Actinomycetales, Nocardiaceae
5.26	<0.001	48	Proteobacteria, Alphaproteobacteria, Rhodospirillales, Acetobacteraceae

5.06	<0.001	122	Proteobacteria, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae
4.83	<0.001	29	Actinobacteria, Actinobacteria, Actinomycetales, Microbacteriaceae
4.59	0.001	41.5	Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae
4.39	0.001	25.5	Proteobacteria, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae, Kaistobacter
4.67	0.001	29.14	Bacteroidetes, Saprospirae, Saprospirales, Chitinophagaceae
4.82	0.001	37.11	Bacteroidetes, Cytophagia, Cytophagales, Cytophagaceae, Hymenobacter
3.72	0.002	8.85	Proteobacteria, Alphaproteobacteria, Rhodospirillales, Acetobacteraceae
4.18	0.002	13.07	Proteobacteria, Alphaproteobacteria, Rhizobiales
3.95	0.003	24.5	Proteobacteria, Alphaproteobacteria, Rhodospirillales, Acetobacteraceae
3.92	0.003	23	Proteobacteria, Gammaproteobacteria
3.51	0.005	10	Actinobacteria, Acidimicrobiia, Acidimicrobiales
3.88	0.005	11.35	Proteobacteria, Alphaproteobacteria, Rhodospirillales, Acetobacteraceae
3.89	0.005	18.79	Proteobacteria, Alphaproteobacteria, Rhodospirillales, Acetobacteraceae
3.68	0.009	12.06	Proteobacteria, Alphaproteobacteria, Rhodospirillales, Acetobacteraceae, Roseomonas

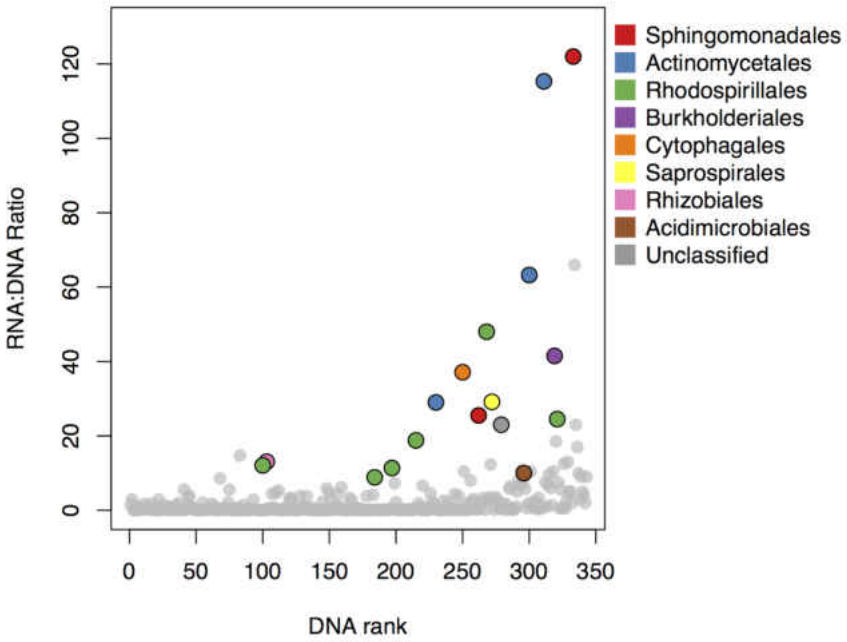

Figure 13. OTUs with highest RNA:DNA ratio are rare in DNA community. OTUs significantly overrepresented in RNA community (DESeq2, $p < 0.01$) are colored by taxonomic order.

4. Discussion

4.1. Rhodospirillales were abundant in both the total and potentially active communities

Culture-independent tools are making it possible to begin mapping the bacterial composition of the atmosphere on a global scale. While this endeavor remains in its infancy, some general patterns are beginning to emerge. Our DNA-based bacterial community data is consistent with prior studies, and includes several taxonomic orders commonly found in air samples. For example, the Actinomycetales, which were abundant in our samples, are frequently found in the atmosphere (e.g. Gandolfi et al., 2015; Brodie et al., 2007; Bowers et al., 2013) and have been found to be abundant in other studies at high elevations sites (Bowers et al., 2012) including MBO (Smith et al., 2013). There are two reasons to expect this order to be abundant in the atmosphere. First, Actinomycetales are ubiquitous in soil and freshwater (Ventura et al., 2007), and thus there is a large terrestrial source pool. Second, they produce small spores (Reponen et al., 1998), which likely have a long residence time in the atmosphere. Several other taxonomic groups found in samples from this study, which have been previously detected in air samples

collected at MBO, include Pseudomonadales, Burkholderiales, and Sphingomonadales (Smith et al., 2013). One pattern that is unique to our data set is the prevalence of Rhodospirillales. In other studies of airborne bacterial communities, Rhodospirillales have been detected low relative abundance and have never been found in high relative abundance (Bowers et al., 2013; Polymenakou et al., 2008). One potential mechanism driving the abundance of Rhodospirillales at MBO could be the nearby marine source. MBO is approximately 200 km from the Pacific Ocean, and most air masses travel over the Pacific for several days before reaching the summit (Weiss-Penzias et al., 2006). Rhodospirillales are frequently found in marine samples (e.g. Yin et al., 2013; Feingersch et al., 2010; Li et al., 2014), and their presence in the atmosphere has been reported in a study of communities in the upper levels of the atmosphere (~ 8-10 km above sea level) (DeLeon-Rodriguez et al., 2013). Researchers found that Rhodospirillales were enriched in samples collected during a hurricane (DeLeon-Rodriguez et al., 2013) suggesting that, under certain conditions, marine bacteria can be aerosolized and reach the upper levels of the atmosphere.

To our knowledge, this is the first RNA-based analysis of the atmosphere. Consistent with what has been reported in other environmental systems (e.g. soil (Baldrian et al., 2012), and water (Wilhelm et al., 2014)), we found that the RNA- and DNA-community composition differed (Figure 11). Several orders - including RB41 (an uncharacterized order of Acidobacteria), Saprospirales, and Sphingomonadales - were significantly underrepresented in the RNA relative to the DNA community. RB41 and Saprospirales are typically found in soils (King et al., 2010; Janssen, 2006), and Saprospirales has also been detected in air samples (Fierer et al., 2008). Sphingomonadales are commonly detected in air samples (e.g. Bowers et al., 2011; Amato et al., 2007; Després et al., 2012) and are abundant on leaf surfaces (Vorholt, 2012). These results suggest that soil and leaf surfaces are likely substantial sources of bacteria in the atmosphere and may shape the composition of the total community, but perhaps not the active community. There was only one order that was significantly more abundant in the RNA relative to the DNA community, and this was Rhodospirillales. The potential role of metabolically active Rhodospirillales is discussed below.

4.2. Potentially active communities were characterized by both dominance and rarity

Similar to soil, freshwater, and marine systems (e.g. Mikkonen et al., 2014; Zhang et al., 2014; Wilhelm et al., 2014), we found that the diversity of the total and potentially active communities differed in the atmosphere. Shannon diversity and evenness was higher in the DNA community (Figure 12). In contrast, the RNA community was more characterized by dominance and rarity (i.e. there were a few abundant organisms and many rare organisms). This pattern may be driven by a differential in the activity of taxa, given that a few taxa were highly active and the majority of taxa had low activity levels. Most comparative DNA- and RNA-community studies do not report on evenness. However, two studies of active and total communities in soils yielded conflicting results, with one reporting higher evenness in the DNA community (Gremion, Chatzinotas, and Harms, 2003), and the other reporting higher evenness in the RNA community (Mikkonen et al., 2014). The lack of consistent patterns across studies could reflect differences in ecosystem properties. Microbial rank abundance curves are dynamic (Lennon and Jones, 2011). Differences in the structure of active and total communities depend on ecosystem characteristics including resource availability, perturbation regime, and residence time, which change through time and can drive changes in the microbial diversity patterns.

4.3. Rare taxa in the total community were disproportionately active

Across all OTUs, abundance in DNA was correlated with abundance in RNA. However, OTUs with the highest RNA:DNA ratio were rare members of DNA community. In other words, the potential activity of these OTUs was negatively related to abundance in the DNA community (Figure 13). This pattern has been observed in other environments including marine (Hugoni et al., 2013; Campbell et al., 2011; Hunt et al., 2013), freshwater (Wilhelm et al., 2014), and soil systems (Gremion, Chatzinotas, and Harms, 2003). Suggesting that across environments, the bacterial taxa that contribute to ecosystem functioning appear rare in DNA-based surveys, so their importance may not be recognized. This highlights the importance of RNA-based surveys in linking microbial community composition to ecosystem function, particularly in relatively uncharacterized environments such as the atmosphere.

4.4. Rhodospirillales are potentially active in the atmosphere

Potentially active taxa were identified as belonging to several taxonomic groups. Members of the order Rhodospirillales were consistently identified as being active across all analyses. At the family level, potentially active individuals include members of the following taxonomic groups: Acetobacteraceae; Nocardiaceae; Ellin6075 species; Kaistobacter species. Of 343 shared OTUs, 59 significantly differed in abundance. Of those, 14 OTUs belonging to the family Acetobacteraceae were more abundant in the RNA community. The Acetobacteraceae are members of the order Rhodospirillales. They are the acetic acid bacteria, and their metabolism is characterized by the fermentation of ethanol to acetic acid (Komagata, Iino, and Yamada, 2014). Ethanol is a common chemical in the atmosphere and is a precursor of acetaldehyde and peroxyacetyl nitrate, which are both components of smog. This suggests that bacteria in the atmosphere may be involved in the cycling of compounds that are relevant to human health.

The three Acetobacteraceae OTUs with the greatest difference in abundance were all identified by BLAST (Altschul et al., 1990) as *Acidisphaera rubrifaciens. A. rubrifaciens* is an aerobic, chemoorganoheterotroph and facultative phototroph which was isolated from an acidic hot spring. It produces bacteriochlorophyll a and carotenoid pigments, which could protect against UV damage in the atmosphere. Research has shown that the optimal growth of *A. rubrifaciens* occurs in the light with simple organic compounds as energy and carbon sources. More specifically, growth can occur on the conjugate bases of organic acids (Hiraishi et al., 2000) found in the atmosphere such as fumarate, gluconate, lactate, malate, pyruvate, and succinate (Finlayson-Pitts and Pitts Jr 1999). It is possible that succinate concentrations were elevated during the time of sampling at the MBO site, as there were several active wildfires in the region, and concentrations succinic acid are often elevated in the atmosphere during biomass burning (Kundu et al., 2010; Falkovich et al., 2005). Furthermore, microbes isolated in the atmosphere can degrade organic acids, including succinate, and this biological process may be more important than abiotic chemical cycling (i.e. photodegradation) (Vaïtilingom et al., 2011). The potential ability of airborne bacteria, such as *A. rubrifaciens*, to grow using organic acids has implications for biogeochemical cycling in the atmosphere.

5. Conclusion

Airborne metabolically active bacteria may alter the chemistry of the atmosphere through the biogeochemical cycling of organic compounds. However, little is currently known about which taxa may be active and their potential functions. Our study represents the first to use both RNA- and DNA-based methods to identify potentially active bacteria in the atmosphere. We found that the RNA community was characterized by the presence of a few highly active taxa and many taxa with low activity levels, and taxa that were rare in the DNA community were the most likely to be metabolically active. Potentially active taxa in the atmosphere included members of the order Rhodospirillales, specifically, *Acidisphaera rubrifaciens*. *A. rubrifaciens* may be well-suited for growth in the atmosphere because it has pigments which can mitigate UV damage, and it grows well on simple organic compounds including some common in the atmosphere. We suggest future research should combine both culture-independent and culture-dependent approaches to assess the potential activity of bacteria in the atmosphere. Culture-independent approaches could be used to identify potentially active taxa, and then culture-dependent methods could be used to isolate organisms and study their physiology under various conditions possibly using aerosolization chambers. As with any environment, understanding both the structure and function of microbial communities in the atmosphere is needed to assess their potential impact on ecosystem processes such as carbon cycling. This study opens the door for future investigations of the diversity and function of bacterial communities in the atmosphere.

CHAPTER VI
CONCLUSION

This dissertation contributes to the fields of microbiology and ecology by examining the microbial life in an understudied environment, the atmosphere. It expands the scope of inquiry around microbes in the atmosphere by considering the possibility that the atmosphere could be a habitat for microbial life and provides a foundation for further inquiry into the identity and function of metabolically active populations of microbes in the atmosphere. By measuring temporal variation in bacterial communities in the atmosphere, this work also contributes to our understanding of the role of the forces structuring microbial communities in the atmosphere.

Summary of Results

Chapter II

The study of the microbial diversity of the atmosphere has the potential to greatly expand how we think about the distribution of life on Earth. Aerobiology has a rich history of study, but technical and conceptual limitations have hindered the study of microbial communities in the atmosphere. Advances in molecular biology techniques and DNA sequencing have made it possible to study the diversity of airborne communities using culture-independent methods. Conceptual limitations can be overcome by viewing the atmosphere as a potential habitat for microorganisms and by designing experiments accordingly.

Chapter III

In the atmosphere over the Amazon rainforest, the total and metabolically active fungal communities differed the relative abundances of the dominant phyla, Ascomycota and Basidiomycota. These differences were predicted using a mass-balance model, which took into account differences in the size and abundance of cells from each phylum. The metabolically active community more closely resembled communities in potential source environments than did the total community. Lichen fungi were abundant members of the active community and their abundance in the total community may be underestimated.

Lichen fungi are efficient ice nucleators, so their abundance in the atmosphere over the Amazon rainforest has implications for precipitation development in this globally important region.

Chapter IV

At Mt. Bachelor Observatory, the diversity and composition of microbial communities in the atmosphere varies over multiple time scales. The composition of bacterial communities in the atmosphere varied within days and may be related to diurnal fluxes of bacteria from local sources including rock surfaces. In contrast to the pronounced diurnal variability, community composition was relatively stable across days. Diversity varied over both within and across days. Changes in diversity across days could be due to influence of distinct air masses that arrived at the summit during sampling including air masses from high elevations in the atmosphere and air masses which were impacted by wildfires.

Chapter V

Comparative DNA- and RNA-based evidence suggests that bacteria in the atmosphere are metabolically active. At Mt. Bachelor Observatory, the total and active communities significantly differed. The species abundance distribution of the active community was characterized by dominance and rarity with a few abundant taxa and many rare taxa whereas the total community was characterized by more even relative abundances of taxa. The total and active communities also differed in composition. As has been observed in soil and aquatic environments, taxa that were rare in the total community were more like to be metabolically active highlighting the importance of RNA-based surveys in studying the function of microbial communities. The order Rhodospirillales was overrepresented in the active community and members of this community were differentially abundant in the active and total communities. Members of this order including *Acidisphaera rubrifaciens* may be well suited for growth in the atmosphere.

Future Directions

The study of microorganisms in the atmosphere sheds light on the processes underlying the distribution of microbial life on Earth and contributes to our understanding of the roles microorganisms play in atmospheric processes. A productive approach for future research may employ both culture-independent approaches as well as culture-based, manipulative experiments to study the diversity and function of microbial communities in the atmosphere. For example, experiments might involve the analysis of DNA sequence data from high resolution time series samples and samples local source environments in order to better understand the links between local sources and environmental conditions in structuring microbial communities in the atmosphere. This type of culture-independent data about microbial communities in the atmosphere can also be used to inform culture-dependent experiments in the laboratory. For example, RNA-based sequence data revealed that lichen fungi might be abundant in the atmosphere over the Amazon rainforest. The next step could be to isolate lichen fungi from the atmosphere over the Amazon and test the ability of these organisms to nucleate ice in order to better assess the importance of lichen fungi on precipitation development in the Amazon. Similarly, comparative DNA- and RNA-based analyses suggested that members of the bacterial order Rhodospirillales might be metabolically active in the atmosphere. Future work could involve culturing Rhodospirillales taxa in the laboratory and testing their ability to grow using substrates found in the atmosphere. Such studies might involve aerosolization-chambers and other methods for recreating the atmospheric environment in the lab. These and other experimental approaches have the potential to expand our understanding the distribution and function microorganisms in their habitats. This will be an important step toward an integrated understanding of microbial life on Earth connecting the lithosphere, hydrosphere, and the atmosphere.

APPENDIX A
MASS-BALANCE MODEL FOR CHAPTER III

We use a global, well-mixed, one-box material-balance model to explain the relative abundances of fungal cells measured as gene copies sampled in the active and total portions of atmospheric bioaerosols. By material-balance, for any taxon *i* within a biological community, the change in time in the abundance of fungal gene copies, N_i, must be equal to the difference in source and sinks:

$$\frac{dN_i}{dt} = \sum sources - \sum sinks \quad \text{(A1)}$$

Here we assume sources are equal to the emission of fungal gene copies from the Earth's surface into the atmosphere, E_i (gene copies/hour). We assume sinks are equal to deposition of fungal gene copies out of the atmosphere back to the Earth's surface, $D_i = N_i k_i$, (gene copies/hour), where k_i (1/hour) represents a first order deposition coefficient. We can rewrite Equation (A1) as:

$$\frac{dN_i}{dt} = E_i - N_i k_i$$

We expect the terms E_i and k_i to vary as a function of life history traits including the method of cell release into the atmosphere, the physiological state of sampled cells, and the aerodynamic diameter of fungal taxa. In this case, Equation (A2) does not directly represent the entire airborne fungal gene copy abundance. We assume that a first order approximation of fungal bioaerosol behavior may be obtained by subdividing the particle distribution into two modes: vegetative cells, $N_{i,veg}$, and spores, $N_{i,spores}$. We thus model fungal gene copy abundance as:

$$N_i = N_{i,veg} + N_{i,spores}$$

We can then write and solve parallel versions of Equation (A2) for each mode. At steady state, the expected gene copy abundance taxa *i* in each mode is:

$$N_{i,veg} = \frac{E_{i,veg}}{k_{i,veg}}$$

$$N_{i,spore} = \frac{E_{i,spore}}{k_{i,spore}}$$

Our interest lies in the two most common fungal phyla sampled in the atmosphere: Ascomycota, N_A, and Basidiomycota, N_B. To make predictions about the expected relative abundance of gene copies in these two groups, we make informed assumptions about the relative magnitude of their respective emission and deposition rates. We begin by considering fungal spores. Although a few empirical studies have suggested that Ascomycota are more abundant than Basidiomycota in likely source environments including tropical soils (Kerekes et al., 2013) and leaf surfaces (Kembel and Mueller, 2014), Basidiomycota (e.g. Agaricomycetes, the most abundant class of Basidiomycota in our samples) produce orders of magnitude more spores per individual than Ascomycota (Elbert et al., 2007; Pringle, 2013). For this reason, we assume the emission rate of Basidiomycota spores is much greater than that of Ascomycota spores:

$$E_{A,spores} \ll E_{B,spores}$$

Culture-based microscopy data suggests that spores of Ascomycota are typically larger than spores of Basidiomycota (Elbert et al., 2007; Ingold, 2001; Yamamoto et al., 2014). Owing to the difference in spore size, we expect deposition rate of Ascomycota spores to be greater than that of Basidiomycota spores:

$$k_{d,A,spores} > k_{d,B,spores}$$

Based on these assumptions, it follows that the expected number of Ascomycota spores in the atmosphere will be less than the number of Basidiomycota spores:

$$\frac{E_{A,spore}}{k_{A,spore}} \ll \frac{E_{B,spore}}{k_{B,spore}}$$

or

$$N_{A,spores} \ll N_{B,spores}$$

We next consider fungal vegetative cells. Vegetative forms of Ascomycota are generally smaller than vegetative forms of Basidiomycota (Moore et al., 2011). Many Ascomycota grow as filaments or single cells that are small enough to be aerosolized (Després et al., 2012). In contrast, many Basidiomycota grow as mushrooms, which are too large to be aerosolized (although debris from mushrooms and their mycelia can be aerosolized). Due

to this difference in the vegetative forms of each group, we expect emission rate of vegetative Ascomycota to be greater than Basidiomycota:

$$E_{A,veg} > E_{B,veg}$$

No comparative data currently exists on the relative deposition rate of vegetative cells across fungal taxa. Research has shown that at the phylum level, the aerodynamic diameter of Ascomycota is greater than that of Basidiomycota, resulting in a greater deposition rate overall for Ascomycota (Yamamoto et al., 2014). However this work did not differentiate between vegetative cells and spores, and there is no *a priori* reason to assume that the deposition rate of Ascomycota vegetative cells are less than or greater to that of Basidiomycota cells. For this reason, we make the null assumption that the deposition rate of each group is equal:

$$k_{d,A,veg} = k_{d,B,veg}$$

Based on these assumptions, we expect the number of vegetative Ascomycota genes to be greater than the number of vegetative Basidiomycota genes:

$$\frac{E_{A,veg}}{k_{A,veg}} > \frac{E_{B,veg}}{k_{B,veg}}$$

or

$$N_{A,veg} > N_{B,veg} \tag{A3}$$

Equation (A3) predicts that Ascomycota will dominate the active fungal community in the atmosphere.

Finally, we relate the abundance of Ascomycota and Basiodiomycota gene copies in their totality to ask if $N_A < N_B$ or $N_A \geq N_B$. $N_A < N_B$ if and only if:

$$N_{A,veg} + N_{A,spores} < N_{B,veg} + N_{B,spores}$$

Rearranging terms and dividing both sides of the equation by $N_{B,spores}$ yields the inequality:

$$\frac{N_{A,veg} - N_{B,veg}}{N_{B,spores}} + \frac{N_{A,spores}}{N_{B,spores}} < 1$$

or

$$\frac{N_{A,veg} - N_{B,veg}}{N_{B,spores}} < 1 - \varepsilon$$

where $\varepsilon = \frac{N_{A,spores}}{N_{B,spores}}$. Empirical data on the discharge of Ascomycota and Basidiomycota spores from fruiting bodies suggests that $\varepsilon \leq 0.01$ (Elbert et al., 2007). In this case $N_A < N_B$ if and only if:

$$\frac{N_{A,veg} - N_{B,veg}}{N_{B,spores}} < 0.99 \quad \text{(A4)}$$

We expect Equation A4 to hold due to the likelihood that spores greatly out number vegetative cells in the atmosphere in both phyla. Spores can be actively discharged into the air, whereas vegetative cells are not actively propelled into the atmosphere and require aerosolization by mechanical forces like wind. Furthermore, empirical data suggests that vegetative cell fragments constitute a small fraction (0.2-16% (Green et al., 2011)) of the total fungal biomass in the atmosphere. For these reasons, we predict that

$$N_A < N_B$$

Based on the conclusions of this model, we expect Basidiomycota will dominate the total community, and Ascomycota will dominate the active community in the atmosphere. We note there are many limitations to our model. First, we model fungal gene copy abundances assuming a well-mixed atmosphere at steady state. Yet the atmosphere is a highly heterogeneous and dynamic environment; the sampled air volume was likely neither well mixed nor at steady state over the time intervals we measured. Second, we use a global model with emission and deposition as the sole input and output, whereas a local model that incorporated site-specific environmental fate and transport terms would likely provide more accurate expectations. Third, due to a paucity of data, our estimates of fungal gene abundance levels rely on assumptions about the emission and deposition rates of vegetative cells and spores across fungal taxonomic groups. Empirically derived estimates of these model parameters would significantly improve our approach. Fourth, we do not know to what extent vegetative cells and spores are associated with other particulate matter and how this affects their deposition and emission rates. Adopting an

approach to empirically estimate the aerodynamic diameter of these fungal cell types across taxonomic groups would allow for improved estimates of deposition rates (Yamamoto et al., 2014).

APPENDIX B

SUPPLEMENTAL INFORMATION FOR CHAPTER III

Table 3. Barcodes and adapter/sequencing primers for metatranscriptome

Sample	Barcode	5' adapter/sequencing primer	3' adapter/sequencing primer
Dec .8	AAACT	AGTTTAGATCGGAAGAGCG GTTCAGCAGGAATGCCGAG	ACACTCTTTCCCTACACGA CGCTCTTCCCATCTAAACT
Dec. 9	AAAGC	AGTTTAGATCGGAAGAGCG GTTCAGCAGGAATGCCGAG	ACACTCTTTCCCTACACGA CGCTCTTCCCATCTAAACT
Dec. 10	AACAA	AGTTTAGATCGGAAGAGCG GTTCAGCAGGAATGCCGAG	ACACTCTTTCCCTACACGA CGCTCTTCCCATCTAAACT
Dec. 11	AACTG	AGTTTAGATCGGAAGAGCG GTTCAGCAGGAATGCCGAG	ACACTCTTTCCCTACACGA CGCTCTTCCCATCTAAACT

Table 4. LSU barcodes and primers

Sample	Forward barcode	Reverse barcode	Full barcode	Forward primer (LR0R)	Reverse primer (LR3)
Dec .8	TAATTC	AATATC	AATATC TAATTC	ACCCGCTGA ACTTAAGC	CCGTGTTTC AAGACGGG
Dec. 9	TACACA	AATATC	AATATC TACACA	ACCCGCTGA ACTTAAGC	CCGTGTTTC AAGACGGG
Dec. 10	CCCTAA	AATATC	AATATC CCCTAA	ACCCGCTGA ACTTAAGC	CCGTGTTTC AAGACGGG
Dec. 11	CCGAGG	AATATC	AATATC CCGAGG	ACCCGCTGA ACTTAAGC	CCGTGTTTC AAGACGGG

Table 5. Air sequence summary statistics

Sample	# Metatranscriptome Sequences	# rRNA sequences (metatranscriptome)	# LSU amplicon sequences
Dec. 8	331,765	51,361	5,377
Dec. 9	601,828	97,871	20,766
Dec. 10	0	0	18,778
Dec. 11	4,231,592	1,766,762	10,493

Table 6. Multi-environment sequence summary statistics

Study	# Samples	# Sequences	Biome
Barnard et al., 2013	60	151,125	Temperate grassland soil
Kerekes et al., 2013	36	26,390	Tropical forest soil
Kembel and Mueller, 2014	100	771,562	Tropical forest phyllosphere
Penton et al., 2013	16	82,618	Tundra soil
Penton et al., 2013	12	93,994	Temperate grassland soil
Penton et al., 2014	48	451,769	Temperate grassland soil
This study (amplicons)	4	55,414	Tropical forest air (DNA)
This study (metatranscriptome)	3	1,915,994	Tropical forest air (RNA)

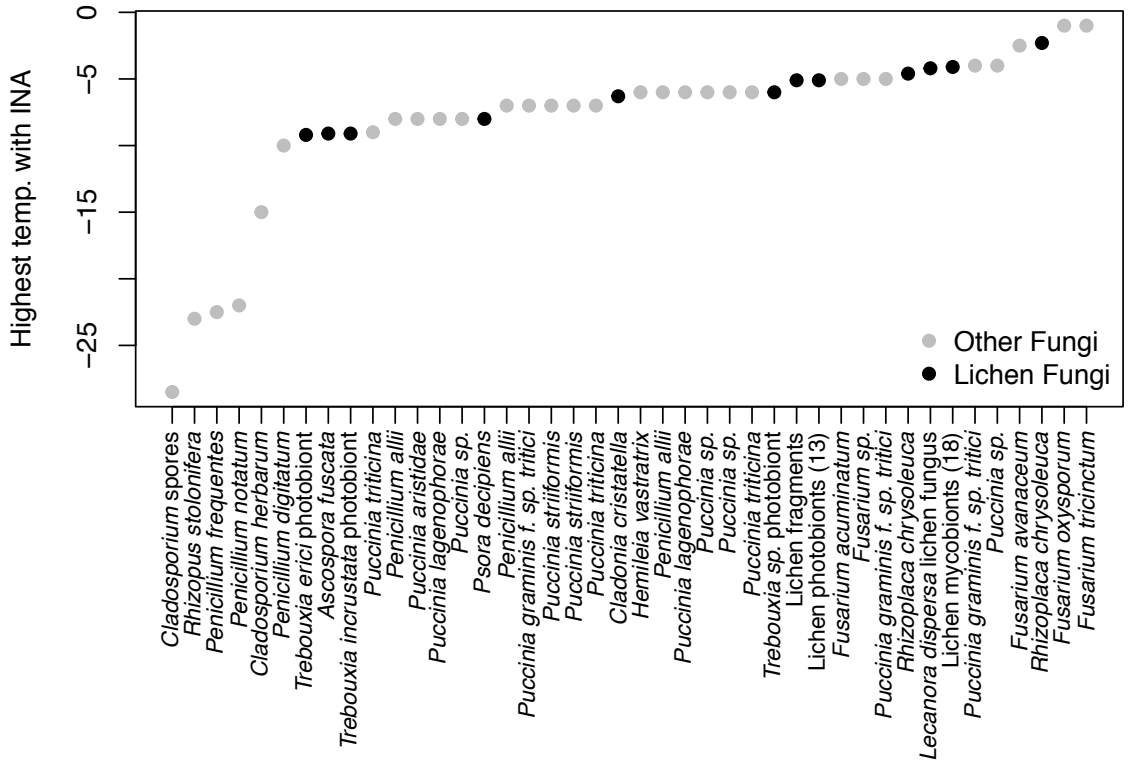

Figure 14. Highest temperatures with ice nucleation activity in lichens and other fungi. Data compiled from Després et al., 2012 and C. E. Morris et al., 2013.

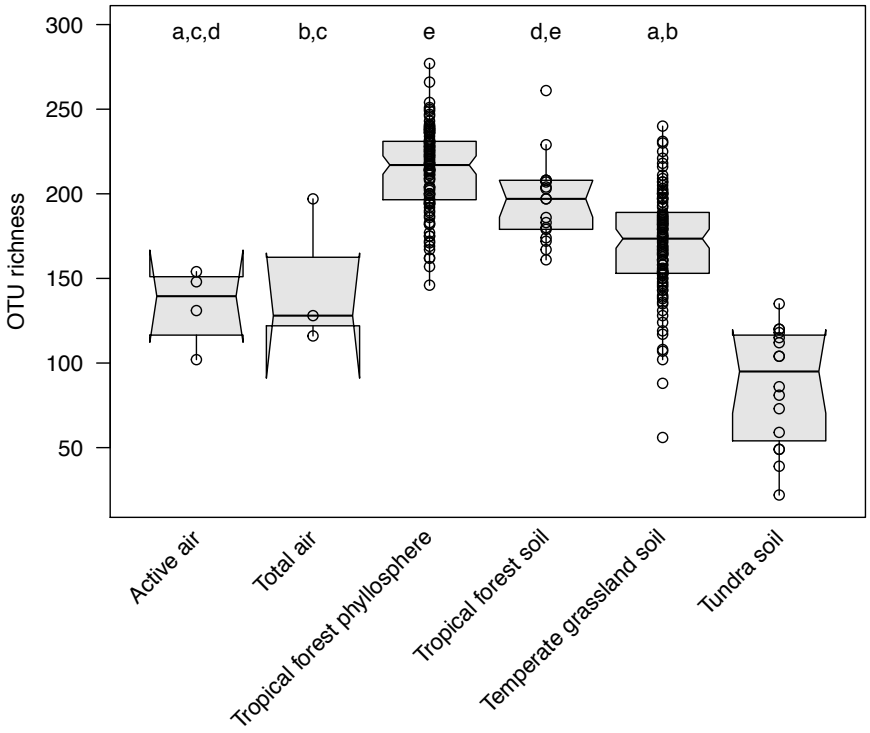

Figure 15. OTU richness significantly varied among environment types. Letters above each box indicate non-significant pairs after Tukey's test (adjusted p-value > 0.05).

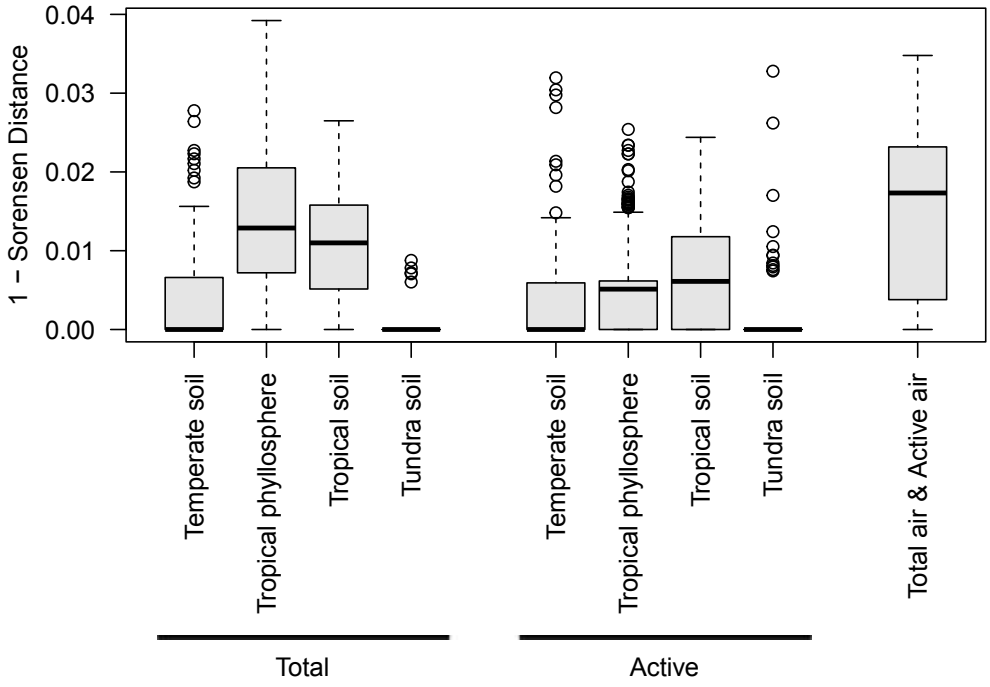

Figure 16. Total and active air communities were most similar to tropical phyllosphere tropical soil communities.

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