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Long Range Transport of Microorganisms in the Upper Atmosphere

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Abstract

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Microorganisms are abundant in the atmosphere and can be transported globally on winds. In general, the upper atmosphere is an understudied, extremely harsh environment where the limits of life can be tested unlike anywhere else on our planet. However, the challenge of collecting samples (and historic reliance upon culture-based characterization methods) has prevented a comprehensive understanding of microbes at high altitudes. The goal of my dissertation is to examine the airborne transport of microorganisms across the Pacific Ocean using surface-, satellite-, aircraft-, and model-based observations. A variety of techniques were employed to achieve maximum sensitivity to airborne microbes (including dead and non-cultivable cells). Air was collected at the summit of the Mt. Bachelor Observatory (2.7 km above sea level in Oregon, USA) where free tropospheric plumes routinely arrive from distant, transpacific sources. Microbial abundance and richness values were derived from qPCR measurements and 16S rRNA microarray analysis. Thousands of distinct bacterial taxa (and 6 archaeal species) were detected in Asian air samples, spanning a wide range of phyla and surface environments. Viable cells (including presumptive plant pathogens) were identified by rRNA gene sequencing. Next, we flew a high-altitude (20 km) sampling mission over the Pacific Ocean in a period that coincided

with intercontinental dust plumes. Several bacterial and fungal isolates were recovered and later exposed to a series of stratosphere simulations to evaluate survivability during transport. Most (99.9%) of cells were killed by ultraviolet irradiation within several hours, indicating that the upper atmosphere can be a critical barrier to long-distance microbial dispersal. Overall, we discovered the upper atmosphere contains a surprising degree of microbial richness and that atmospheric winds can bridge populations (containing living and dead cells) between Asia and North America. Our results inform a number of important environmental and population health problems on both sides of the Pacific. Furthermore, we demonstrate that microbial biogeography is a useful tool for validating aerosol source regions and global transport models. Ultimately, studying life in the upper atmosphere redefines our concept of the biosphere and can provide new insights about the diversity, distribution, and evolution of life on Earth.

TABLE OF CONTENTS

Page

List of Figures iii
List of Tables iv
Chapter 1: The High Life: Transport of Microbes in the Atmosphere 1
1.1 Introduction 1
1.2 Hard to Kill 2
1.3 Aerobiological Cycles 4
1.4 Implications 5
1.5 Long-Term Sampling 7
1.6 Acknowledgements 9
Chapter 2: Free Tropospheric Transport of Microorganisms from Asia to North America 10
2.1 Abstract 10
2.2 Introduction 11
2.3 Materials and Methods 15
2.4 Results 22
2.5 Discussion 29
2.6 Acknowledgements 35
Chapter 3: Intercontinental Dispersal of Bacteria and Archaea in Transpacific Winds 37
3.1 Abstract 37
3.2 Introduction 38
3.3 Materials and Methods 39
3.4 Results and Discussion 43
3.5 Acknowledgements 53
3.6 Supplementary Information 54
Chapter 4: Stratospheric Microbiology at 20 km over the Pacific Ocean 61
4.1 Abstract 61
4.2 Introduction 62
4.3 Materials and Methods 65
4.4 Results 71
4.5 Discussion 74
4.6 Acknowledgements 77
Chapter 5: Microbial Survival in the Stratosphere and Implications for Global Dispersal 79
5.1 Abstract 79
5.2 Introduction 80
5.3 Materials and Methods 84
5.4 Results 92
5.5 Discussion 97
5.6 Acknowledgements 102

Chapter 6: Conclusions 104
6.1 Synthesis 104
6.2 Future Work 105

LIST OF FIGURES

Page

Figure Number

1.1 Scanning electron micrograph (SEM) of bacterial spores and dust particle 3
1.2 Mt. Bachelor Observatory (MBO) in central Oregon 8
2.1 Air sampling apparatus at the MBO 14
2.2 Microbial qPCR data from 1 March to 11 April 2011.....21
2.3 Microbial qPCR data from 11 April to 20 May 2011.....22
2.4 SEM of filters used for capturing airborne microorganisms at MBO. 23
2.5 Seasonal dust measured by sub-micron aerosol mass concentration 28
2.6 Profile of the 22-23 April 2011 long range transport event 29
2.7 Profile of the 13-15 May 2011 long range transport event 32
3.1 HYSPLIT kinematic back trajectories for air arriving at MBO 44
3.2 Partitioning plume samples and background samples using incidence data 48
3.3 Relative abundance of fifteen common bacterial families across plumes 49
3.4 Significant abundance variations in taxa 51
S3.1 Enhancement ratios of THg and CO during plumes 54
S3.2 Soil, NH_4SO_4, and aerosol data for the 22-24 April 2011 episode 55
S3.3 NAAPS modeled aerosol plumes 56
S3.4 Soil, NH_4SO_4, and aerosol data for the 12-15 May 2011 episode 57
S3.5 Partitioning plume samples and background samples using abundance data 58
S3.6 Principle coordinate analysis 59
S3.7 Circular tree displaying taxonomic relationship and changes in abundance 60
4.1 Flight hardware 64
4.2 Sampling flight path 66
4.3 Jet stream trajectory 67
4.4 Environmental data at stratosphere sampling location 68
4.5 Post-flight processing sequence and hardware nomenclature 69
5.1 Hypobaric environmental chamber used for stratosphere simulation experiments 90
5.2 Energy-dispersive x-ray spectrometry analysis performed on bacterial coupons 92
5.3 Scanning electron microscopy of bacterial coupons 94
5.4 Ultraviolet light values 94
5.5 Simulated stratosphere experiments 96
6.1 Microorganisms in the Stratosphere (MIST) mission patch.....106

LIST OF TABLES

Page

Table Number

2.1 Reaction Conditions and qPCR Primers 20
2.2 Microbial and Meteorological Averages +/- Standard Deviation 24
2.3 Frequency and Best Match of Bacteria Recovered 26
2.4 Frequency and Best Match of Fungi Recovered 27
3.1 April Plume Sample Manifest 45
3.2 May Plume Sample Manifest 45
3.3 Archaea Detected in April and May Events 47
4.1 Bacterial Growth 72
4.2 Fungal Growth 73
5.1 UV Fluence Rates for Experiments 88
5.2 Description of Experimental Groups in Stratosphere Simulation 88

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DEDICATION

To Charles Duchen
(1918-2012)

Chapter 1

THE HIGH LIFE: TRANSPORT OF MICROBES IN THE ATMOSPHERE

Chapter 1 introduces readers to the interdisciplinary science of aerobiology and my overarching research questions. Portions of this chapter were originally published in collaboration with Dale W. Griffin and Daniel A. Jaffe in the July 2011 edition of the newsletter *Eos* (Smith et al., 2011, *Eos*, Vol. 92(30), pp. 249-250) (©2011 *Eos, Transactions*, American Geophysical Union), and are reproduced by permission of American Geophysical Union.

1.1 Introduction

Microbes (bacteria, fungi, algae, and viruses) are the most successful type of life on Earth because of their ability to adapt to new environments, reproduce quickly, and disperse globally. Dispersal occurs through a number of vectors, such as migrating animals or the hydrological cycle, but transportation by wind may be the most common way microbes spread.

General awareness of airborne microbes predates the science of microbiology. People took advantage of wild airborne yeasts to cultivate lighter, more desirable bread as far back as Ancient Egypt by simply leaving a mixture of grain and liquids near an open window. In 1862, Louis Pasteur's quest to disprove spontaneous generation resulted in the discovery that microbes were actually single-celled, living creatures, prevalent in the environment and easily killed with heat (Pasteurization). His rudimentary experiments determined that any nutrient medium left open to the air would eventually be teeming with microbial life because of free-floating, colonizing cells. The same occurrence can happen in a kitchen; opportunistic fungal and bacterial cells cause food items briefly exposed to the air to eventually spoil.

Unknowingly, Pasteur founded the field that is referred to today as aerobiology, the science that studies the diversity, influence, and survival of airborne microorganisms. Scientists now have the ability to monitor the movement of atmospheric microorganisms on a global scale. But long-term, molecular-based measurements of microbial concentrations are still missing—such information is needed to improve our understanding of microbial ecology, the spread of disease, weather patterns, and atmospheric circulation models.

1.2 Hard to Kill

Single-celled microorganisms have direct contact with the outside environment through a relatively thin plasma cell membrane, which allows them to be extremely efficient metabolic machines. The tradeoff is that when environmental conditions worsen (e.g., the disappearance of water or nutrients, increased exposure to solar radiation, etc.), the slim barrier is all that stands between life and death.

Bacteria have evolved a variety of defense mechanisms that have enabled them to endure the physical threats associated with airborne transport. For example, one of the most successful protective strategies for some bacteria during periods of stress is to form a dormant endospore (more commonly referred to as 'spore,' see Figure 1.1), a phase somewhat analogous to hibernation in animals. The metamorphosis from a normal parent cell into a dormant spore is controlled by the entire cell population (via 'quorum-sensing' pathways). During the spore-building process, cells shrink and harden, intercellular contents are dehydrated and an impermeable cell-wall coating is reinforced to shield the interior. The rate of sporulation is temperature and species dependent, and when completed, parent bacterial cells have been transformed from a size of 3–5 micrometers down to ~1 micrometer. In many regards, a spore is

a microbiological fortress, completely dormant with no active growth or metabolism, constructed for the purpose of indefinitely protecting DNA and conserving energy.

But spores can rapidly reactivate and resume normal cellular activity upon contact with water or nutrients, provided that certain biomolecules were not damaged during the dormancy period. Ultraviolet (UV) radiation (particularly in the wavelength range of 200 – 315 nm) tends to be the main lethal factor for airborne microorganisms, but spore-forming bacteria have evolved defenses against that problem too. Many species have the ability to stabilize DNA strands with small acid-soluble proteins (SASP), perform active repairs to damaged macromolecules, or synthesize photoprotective pigments. Non-spore-forming bacteria (the vast majority of bacterial species, in fact) must rely on other tricks to survive atmospheric stress; for example, the ability to efficiently scavenge extra cellular water and nutrients, generating higher concentrations of

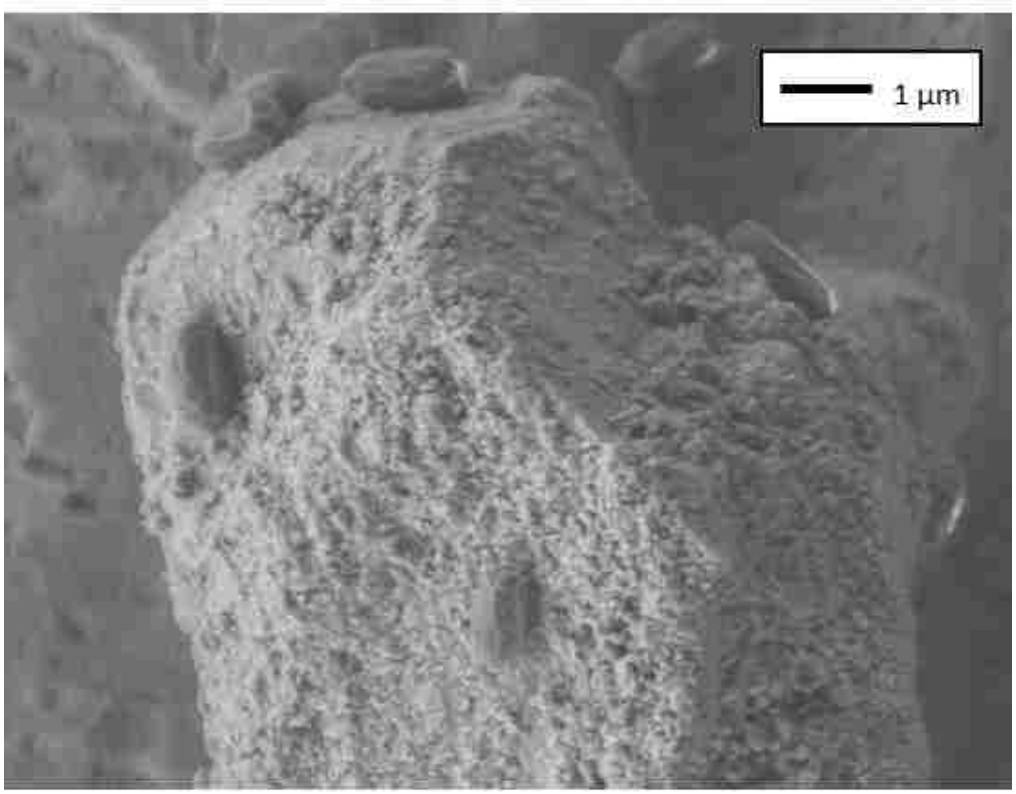

Figure 1.1 Tiny (1µm) ovoid-shaped bacterial spores sampled from the atmosphere are attached to a large dust particle.

pigmentation, or repairing molecules damaged by radiation. Both spore-forming and non-spore-forming bacteria are well represented in aerobiology literature, though viable non-spore-forming species tend to be less common at higher altitudes where UV intensity is greater (Smith et al., 2010). Other airborne biota such as viruses and reproductive fungal spores may depend on associations with particulate matter to prevent the degradation of DNA. Shielding can be provided by organic (e.g. pollen) and inorganic (e.g. dust) particles, or even surrounding layers of dead cells.

1.3 Aerobiological Cycles

Wind is responsible for lofting of most atmospheric microbes from land or oceanic sources, but it is difficult to determine precisely how cells become and remain airborne. Aerobiologists have identified a variety of possible lofting mechanisms, including but not limited to: dust storms; wave action on the ocean surface; smoke plumes from forest fires; sewage plant emissions; and aerosolized topsoil associated with agriculture. While seasonal dust storms from the largest deserts on the planet in regions of North Africa and Asia may be the primary sources of airborne biomass (see Kellogg & Griffin, 2006), oceans, lakes, grasslands, forests and agricultural fields are other regularly contributing ecosystems.

Burrows et al. (2009b) reviewed the sources and sinks of airborne microbiology and estimated that the annual flux of bacteria to the atmosphere is 40–1800 billion grams. This profusion would explain why, in recent decades, the aerobiology community has obtained microbial samples from many different atmospheric environments, ranging from urban centers to remote continental areas, and even at altitudes up to 77 kilometers in the mesosphere (see Smith et al., 2010). Historically, aerobiologists have probably undervalued the amount of airborne

biology by using enumeration techniques that depend on the cultivation of microbial strains in the laboratory. My research will demonstrate that molecular-based assays can generate more accurate abundance estimates.

1.4 Implications

The distance microorganisms can travel before returning to the surface depends primarily on (1) the size of the cell and any associated particles, (2) rates of removal by wet deposition and (3) vertical or horizontal winds. Most microbes larger than a few micrometers that find their way into the troposphere (below an altitude of 12 kilometers) fall out relatively quickly due to gravitational settling or precipitation. Numerous studies (Burrows et al., 2009a, and references therein) have shown that many cloud condensation nuclei (CCN) and ice condensation nuclei (ICN) responsible for climate and precipitation patterns are in fact airborne microorganisms (living or dead). It is anticipated that more dust (and microbes) will be introduced into the atmosphere with each passing year as worldwide deforestation increases desert acreage. Exactly how higher concentrations of airborne microorganisms will interact with other variables that drive weather and precipitation (temperature, location, winds, and season) is another major unknown in the climate change equation.

While lower tropospheric microorganisms fall or rain out, cells that reach the upper troposphere or the stratosphere (between 12 and 45 kilometers in altitude) can stay aloft much longer and travel significantly greater distances around the globe. Though no stratospheric sampling mission has been able to identify the exact source of sampled microbes or measure precise residence times at those higher altitudes, it is thought that volcanic eruptions, strong storms (thunderstorms, hurricanes, and monsoons) and airplane traffic probably all contribute to

the biological content in the upper atmosphere. The residence times for microorganisms might depend on dominant atmospheric circulation patterns (e.g. Brewer-Dobson cycles that eventually send stratospheric air back to the surface at the poles). Micron-sized stratospheric aerosols were observed during the 1991 eruption of Mt. Pinatubo, with some particles remaining airborne for five years before falling out. If used as a proxy, this event demonstrates the potential for stratospheric microorganisms to stay aloft for years and be globally dispersed (Smith et al., 2010). However, to test ecological hypotheses related to this possibility, more frequent missions to the stratosphere measuring microbial origin, concentration, and viability are needed. In summary, the residence time of airborne microbes could be as short as a few hours or as long as several years, depending on where, when, and how the cells were lofted into the atmosphere.

The potentially long residence time of microorganisms in the atmosphere is important to the health of human populations, crops, and livestock because it only takes one viable microbial pathogen to propagate disease. As a result, understanding airborne transport, movement, and dilution of microbes is an interesting and relevant scientific problem. Already there are documented examples of long-distance, pathogen dispersal in wind. For example the foot-and-mouth disease virus (FMDV) has traveled across the English Channel (Griffin et al., 2001); wheat stem rust has floated from the Mississippi Valley to regions of Canada; and more recently, scientists identified elevated concentrations of influenza viruses in the atmosphere when dust emanating from China reached the island of Taiwan (Chen et al., 2010).

Though the ability to identify and isolate the spread of disease has improved substantially in the last few decades, the genetic diversity of modern crops is smaller than any time in recent history. Most industrial agriculture is based on growing monocultures – producing one single crop over a large area – often clones with identical genes. Whereas naturally diverse plant

populations can have genetic variants resistant to disease, the homogeneity in agricultural fields allows for invading pathogens to conquer quickly. From a national security perspective, if context is shifted from agriculture to biological warfare, then there is a clear need to research, develop, and implement measures that identify and mitigate the spread of airborne pathogens. This may include improving the ability to track the movement of airborne microbes with fixed site monitoring, aircraft and satellite technologies.

1.5 Long-Term Sampling

Airborne microbes can be sampled through high-altitude aircraft, balloons, and rockets, but none of these platforms can provide the data needed for long term observations—preferably spanning seasons but ideally spanning years. To fill this data void (for further details, see Burrows et al., 2009a, 2009b), well-established atmospheric research sites from numerous locations around the globe should begin taking aerobiological measurements. Here in North America, it would be prudent to establish a network of monitoring sites on the West Coast, Rocky Mountains, and East Coast, in order to tracks the spatial distribution and movement of airborne microbial populations.

In 2009, I began collecting airborne microorganisms at the Mt. Bachelor Observatory (MBO) (http://www.atmos.washington.edu/jaffegroup/modules/MBO/) in central Oregon (43.98 ºN, 121.69 ºW located 2.8 km above sea level) (Figure 1.2). This research station has been operated by Daniel Jaffe at the University of Washington since 2004 and is equipped with instruments for measuring atmospheric composition (carbon monoxide, ozone, aerosols, nitrogen oxides, etc.) and other relevant meteorological variables. Published literature from MBO has identified trans-Pacific pollutants from Asia (e.g., Weiss-Penzias et al., 2007) and Asian dust (e.g., Fischer et al.,

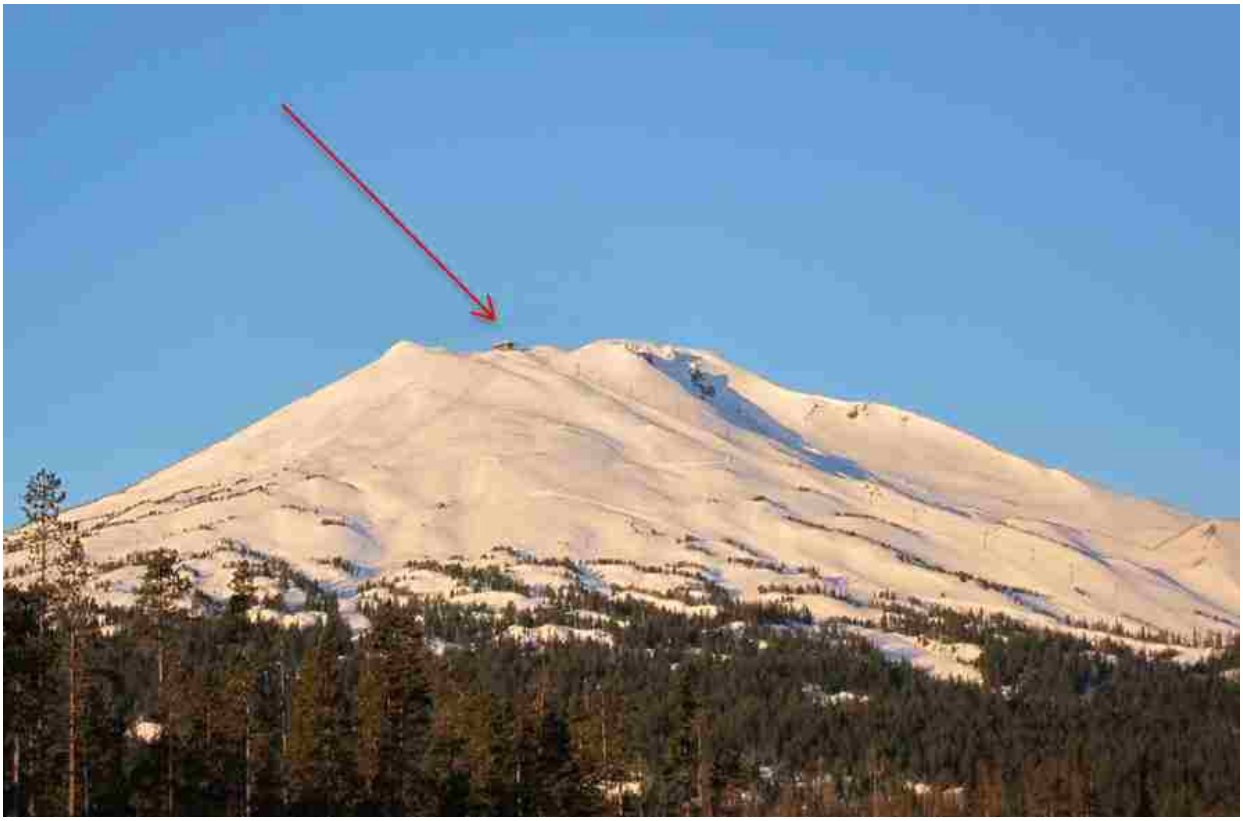

Figure 1.2 Red arrow points to the Mt. Bachelor Observatory (MBO) located in central Oregon at 43.98 ºN, 121.69 ºW.

2009), in addition to short-lived episodes of upper troposphere / lower stratosphere air intrusions. The diverse types and origins of air at MBO make it an ideal location to study the seasonal abundance and diversity of microbes at high altitudes.

In springtime, Asian dust and air pollution are the dominant aerosol type at MBO. Many aerosols collected can be traced back to Asian industry, fires, deserts, or other sources of emissions. Unlike previous aerobiology studies that relied upon culturing methods (i.e., viable cells), the use of molecular-based assays at MBO allows absolute quantification of airborne biological material, both living and dead. Overall, microbial abundances are expected to correlate with dust concentrations, and it is also expected that diurnal and seasonal variations will be observed (e.g., Burrows et al., 2009a). DNA from isolated microorganisms may help

determine the origin of air samples arriving to central Oregon and provide a new tool to trace atmospheric transport pathways.

These studies, if carried out for multiple seasons, may help answer some fundamental aerobiological questions. For example, what are the concentrations and fluxes of microbes in the free troposphere? How do abundances in stratospheric and tropospheric air compare? What meteorological conditions, aerosols, or pathogens are associated with the measured biomass over time? By studying a specific (transpacific) atmospheric pathway with surface-, satellite-, aircraft, and model-based observations, we can generate an unprecedented analysis of the transport of airborne microorganisms between continents, something that can be used as a case study for any number of the important environmental and population health issues that involve aerobiology.

1.6 Acknowledgments

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Chapter 2

FREE TROPOSPHERIC TRANSPORT OF MICROORGANISMS FROM ASIA TO NORTH AMERICA

Chapter 2 describes the abundance, diversity, and viability of bacteria and fungi sampled at the Mt. Bachelor Observatory using a combination of culture- and molecular-based characterization methods. Atmospheric chemistry data from two well-defined plumes of interest are included to demonstrate Asian long range transport. Portions of this chapter were originally published in collaboration with Daniel A. Jaffe, Michele N. Birmele, Dale W. Griffin, Andrew C. Schuerger, Jonathan Hee, and Michael S. Roberts in the July 2012 edition of *Microbial Ecology* (Smith et al., 2012, *Microbial Ecology*, 64:973-985) (©2012 *Springer Science+Business Media, LLC*), and are reproduced below with permission of Springer Science+Business Media, LLC.

2.1 Abstract

Microorganisms are abundant in the troposphere and can be transported vast distances on prevailing winds. This study measures the abundance and diversity of airborne bacteria and fungi sampled at the Mt. Bachelor Observatory (located 2.7 km above sea level in North America) where incoming free tropospheric air routinely arrives from distant sources across the Pacific Ocean, including Asia. Overall deoxyribonucleic acid (DNA) concentrations for microorganisms in the free troposphere, derived from quantitative polymerase chain reaction assays, averaged 4.94 x 10^{-5} ng DNA m^{-3} for bacteria and 4.77 x 10^{-3} ng DNA m^{-3} for fungi. Aerosols occasionally corresponded with microbial abundance, most often in the springtime.

Viable cells were recovered from 27.4% of bacterial and 47.6% of fungal samples (N = 124), with 49 different species identified by ribosomal RNA gene sequencing. The number of microbial isolates rose significantly above baseline values on 22-23 April and 13-15 May. Both events were analyzed in detail, revealing distinct free tropospheric chemistries (e.g., low water vapor; high aerosols, carbon monoxide and ozone) useful for ruling out boundary layer contamination. Kinematic back trajectory modeling suggested air from these events probably originated near China or Japan. Even after traveling for 10 days across the Pacific Ocean in the free troposphere, diverse and viable microbial populations, including presumptive plant pathogens *Alternaria infectoria* and *Chaetomium globosum*, were detected in Asian air samples. Establishing a connection between the intercontinental transport of microorganisms and specific diseases in North America will require follow-up investigations on both sides of the Pacific Ocean.

2.2 Introduction

Fronts and convective lofting can push microorganisms out of the boundary layer in the same way aerosols, dust and other types of pollution gain altitude in the troposphere. Although airborne bacteria and fungi have been sampled from diverse locations around the world for hundreds of years, many questions remain about the nature of microbial "pollution" – particularly on the topic of global dispersion and how far microorganisms can travel from points of origin. Current knowledge of the sources, sinks, residence time and distribution of atmospheric bacteria and fungi have been summarized elsewhere (Burrows et al., 2009a; Burrows et al., 2009b; Fröhlich-Nowoisky et al., 2009; Horneck et al., 2010; Womack et al., 2010). Atmospheric transport can impact life on the surface in many critical ways. Dobson et al.

(2000) hypothesized organic aerosols could have been essential prebiotic chemical reactors on early Earth, and once microbial life was underway, time aloft in the radiation rich atmosphere probably accelerated natural selection and speciation (Smith et al., 2011b). Perhaps the persistence of microbial groups through extinction events and the cosmopolitan distribution of many species today could be explained, in part, by wind dispersal over geographic barriers (Martiny et al., 2006; Womack et al., 2010). Recently, numerous diseases and allergens have been associated with the arrival of dust clouds (Simmer & Volz, 1993; Brown & Hovmøller, 2002; Lacey & West, 2006; Griffin, 2007) and the influence of microorganisms on atmospheric chemistry, cloud formation and precipitation is gaining attention (Ariya et al., 2009; Vaïtilingom et al., 2010).

Seasonal reports published in the past decade (e.g., Shelton et al., 2002; Fröhlich-Nowoisky et al., 2009; Fahlgren et al., 2010) have demonstrated that complex factors influence airborne microbial abundance, including: sampling site, weather, time of day, altitude and topography. Previous studies usually allude to global dispersion, but spreading patterns have never been systematically measured because of challenges associated with sampling in the free troposphere where most long range (> 500 km) microbial transport occurs (Weiss-Penzias et al., 2006). Several aerobiology research teams (Bovallius et al., 1980; Prospero et al., 2005; Pearce et al., 2009; Fahlgren et al., 2010) have sampled at isolated coastal locations with the objective to capture viable, airborne microbial populations that originated from distant continental sources. Field sites in these studies, however, were less than 5 m above sea level and the sampled populations may have included microorganisms dispersed in sea spray. In addition, back trajectory modeling of transport histories from lower elevation coastal areas is difficult because boundary layer air is heavily mixed and becomes untraceable over time (Weiss-Penzias et al.,

2006). Although earlier aerobiology surveys may have included bacteria and fungi from distant locations, boundary layer influences cannot be ruled out. Furthermore, the use of culture-based recovery methods in these studies (Bovallius et al., 1980; Prospero et al., 2005; Pearce et al., 2009; Fahlgren et al., 2010) would have only partially assessed microbial abundance since most microorganisms cannot be cultivated in the laboratory (Gillespie et al., 2005; Burrows et al., 2009a).

Large deserts in Asia, including the Gobi, Takla Makan and Badain Juran, are major sources of dust injected into the global atmosphere (Kellogg & Griffin, 2006) and recent literature describes the concentration and diversity of Asian microorganisms mobilized by wind storms (Maki et al., 2008; Maki et al., 2010; Yamada et al., 2010; Jeon et al., 2011; Wang et al., 2011; Hara & Zhang, 2012). Aerosols larger than microbes (up to 10 μm) routinely travel thousands of kilometers across the Pacific Ocean (Betzer et al., 1989), leading some researchers (Hammond et al., 1989; Brown & Hovmøller, 2002; Kellogg & Griffin, 2006; Griffin, 2007) to propose that Asian microorganisms, too, might be blown towards North America on prevailing westerly winds. Surprisingly, no study was found in the literature that documented the long range dispersal of microorganisms downwind (i.e., east) of Japan. At the Mauna Loa Observatory in Hawaii, Mims & Mims (2004) captured viable spores in ash thought to have originated from Asian fires; however, the authors did not address background levels of microorganisms in the air before the ash arrived. Smith et al. (2010) might have gathered evidence of trans-Pacific microorganisms during a single high-altitude flight over the open ocean, but limited amounts of dust and atmospheric data prevented definitive statements about the source of sampled air.

In the spring and summer months, air traveling eastward from Asia gains height over the Pacific due to convection and atmospheric warm conveyor belts (Weiss-Penzias et al., 2006).

Anywhere from 5 to 10 days later, it can arrive at the North American coastline and subside to lower elevations (Jaffe et al., 2003). In this study we test the hypothesis that microorganisms – like other types of free tropospheric pollution from Asia – periodically cross the Pacific Ocean and reach North America. The Mt. Bachelor Observatory (MBO) is a mountaintop atmospheric facility in central Oregon (43.98 ºN, 121.7 ºW) that has been used to measure incoming Asian pollution since 2004 (Jaffe et al., 2005; Weiss-Penzias et al., 2006; Fischer et al., 2009) and is an ideal location to test our hypothesis. The research station is positioned on the summit of an inactive volcano 2763 m above sea level and is operated with cooperation from the Mt. Bachelor Ski Resort. Using a variety of atmospheric and chemistry data, the site has been shown to frequently (> 50%) sample air from the free troposphere with no recent contamination from the

Figure 2.1 Air sampling apparatus at the Mt. Bachelor Observatory. (a) A high vacuum pump, electronics board and 4 separate filter housings were connected to intake pipes through holes at the base of the device. (b) Copper intake pipes emerged outside the building, facing into prevailing winds. (c) Polyethersulfone filter membrane (254 cm^2, 0.8 µm pores) inside plastic housing after sampling period.

boundary layer or local emissions (Jaffe et al., 2005; Weiss-Penzias et al., 2006; Fischer et al., 2009; Reidmiller et al., 2010). Herein we report results from the 2011 field season at MBO from 1 March to 20 May, including: (1) measurements of the abundance, diversity and viability of airborne bacteria and fungi; (2) evidence for the long range transport of microorganisms from Asia to North America during two separate events; (3) atmospheric data useful for understanding the origin and transport history of air from distant sources; and (4) aerobiology methods which permit molecular assays despite low densities of microorganisms in the free troposphere.

2.3 Materials and Methods

Operations at MBO

A specialized air sampling device (Fig. 2.1) was constructed to operate at the summit of MBO which frequently experiences extreme winter weather. Our apparatus had 4 separate copper intake pipes (1.5 cm inner diameter) each coupled with filters on a rotating cassette. The bulk of the apparatus was sheltered inside with only the intake pipes exposed to the outside environment through drilled holes on the uppermost level of the building. Intakes were unobstructed and faced in the general direction of prevailing winds. Sterile, 254 cm^2 polyethersulfone (PES) filters with 0.8 µm pores (PES009025, Sterlitech Corporation, Kent, WA) were placed inside invidivual plastic housing connected to the copper intake pipes via polyvinyl chloride joints. A high volume vacuum pump (Windjammer model # 117417-01, Ametek, Kent, OH) pulled ~ 0.5 m^3 min^{-1} of air through the filters (measured by an air velocity instrument, model FMA-905-VSR, Omega Engineering Inc., Stamford, CT). Prior to filter capture, the only other contact surface for the air was with the copper intake tubes. To minimize the possibility of contamination, the intake pipes were routinely washed with a bleach (NaOCl) solution. The sampler was controlled

with a laptop and Loggernet^{TM} software connected to a CR10x data logger (Campbell Scientific, Logan, UT). The sampler was programmed to switch to a new filter position every 12 hrs, except for the fourth and final position which ran until manual shut down. This allowed for continuous air sampling even during poor weather conditions which sometimes prevented access to MBO. Thus, certain filters collected air for periods lasting longer than 12 hrs but most samples were of that exact duration. After the sampling interval, filters were removed from the apparatus using aseptic techniques and placed in separate sterile Whirl-Pak® bags (product B00736WA, Nasco, Modesto, CA), then transported on ice to a -80 °C freezer within 2 hrs. Fresh filters were quickly re-loaded into the sampler, leaving only a small gap (< 45 min) in time coverage. Blank filters were periodically loaded and left in housings (with no air flow) to ensure sterility of the equipment, the building environment and the lag time prior to sampling.

Meteorological and atmospheric chemistry data were collected throughout the season to determine the composition and origin of arriving air. Measurements included concentrations of aerosols (dust), ozone (O_3), carbon monoxide (CO), water (H_2O) vapor mixing ratio, temperature, atmospheric pressure, wind speed and direction. Sub-micron aerosol mass concentration ($\mu g\ m^{-3}$) was calculated from the sub-micron scattering data (at 530 nm) using a dry scattering efficiency of 3 $m^2\ g^{-1}$. A detailed description of the instruments and calibrations used has been published elsewhere (Jaffe et al., 2005; Weiss-Penzias et al., 2006). Due to ski resort operations (e.g., snow clearing activities), nearby combustion sometimes contaminated air at MBO, but the periods were readily identifiable through short-lived spikes in aerosols and CO several orders of magnitude above baseline values. For sampling periods of particular interest, 10-day kinematic back-trajectories were calculated using the Hybrid Single-Particle Lagrangian Integrated Trajectory (HYSPLIT-4) model (Draxler & Rolph, 2003) available through the

National Oceanic and Atmospheric Administration (NOAA). Fischer et al. (2011) has discussed the model inputs and error associated with using the Global Data Assimilation System (GDAS) meteorological archive for calculating back trajectories from MBO.

Imaging

In order to assess the distribution and morphology of particulates and microorganisms on air filters, samples from a period of variable dust concentration (18 to 20 April 2011) were imaged using a Zeiss EVO 60 environmental scanning electron microscope (SEM) with a tungsten filament (Carl Zeiss Microscopy, Peabody, MA). To prevent charges from building during high magnification imaging, filters were first sputter-coated with a gold/palladium film (~220 Å thick) using a Denton Vacuum Desk IV system (Denton Vacuum, LCC, Moorestown, NJ) and Maxtek TM-400 Thickness Monitor (Maxtek, Inc., Cypress, CA). Sputter coating was performed at high vacuum (7 mtorr) for a 60 sec interval with samples rotating at 10 RPM. Coated samples were immediately mounted onto the SEM stage for high resolution imaging using carbon tape at a working distance of 11 mm.

Culturing

Microbe filters inside Whirl-Pak® bags were brought to room temperature in a laminar flow hood. Using sterile forceps and scissors, filters were cut into quarters, only one of which was used for microbial growth and recovery. The remaining pieces were returned to storage at -80 °C for use in other assays. Each quartered filter was halved to permit two different culturing approaches. The first approach put untreated samples directly onto selective Difco™ R2A and potato dextrose agar (PDA) (Difco, Fisher Scientific, Pittsburg, PA). To encourage the recovery

of spore-forming bacteria while preventing the growth of fungi that can quickly overtake mixed cultures, the second approach heat-shocked samples before placement onto solid media (again R2A and PDA). Heat shock treatment consisted of immersing filters in 5 ml sterile deionized H_2O and heating at 80 °C for 10 min. Plates from both cultivation approaches were wrapped with Parafilm® and incubated in the dark at 25 °C, given 14 days to shows signs of growth. Soon after initial colonies became visible, unique morphologies were sub-cultured and kept fresh until isolation.

Identification of Recovered Isolates

Bacterial sequencing of recovered isolates was performed with the MicroSeq® 500 16S ribosomal RNA (rRNA) kit while fungal identification was performed with the MicroSeq® D2 large-subunit (LSU) rRNA kit (Applied Biosystems (ABI), Life Technologies Corp., Carlsbad, CA). The first 500 base pairs of the bacterial 16S rRNA and the D2 region of fungal LSU rRNA genes were sequenced, analyzed and compared to a library using MicroSeq® ID Analysis Software Version 2.0. A working stock of deoxyribonucleic acid (DNA) was made at a 1:100 dilution, except for several samples with limited amounts of isolated DNA that required a 1:10 dilution instead. Otherwise, all downstream steps for polymerase chain reaction (PCR), purification, electrophoresis on the ABI 3130 Genetic Analyzer and sequencing were done according to the MicroSeq® kit protocol.

DNA Extraction from Filter Samples

DNA isolation for quantitative PCR (qPCR) experiments was achieved using MO BIO PowerWater® kits (product 14900-100-NF, MO BIO Laboratories, Inc., Carlsbad, CA) with a

modified protocol to increase yield. For each sample, one quarter of a filter was placed inside a 5 ml PowerWater® Bead Tube containing 3 ml of Solution PW1. The tube was sealed and immersed in a 37 kHz sonicator (Elmasonic S 60/(H), Elma, Singen, Germany) for 30 min at 65 °C. Following the incubation, samples were vortexed for 15 min (product 12-812, Fisher Vortex Genie 2™, Fisher Scientific). All subsequent extraction steps followed the PowerWater® protocol, with solution volumes proportionally adjusted to match the initial volume of PW1. A centrifuge (product 5804, Eppendorf, Hauppauge, NY) and PowerVac™ manifold (product 11991, MO-BIO Laboratories, Inc.) were used, along with a wash of 800 µl of 100% ethanol prior to the addition of Solution PW4. The resulting 100 µl DNA was then passed through a MiniElute® Reaction Cleanup Kit (product 28204, Qiagen Inc., Valencia, CA) to improve its quality, measured by a Nanodrop spectrophotometer (ND-1000, Thermo Scientific, Wilmington, DE). The final sample elution volume was 40 µl in Buffer EB.

Quantitative PCR

Primer sets and conditions were modeled after Fierer et al. (2005) and are listed in Table 2.1. For each preparation, a standard 20 µl reaction volume contained: 5 µl extracted template; 10 µl of LightCycler® 480 DNA SYBR Green I Master Mix kit (contains FastStart DNA *Taq* polymerase, reaction buffer, dNTP mix (with dUTP instead of dTTP), SYBR Green I dye and $MgCl_2$); 40 nM of each forward and reverse primer for the bacterial assay or 250 nM of each primer for the fungal assay (Invitrogen, Carlsbad, CA); and 0.4 µl of Bovine Serum Albumin (for bacterial assay only). The remaining reaction volume was adjusted with sterile molecular-grade H_2O. All reagents were obtained from Roche Diagnostics Corp. (Indianapolis, IN) and reactions were performed in a 96-well semi-skirted plate with optical sealing foils.

Table 2.1 Reaction Conditions and qPCR Primers

Group	Amplicon Length (bp)	Forward Primer	Reverse Primer	PCR Conditions
Bacteria	~ 180	Eub338 (forward) 5'-ACTCCTACGGGAG GCAGCAG-3'	Eub518 (reverse) 5'-ATTACCGCGGCTGCTGG-3'	95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s and 72 °C for 20 s
Fungi	~ 300	5.8s (forward) 5'-CGCTGCGTTCTTCATCG-3'	ITS1f (reverse) 5'-TCCGTAGGTGAACCTGCGG-3'	95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 58 °C for 30 s and 72 °C for 30 s

Amplification was conducted in a Roche LightCycler® 480 Real-Time PCR System. Annealing temperatures were experimentally optimized for bacteria and fungi. Each plate had triplicate reactions per sample, appropriate standards (bacterial or fungal) and sterile molecular-grade H_2O as the no template/reagent control. Fluorescence levels were recorded at the end of each amplification cycle, between 28 and 35 cycles, and reaction efficiencies ranged from 1.7 to 2.1. Melt curve analysis of the PCR products was conducted at the end of each assay to verify the quality and specificity of the fluorescence signal. A standard curve was generated by plotting the C_p value for a standard series of genomic DNA concentrations (20 ng μL^{-1} to 200 fg μL^{-1}) extracted from cultures of *Pseudomonas aeruginosa* for the bacterial assay and *Penicillium chrysogenum* for the fungal assay.

Statistical Analyses

Data were analyzed with the statistical program R version 2.14.1 (The R Foundation for Statistical Computing, Vienna, Austria, 2012). Chemical and meteorological values were averaged hourly and any errant signals (e.g., occasional anthropogenic emissions at MBO) were removed. Microbial concentrations were calculated across longer intervals (typically 12 hrs per filter sample) and the data set was divided into six smaller periods: 1-15 March; 15-29 March; 29

March to 11 April; 11-25 April; 25 April to 8 May; and 8-20 May. A multi-variable linear regression analysis was conducted to understand the relationship between airborne microbial abundance and meteorological variables (i.e., aerosols, CO, O_3 and H_2O vapor mixing ratio) for two specific events: 22-23 April and 13-15 May. At a 95% confidence level, the Wilcoxon Test and t-Test were used to measure significant differences between means of independent variables.

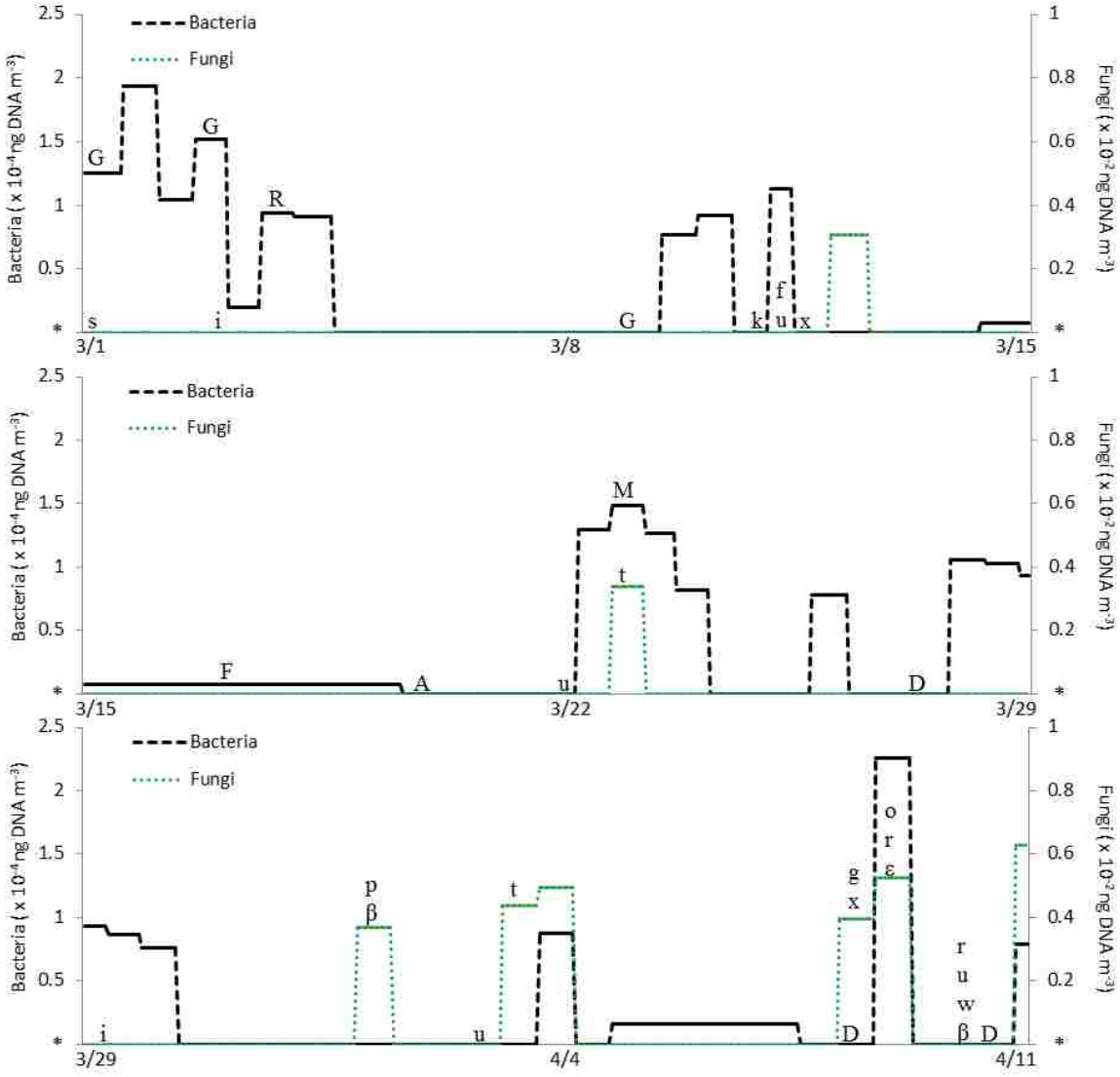

Figure 2.2 Microbial data from 1 March to 11 April. Values for bacteria and fungi are means of triplicate qPCR runs, time-averaged over the sampling interval. Note the difference in scale concentrations between groups. Asterisks represent the lower detection limit of the qPCR assay and letter codes correspond to recovered isolates listed in Tables 2.3 and 2.4.

2.4 Results

Figs. 2.2 and 2.3 depict changes in airborne microbial concentration over time at MBO. Averages are summarized across six smaller periods in Table 2.2 along with meteorological data. The mean concentration of airborne bacteria and fungi over the entire sampling season was 4.94 x 10^{-5} ng DNA m^{-3} and 4.77 x 10^{-3} ng DNA m^{-3}, respectively. Fungal biomass consistently

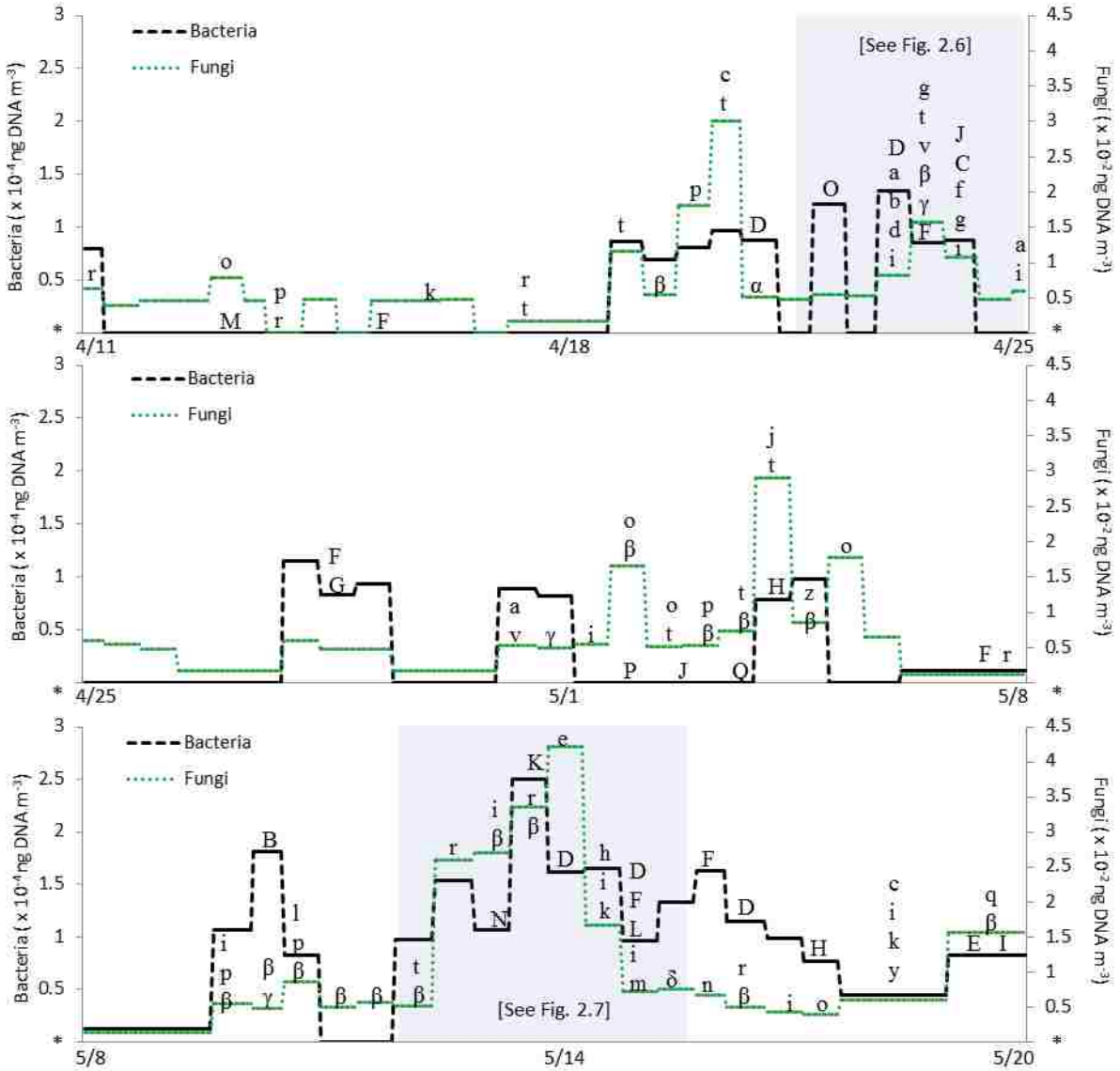

Figure 2.3 Microbial data from 11 April to 20 May. Values for bacteria and fungi are means of triplicate qPCR runs, time averaged over the sampling interval. Note the difference in scale concentrations between groups. Asterisks represent the lower detection limit of the qPCR assay and letter codes correspond to recovered isolates listed in Tables 2.3 and 2.4. Asian, long range transport events are highlighted with blue boxes.

outnumbered bacterial biomass by 2 orders of magnitude, and the concentrations of each group varied significantly over time and in comparison to each other ($P < 0.05$). Bacterial abundance was highly variable throughout the season, with peak concentrations (1.94 to 2.50 x 10^{-4} ng DNA m^{-3}) occurring across three different months. In contrast, fungi were not regularly detected until April but steadily increased thereafter, with concentration peaking on 14 May at 4.21 x 10^{-2} ng DNA m^{-3}. The period with the highest concentration of both bacteria and fungi was 8-20 May. Bacteria were below the sensitivity limit of the qPCR assay in 52.4% of the samples ($N = 124$), while the month of March accounted for most of the failed fungi detections (in 44.4% of the

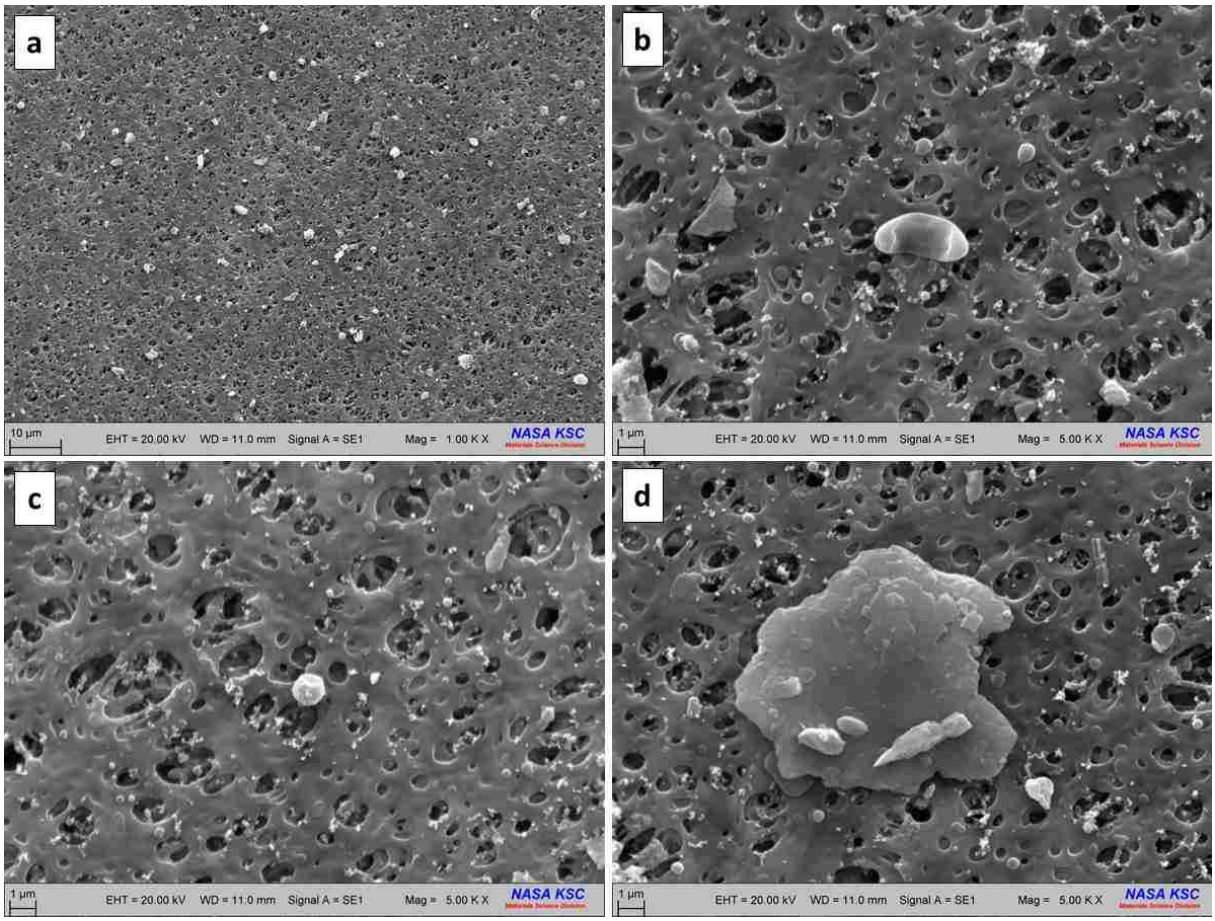

Figure 2.4 Scanning electron micrographs of PES filters used for capturing airborne microorganisms at the Mt. Bachelor Observatory. (a) Typical density and distribution of atmospheric particles and microorganisms which appear as bright forms against the darker filter background. (b) Desiccated, rod-shaped bacterium. (c) Fungal spore in the center of the micrograph. (d) Ovoid-shaped bacterial spore attached to a larger particle.

Table 2.2 Microbial and Meteorological Averages +/- Standard Deviation

Period	N (hrs)	Bacteria (x 10^{-5} ng DNA m^{-3})	Fungi (x 10^{-3} ng DNA m^{-3})	Aerosols (x 10^{-1} µg m^{-3})	CO (ppbv)	O_3 (ppbv)	H_2O Vapor (g kg^{-1})
1-15 March	315	3.93 +/- 5.75	0.130 +/- 0.610	1.20 +/- 2.43	138 +/- 6.36	47.0 +/- 3.91	2.91 +/- 0.488
15-29 March	336	0.251 +/- 4.84	0.122 +/- 0.632	0.251 +/- 0.963	137 +/- 11.9	35.9 +/- 8.45	2.70 +/- 0.451
29 March to 11 April	312	2.49 +/- 5.01	0.956 +/- 1.90	2.53 +/- 4.39	129 +/- 18.9	46.0 +/- 9.95	3.19 +/- 1.24
11-25 April	336	3.17 +/- 4.57	6.39 +/- 6.05	9.14 +/-10.9	135 +/- 13.2	47.4 +/- 8.65	2.99 +/- 0.753
25 April to 8 May	312	2.65 +/- 4.04	6.02 +/- 6.16	4.62 +/- 5.56	128 +/- 8.70	51.5 +/- 7.95	2.66 +/- 0.856
8-20 May	307	9.06 +/- 6.24	10.6 +/- 10.5	8.47 +/-10.0	121 +/- 16.1	46.8 +/- 6.19	3.46 +/- 0.684

samples). All negative control filters (i.e., DNA extractions from blanks) were below the sensitivity limit of the qPCR assay. Fig. 2.4a depicts the typical density of dust and microorganisms trapped on filter membranes, with a large distribution in the shape and size of captured particles. Fungal spores and bacteria were identifiable by size (1 to 10 µm) and shape, with the latter appearing in cocci, bacilli, and spirilla forms. Most microorganisms showed evidence of desiccation and were attached to the filter membrane independent of other particles (Fig. 2.4b and 2.4c), though clumping was also observed (Fig. 2.4d). Some particles and fragments of cells smaller than the filter membrane pore size (0.8 µm) were embedded deeper within the PES matrix. Viable bacteria and fungi were recovered from 27.4% and 47.6% of samples (N = 124), respectively, 1 to 7 days after incubation. The two cultivation treatments segregated samples: all bacterial were recovered from heat-shocked filters and all fungi were recovered from untreated filters. Some of the filters (28.2%; N = 124) had more than one viable

microorganism. For both groups, viability was more common in the later months, with 8-20 May the period of highest frequency. Viability was not always correlated with overall microbial abundance – in fact, 10 bacterial and 9 fungal isolates were recovered from filters that were below the qPCR assay detection limit.

Best matches and frequencies of isolated microorganisms inferred through rRNA gene sequencing are listed in Tables 2.3 and 2.4, including a code which corresponds to sampling dates in Figs. 2.2 and 2.3. For bacteria, 18 unique species from 6 genera were recovered during the sampling season. *Bacillus megaterium* (N = 8), *Bacillus pumilus/safensis* (N = 7) and *Bacillus simplex* (N = 4) were the most commonly identified bacterial species. For fungi, 31 unique species from 26 genera were recovered. *Stromatinia narcissi* was the most commonly identified fungi (N = 18), followed by *Cladosporium* sp. / *Mycosphaerella aronici* (N = 12), *Penicillium chrysogenum* (N = 10) and *Neosartorya fischeri* (N = 9). Several bacterial and fungal isolates had < 92% rRNA sequence similarity (*Amphibacillus tropicus* and *Paenibacillus curdlanolyticus*; *Drepanopeziza populorum*, *Lophodermium pinastri* and *Nigrospora oryzae*), making species identifications more uncertain in such instances. Microbial diversity increased significantly from March to May ($P < 0.05$), with higher levels of species richness occurring in the latter half of the sampling season. The greatest amount of biodiversity was sampled on 22 April and 15 May (during two events discussed in greater detail below). Negative controls implemented in this study (blank filters and storage bags) did not result in microbial growth, and positive controls during sequencing methods for bacteria (*Escherichia coli* ATCC = 11303) and fungi (*Saccharomyces cerevisiae* ATTC=18824) confirmed the accuracy of techniques.

Carbon monoxide, O_3 and H_2O vapor (also summarized in Table 2.2) were highly variable throughout the season, averaging 131.7 ppbv, 45.7 ppbv and 2.98 g kg^{-1}, respectively. Means for

Table 2.3 Frequency and Best Match of Bacteria Recovered

Species	% Identity	Samples (*N*)	Figure Code
Amphibacillus tropicus	90.6	1	A
Bacillus altitudinis	100	1	B
Bacillus atrophaeus	99.9	1	C
Bacillus megaterium	99.9-100	8	D
Bacillus mojavensis	100	1	E
Bacillus pumilus	97.5-100	7	F
or			
Bacillus safensis			
Bacillus simplex	100	4	G
Bacillus soli	97.9-98.3	2	H
Bacillus subtilis spizizenii DSM=15029	100	1	I
Bacillus subtilis subtilis ATCC=6051	100	2	J
Bacillus vallismortis	99.7	1	K
Brevibacillus choshinensis	99.9	1	L
Brevibacillus ginsengisoli	97.0-97.2	2	M
Paenibacillus alginolyticus	97.6	1	N
Paenibacillus borealis	95.7	1	O
Paenibacillus curdlanolyticus	88.8	1	P
Pseudomonas frederiksbergensis	100	1	Q
Streptomyces lienomycini	99.4	1	R

these variables did not change significantly across the periods and concentrations were generally not a predictor for microbial abundance. Wind speed, temperature, relative humidity, atmospheric pressure, CO_2, Hg and NO_X data were not included in this study but are available at http://www.atmos.washington.edu/jaffegroup/modules/MBO/. It was an unusually stormy period in the Pacific Northwest in 2011, with frequent precipitation and cloud cover. Average relative humidity and sub-micron aerosol concentrations for the spring were 88% and 0.5 μg m^{-3}, compared to 78% and 1.0 μg m^{-3} for the spring average from 2004-2010. From 1 March to 20 May, aerosol concentrations averaged 0.44 μg m^{-3} with values increasing significantly over the course of the season (Fig. 2.5). Initially, there was no correlation between fungi and aerosols (R^2 = 0.087 for 11-25 April), but it steadily increased (R^2 = 0.13 for 25 April to 8 May) until the final time period which exhibited the strongest correlation between the variables (R^2 = 0.58 for 8-20

Table 2.4 Frequency and Best Match of Fungi Recovered

Species	% Identity	Samples (*N*)	Figure Code
Allewia eureka	99.6	3	a
Alternaria infectoria	100	1	b
Alternaria longissima	99.7	2	c
Aspergillus parvathecius	99.9	1	d
Aspergillus niger ficuum DSM=932 or *Aspergillus phoenicis*	100	1	e
Arthrinium phaeospermum	93.0	2	f
Botryotinia sp. or *Botrytis* sp.	95.5-100	3	g
Chaetomium globosum CBS=145.38	98.9	1	h
Cladosporium sp. or *Mycosphaerella aronici*	100	12	i
Cryptococcus albidus	95.7	1	j
Cryptococcus dimennae	86.4-91.2	4	k
Dendrostilbella mycophila	93.6	1	l
Drepanopeziza populorum	85.5	1	m
Epicoccum nigrum	100	1	n
Lecythophora mutablis	97.3-97.7	6	o
Lophodermium pinastri	79.8	6	p
Malassezia restricta	99.7	1	q
Neosartorya fischeri	98.6	9	r
Nigrospora oryzae	89.1	1	s
Penicillium chrysogenum	100	10	t
Penicillium citrinum	99.8	4	u
Penicillium oxalicum	98.3	2	v
Phialocephala fortinii	96.4	1	w
Pithomyces atro-olivaceus	99.3	2	x
Pleospora papaveracea	99.7	1	y
Rhodotorula minuta	94.8	1	z
Sclerotinia sclerotiorum	99.7	1	α
Stromatinia narcissi	94.9-95.3	18	β
Sydowia polyspora	99.3-99.7	3	γ
Ulocladium sp.	100	1	δ
Venturia inaequalis	98.3	1	ε

May). The relationship between aerosols and bacteria, in comparison, was weaker but still noteworthy from 15-29 March ($R^2 = 0.22$) and 8-20 May ($R^2 = 0.24$).

Elevated aerosol levels on 22-23 April and 13-15 May (highlighted in Fig. 2.5) were analyzed in detail along with changes in CO, O_3 and H_2O vapor concentration in Figures 2.6 and

2.7. The 22-23 April event lasted nearly 48 hrs and was characterized by abrupt increases in aerosol, CO and O_3 levels along with a corresponding drop in H_2O vapor values. Multi-variable linear modeling measured a strong relationship between the atmospheric variables (R^2 = 0.92) and while overall microbial abundance (ng DNA m^{-3}) was not higher than in the days preceding the event, the rise in number of cultured species was significant ($P < 0.05$). In comparison, the 13-15 May event was longer-lasting and less sharply defined with a lower correlation between all atmospheric variables (R^2 = 0.44). However, the linear relationship between just microbial abundance, aerosols and CO was more substantial (R^2 = 0.75). Similar to the April event, microbial diversity was significantly higher than baseline values ($P < 0.05$). Kinematic back trajectories over a range of altitudes were plotted to understand the transport history of air from these periods of interest. Air arriving to the site at 1600 UTC on 22 April showed transport from Asia (near China and Japan) at 2700 and 3200 m (Fig. 2.6). It took 10 days for the air to cross the Pacific Ocean in the free troposphere (reaching altitudes up to 6 km) before subsiding to

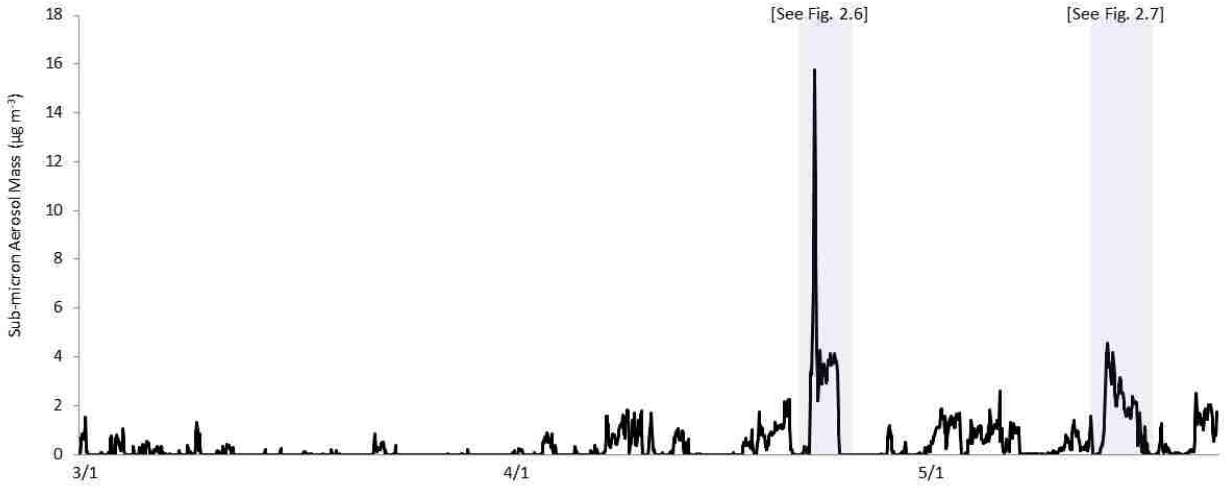

Figure 2.5 Seasonal dust measured by sub-micron aerosol mass concentration (µg m^{-3}). Stormy weather in earlier months kept dust levels low, but values increased towards springtime. Asian, long range transport events are highlighted with blue boxes.

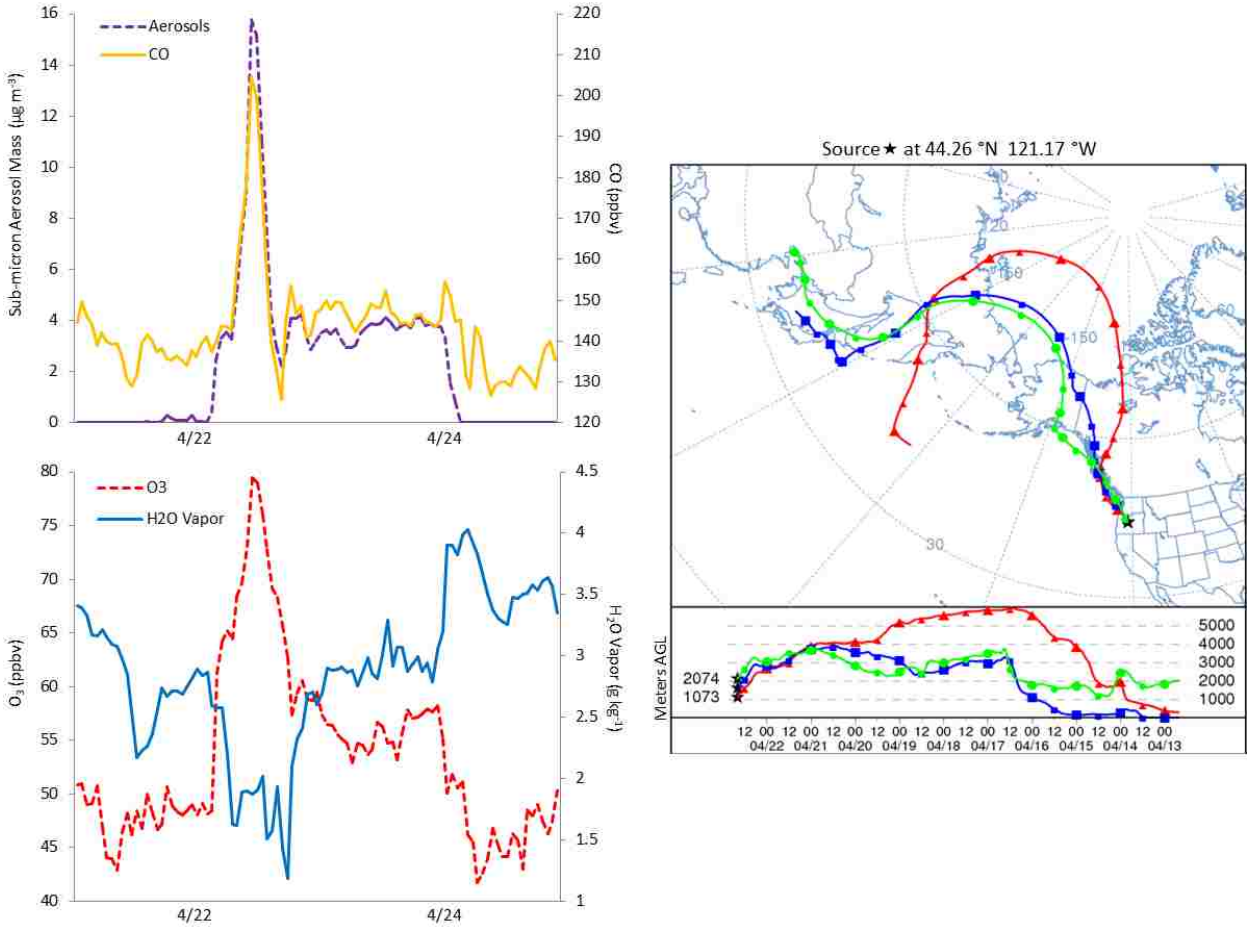

Figure 2.6 Profile of the 22-23 April long range transport event. Atmospheric data change significantly over the event ($P < 0.05$) and exhibit free tropospheric signatures (i.e., dry, aerosol/CO/O_3 enriched air). Kinematic back trajectories ending at 1600 UTC were calculated at 2200 (red line), 2700 (blue line) and 3200 (green line) m and suggested trans-Pacific transport from source regions near Asia.

MBO. Similarly, the trajectories plotted for air arriving at 0000 UTC on 13 May (Fig. 2.7) showed strong agreement, originating near Japan before traveling across the Pacific Ocean and reaching central Oregon.

2.5 Discussion

The objective of this study was to measure the concentration of microorganisms in the free troposphere – critical for understanding global dispersion, microbial biogeography and the possibility of airborne bacteria and fungi traveling across the Pacific Ocean in an atmospheric

bridge. By combining microbial observations with atmospheric chemistry, we closed a spatial/temporal gap acknowledged in the aerobiology literature (Burrows et al., 2009b; Womack et al., 2010). Overall, our seasonal data suggest that average microbial abundance in the free troposphere is low, consistent with earlier estimates by Spooner & Roberts (2005). Bacterial abundance levels at MBO averaged 49.4 fg DNA m^{-3}. This could represent 6-20 genomes m^{-3} assuming intact cells were sampled and considering bacterial genomes can range from 2-8 fg DNA (typically). While the relative amount of fungi was much greater than bacteria (in agreement with global flux projections (Burrows et al., 2009b; Fröhlich-Nowoisky et al., 2009)), mass-based statistics can be misleading since fungal spores usually contain much more DNA than bacterial spores (Johnston & Young, 1972; Gadkar & Rillig, 2005). It is possible that the ratio of whole airborne fungi and bacteria cells may be closer to 1:1, but the large variation in the amount of DNA between species and individual cells makes such calculations challenging.

All of the bacterial species recovered in this study (except *Pseudomonas frederiksbergensis*) are gram-positive, capable of forming endospores and commonly found in soils. Surely cells of all types can be lofted into the atmosphere, but endospore formers may be uniquely capable of surviving atmospheric transport and desiccation on air sampling devices. Endospore formation in *Bacillus* sp. provides high resistance to ultraviolet radiation or desiccation (Smith et al., 2011a) and the genus is quite common in the aerobiology literature (e.g., Hua et al., 2007; Maki et al., 2008; Maki et al., 2010; Jeon et al., 2011; Wang et al., 2011). Similarly, the pigmentation of *Bacillus atrophaeus* and high guanine and cytosine content of *Streptomyces lienomycini* might protect DNA during radiation exposure in the atmosphere (Smith et al., 2011b). Two species, *Bacillus mojavensis and Bacillus vallismortis*, have been characterized by our team in the past from desert soil samples (Roberts et al., 1994; Roberts et al., 1996). *Bacillus altitudinis* was

captured during balloon experiments in the stratosphere by Shivaji et al. (2006) and finding it again here suggests this species is well-suited for surviving high altitude transport. *Pseudomonas frederiksbergensis* was first isolated from coal gasification emission sites in Europe (Anderson et al., 2000) and may be another species common to airborne communities. *Amphibacillus tropicus* and *Brevibacillus ginsengisoli* have been sampled primarily from Russia (Zhilina et al., 2001) and South Korea (Baek et al., 2006), respectively, though the identity of the former was less certain based on rRNA sequence divergence (90.6%). *Paenibacillus borealis* is often associated with forest humus (Elo et al., 2001) and could represent local influences on 21 April.

A broad range of fungi were recovered in this study (26 genera), including molds, yeasts, cup and sac fungi with diverse ecological distributions, mostly from soils and plant debris. Many of the sampled genera release spores in the spring time (Lacey & West, 2006), explaining the fungal biomass 'burst' measured in our study (beginning in April) and others (Fröhlich-Nowoisky et al., 2009). Some of the 31 species recovered are presumptive allergens or plant pathogens (e.g., *Alternaria infectoria, Alternaria longissima, Botryotinia* sp., *Chaetomium globosum, Drepanopeziza populorum, Epicoccum nigrum, Lophodermium pinastri, Nigrospora oryzae, Penicillium oxalicum, Pleospora papaveracea, Sclerotinia sclerotiorum, Sydowia polyspora* and *Venturia inaequalis*) (Spooner & Roberts, 2005). While the possibility of sampling local microorganisms was equally likely with bacteria, it may be easier to identify with fungi due to highly specific ecological associations. For example, *Phialocephala fortinii* and *Sydowia polyspora* are typically found on pine trees (Spooner & Roberts, 2005) which are abundant in the surrounding MBO wilderness. *Lophodermium pinastri* is another pine associated fungus, but the rRNA identity match of the 6 isolated samples was low (79.8%).

Other possible contaminants include *Malassezia restricta* that is routinely found on the skin of animals (Aspiroz et al., 1999).

Sampling at high altitude in the remote mountain wilderness created unique challenges which may have underestimated the actual abundance and diversity of microorganisms characterized in this study. For instance, air filters had to be frozen at -80 °C prior to recovery in the laboratory which may have killed a subset of culturable microorganisms (preferentially afflicting gram negative bacteria more than gram positive bacteria). To permit molecular methods, long-lasting

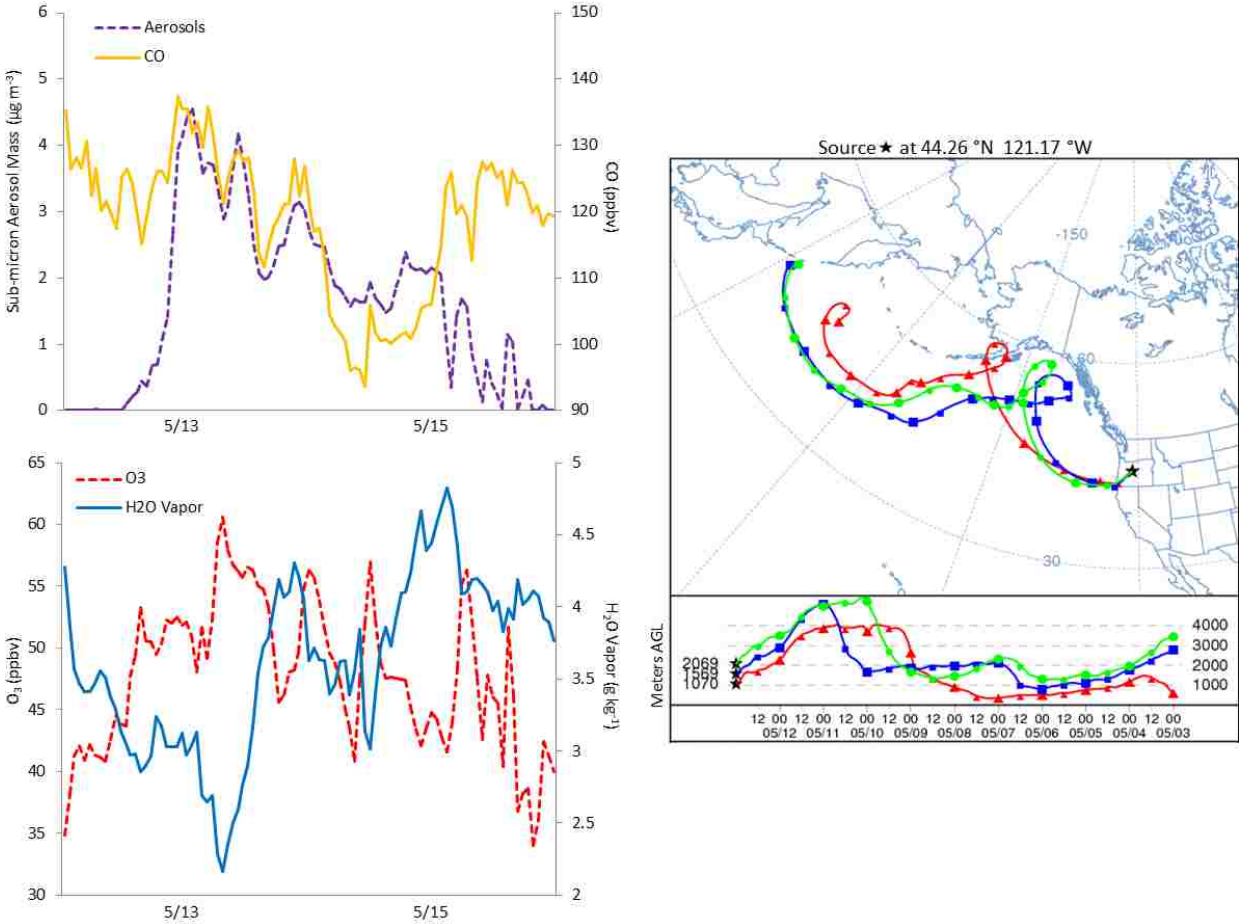

Figure 2.7 Profile of the 13-15 May long range transport event. Atmospheric data change significantly over the event ($P < 0.05$) and exhibit free tropospheric signatures (i.e., dry, aerosol/CO/O_3 enriched air). Kinematic back trajectories ending at 0000 UTC were calculated at 2200 (red line), 2700 (blue line) and 3200 (green line) m and suggested trans-Pacific transport from source regions near Asia.

sampling periods were necessary for capturing sufficient airborne biomass, but extended air flow can dehydrate cells (as observed in the SEM analysis) and select only the most resilient species to be recovered (Pearce et al., 2009; Womack et al., 2010). Robust PES filters were used to withstand high air flow rates without tearing, however this made DNA extraction procedures more difficult since the dense membrane also trapped many particles and cellular fragments (< 0.8 μm) deep inside its matrix. Extended bead beating, sonication and incubation with the lysing agent were needed to remove DNA and in doing so may have degraded its quantity and quality. Even with steps to maximize yield from samples, DNA quantities were still sometimes below the sensitivity limit for qPCR (Hospodsky et al., 2010). This explains why several bacterial and fungal isolates were recovered from samples with no measurable amplification signal. Future free troposphere studies using qPCR should employ even higher air pump flow rates and consider using liquid impingement to eliminate extraction inefficiencies associated with filter membranes (Griffin et al., 2011).

Microorganisms and other biological particulates can act as cloud and ice condensation nuclei (Ariya et al., 2009; Burrows et al., 2009a), and the high incidence of storms during the sampling season at MBO could have led to a substantial fallout/washout of airborne cells, reducing overall microbial concentrations. When the weather improved later in the season, aerosol levels rose and so did microbial abundance. Once correlations became stronger, other atmospheric data were used to analyze the composition and origin of arriving air. Weiss-Penzias et al. (2006) has described the approach for distinguishing between the North American boundary layer and free tropospheric, long range transported air. The latter tends to be drier and O_3-enriched compared to the boundary layer. On average, daytime measurements reflected a mixture of boundary layer and free troposphere air whereas nighttime samples were mostly free

tropospheric air with no local influence (Reidmiller et al., 2010). By focusing on two well-defined events with characteristic free tropospheric chemical compositions and coherent transport histories, we make an argument for sampling air from distant source regions. On 22-23 April the enhancement of O_3 from 50 to 80 ppbv and the depletion of H_2O vapor from 3 to 1 g kg^{-1} signaled the arrival of non-local, free tropospheric air. Simultaneously, aerosols jumped from 0 to almost 50 Mm^{-1} while CO rose almost 60 ppbv above baseline values. A similar (but less pronounced) pattern occurred on 13-15 May. In both cases, back trajectories showed transoceanic, free tropospheric transport beginning near China or Japan. Together, the chemical and meteorological data suggest local emissions were not the primary source of enhancements and depletions measured on 22-23 April and 13-15 May.

Richer levels of bacteria and fungi diversity coincided with the arrival of Asian air during the 22-23 April and 13-15 May events. We take a conservative approach identifying candidate Asian microorganisms. If a species was not recovered at any other time of the sampling season, it seems reasonable to suggest it traveled along with trans-Pacific free tropospheric pollution given the history of Asian emissions arriving at MBO (Jaffe et al., 2003; Jaffe et al., 2005; Fischer et al., 2009) and other locations in North America (VanCuren & Cahill, 2002). The event on 22-23 April delivered at least 9 different species of fungi and 4 different species of bacteria and was the only occurrence in which *Alternaria infectoria, Aspergillus parvathecius* and *Bacillus atrophaeus* were detected. Meanwhile the 13-15 May spike in diversity equaled that of April but delivered even more unique species, including: *Aspergillus niger ficuum* / *Aspergillus phoenicis* and *Chaetomium globosum*; *Bacillus vallismortis, Brevibacillus choshinensis* and *Paenibacillus alginolyticus*. *Drepanopeziza populorum* was also recovered but with less certainty based on rRNA sequence identity match (85.5%). Pinpointing exact

microbial emission source regions in Asia could soon be enabled through (1) DNA microarray data that can reveal many bacterial species ignored by culturing methods and (2) additional atmospheric transport analyses. Follow-up sampling seasons at MBO would be valuable given that overall biomass concentrations may have been reduced due to stormy weather and microbial extraction inefficiencies. For now, the detection of viable, presumptive plant pathogens (*Alternaria infectoria* and *Chaetomium globosum*; and possibly *Drepanopeziza populorum*) in two separate Asian free tropospheric transport events has significant implications for epidemiology. While we are unaware of plant infections by these pathogens in North America, our results might inspire follow-up DNA fingerprinting studies (e.g., Milgroom et al., 1996) to trace the relationship of pathogens on distant continents. With the health of ecosystems, agriculture and human populations at stake, intercontinental aerobiology studies must ultimately work towards developing predictive models for disease dispersal.

2.6 Acknowledgments

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Chapter 3

INTERCONTINENTAL DISPERSAL OF BACTERIA AND ARCHAEA IN TRANSPACIFIC WINDS

Chapter 3 describes the abundance and species richness of bacteria and archaea sampled at the Mt. Bachelor Observatory using a 16S rRNA microarray – a comprehensive, culture-independent tool sensitive to microorganisms that are dead or cannot be cultivated in the laboratory. The study focuses on the same plumes characterized in Chapter 2 and incorporates new atmospheric data to support Asian long range transport. Results were generated and written in collaboration with Hilkka J. Timonen, Daniel A. Jaffe, Michele N. Birmele, Dale W. Griffin, Kevin D. Perry, Peter D. Ward, and Michael S. Roberts. When this dissertation was submitted, portions of this chapter were in-press (AEM03029-12R1) by *Applied and Environmental Microbiology* (© American Society for Microbiology). Microarray data can be accessed at http://greengenes.secondgenome.com/downloads/phylochip_datasets.

3.1 Abstract

Microorganisms are abundant in the upper atmosphere, particularly downwind of arid regions where winds can mobilize large amounts of topsoil and dust. However, the challenge of collecting samples from the upper atmosphere and reliance upon culture-based characterization methods has prevented a comprehensive understanding of globally-dispersed airborne microbes. In spring 2011 at the Mt. Bachelor Observatory in North America (2.8 km above sea level), we captured enough microbial biomass in two transpacific air plumes to permit a microarray analysis using 16S rRNA genes. Thousands of distinct bacterial taxa spanning a wide range of phyla and

surface environments were detected before, during and after each Asian long range transport event. Interestingly, the transpacific plumes delivered higher concentrations taxa already in the background air (particularly *Proteobacteria*, *Actinobacteria*, and *Firmicutes*). While some bacterial families and a few marine archaea appeared for the first and only time during the plumes, the microbial community composition was similar despite unique transport histories of the air masses. It seems plausible, when coupled with atmospheric modeling and chemical analysis, that microbial biogeography can be used to pinpoint the source of intercontinental dust plumes. Given the degree of richness measured in our study, the overall contribution of Asian aerosols to microbial species in North American air warrants additional investigation.

3.2 Introduction

Air samples from the lower troposphere contain a substantial microbial component that originates from a variety of marine/terrestrial sources (Brodie et al., 2007; Burrows et al., 2009a; Fahlgren et al., 2010). Airborne cells can spread genes to distant environments and even influence weather as cloud/ice condensation nuclei (Christner et al., 2008). But very little is known about microbial diversity and abundance at higher altitudes where long-range atmospheric transport (i.e., global dispersal) is more efficient (Weiss-Penzias et al., 2006). Mountaintop observatories can provide access to the upper troposphere and lower stratosphere, making it feasible to capture enough biomass to employ modern molecular assays. Seasonal measurements at such platforms may help clarify the influence of microorganisms on patterns of climate (Christner et al., 2008; Burrows et al., 2009a), epidemiology (Brown & Hovmøller, 2002; Kellogg & Griffin, 2006; Griffin, 2007), and biogeography (Womack et al., 2010).

We collected samples from the Mt. Bachelor Observatory (MBO), a research station 2.8 km above sea level on the summit of an extinct volcano in central Oregon, USA (43.98 ºN, 121.7 ºW). In the springtime, windblown plumes of pollution, smoke, and dust from Asia routinely reach the field site after crossing the Pacific Ocean in 7 to 10 days (Jaffe et al., 2005; Weiss-Penzias et al., 2006; Fischer et al., 2009). Annually, as much as 64 Tg of Asian aerosols are transported to North America (Yu et al., 2012). Recently (Smith et al., 2012), we described two major Asian long-range transport (ALRT) plumes with high concentrations of particulate matter (mostly dust, but also anthropogenic pollution) arriving at MBO. The first event began at 2:00 UTC on 22 April 2011 and lasted 51 hours; the second started at 12:00 UTC on 12 May 2011 and ended 80 hours later (see Tables 3.1 and 3.2). Airborne bacteria concentrations were measured by qPCR and rRNA sequencing was used to identify cultured species. Average bacterial genomes ranged from 1 to 4 m^{-3} across the April episode and 2 to 7 m^{-3} across the May episode, assuming intact cells (containing 2 to 8 fg DNA) were captured. Several gram-positive bacterial isolates were identified using culture-based recovery methods, but since so few species can actually be cultivated (Womack et al., 2010; Smith et al., 2012), our goal was to reexamine the air samples with a more comprehensive molecular tool testing the hypothesis that transpacific plumes deliver rich microbial populations to North America.

3.3 Materials and Methods

Sample Collection

Microbes were collected on sterile polyethersulfone filters (0.8 μm pore size) connected to a previously described air-sampling device at MBO (Smith et al., 2012). Briefly, a high-volume pump pulled ~ 0.5 m^3 min^{-1} of air through individual filters over 12-h intervals, then samples

were removed from the device and stored at -80 °C. During sampling periods, meteorological and atmospheric chemistry data were collected for aerosol elemental composition (e.g., ammonium sulfate (NH_4SO_4), soil, and trace metals), carbon monoxide (CO), ozone (O_3), water (H_2O) vapor, total gaseous mercury (THg), temperature, atmospheric pressure, wind speed, and direction. Details of MBO instruments, calibrations, and element concentration calculations have been published elsewhere (Jaffe et al., 2005; Weiss-Penzias et al., 2006; Smith et al., 2012).

Atmospheric Modeling

We calculated 240-h backward trajectories initialized from MBO during peak aerosol periods to establish the long-range transport history of arriving air masses. Trajectories were calculated with the Hybrid Single-Particle Lagrangian Integrated Trajectory (HYSPLIT) model (Draxler & Rolph, 2003) version 4, which uses global meteorological data from the Global Data Assimilation System archive. The dataset has a time resolution of 3 h, a spatial resolution of 1° latitude by 1° longitude, and a vertical resolution of 23 pressure surfaces between 1000 and 20 hPa. Trajectories were run at multiple heights (1200, 1500, and 1800 m above ground level) surrounding the summit of MBO (Weiss-Penzias et al., 2006).

Transoceanic aerosol plumes were also modeled with the Navy Research Laboratory Aerosol Analysis and Prediction System (NAAPS) (http://www.nrlmry.navy.mil/aerosol/) to compare with HYSPLIT-4 long-range transport patterns. The NAAPS model produces a total aerosol forecast at optical depth 550 nm that includes sulfate, smoke, dust, and sea salt mass concentrations. We examined periods from 15 to 25 April 2011 and from 7 to 17 May 2011.

DNA Extraction and PCR Amplification

To extract DNA, samples were processed with MO BIO PowerWater® kits (product 14900-100-NF, MO BIO Laboratories, Inc., Carlsbad, CA). Each quartered filter was placed inside a 5-ml PowerWater® Bead Tube containing 3 ml of Solution PW1, and the tube was incubated for 30 min at 65 °C, followed by a 15-min vortex (product 12-812, Fisher Vortex Genie 2™, Fisher Scientific). All subsequent extraction steps followed the PowerWater® manufacturer guidelines, with solution volumes proportionally adjusted to match the boosted volume of PW1. A centrifuge (product 5804, Eppendorf, Hauppauge, NY) and PowerVac™ manifold (product 11991, MO-BIO Laboratories, Inc.) was used, along with a wash of 800 µl of 100% ethanol prior to the addition of Solution PW4. The final sample elution volume was 40 µl in Buffer EB. DNA samples were quantified using Nanodrop spectrophotometery (ND-1000, Thermo Scientific, Wilmington, DE) and PicoGreen® (Life Technologies™, Grand Island, NY) methods. Next, 16S rRNA gene PCR amplification was performed: bacterial 16S rRNA genes were amplified using forward primer 27F.1 (5'-AGRGTTTGATCMTGGCTCAG-3') and reverse primer 1492R.jgi (5'-GGTTACCTTGTTACGACTT-3'); archaeal 16S rRNA genes were amplified using forward primer 4Fa (5'- TCCGGTTGATCCTGCCRG-3') and reverse primer 1492R.jgi (Hazen et al., 2010). Thermocycling program conditions were 95 °C (3 min), followed by 35 cycles at 95 °C (30 sec), 50 °C (30 sec), 72 °C (2 min), and an extension at 72 °C (10 min) before holding at 4 °C. Amplified products were concentrated and quantified by electrophoresis using an Agilent 2100 Bioanalyzer®.

Microarray Design, Experiment, and Scoring

PhyloChip™ microarrays are commercially available through Second Genome, Inc. (San Bruno, CA) and have been described extensively elsewhere (Wilson et al., 2002; DeSantis et al., 2005;

DeSantis et al., 2007; Hazen et al., 2010). Amplified and purified DNA products were fragmented and biotin labeled. Each reaction mixture was injected into the hybridization chamber of version G3 of a PhyloChip™ Array (Hazen et al., 2010). A PhyloChip Control Mix™ was included for scaling and normalization. Oligonucleotide targets and probes on PhyloChip™ version G3 were synthesized by photolithography, representing publicly available bacteria and archaea 16S rRNA genes. Other relevant technical details, quality/processing controls, and reproducibility tests for the PhyloChip™ version G3 have been published (Hazen et al., 2010). Operational taxonomic units (OTUs) were defined as a group of highly similar 16S rRNA gene sequences (< 0.5% divergence). In total, there were 59,959 potential clusters, spanning 2 domains (archaea and bacteria), 147 phyla, 1,123 classes, and 1,219 orders. Each OTU was assigned to one of 1,464 families (Hugenholtz, 2002). Experimentally, hybridization took place for 16 h in an oven at 48°C and 60 RPM before the PhyloChip™ was washed, stained, and scanned using a GeneChip Scanner 3000 7G (Affymetrix, Santa Clara, CA). Fluorescence intensity was captured using Affymetrix software (GeneChip® Microarray Analysis Suite) and calculated using pre-established formulas (DeSantis et al., 2005).

Statistical Analysis

Hybridization scores (HybScores) were calculated for each OTU using probe fluorescent intensities. The relative abundance of taxa was measured by comparing intensities against the PhyloChip Control Mix™ after subtracting from background values. Multivariate statistical analyses were performed with PhyCA-Stats™ software (Second Genome, Inc.) and algorithms compared the relationship between probes and taxa. Maximum and minimum HybScores were discarded before averaging and scaling values so that a HybScore change of 1000 represented a

doubling in probe fluorescent intensity. Data were reduced to a series of pre-defined filters (Hazen et al., 2010) based on significant taxa patterns, established through either parametric Welch tests or Adonis tests to generate p-values. Presence and absence (i.e., incidence) data were transformed into binary metrics, which used the Unifrac distance method for examining changes in communities (Clark & Ainsworth, 1993; Lozupone et al., 2006). Abundance metrics used HybScore data for OTUs and a weighted Unifrac method for measuring sample-to-sample distance.

The entire microarray dataset with OTU annotations can be accessed at: http://greengenes.secondgenome.com/downloads/phylochip_datasets.

3.4 Results and Discussion

First, it was essential to establish possible source regions and transport histories for the events of interest using meteorological and chemical data. Kinematic back trajectories were modeled for each episode with HYSPLIT, revealing key differences in origin, mixing, and vertical transport. Ten-day back trajectories for the April event began near China, Korea, or Japan and showed the air mass rapidly lifting to an altitude of ~8 km (Fig. 3.1a). Low humidity and high O_3 indicate that the pollution plume may have mixed with air from the upper troposphere/lower stratosphere during transport (Smith et al., 2012). Airborne THg and CO can be used as tracers for ALRT (Weiss-Penzias et al., 2006), and enhancement ratios between the species (Fig. S3.1) in the April plume were consistent with previously identified Asian pollution events at the collection site (Jaffe et al., 2005). Soil and NH_4SO_4 concentrations were also correlated (Fig. S3.2), indicating a similar origin and an airborne time sufficient for homogenization (Jaffe et al., 2003). The NAAPS model provided another check for ALRT by depicting the transpacific migration of

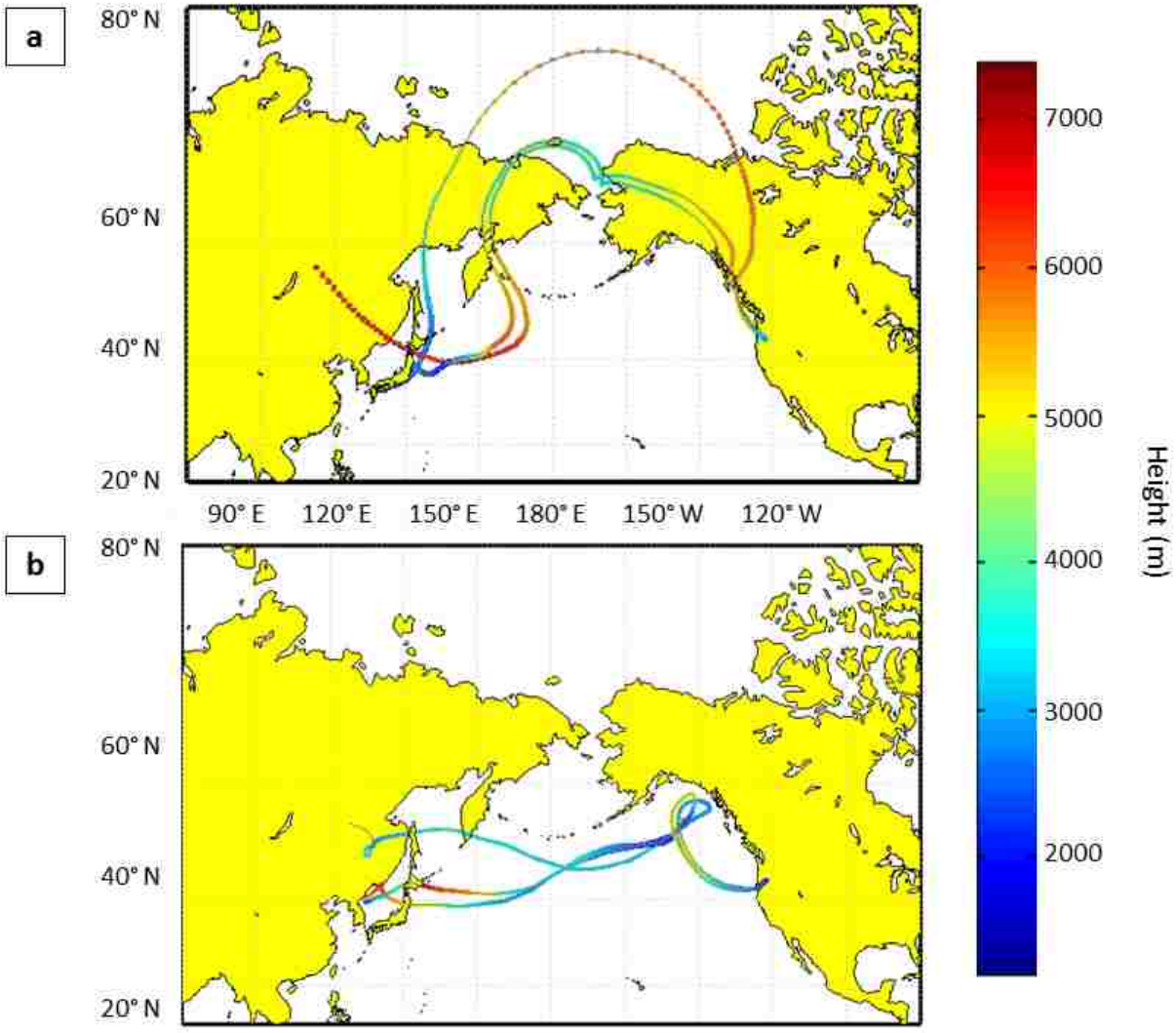


Figure 3.1 Ten-day kinematic back trajectories for air arriving at MBO (43.98 ºN, 121.7 ºW), depicting transpacific transport patterns from Asia (left) to North America (right). Calculated for both episodes using the HYSPLIT model at peak plume hour (highest aerosol, CO, and O_3 levels). Colored scale bar represents trajectory height (m above sea level). (a) Trajectories ending 22 April 2011 at 12:00 UTC, showing a rapid ascent to the upper troposphere / lower stratosphere prior to sampling (b) Trajectories ending 13 May 2011 at 00:00 UTC, showing possible marine boundary layer mixing and a storm loop off the Alaskan coast prior to sampling.

Table 3.1 April Plume Sample Manifest

Sample	Date and Time (UTC)	Category	Aerosol avg ($\mu g\ m^{-3}$)	DNA concn ($ng\ \mu l^{-1}$)	PCR yield (ng)	No. OTUs detected
Abk142	4/21/2011 20:00 to 4/22/2011 08:00	Background	1.56	2.2	1394	2325
Adu143	4/22/2011 08:00 to 4/22/2011 19:00	Plume	23.3	15.3	1442	2197
Adu144	4/22/2011 19:00 to 4/23/2011 07:00	Plume	10.3	1.5	1328	2808
Adu145	4/23/2011 07:00 to 4/23/2011 19:00	Plume	10.7	1.7	1347	2620
Adu146	4/23/2011 19:00 to 4/24/2011 07:00	Plume	10.2	1.8	1330	2742
Abk147	4/24/2011 07:00 to 4/24/2011 19:00	Background	0	2.0	991	2114

Table 3.2 May Plume Sample Manifest

Sample	Date and Time (UTC)	Category	Aerosol avg ($\mu g\ m^{-3}$)	DNA conc ($ng\ \mu l^{-1}$)	PCR yield (ng)	No. OTUs detected
Tbk175	5/12/2011 06:00 to 5/12/2011 19:00	Background	0.408	1.6	1339	2311
Tdu176	5/12/2011 19:00 to 5/13/2011 08:00	Plume	9.52	0.5	1331	2155
Tdu177	5/13/2011 08:00 to 5/13/2011 20:00	Plume	8.70	2.6	1314	2682
Tdu178	5/13/2011 20:00 to 5/14/2011 08:00	Plume	7.82	1.4	1115	2864
Tdu179	5/14/2011 08:00 to 5/14/2011 20:00	Plume	5.05	1.1	1361	2841
Tdu180	5/14/2011 20:00 to 5/15/2011 08:00	Plume	5.56	1.2	1309	2656
Tbk181	5/15/2011 08:00 to 5/15/2011 20:00	Background	1.99	1.3	1165	2516
Tbk182	5/15/2011 20:00 to 5/16/2011 08:00	Background	0.161	6.7	1259	2235

airborne sulfate, dust, smoke, and sea salt at a total aerosol optical depth of 0.1 to 0.2 (Fig. S3.3a). For the May episode, 10-day HYSPLIT back trajectories show an air mass originating over the Pacific Ocean and mixing into the marine boundary layer (Fig. 3.1b). According to the model, the air was swept through a storm loop off the coast of Alaska before ascending into the free troposphere and MBO. Depleted levels of THg/CO (Fig. S3.1) and NH_4SO_4/soil (Fig. S3.4) align with the possibility of a boundary layer excursion. Compared to what was observed in the April episode, the May NAAPS data (Fig. S3.3b) show denser emissions in Asia and a larger transpacific plume.

Air samples (spanning 12-h intervals) from before, during, and after ALRT events were analyzed for microbial richness and abundance using a PhyloChip^{TM} 16S rRNA microarray (Wilson et al., 2002; DeSantis et al., 2005; Brodie et al., 2007; DeSantis et al., 2007; Hazen et al., 2010). Tables 3.1 and 3.2 provide the aerosol level, microbial concentration (ng DNA) and community richness (number of OTUs) for each sampling interval and category (plume or background). Overall, 2,808 bacterial OTUs were detected at the peak of the April episode (694 above background levels); 2,864 bacterial OTUs were detected at the peak of the May episode (629 above background levels). Based on incidence data alone, bacterial richness was highest during plumes and lowest before and after (i.e., from background air). Bacterial taxa spanned a broad range of phyla, including *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes*. Six species of archaea were also measured in 8 samples (see Table 3.3).

Principle Coordinate Analysis (PCoA) was used to measure dissimilarity in ALRT plumes. Each point on the ordination plots represents an entire microbial community sample. Figure 3.2 assembled incidence data from 38,546 possible taxa and partitioned the samples into two distinct

Table 3.3 Archaea Detected in April and May Events

Sample	Class	Order	Family	Genus
Abk142	Methanomicrobia	Methanosarcinales	Unclassified	sfD
Adu144	Methanomicrobia	Methanosarcinales	Unclassified	sfD
Adu146	Methanomicrobia	Methanosarcinales	Unclassified	sfD
Tdu177	Methanomicrobia	Methanosarcinales	Unclassified	sfF
	pMC2A209	Unclassified	Unclassified	sfC
Tdu178	Methanomicrobia	Methanosarcinales	Unclassified	sfF
	Methanobacteria	Methanobacteriales	WSA2	unclassified
Tdu179	Methanomicrobia	Methanosarcinales	Unclassified	sfD
	Methanomicrobia	Methanosarcinales	Unclassified	sfF
Tdu180	Thermoprotei	Desulfurococcales	Desulfurococcaceae	Aeropyrum
Tbk182	Methanomicrobia	Methanosarcinales	Unclassified	sfF

categories: plume and background. Significant clusters (P = 0.032) emerged with (a) April and May background samples and (b) April and May plume samples; supporting the idea that ALRT events were more similar than different. Comparable clustering was observed (P = 0.044) in a follow-up analysis using abundance data from 2,514 possible taxa (Fig. S3.5). Only incidence data for 86 selected taxa at peak plume sampling intervals (Adu143-145; Tdu177-179) revealed a microbiome contrast between April and May plumes (Fig. S3.6).

Figure 3.3 supports the idea that ALRT plumes delivered similar microbiota despite unique transport histories in April and May: although species richness levels varied between the events (Tables 3.1 and 3.2), the proportion of 15 common bacterial families was essentially parallel. An important point, however, is that while the relative abundance remained steady across ALRT plumes, the absolute abundance of 312 OTUs changed significantly (P values < 0.001). To illustrate this observation, a circular tree (Fig. S3.7) was constructed to display differentially

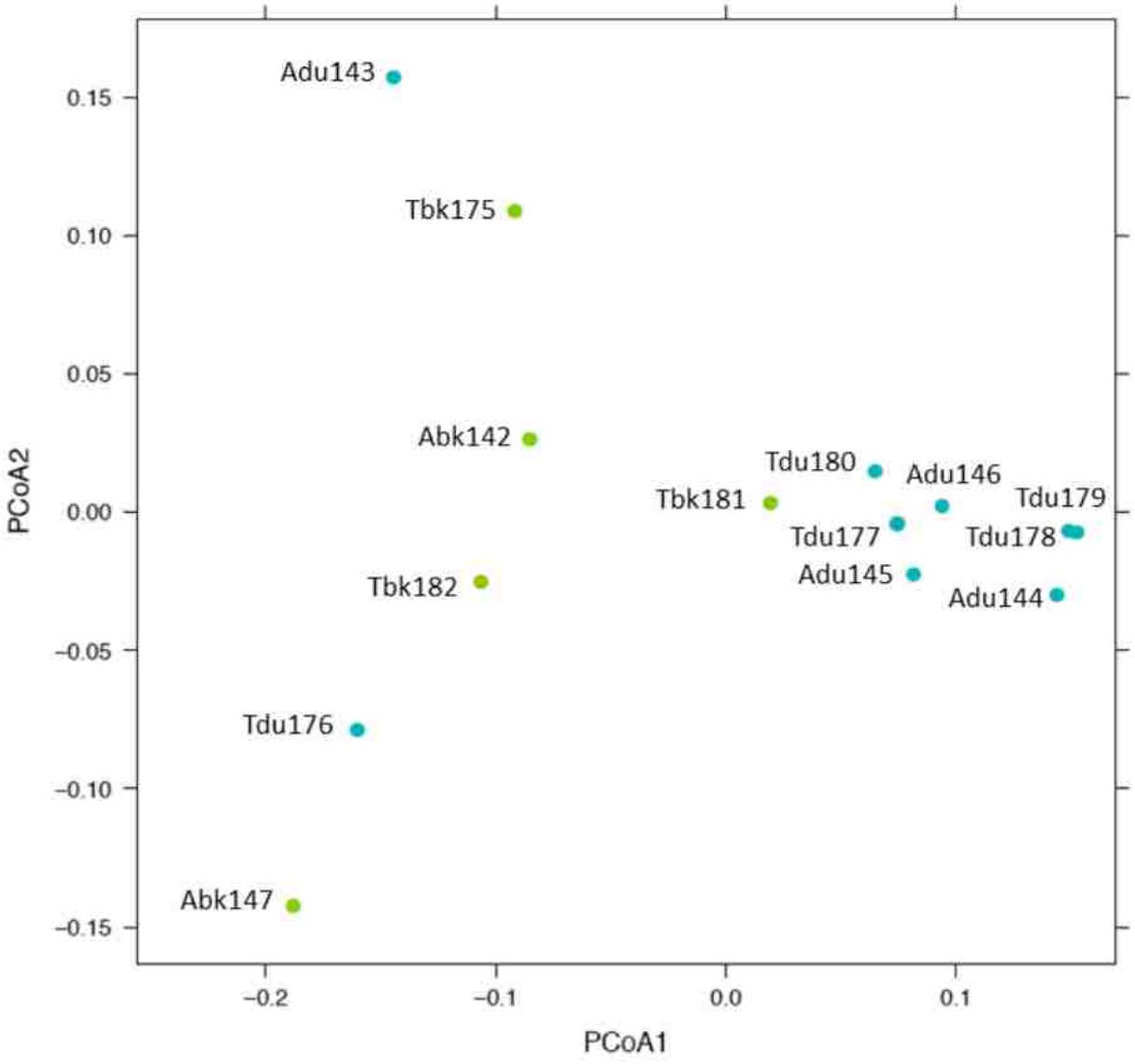
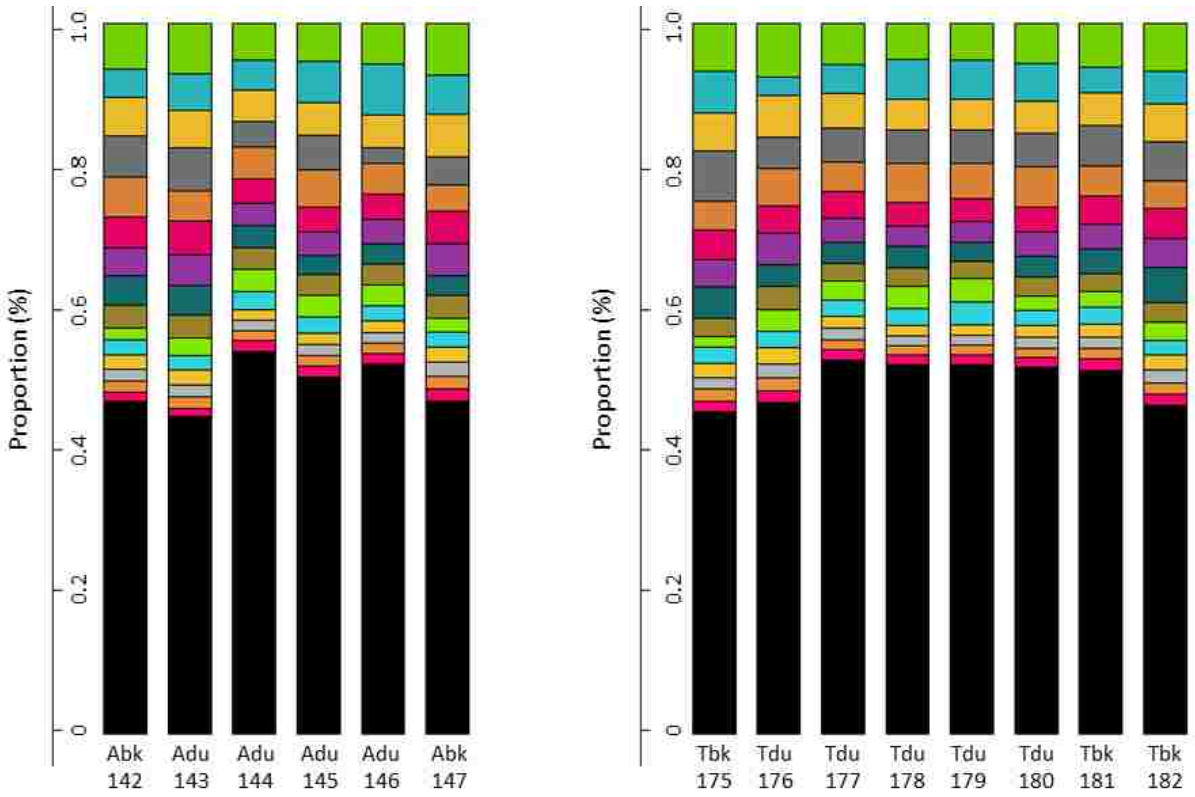


Figure 3.2 Principle coordinate analysis of background samples (green) and plume samples (blue). Analysis was based on unweighted unifrac distance between samples from 38,546 possible taxa with incidence differences. Axis 1: 29% of variation explained; Axis 2: 9% of variation explained. The partitioning shows the similarity in community composition between plume samples (-du-) and the similarity in community composition background samples (-bk-), regardless of plume timing (April or May). Note: Adu143 and Tdu176 were transitional samples at the onset of a plume (i.e., a mixture of background and plume).



Color	Domain	Phylum	Class	Order	Family
	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae
	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae
	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae
	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae
	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae
	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Corynebacteriaceae
	Bacteria	Firmicutes	Bacilli	Bacillales	Staphylococcaceae
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae
	Bacteria	Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae
	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Streptomycetaceae
	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae
	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Aquabacteriaceae
	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae
	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae
	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae
	Other				

Figure 3.3 Relative abundance of fifteen common bacterial families across April plume (left) and May plume (right). The size of each color block (assigned to families in the table below) represents the number of detected OTUs in the family relative to the total number of OTUs detected in that sample. For example, *Bacillaceae* OTUs accounted for 6.5% of the total OTUs detected in the first April sample (Abk142). Generally, family proportions remained constant across both episodes.

abundant OTUs and their taxonomic relationship based on 16S rRNA gene alignment. Welch test p-values were used to reduce the number of significantly different families to 83, and the one OTU with the greatest abundance difference from each family was included in the tree. Heat-map values revealed increases within the class *Clostridia* and families *Sphingomonadaceae*, *Rhodobacteraceae*, and *Isosphaeraceae* relative to the combined means of baseline samples. Altogether, *Proteobacteria* (N = 29), *Actinobacteria* (N = 19) and *Firmicutes* (N = 19) totaled 80% of the phyla whose abundance increased during ALRT plumes. A higher level of Actinobacteria and Firmicutes is noteworthy because the families include many spore-forming and gram-positive species capable of surviving extreme conditions associated with long-range upper atmospheric transport. Curiously, 4 out of 5 of the families that decreased in abundance during plumes were *Proteobacteria* from marine environments (including *Alteromonadaceae*, *Vibrionaceae*, and the *OM60* family within *Oceanospirillales*).

After establishing ALRT plumes delivered higher concentrations of microbes already present in the North American background air, we focused on variations within specific taxa using Prediction Analysis for Microarrays (PAM) (Tibshirani et al., 2002). Figure 3.4 highlights taxa with possible Asian or oceanic origins, including isolates from a Chinese forest (OTU 51259), Dongping Lake sediments (OTU 51013), and marine microbial mats (OTU 75349). The alignment between taxa source regions and probable emission sites identified by atmospheric data was striking; however, annotations from the 16S rRNA sequence database can be inaccurate. Source verification of probe-detected taxa would require sample sequencing (outside the scope of our current study). Another possible agreement between atmospheric transport models and microbial biogeography was the detection of *Aeropyrum* sp. in the May episode. Finding marine archaea (Sako et al., 1996) in the free troposphere above central Oregon also

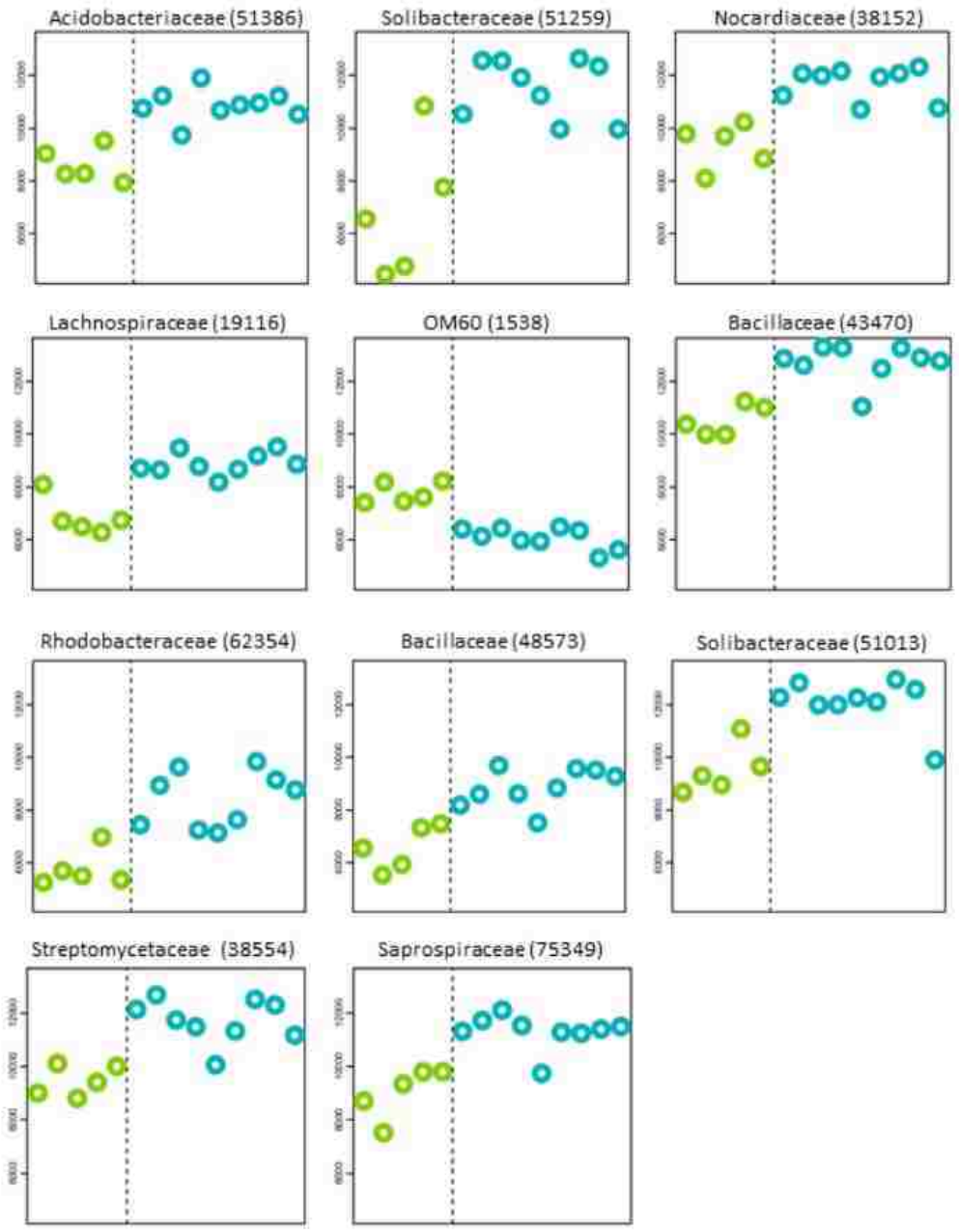


Figure 3.4 Significant abundance variations in specific taxa between background (green) and plume (blue) periods. Combined data for April and May events. HybScores are on the y-axis and sample order (from left to right) follows the order in Tables 3.1 and 3.2 (e.g., Abk142 is the leftmost data point). Numbers in parentheses are OTU identification numbers.

supports microbial ALRT. But biogeography, alone, cannot be the only means of inferring distant provenance. Only after considering the location of our field site, prevailing wind direction and long range transport validation through a number of independent atmospheric data sets (including: (1) HYSPLIT-4 kinematic back trajectories; (2) aged aerosol data (i.e., NH_4SO_4 and soil); (3) plume chemical composition (i.e., CO and THg); and (4) the NAAPS model) could we be confident about the transoceanic origin of air samples.

Soon, it may be useful to think about microorganisms as air pollution (e.g., how aerosols were depicted in Fig. S3.3: moving in plumes through a global background layer). Our main finding – transpacific dust plumes delivering elevated levels of species already in the background air – suggests microbes pool like other types of pollution over the Pacific Ocean. However, a transpacific monitoring network with sampling sites in eastern Asia and western North America is needed to establish an aerobiology dataset comparable to the NAAPS model. Such an undertaking would require seasonal measurements from a variety of natural (desert dust, marine sea spray, etc.) and artificial (livestock feedlots, wastewater-treatment facilities, etc.) upwind emission sources (Burrows et al., 2009b), monitored, ideally through a combination of ground- and aircraft-based platforms. Global sampling efforts using rRNA microarrays might consider employing the same commercial to reduce false hybridizations and other sources of variation (DeSantis et al., 2005). Standardizing air collection techniques, DNA extraction, PCR amplification, and microarray protocols would be useful for comparisons between field sites. Even though microarrays offer improved sensitivity to microbial taxa (our first investigation of the same ALRT samples detected only 18 species of bacteria (Smith et al., 2012)), culture-based aerobiology data still have value: understanding what species remain viable after intercontinental atmospheric transport informs questions related to disease propagation and microbial speciation.

Airborne microorganisms originate from the surface and must eventually return to it. Consequently, the atmosphere has generally been considered a conduit for life rather than a true ecosystem. However, our study revealed a microbial richness that rivals surface ecosystems and the presence of many phyla with adaptations for extended viability during atmospheric transport (e.g., spore forming and gram-positive bacteria). In addition, the potential for dynamic microbial interactions with the environment, such as *in-situ* metabolism (Sattler et al., 2001), the stimulation of cloud formation and precipitation (Christner et al., 2008), and selection pressures from ultraviolet radiation (Smith et al., 2011) all support the idea that the atmosphere might be considered an ecosystem in its own right. No matter how it is classified, as desertification injects more dust into the atmosphere (Kellogg & Griffin, 2006; Yu et al., 2012) and humans grow increasingly vulnerable to changing patterns of weather and disease, it will be important to monitor microbial populations on intercontinental winds.

3.5 Acknowledgements

Research funding was provided by National Science Foundation (NSF) Integrative Graduate Education and Research Traineeship (IGERT) at the University of Washington (UW) Astrobiology Program, National Geographic Society Waitt Grants Program (#W177-11), NASA Astrobiology Institute Director's Discretionary Fund, and the Virtual Planetary Lab at UW. NSF Grant ATM-0724327 funded the MBO atmospheric chemistry measurements. We thank J. Hee, B. Hicks, P. Ball, C. Higginbotham, T. Lomax, J. Baross, T. DeSantis, B. Lidz, C. Kellogg, A. Schuerger, T. Bradshaw, R. Wheeler, and the staff at Mt. Bachelor Ski Resort for support. We are also grateful to three anonymous reviewers for their time and constructive feedback.

3.6 Supplementary Information

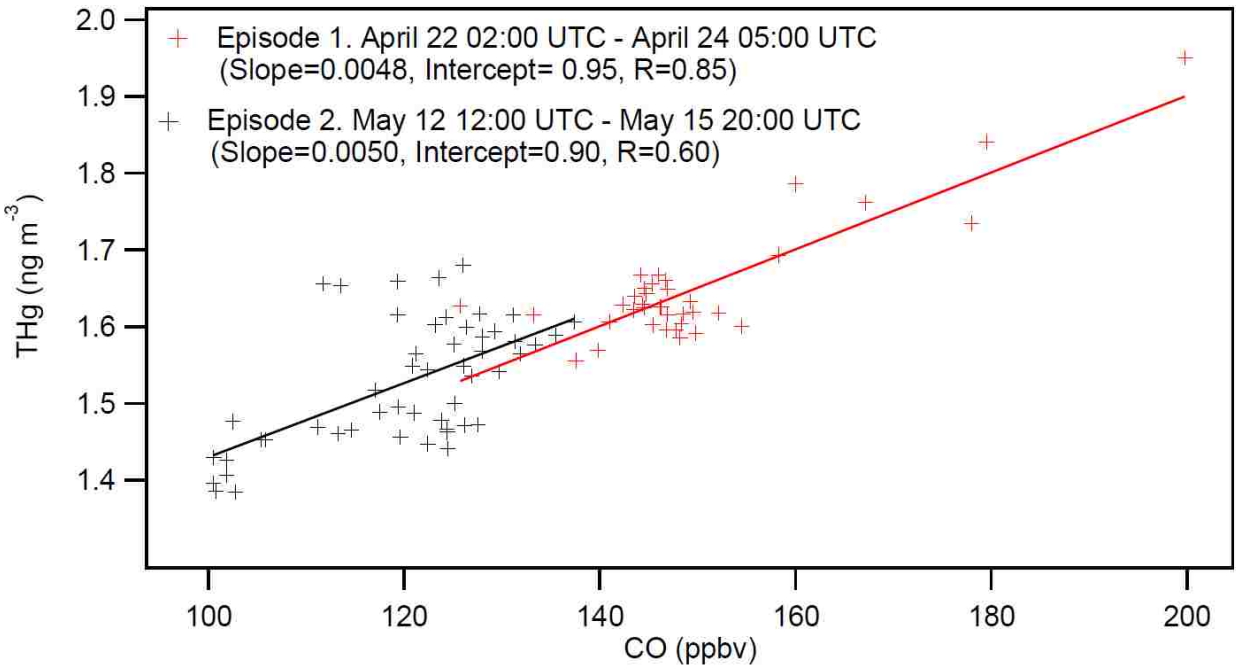
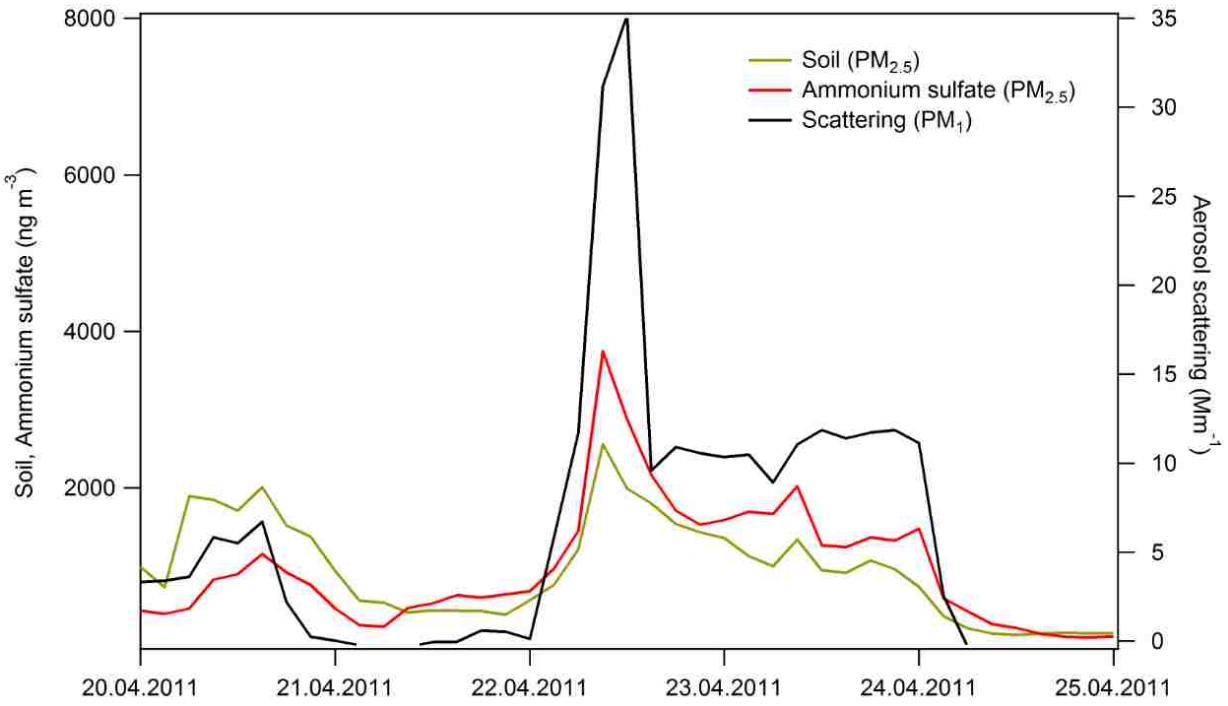


Figure S3.1 Total gaseous mercury (THg) and carbon monoxide (CO) data for 22-24 April (red) and 12-15 May (black) events. Enhancement ratios for both periods were indicative of Asian long-range transport based on analyses of similar pollution episodes measured at MBO (Jaffe et al., 2005).

a



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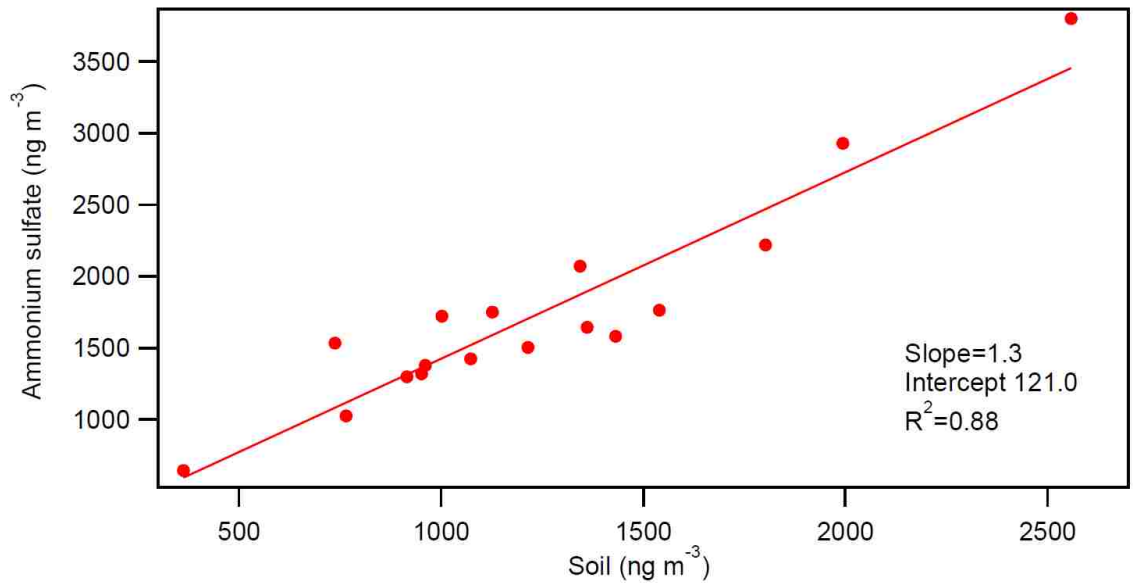
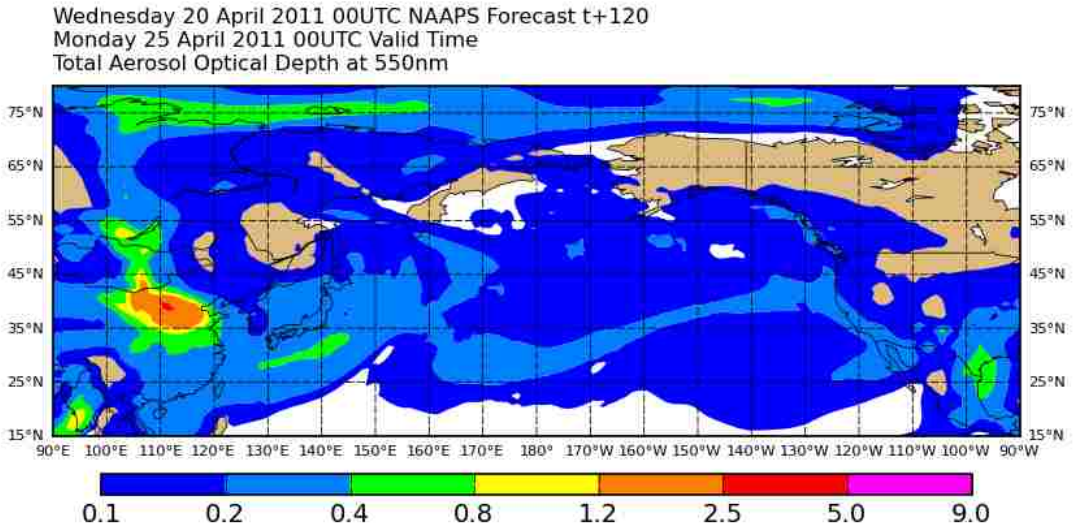


Figure S3.2 Soil (yellow), NH_4SO_4 (red), and aerosol (black) data for the April episode. Soil and NH_4SO_4 values were calculated using the Interagency Monitoring of Protected Visual Environments (IMPROVE) equations (Malm et al., 1994): [soil] = 2.2[Al] + 2.49[Si] + 1.63[Ca] + 2.42[Fe] + 1.94[Ti]; [NH_4SO_4] = 4.125*[S]. (a) Enhancements show the beginning, transition, and end of the event. (b) A high correlation coefficient between soil and NH_4SO_4 was observed (R^2 = 0.88), meaning the sampled air mass was aloft long enough for the species to mix (Jaffe et al., 2003).

a



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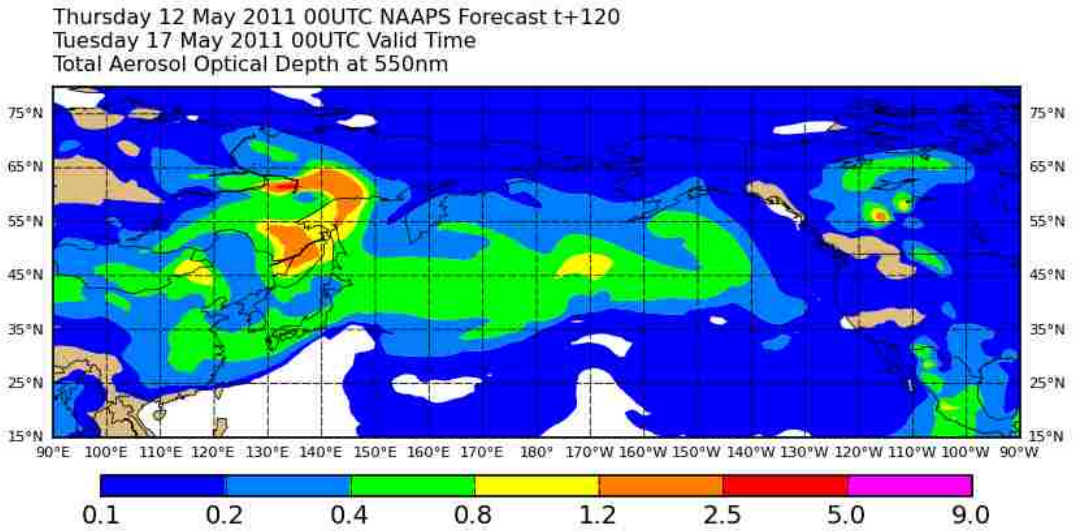
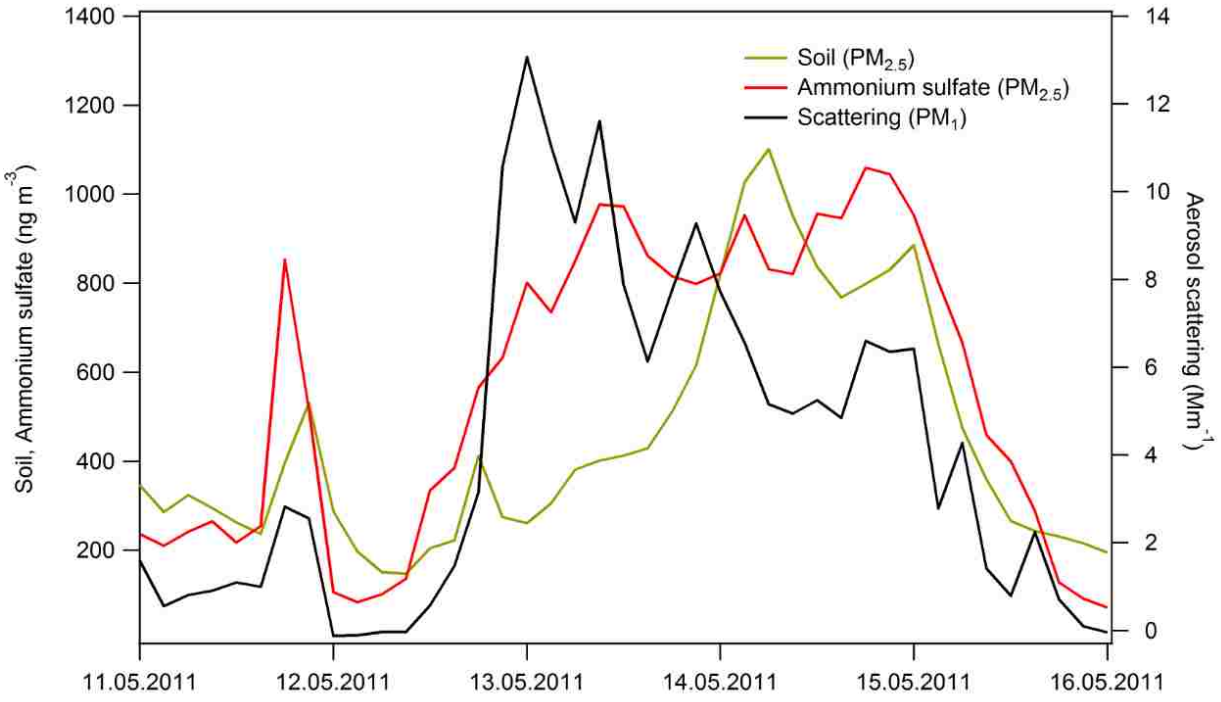


Figure S3.3 Transoceanic aerosol plumes modeled with the Navy Research Laboratory Aerosol Analysis and Prediction System (NAAPS). Total aerosol at optical depth 550 nm includes sulfate, smoke, dust, and sea salt mass concentrations. (a) Selected panel from 15 to 25 April 2011. (b) Selected panel from 7 to 17 May 2011.

a



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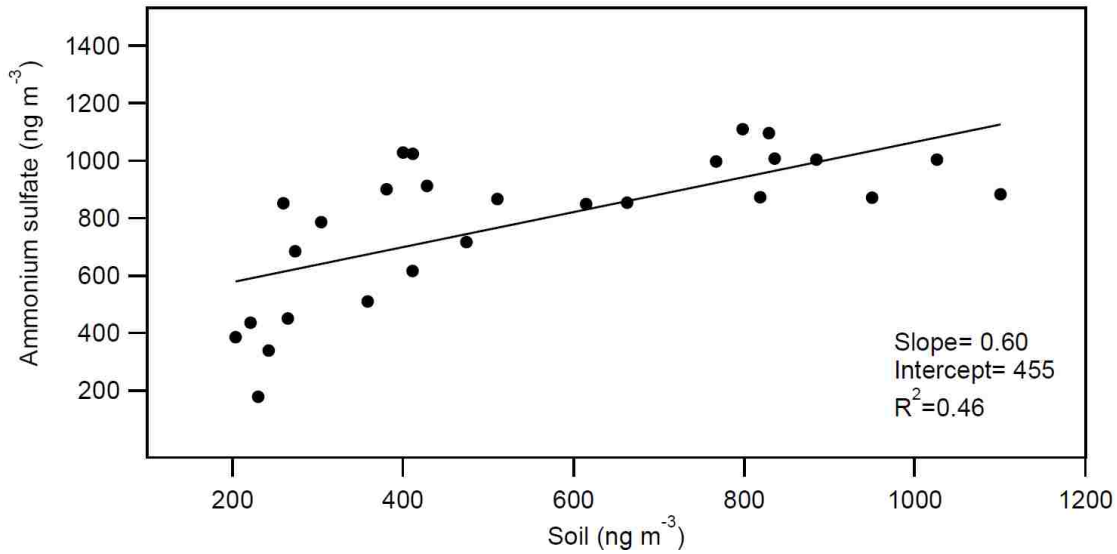

Figure S3.4 Soil (yellow), NH_4SO_4 (red), and aerosol (black) data for the May episode. Soil and NH_4SO_4 values were calculated using the Interagency Monitoring of Protected Visual Environments (IMPROVE) equations (Malm et al., 1994): [soil] = 2.2[Al] + 2.49[Si] + 1.63[Ca] + 2.42[Fe] + 1.94[Ti]; [NH_4SO_4] = 4.125*[S]. (a) Enhancements show the beginning, transition, and end of the event. (b) Compared to the April episode, a much lower correlation coefficient between soil and NH_4SO_4 (was observed (R^2 = 0.46), indicating substantial boundary layer influence during transpacific transport (Jaffe et al., 2003).

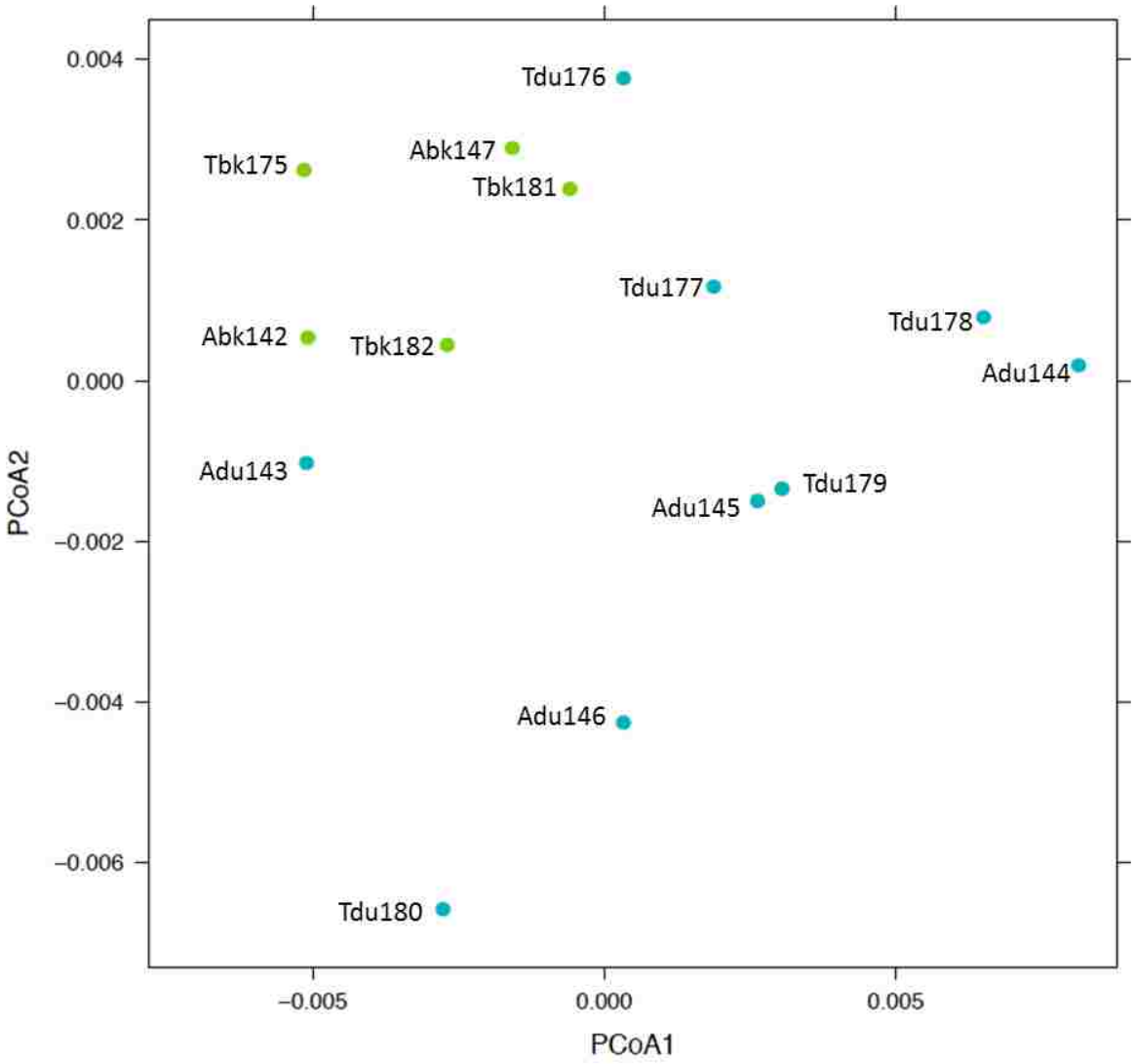

Figure S3.5 Principle coordinate analysis of background samples (green) and plume samples (blue). Analysis was based on weighted unifrac distance between samples from 2514 taxa with significant abundance differences. Axis 1: 50% of variation explained; Axis 2: 23% of variation explained. The partitioning shows the similarity in community composition between plume samples (-du-) and the similarity in community composition background samples (-bk-), regardless of plume timing (April or May). Note: Adu143 and Tdu176 were transitional samples at the onset of a plume (i.e., a mixture of background and plume).

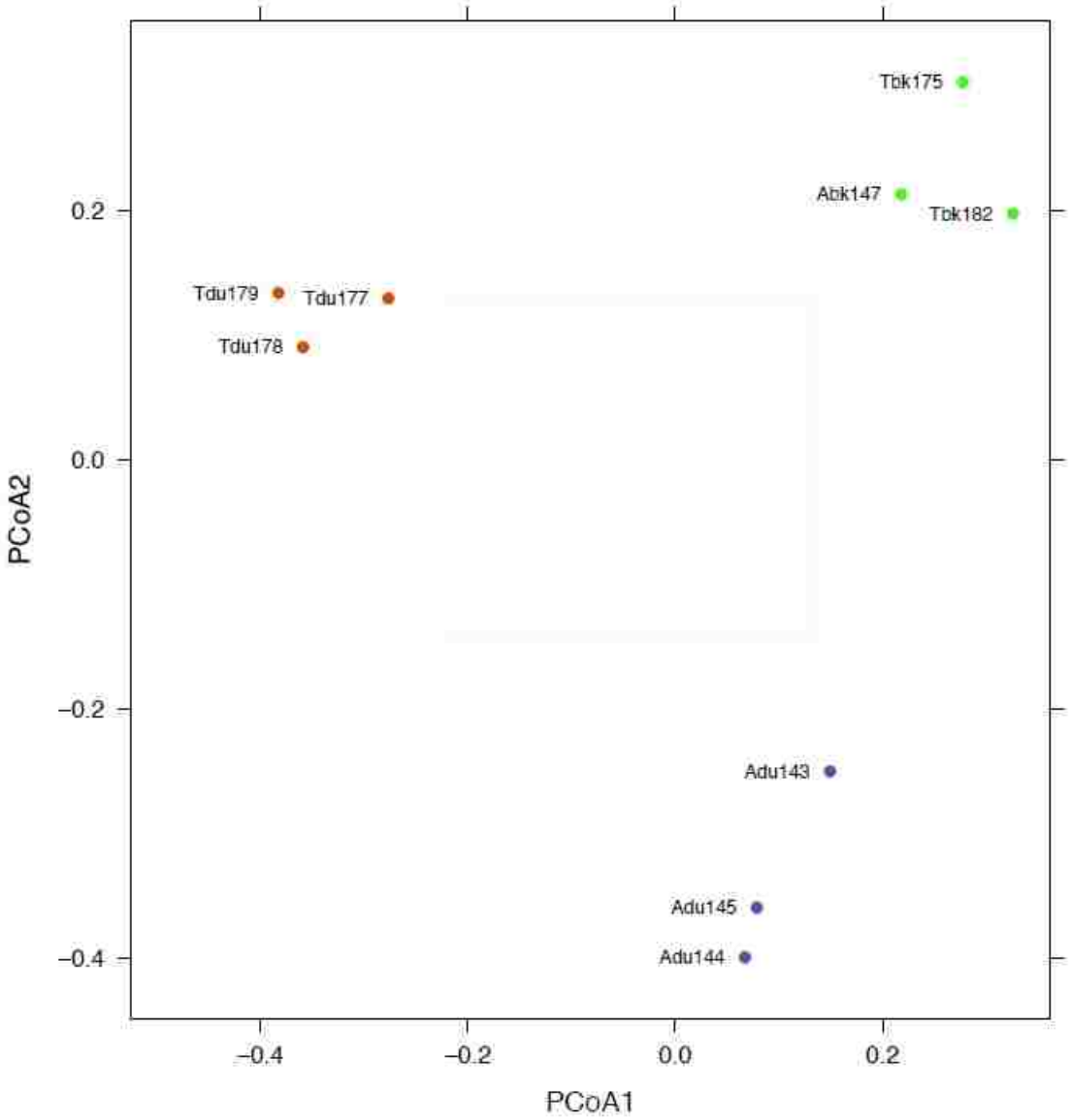

Figure S3.6 Principle coordinate analysis of April plume samples (purple), May plume samples (brown), and Background samples (green). Analysis was based on unweighted unifrac distance between samples from 86 selected taxa with incidence differences at peak plume intervals. Axis 1: 28% of variation explained; Axis 2: 26% of variation explained. The partitioning shows a microbiome contrast between the April and May plumes. Note: all samples were included in analysis but transitional points (i.e., a mixture of background and plume) were removed from the plot for clarity.

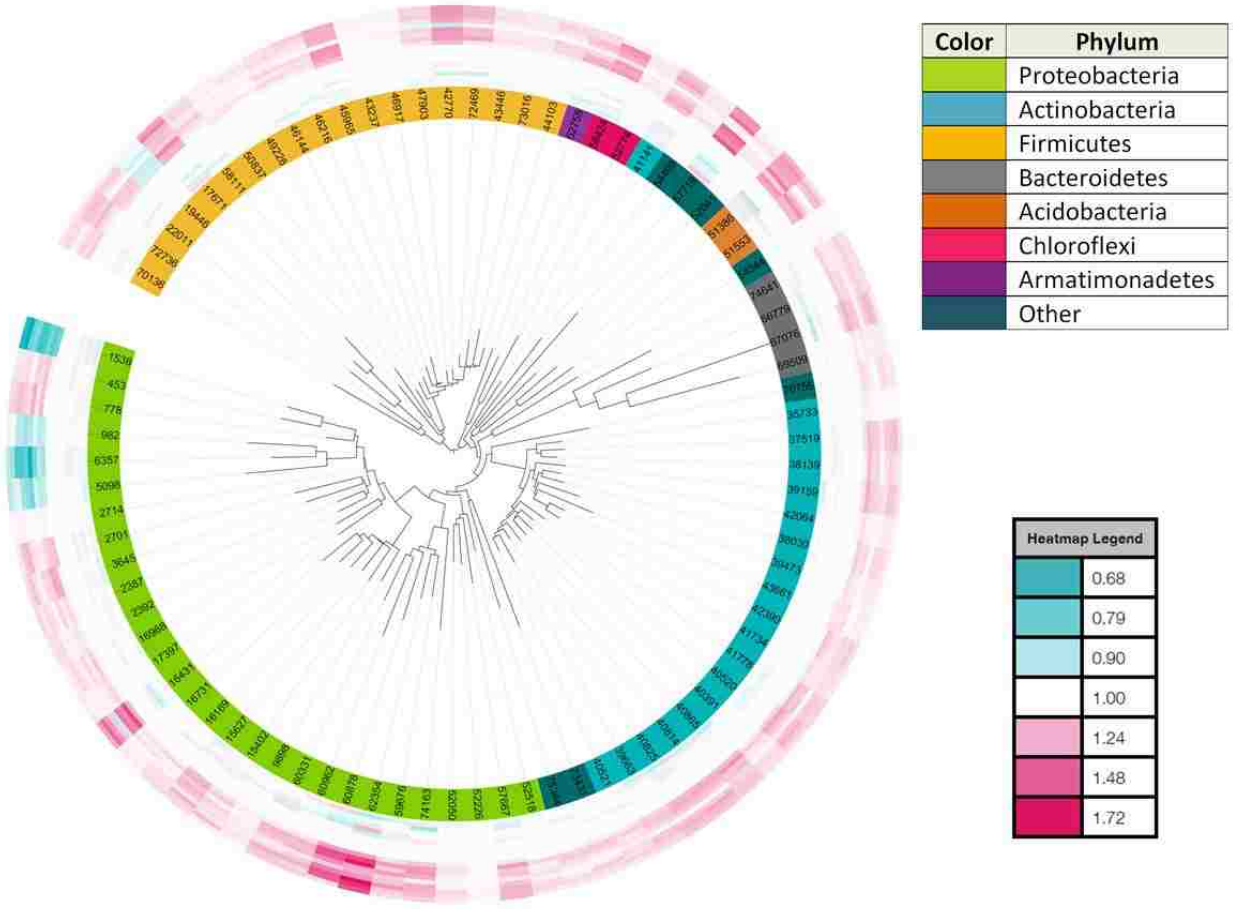

Figure S3.7 Circular tree displaying taxonomic relationship of differentially abundant OTUs based on 16S rRNA gene alignment. Families with significant abundance differences between background and plume samples from April and May events (Welch test *P* values < 0.001). Heat-map rings around the tree show increases or decreases in microbial abundance relative to combined category means: red indicates an increase in OTU abundance; blue indicates a decrease in abundance; and color saturation represents difference in intensity. Background samples are inner rings and plume samples are outer rings. Ring layer order follows the sample order in Tables 3.1 and 3.2 (e.g., the innermost outer ring is Adu143 and the outermost outer ring is Tdu180). Individual leaves on the tree, representing 83 families, have OTU identification numbers than can be used to locate more information at the microarray dataset archive (http://greengenes.secondgenome.com/downloads/phylochip_datasets).

Chapter 4

STRATOSPHERIC MICROBIOLOGY AT 20 KM OVER THE PACIFIC OCEAN

Chapter 4 characterizes microorganisms collected from a high-altitude flight over the Pacific Ocean. Several isolates of bacteria and fungi were recovered in the laboratory and possible origins are discussed. Portions of this chapter were originally published in collaboration with Dale W. Griffin and Andrew C. Schuerger in the November 2009 edition of *Aerobiologia* (Smith et al., 2010, *Aerobiologia*, 26:35-46) (©2009 *Springer Science+Business Media, LLC*), and are reproduced below with permission of Springer Science+Business Media, LLC.

4.1 Abstract

An aerobiology sampling flight at 20 km was conducted on 28 April 2008 over the Pacific Ocean (36.5° N, 118-149° W), a period of time that coincided with the movement of Asian dust across the ocean. The aim of this study was to confirm the presence of viable bacteria and fungi within a transoceanic, atmospheric bridge and to improve the resolution of flight hardware processing techniques. Isolates of the microbial strains recovered were analyzed with ribosomal ribonucleic acid (rRNA) sequencing to identify bacterial species *Bacillus* sp., *Bacillus subtilis*, *Bacillus endophyticus*, and the fungal genus *Penicillium*. Satellite imagery and ground-based radiosonde observations were used to measure dust movement and characterize the high-altitude environment at the time of collection. Considering the atmospheric residence time (7 to 10 days), the extreme temperature regime of the environment (-75° C), and the absence of a mechanism that could sustain particulates at high altitude, it is unlikely that our samples indicate a permanent, stratospheric ecosystem. However, the presence of viable fungi and bacteria in

transoceanic stratosphere remains relevant to understanding the distribution and extent of microbial life on Earth.

4.2 Introduction

Microbial diversity in Earth's upper atmosphere remains largely unexplored, despite its relevance to a number of scientific disciplines. Bacteria and fungi are injected into the atmosphere primarily by dust storms over arid regions of the planet, but hurricanes, volcanoes, fire, and anthropogenic sources also contribute to the total amount of biological material in the air. Meteorological factors control the altitude, distribution, and residence time of particles aloft (Deshler et al., 1993; Griffin et al., 2001; Bauman et al., 2003; Kellogg & Griffin, 2006; Wainwright et al., 2006; Griffin, 2007). Lower atmosphere (altitude 0 – 10 km) collections have documented how microorganisms can affect local ecology and climate. Global patterns of atmospheric dust and long-range dispersion can influence the spread of plant and animal pathogens (Griffin et al., 2001; Brown & Hovmøller, 2002; Griffin, 2007). Microbial cells contribute to precipitation as cloud condensation nuclei (CCN) or ice condensation nuclei (ICN), as discussed by Schnell & Vali (1976), Maki & Willoughby (1978), Szyrmer & Zawadzki (1997), Hamilton & Lenton (1998), Sun & Ariya (2006), Pratt et al. (2009), and Prenni et al. (2009). While most airborne microbes remain dormant during atmospheric transport, Sattler et al. (2001) demonstrated growth of bacteria in supercooled cloud droplets (at or below 0° C) from samples collected at 3 km.

Clearly bacteria and fungi are abundant in the troposphere, but what about even farther above the Earth's surface? The biologically challenging environment in the stratosphere (10 km – 50 km) would suggest that there would be lower viable biomass as altitude increases. For instance,

the stratosphere has greater extremes of ultraviolet (UV) irradiation, desiccation, cold temperatures, and nutrient deprivation than the troposphere (Brasseur & Solomon, 1986; Lysenko & Demina, 1992; Nicholson et al., 2000). Yet several studies conducted in the stratosphere have reported viable, airborne microbes (Imshenetsky et al., 1978; Lysenko, 1980; Harris et al., 2002; Wainwright et al., 2002; Narlikar et al., 2003; Griffin, 2004; Griffin, 2008), including novel species (Shivaji et al., 2006). Missions to sample stratospheric biota have been historically infrequent, making it difficult to answer even basic ecological questions. For example, What is the diversity and distribution of microbial species at high altitudes? Is there a limit to their dispersion (vertical and horizontal)? What influences cell viability at high altitudes? Finally, and perhaps most interestingly, can growth and/or reproduction occur *in-situ* at altitude? Investigating these topics with subsequent stratosphere collections is critical to understanding the true boundaries of the terrestrial biosphere.

The environmental factor most relevant to each question above is residence time – defined here as the total time that microbes remain aloft in the upper atmosphere. To date, no study has been able to determine whether residence time is on the order of days, months, or possibly even longer. Although satellites and ground instruments can track dust storms, neither can remotely detect airborne microbes. Our group recognized that sampling an air mass above the open ocean would be an effective way to assign a residence time by (1) eliminating the possibility of local dust injections and (2) comparing satellite-imaged dust trajectories with DNA sequence information to infer place of origin. In Asia, dust generated by the Gobi, Takla Makan, and Badain Jaran deserts blows seasonally across the Pacific Ocean from approximately March to May (Duce et al., 1980; Kar & Takeuchi, 2004), eventually reaching the west coast of North America. Chemical measurements (Jaffe et al., 2003) and satellite imagery (Griffin, 2007) have

been used to estimate that Asian dust typically takes 7 to 10 days to cross the Pacific. Along a transoceanic route, some fraction of microbes will be removed from the air by precipitation or gravitational settling (Griffin et al., 2006). Collecting dust deposited at lower elevations in North America would subject microbes to heavy dilution and possible contamination from local sources. To avoid this problem, our group sampled an air mass that was still in transit over the Pacific Ocean using a high altitude aircraft that offered precise instrumental control and a large collection range. We hypothesized that most microbial species recovered from the transoceanic air samples would trace back to Asian isolates, several thousand kilometers from the desert source, due to the timing and location of our collection.

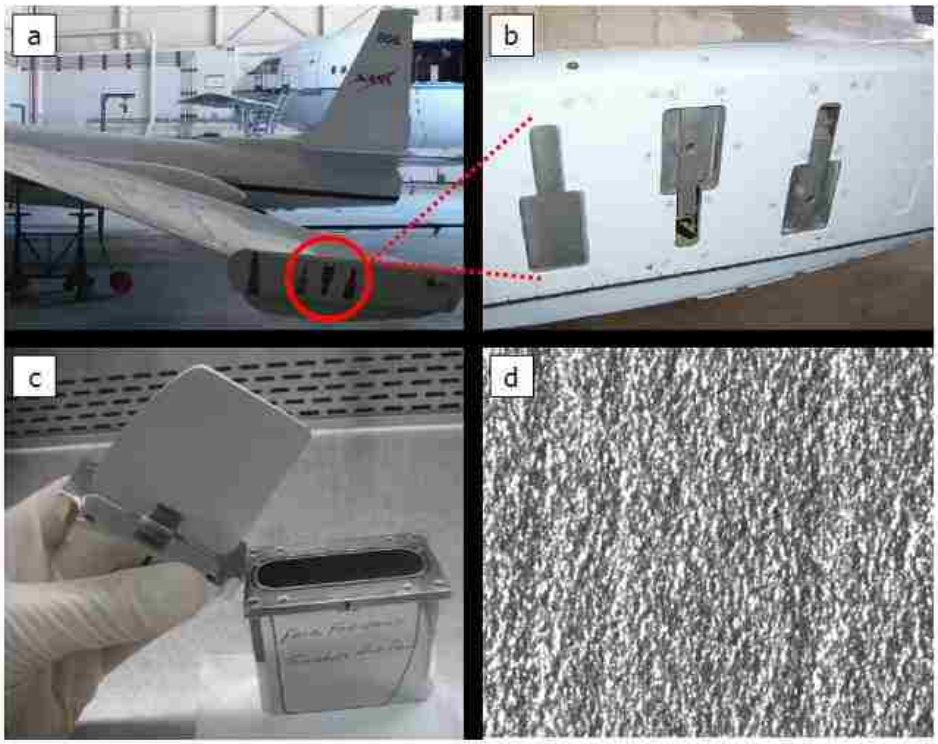

Figure 4.1 Flight hardware (a) The flight hardware system (flag in housing) was loaded into the wingtip payload bay of the Lockheed Martin ER-2 high altitude research aircraft at NASA Dryden Flight Research Center (b) Upon pilot command, the hardware was deployed from the wingtip, extending the flag into the stratosphere (c) View of the flag face if separated from housing to demonstrate individual system components (d) Microscopic view of flag face surface after sandblasting treatment designed to increase adhesion of impacting atmosphere particles.

4.3 Materials and Methods

Flight Hardware

A modified impactor plate from the NASA Cosmic Dust Group at Johnson Space Center was designed to interface with a pressure-sealed, metal housing (serial number U2-144) that mounted onto the wingtip of a high altitude ER-2 aircraft (Lockheed Martin Corp. at NASA Dryden Flight Research Center) and was deployed by pilot command (Fig. 4.1). Details of the flight hardware have been published elsewhere (Griffin, 2004; Griffin, 2008). An exact replicate of the acrylic Cosmic Dust Group impactor plate (hereafter referred to as a 'flag' for its likeness in shape), measuring roughly 5.6 x 5.8 cm, was machined out of lightweight aluminum in order to make the system compatible with autoclaving. The aluminum flag was then textured on its leading-edge side with a sandblaster to increase its surface area and create additional attachment troughs for impacting atmosphere particles. Surface topography was confirmed with a high-resolution video microscope (model VH-7000, Keyence Corp. of America, Woodcliff Lake, NJ, USA).

Microbiology Procedures, Hardware Preparation, and Sampling Flight

Difco™ R2A (18.2 g added to 1 L of deionized water) and potato dextrose agar (PDA) (39 g added to 1 L of deionized water; Difco media, Fisher Scientific, Pittsburg, PA) were used for solid growth of bacteria and fungi, respectively. A 10% dilution (3 g added to 1 L of deionized water) of tryptic soy broth (TSB) (Becton Dickinson and Company Sparks, MD) in 10 mL tubes was used for liquid growth of both bacteria and fungi.

Before flying, a series of hardware assembly/disassembly tests were conducted in the Space Life Sciences Laboratory at NASA Kennedy Space Center (FL, USA) to verify the sterility of

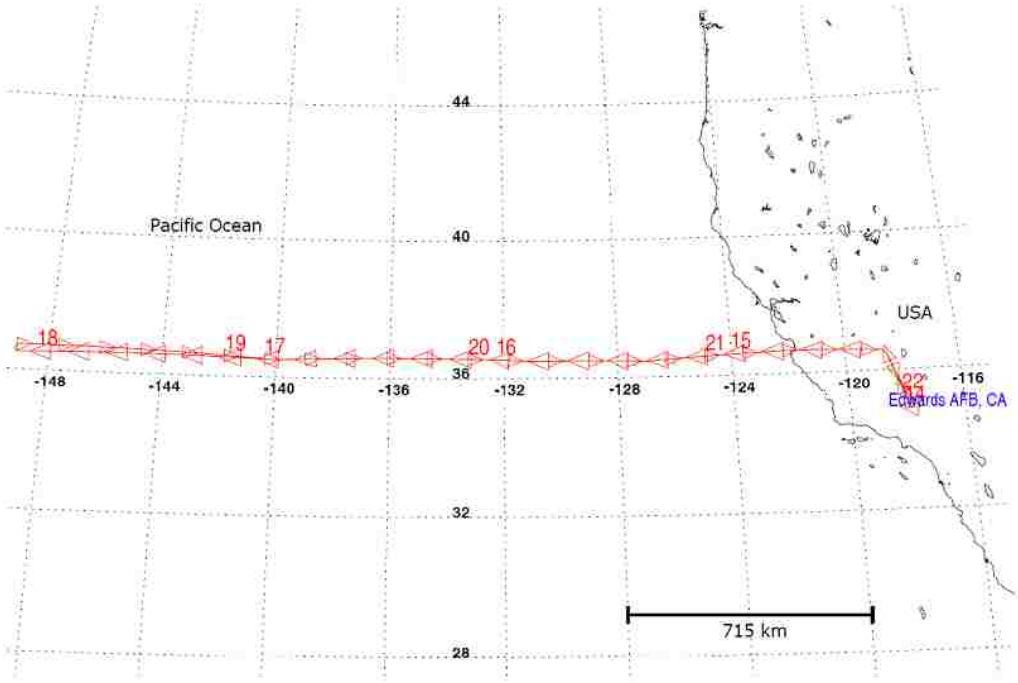

Figure 4.2 Sampling flight path. On 28 April 2008, the ER-2 aircraft (sortie # 08-6017, depicted in red) departed from Edwards Air Force Base (CA, USA) and traveled over the Pacific Ocean (36.5° N, 118-149° W) at an altitude of 20 km.

autoclaved equipment, the sealed housing, and the hardware processing environment. All procedures were conducted under aseptic conditions, using a laminar flow hood (NuAire Inc., model NU-602-400, Plymouth, MN, USA) equipped with a UV irradiation light source. Prior to assembly, the flag and the metal housing were washed with 70% isopropanol, mated, placed in a tape-sealed sterilization pouch (Tower Dual Peel® Sterilization Pouch, Baxter Healthcare Corporation, Deerfield, IL, USA) to reduce moisture, and double autoclaved at 121° C for 20 minutes along with other tools required for the procedure; notably, razor blades and Allen wrenches required for the housing screw mounts. The unit was then disassembled so that a pre-sterilized 15 µL aliquot of glycerol could be applied to the surface of the flag, using a micropipette and razor blade for uniform spreading. Once re-assembled and autoclaved, the unit was placed in the laminar flow hood for 48 hrs to simulate pre-flight transportation/idle time.

Using sterile techniques, the flag was then removed from the housing and left exposed in the laminar flow hood for 8 hrs to mimic a typical flight-sampling period. After exposure, the flag was placed face-down (see below for detail) on R2A media for a two-week incubation period in a 30° C growth chamber (Innova 4230, New Brunswick Scientific, Edison, NJ, USA), and no growth was observed.

The assembly method (described above) was repeated for the hardware intended for flight. The pre-sterilized glycerol aliquot spread onto the flag was also applied onto R2A plates for negative control testing and no growth occurred over a four-week incubation period. The hardware was shipped to NASA Dryden Flight Research Center where the complete system was mounted onto the wing payload bay of the ER-2 aircraft. On 28 April 2008, the aircraft departed

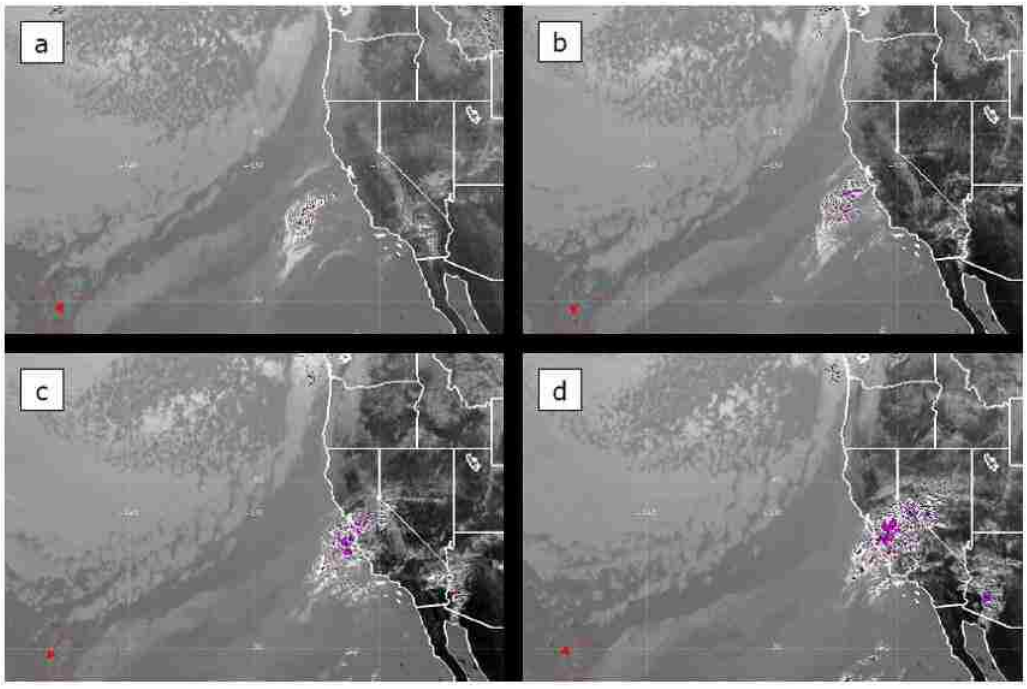

Figure 4.3 Jet stream trajectory. Satellite images of atmosphere at 20 km on 28 April 2008, taken at IR wavelength by METEOSAT-5 and provided courtesy of NOAA/NASA. The west-to-east movement of the air mass can be observed by following the water content (highlighted in purple) over time (indicated by the red arrow) (a) 1615 Zulu (b) 1815 Zulu (c) 2015 Zulu (d) 2215 Zulu.

(sortie # 08-6017) from Edwards Air Force Base (CA, USA) and deployed the flag at a sustained altitude of 20 km while maintaining an east-to-west transect (36.5° N, 118-149° W) over the Pacific Ocean (Fig. 4.2). The exposure to the stratosphere lasted for 7.5 hr (opened 1416 Zulu and closed 2154 Zulu). The Pacific Ocean jet stream along the flight path was analyzed through infrared satellite images provided by the National Oceanic and Atmospheric Administration (NOAA) (Fig. 4.3). Environmental conditions at the sampling altitude were modeled using radiosonde observations and geopotential height contour maps provided by the Naval Research Laboratory and the California Regional Weather Server (D. Westphal and D. Dempsey, personal communication, June 2008) from the nearest land-based station, KOAK (WMO identification # 72493), located at 37.73° N, 122.22° W (Fig. 4.4).

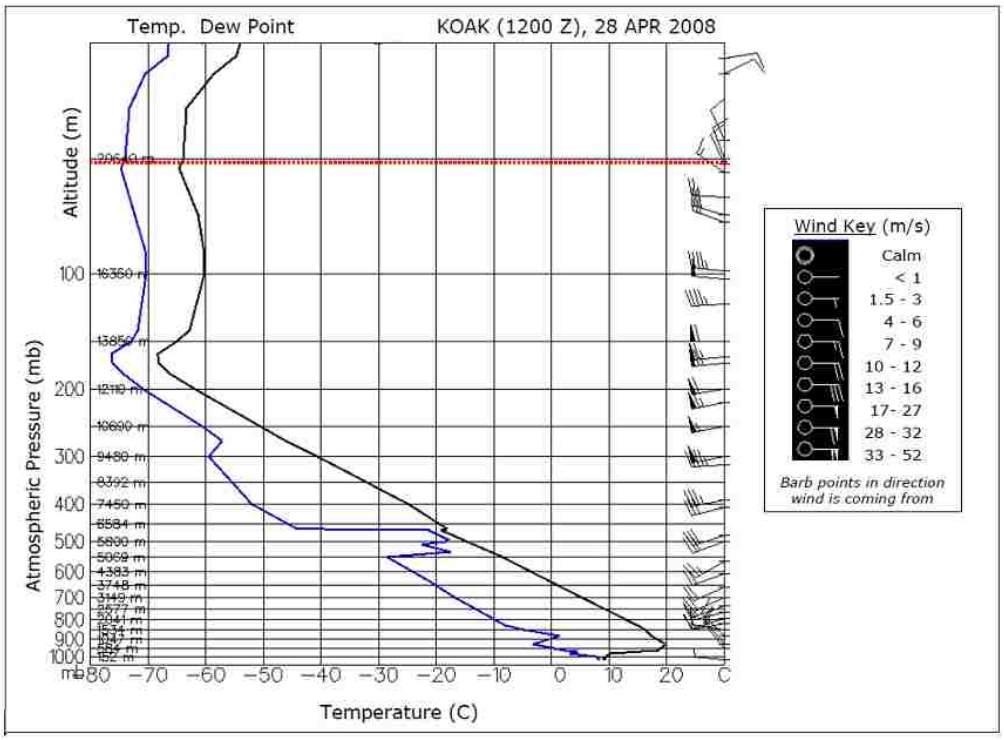

Figure 4.4 Environmental data at stratosphere sampling location. Radiosonde observations from station KOAK (37.73° N, 122.22° W) depict the temperature and dew point changes with altitude gain. At the 20 km sampling altitude (indicated in red) the atmospheric pressure was estimated at 50 mb, with temperature and dew point at -75° C and -64° C, respectively. Wind was on a northeast heading at 4-9 m/s, blowing towards the coast of North America.

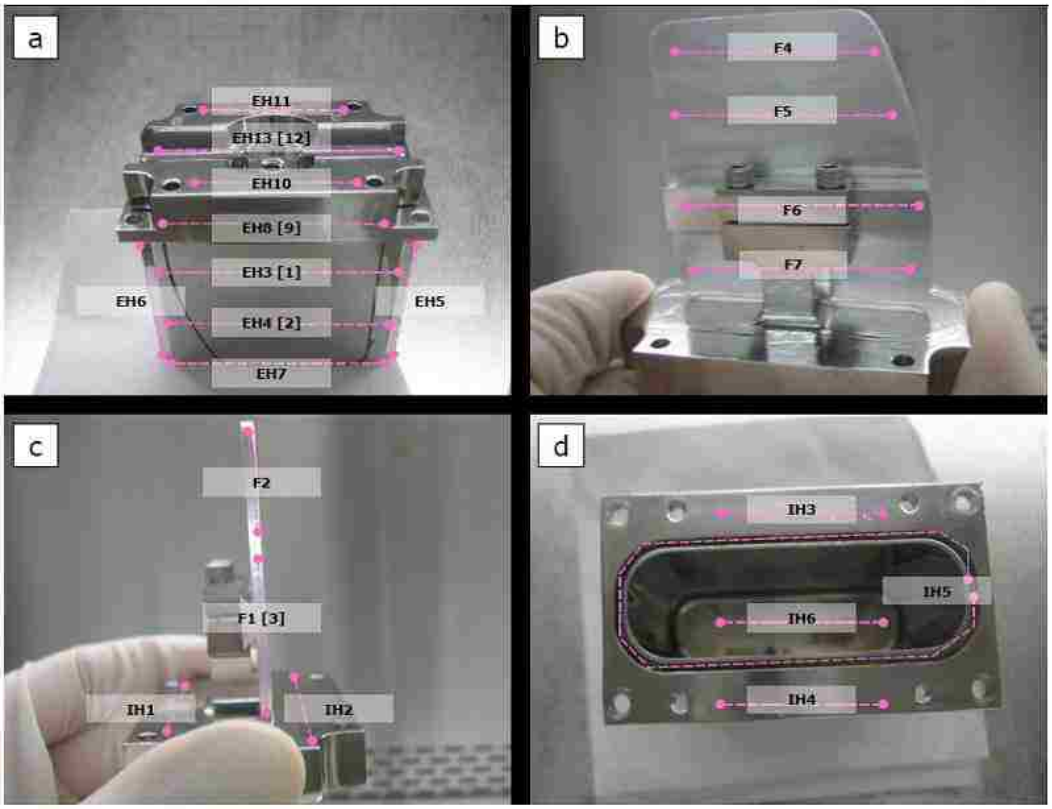

Figure 4.5 Post-flight processing sequence and hardware nomenclature (Note: numbers in brackets correspond to equivalent location on opposite side of hardware, not pictured). (a) External housing (EH) was sampled immediately after removing the flight hardware from the shipping container (b) Once the housing was opened, the leeward side of the flag (F) was processed (c) Next, the perimeter of the flag and internal housing (IH) lid were sampled and then the flag was detached from the housing and placed face-down on R2A growth medium (d) The remaining IH sites were processed to complete the processing procedure.

Post-Flight Processing

Upon receiving the flight hardware, the assembly was unpackaged in a UV disinfected (12 hr) laminar flow hood. A novel swabbing procedure was employed on the flight hardware which (1) increased the number of samples compared to historical assays (Griffin, 2004; Griffin, 2008), (2) used several growth media (liquid and solid) to encourage diverse cell recovery, and (3) would provide a useful comparison between the flag and the internal/external surfaces of the flight housing, divided into various sampling sections: F (flag), EH (external housing), and IH (internal housing) (Fig. 4.5). With the housing still closed, EH samples were taken with autoclaved 10-

cm cotton applicators that were moistened in sterile, deionized H_2O. Each swab consisted of a surface rub of the cotton tip over the application spot. The contact side of the applicator was swabbed onto R2A media, then placed into 10 mL test tubes containing liquid TSB for a second growth opportunity. Negative controls with cotton applicators were performed periodically (N = 10) and there was no growth in liquid or solid media. Swabs were taken along the flag aluminum perimeter and backside before it was placed face-down on R2A. Finally, IH locations were swabbed, plated, and also introduced into liquid media. Test tubes and plates (parafilmed to prevent drying) were stored in a dark incubator at 30° C and monitored for growth. The face-down flag was transferred onto new R2A plates after time-steps of 1, 2, 4, 7, and 56 d.

Genetic Identification

Bacterial cultures were isolated and archived at -20° C for genetic analysis. Subsets to be identified were grouped according to growth rate, medium preference, colony size, colony shape, pigmentation, and location on the sampling hardware. Deoxyribonucleic acid (DNA) extraction was performed, followed by the polymerase chain reaction (PCR) amplification of 16S ribosomal ribonucleic acid (rRNA), according to previously described protocols (Griffin, 2004). Universal primers listed in Grasby et al. (2003) were used for eight bacterial isolates, generating 1538 bp amplicons that were directly sequenced by Northwoods DNA, Inc. (Becida, MN). GenBank Blast (http://www.ncbi.nlm.nih.gov/BLAST/) was utilized for sequence/isolate identification. Furthermore, three bacteria isolates were identified with a 466 bp 16S rRNA primer set described by Nadkarni et al. (2002) due to repeated sequencing errors noted with the larger primer set from Grasby et al. (2003). Table 4.1 provides the primer sets used for each bacterial isolate.

Fungal growth in liquid or solid media was immediately transferred to PDA and archived at 4° C for identification, noting growth rate and morphology for hyphae and fruiting bodies. After trying several primer sets in PCR, 18S rRNA for all fungal isolates were successfully amplified using primers NS3 and NS4 from White et al. (1990). Amplicons were directly sequenced and identified as described above with GenBank Blast.

4.4 Results

Bacteria were recovered from 6 of 27 possible locations on the flight hardware. The positive growth was from 4 F-sites and 2 EH-sites. Signs of growth in liquid media ranged from 24-96 hr. Subsequently plated colonies were small and medium sized, non-pigmented, and had either a smooth/round or sharp/jagged appearance. Analysis by 16S rRNA for F-bacteria showed strong homology with *Bacillus* sp. at the genus level, with most species matched to *Bacillus subtilis*. The EH cultures were recovered from the lid (EH11) and base (EH7) of the housing, on solid media from the swabbed locations. The EH7 colonies appeared to share morphological features with F-isolates, but sequence analysis revealed a relationship to *Bacillus endophyticus*. More surprising was the EH11 bacterium, which took nearly 3 weeks to recover on R2A and formed small colonies with red pigmentation. However, sequencing could only match the isolate to the genus-level of *Bacillus* sp. Interestingly, at least one (and in some cases, many) of the closest GenBank neighbors were previously identified in China, Japan, Korea, or India (see Table 4.1).

Fungi were recovered from 8 of the 27 locations on the flight hardware: 4 isolates came from F-sites, while EH and IH-sites each yielded 2 isolates. When the flag was placed face down on R2A it produced 2 adjacent fungal colonies near its perimeter after 72 hrs, which were subsequently removed to allow for additional microbe recovery. However, no additional growth

Table 4. 1 Bacterial Growth

Site*	Recovery Media	Time (hr)	Colony Description	Primers	% Homology (seq. length)	GenBank Closest Neighbor	Accession Number	Other GenBank Information
F1	TSB, R2A	24	Small, white, round	Grasby et al. (2003)	100 (560/560)	*Bacillus* sp_ fj427970.1	FJ649341	Multiple *Bacillus* hits, primarily *subtilis*
	TSB, R2A	24	Small, white, jagged	Grasby et al. (2003)	99 (797/800)	*Bacillus* sp. eu168188.1	FJ649340	Matched isolate found in Chinese oil field
F3	TSB, R2A	72	Small, white, jagged	Grasby et al. (2003)	99 (559/560)	*Bacillus* sp fj427970.1	FJ649337	Multiple *Bacillus* hits, primarily *subtilis*
F6	TSB, R2A	72	Medium, white, round	Grasby et al. (2003)	99 (560/561)	*Bacillus* sp. fj427970.1	FJ649339	Multiple *Bacillus* hits, primarily *subtilis*
	TSB, R2A	72	Small, white, round	Grasby et al. (2003)	99 (797/800)	*B. subtilis* dq846632.1	FJ649342	Matched isolate found in Korea
	TSB, R2A	72	Small, white, jagged	Nadkarni et al. (2002)	99 (407/408)	*B. subtilis* eu532192.1	FJ649343	Matched Indian isolate associated w/ jute plant
F7	TSB, R2A	72	Medium, white, round	Grasby et al. (2003)	99 (518/520)	*Bacillus* sp. FJ393325.1	FJ649338	Multiple *Bacillus* hits, primarily *subtilis*
	TSB, R2A	96	Medium, white, round	Nadkarni et al. (2002) Grasby et al. (2003)	99 (420/423)	*Bacillus* sp. fj434652.1	FJ649344	Multiple *Bacillus* hits, primarily *subtilis*
	TSB, R2A	96	Small, white, jagged		99 (679/680)	*Bacillus* sp FJ393325.1	FJ649345	Multiple *Bacillus* hits, primarily *subtilis*
EH7	R2A	72	Medium, white, round	Grasby et al. (2003)	98 (588/560)	*B. endophyticus* dq485415.1	FJ649336	4 same score hits with Chinese isolates
EH11	R2A	552	Very small, red, round	Nadkarni et al. (2002)	99 (407/409)	*Bacillus* sp. ab242649.1	FJ649346	Matched Chinese and Japanese isolates

* Cells not recovered from the following sites: Flag face, F2, F4, F5, EH1, EH2, EH3, EH4, EH5, EH6, EH8, EH9, EH10, EH12, EH13, IH1, IH2, IH3, IH4, IH5, IH6

Table 4.2 Fungal Growth

Site*	Recovery Media	Time (hr)	Growth Description	Primers	% Homology (seq. length)	GenBank Closest Neighbor	Accession Number	Other GenBank Information
Flag Face	R2A, PDA	72	Blue/green fruiting bodies, white mycelium	White et al. (1990)	99 (537/539)	*Penicillium* sp. EU827607.1	FJ649347	Closest 3 hits matched Chinese and Japanese isolates
F4	TSB, R2A, PDA	96	Blue/green fruiting bodies, white mycelium	White et al. (1990)	100 (200/200)	*Eurotiomycetes* sp. FJ458446.1	FJ649350	Matched numerous *Eurotiomycetes* sp.
F5	TSB, R2A, PDA	96	Blue/green fruiting bodies, white mycelium	White et al, (1990)	100 (489/489)	*Penicillium* sp. EU827607.1	FJ649353	Closest 3 hits matched Chinese and Japanese isolates
F6	R2A, PDA	552	Blue/green fruiting bodies, white mycelium	White et al. (1990)	100 (200/200)	*Eurotiomycetes* sp. FJ458446.1	FJ649348	Matched numerous *Eurotiomycetes* sp.
EH3	TSB, R2A, PDA	96	Blue/green fruiting bodies, white mycelium	White et al. (1990)	99 (497/500)	*Penicillium* sp. EU827607.1	FJ649352	Closest 3 hits matched Chinese and Japanese isolates
EH11	TSB, R2A, PDA	120	Blue/green fruiting bodies, white mycelium	White et al. (1990)	100 (486/486)	*Penicillium* sp. EU827607.1	FJ649354	Closest 3 hits matched Chinese and Japanese isolates
IH3	TSB, R2A, PDA	96	Blue/green fruiting bodies, white mycelium	White et al. (1990)	96 (185/192)	*Penicillium* sp. EU827607.1	FJ649351	Closest 3 hits matched Chinese and Japanese isolates
IH4	TSB, R2A, PDA	120	Blue/green fruiting bodies, white mycelium	White et al. (1990)	99 (537/538)	*Penicillium* sp. EU827607.1	FJ649349	Closest 3 hits matched Chinese and Japanese isolates

* Cells not recovered from the following sites: F1, F2, F3, F7, EH1, EH2, EH4, EH5, EH6, EH7, EH8, EH9, EH10, EH12, EH13

was observed. Nor did growth occur when the flag was transferred onto fresh R2A plates at 2, 4, 7, and 56 d time-steps. Swab-based cultures recovered in liquid media showed first signs of growth between 96-120 hr, while cells on solid media generated a wider range of growth time, 72-552 hr. On both R2A and PDA media, fungi grew rapidly and were characterized by blue/green fruiting bodies and white mycelia. Sequences submitted to GenBank identified isolates F4 and F6 (located on the leeward side of the flag) to class Eurotiomycetes, using 200 bp sequence reads. The remaining 6 fungal isolates were matched to the same species of *Penicillium* sp., whose closest neighbors in GenBank originated from China or Japan (see Table 4.2). The positive growth at IH locations was close to the attachment point of the flag on the housing lid, but neither IH1 nor IH2 yielded fungi. Only one non-flag associated site, EH11, produced both bacterial and fungal cultures.

4.5 Discussion

Our study confirmed the presence of viable microbes in the stratosphere, supporting previous reports by Imshenetsky et al. (1978), Lysenko (1980), Harris et al. (2002), Wainwright et al. (2002), Narlikar et al. (2003), Griffin (2004), Shivaji et al. (2006), and Griffin (2008). However, our samples are the first documentation of microbiota recovered from a high altitude location directly over the Pacific Ocean. Infrared satellite imagery from METEOSAT-5 showed air with an eastward movement across the ocean during the sampling time. Independently, the closest neighbors of many bacterial and fungal samples submitted to GenBank were Chinese, Japanese, Korean, and Indian-derived isolates; primarily *Bacillus* sp. and *B. subtilis* for bacterial species and *Penicillium* sp. and class Eurotiomycetes for fungi. The bacterial isolates reported in this study are all spore forming species and have been collected in prior stratospheric assays, at

genus-level taxonomy (Imshenetsky et al., 1978; Wainwright et al., 2002; Narlikar et al., 2003; Griffin, 2004). Although non spore forming eubacterial species have been recovered from the stratosphere (Griffin, 2008), sporulation likely enhances resistance to high altitude stress (Riesenman & Nicholson, 2000; Nicholson, 2002).

The abundance of cells recovered in this study (12 out of 27 possible flight hardware sites) was surprising based on the previously described dilution effect on cells in the stratosphere (Griffin, 2007). Either our extended horizontal range and modifications to the surface of the flag actually improved the collection efficiency, *or* the impactor collected spores that subsequently germinated and propagated inside the housing prior to recovery back at the laboratory. The spatial correlation of positive growth areas on the hardware would suggest the possibility of post-flight growth. While the recovery of bacteria and fungi was most abundant on flag-associated regions, the presence of *B. endophyticus*, *Bacillus* sp. and *Penicillium* sp. on the external housing must also be considered. This area was exposed to the atmosphere when attached to the aircraft wing, possibly allowing low altitude contamination of the surface during (1) hardware installation/removal or (2) while the aircraft was on the ground or en route to 20 km altitude. It is interesting that the major bacterial outliers reported in this study – the sole representative of *B. endophyticus* and the only red-pigmented species of *Bacillus* sp. – both came from EH locations. Therefore, the highest confidence of stratosphere-sampled isolates should be reserved strictly for microbes recovered from the flag and IH locations (*Bacillus* sp., *B. subtilis*, and *Penicillium* sp.) since these areas were pressure-sealed inside the housing during the brief contamination windows discussed above. Nevertheless, ground contamination cannot explain the strong DNA sequence homology of microbes recovered in this study with previously described Asian isolates located on the other side of the Pacific Ocean. Moreover, if ground contamination was a factor

one might expect an even greater diversity of species recovered, including human associated microbes.

While this study has provided evidence for a long-distance, stratospheric transport of microbes across the open ocean, it is important to emphasize that it does not indicate an independent, airborne microbial ecosystem. Rather, the viable bacteria and fungi reported herein were most likely associated with temporary, trans-Pacific dust events before particles fall out of the atmosphere. While Narlikar et al. (2003), Shivaji et al. (2006) and Wainwright et al. (2006) use the discovery of non-culturable particulates and novel species as evidence of a distinct stratosphere ecology, no mechanism has been presented to explain how microbes could remain aloft, growing or replicating. The possibility of a permanent, upper-atmosphere ecosystem seems unlikely based on several lines of evidence. First, the very low temperature of the sampling environment (-75° C) in this study was far below -20° C, the minimum temperature for microbial growth reported to date (Price & Sowers, 2004; Junge et al., 2006). Although there is a temperature inversion with increasing altitude in the stratosphere (Ramaswamy et al., 2001), the extra warmth is gained due to absorption of solar radiation, including potentially biocidal UV wavelengths. Second, without a means of controlling altitude, airborne species should eventually (< 5 yr) fall out of the atmosphere, if aerosol extinction rates from the volcanic eruption of Mt. Pinatubo are used as a proxy (Bauman et al., 2003). Upper and lower atmosphere layers can mix according to Deshler et al. (1993), therefore how could microbes reported by Narlikar et al. (2003), Shivaji et al. (2006) and Wainwright et al. (2006) remain completely independent of terrestrial samples? The simplest way of explaining the unique stratospheric assemblages described by Narlikar et al. (2003), Shivaji et al. (2006) and Wainwright et al. (2006) is that these populations exist in terrestrial or aquatic ecosystems but

have not yet been identified. However, in this study and others similar to it (Imshenetsky et al., 1978; Lysenko, 1980; Harris et al., 2002; Narlikar et al., 2003; Griffin, 2004; Griffin, 2008) there was a strong genetic relationship between microorganisms recovered at high altitudes with those common in terrestrial or aquatic environments.

Significant improvements to the efficiency and accessibility of upper atmosphere sampling will be necessary to advance the study of high altitude aerobiology. Hard surface impactors deployed by aircraft have demonstrated utility, but there is clearly a limit to their use. Assuming that a more robust collecting device can be engineered, special emphasis should be placed on culture-independent processing (Griffin et al., 2001; Griffin, 2007) and measuring UV-induced DNA mutations (Wainwright et al., 2006). The ability to study high altitudes (up to 33 km) with sophisticated, long-duration science payloads may be available soon through ultra-long duration (up to 100 d) balloons developed by the United States Antarctic Program and NASA (Smith, 2004; Gregory & Stepp, 2004). Measuring cells in-situ (Pratt et al., 2009) for weeks, or possibly longer, could resolve the residence time question addressed throughout this study. Ultimately, such information would strengthen our notion of the biosphere – the broad range of microorganisms within it and how the upper atmosphere may have influenced the diversity and distribution of life on Earth.

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Chapter 5

MICROBIAL SURVIVAL IN THE STRATOSPHERE AND IMPLICATIONS FOR GLOBAL DISPERSAL

In Chapter 5, isolates from Chapter 4 were exposed to a series of stratosphere simulations using a hypobaric environmental chamber. Viability of the microbes was evaluated and physical factors limiting survival were identified. We discuss population bottleneck effects and ecological implications. Portions of this chapter were originally published in collaboration with Dale W. Griffin, Richard D. McPeters, Peter D. Ward, and Andrew C. Schuerger in the March 2011 edition of *Aerobiologia* (Smith et al., 2011, *Aerobiologia*, 27:319-332) (©2011 *Springer Science+Business Media, LLC*), and are reproduced below with permission of Springer Science+Business Media, LLC.

5.1 Abstract

Spores of *Bacillus subtilis* were exposed to a series of stratosphere simulations. In total, five distinct treatments measured the effect of reduced pressure, low temperature, high desiccation, and intense ultraviolet (UV) irradiation on stratosphere-isolated and ground-isolated *B. subtilis* strains. Environmental conditions were based on springtime data from a mid-latitude region of the lower stratosphere (20 km). Experimentally, each treatment consisted of the following independent or combined conditions: -70 °C, 56 mb, 10-12% relative humidity and 0.00421, 5.11, and 54.64 W/m^2 of UVC (200-280 nm), UVB (280-315 nm), UVA (315-400 nm), respectively. Bacteria were deposited on metal coupon surfaces in monolayers of ~1 x 10^6 spores and prepared with palagonite (particle size < 20 µm). After 6 hrs of exposure to the

stratosphere environment, 99.9% of *B. subtilis* spores were killed due to UV irradiation. In contrast, temperature, desiccation, and pressure simulations without UV had no effect on spore viability up through 96 hours. There were no differences in survival between the stratosphere-isolated versus ground-isolated *B. subtilis* strains. Inactivation of most bacteria in our simulation indicates that the stratosphere can be a critical barrier to long-distance microbial dispersal and that survival in the upper atmosphere may be constrained by UV irradiation.

5.2 Introduction

Reports of viable bacteria and fungi in the stratosphere have become increasingly common (Imshenetsky et al., 1978; Lysenko, 1980; Harris et al., 2002; Wainwright et al., 2002; Narlikar et al., 2003; Griffin 2004; Shivaji et al,. 2006; Griffin, 2008; Yang et al., 2008a, 2008b; Shivaji et al., 2009; Smith et al., 2010). Microbes are extremely abundant in soil and some have adaptations for aerial dispersal (Papke & Ward, 2004; Kellogg & Griffin, 2006). While limited mixing at the tropopause boundary restricts most airborne microbes to lower altitudes, a wide range of mechanisms can transport aerosols (or biological cells) from the troposphere to the stratosphere, including: volcanic eruptions (Jacob, 1999); Brewer-Dobson atmospheric circulation (Wallace & Hobbs, 2006); dust storms (Kellogg & Griffin, 2006); monsoons (Randel et al., 2010); electrostatic forces created by thunderstorms (Dehel et al., 2008), and rocket launches (Griffin, 2004). It is unknown how long microbes can remain aloft in the stratosphere, but aerosol studies have shown it takes months and sometimes years for particulates to eventually return to the surface (Jacob, 1999; Wallace & Hobbs, 2006). Most stratospheric aerobiology studies have focused only on the characterization of microbes (i.e., identifying species and place of origin), leaving other key ecological questions unaddressed. For example,

how long can cells remain viable in the stratosphere? What atmospheric and biological factors ultimately control the survival of cells? Answers to these questions may provide a critical framework for understanding patterns of microbial biogeography and the evolutionary consequences of long-distance dispersal.

To address survival, one must consider the absence of nutrients, reduced atmospheric pressure, extreme desiccation, low temperatures, toxic chemical species, and intense solar radiation in the stratosphere. Atmospheric pressure is inversely related to altitude; meaning at the tropopause (~18 km) atmospheric pressure can be close to 100 mb, whereas in the stratopause (~50 km) the value is almost two orders of magnitude smaller (Dessler, 2000; Wallace & Hobbs, 2006). Although relative humidity (RH) levels are difficult to determine, dryness helps define the stratospheric boundary layer with the troposphere. One study across North America and Europe reported average RH levels in the stratosphere at ~ 23% (Gierens et al., 1999). Meanwhile, daytime temperatures can be as low as -70 °C in the tropopause and as high as 0 °C in the region of maximum stratospheric ozone (O_3) where absorption of short-wavelength ultraviolet (UV) light generates heat (Brasseur & Solomon, 1986; Jacob, 1999; Dessler, 2000; Wallace & Hobbs, 2006). Understanding UV irradiation is therefore critical to characterizing the stratospheric environment with regard to microbial life. However, generalizing about UV is challenging because conditions vary according to the ozone column (i.e., altitude); latitude; weather; solar zenith angle; and scattering from molecules, aerosols or clouds (Brasseur & Solomon, 1986; Daumont et al., 1992; Malicet et al., 1995; Blumthaler et al., 1997; Dessler, 2000; Schmucki & Philipona, 2002; Kondratyev et al., 2006; Wallace & Hobbs, 2006).

Incredibly, microbes that can form endospores (hereafter referred to as 'spores') appear to survive the harsh environmental conditions described above (Imshenetsky et al., 1978; Harris et al., 2002; Wainwright et al., 2002; Narlikar et al., 2003; Griffin, 2004; Yang et al., 2008a, 2008b; Shivaji et al., 2006, 2009; Smith et al., 2010). Sporulation offers protection against factors that would otherwise threaten vegetative cells (Nicholson et al., 2002). Spores are a dormant stage in the life cycle of a microbe, but viable cells can germinate and resume activity upon contact with liquid water and nutrients at favorable temperatures (Nicholson et al., 2000; Nicholson et al., 2002). The bacterium *Bacillus subtilis* was chosen for this study because the responses of its spore to extreme environments have been well documented (Horneck et al., 1994; Nicholson & Fajardo-Cavazos, 1997; Nicholson et al., 2000; Riesenman & Nicholson, 2000; Slieman & Nicholson, 2000; Dose et al., 2001; Setlow, 2001; Slieman & Nicholson, 2001; Nicholson et al., 2002; Setlow, 2007). In addition, it is a common soil microorganism that has been found previously in the stratosphere (Smith et al., 2010). Nicholson et al. (2000) have reviewed the main physical and molecular defenses that *B. subtilis* uses to protect and repair biomolecules (e.g., deoxyribonucleic acid (DNA)) during dormancy or germination. In brief, spore cores are dehydrated and surrounded by a highly impermeable coat, reducing desiccation and irradiation stress (Setlow, 1995; Riesenman & Nicholson, 2000; Nicholson et al., 2002; Ghosal et al., 2005). Genetic resistance to extreme factors has been associated with several features, including the ability to stabilize DNA strands with small acid-soluble proteins (SASP) (Nicholson et al., 2000), active repairs to damaged macromolecules (Setlow, 1995; Xue & Nicholson, 1996), and the production of photoprotective pigments (Imshenetsky et al., 1979; Nicholson et al., 2002).

A common conclusion in upper atmosphere aerobiology (Imshenetsky et al., 1977; Yang et al., 2008a, 2008b; Shivaji et al., 2006, 2009) is that airborne microbes have higher resistance to

environmental extremes than similar ground strains because the stratosphere has selected for hardier cells (i.e., harsh conditions have preferentially killed off weaker cells). Although intuitive, the claim has not been adequately supported by experimental studies. The first problem with previous microbial resistance studies relates to the simulated UV environment. Imsenetsky et al. (1977) and Yang et al. (2008a, 2008b) used monochromatic 254-nm UV lamps positioned at an arbitrary distance from microbes to evaluate resistance. However, UVC (200-280 nm) sources are a poor approximation of sunlight in natural environments (Xue & Nicholson, 1996; Nicholson et al., 2000; Nicholson et al., 2002) and by focusing only on the 254-nm band those studies failed to address the effect of UVB (280-315 nm) and UVA (315-400 nm) wavelengths which can also contribute significantly to cell death (Xue & Nicholson, 1996; Slieman & Nicholson, 2001). Second, many previous resistance studies have been conducted at ground-normal conditions instead of incorporating stratospheric pressure, desiccation, and temperature. Alone, these factors might have little effect on spore viability (Horneck, 1993; Dose & Klein, 1996; Miyamoto-Shinohara et al., 2006; Osman et al., 2008), but when coupled with UV irradiation the synergistic stress has been shown to amplify the degradation of biomolecules (Nicholson et al., 2000; Saffary et al., 2002; Diaz & Schulze-Makuch, 2006). Only Imshenetsky et al. (1977) evaluated survival in stratospheric temperature and pressure regimes, but did so independently of UV irradiation. Third, Yang et al. (2008a, 2008b) and Shivaji et al. (2006, 2009) relied mainly upon genus-level comparisons of microbial resistance, introducing the possibility of significant genetic variation between samples. Finally, the physical distribution of bacterial spores during previous studies might have generated misleading results. To facilitate dispersal, spores tend to clump onto substrates – and oftentimes other spores. Yang et al. (2008a) acknowledged limited success in dispersing spores evenly during UV experiments.

Neither Imshenetsky et al. (1977) nor Shivaji et al. (2006, 2009) controlled for spore layering, probably exposing dense microbial suspensions to UV. The protective effect of dead and aggregated cells could have provided a significant barrier to irradiation (Horneck et al., 1994; Xue & Nicholson, 1996; Nicholson & Law, 1999; Schuerger et al., 2003; Wainwright et al., 2006).

Our approach was to revisit the question of stratospheric microbial resistance with a more robust environmental simulation. We measured the viability of *B. subtilis* in experiments that simulated exposure to a subtropical altitude of 20 km; i.e., below most of the ozone layer. Simulations consisted of one or multiple stratosphere conditions (UV, temperature, pressure and desiccation) in order to determine how each factor would affect cell survival. Our objectives were fourfold: (1) measure an overall viability fraction to understand the likelihood of long-term microbial survival in the stratosphere; (2) identify critical upper atmosphere environmental factors that limited survival; (3) compare the difference in viability between stratosphere and ground isolates of *B. subtilis*; and (4) evaluate how the presence/absence of dust analog particles affected survival. Collectively, our results addressed the hypothesis that the stratosphere is a barrier to the survival of airborne microbiota during global dispersal events. We predicted that: (1) most spores would be killed during simulations; (2) low pressure and desiccation might contribute to the inactivation of cells, but UV irradiation would be the dominant biocidal factor; (3) greater resistance would be measured in stratosphere isolated *B. subtilis* since it had demonstrated survivability in situ; and (4) dust particles would shield microbes from harmful UV irradiation during simulations.

5.3 Materials and Methods

Microbiological Procedures

Isolate "NASA8" from Smith et al. (2010) was a *Bacillus subtilis* strain (GenBank Accession #FJ649342) collected in the stratosphere at 20 km over the Pacific Ocean. The second bacterium used in this study was the ground isolate "WN696", a *B. subtilis* strain (GenBank Accession #AY260858) obtained from W. Nicholson (University of Florida, USA); originally collected from desert basal outcrop on Sentinel Hill, Arizona, by Benardini et al. (2003). On Bacto™ Tryptic Soy Agar (Becton Dickinson and Co., Sparks, MD, USA), both NASA8 and WN696 displayed growth after 24 hrs as small, round, white colonies. In liquid Bacto™ Tryptic Soy Broth (Becton Dickinson and Co., Sparks, MD, USA), growth (12-72 hr) was distinguishable by culture turbidity; WN696 was denser than NASA8 over the same incubation period and conditions.

Since the experimental equipment was not designed for free-floating spores, aluminum coupons (2 x 1 x 0.1 cm) previously coated with a chromate conversion film (Iridite 14-2, MacDermid, Inc., Waterbury, CT, USA) were used as microbial substrates (see Schuerger et al., 2003; 2005; 2008). Prior to experimentation, spores of NASA8 and WN696 were generated according to the protocol of Mancinelli & Klovstad (2000) and Schuerger et al. (2003). From this stock, spores were diluted in sterile deionized water (SDIW) and quantified using a Spectronic-20 Genesys™ spectrometer (model 4001, Sigma-Aldrich, St. Louis, MO, USA) at 600 nm to achieve optical densities of ~0.32. To create uniform monolayers of spores on the aluminum coupons, quantified suspensions were spotted onto the surfaces in 100 µl aliquots per coupon and left covered for 12 hrs at standard room temperature (25 °C) and pressure (1013 mb) (STP). The number of bacteria applied onto coupons in 100 µl aliquots was measured at ~$1x10^6$ cells. Before experimental use, doped coupons were uncovered and allowed to dry for 2-4 hrs in a dark

laminar flow hood (NuAIRE Biological Safety Cabinet, Class II Type A/B3, Model NU-602-400, Plymouth, MN, USA). All coupons had been dry-heat sterilized overnight at 130 °C and cooled to 24 °C before bacteria spores were deposited as monolayers. The coupon preparation procedure was standardized and used for all experiments.

To determine the effect of atmospheric dust particles on the survivability of *B. subtilis* spores, half of the NASA8 and WN696 coupons were mixed with palagonite collected by A. Schuerger (University of Florida, USA) from the Cerros del Rio volcanic field, New Mexico, USA. Elemental composition of the dust analog was measured by energy-dispersive x-ray spectrometry (EDS) (NORAN Systems SIX, Thermo Electron Corp., Waltham, MA, USA). To prepare the fine-grained particles representative of stratospheric dust (Carder et al., 1986; Betzer et al., 1988), granular palagonite was ball-milled for 24 hrs. Five hundred grams of the crushed palagonite was then passed through a series of dry-sieves (200, 75, 45, 32, and 20 µm) using a shaker apparatus (Retsch AS 200, Newton, PA, USA). The sieved dust product was examined with a scanning electron microscope (SEM) (JSM-7500F, JEOL Ltd., Tokyo, Japan) to assess particle sizes and sterilized at 130 °C for 24 hrs prior to use. To create the aliquot for certain NASA8 and WN696 dust-prepared coupons, 0.10 g of dust was added to 10 L SDIW where it was dispersed with agitation. Then 1 ml of that dust solution was combined with 9 ml SDIW and 10 µl spore stock.

UV Parameters

The UV model was developed to correspond to the sampling site of NASA8 by Smith et al. (2010); i.e., 20 km altitude at a mid-latitude location (36 °N) in boreal springtime. Calculated values were only for direct UV flux at a fixed solar zenith angle of 30°. Bulk solar fluence

values were based on measurements from Nimbus-7 Solar Backscatter UV (SBUV) instrument that took solar spectra during an ascent through the stratosphere in January 1979 (McPeters et al., 1984; McPeters et al., 1993). In order to account for UV attenuation at the simulated altitude, ozone cross sections from McPeters et al. (2007) were averaged and converted to absorption coefficient factors, then applied toward a modified Beers law equation:

$$l_{(p)} = F_0^{\,-s([\alpha * X] + [\beta * P])}$$

where $l_{(p)}$ represented the UV flux, F_0 was the extraterrestrial solar flux, s was the relative optical path (solar zenith angle), α was the ozone absorption coefficient (Daumont et al., 1992; Malicet et al., 1995), X was the cumulative ozone above pressure (P), and β was the Rayleigh scattering coefficient (Bates, 1984). Fluence rates were calculated and integrated to provide a total, instantaneous flux rate (in W/m^2) for UVA, UVB, and UVC. These modeled values of 82.35, 4.16, and 0.0055 W/m^2 for UVA, UVB, and UVC, respectively, became the target fluence rates for environmental simulations (see Table 5.1). Experimentally, bacteria-doped coupons were exposed continuously to UV, meaning that a 4-day exposure more closely resembled an 8-day exposure in nature, based on cumulative dosage principles.

Two separate UV irradiation systems (described by Schuerger et al., 2003; 2006; 2008) were calibrated to represent the stratospheric illumination at 20 km. One system was located on a lab bench and the second was attached to the hypobaric environmental chamber. Each system consisted of a xenon-arc lamp (model 6269, Oriel Instruments, Stratford, CA, USA) at a power setting of 1100 W. Light was passed through a 6-cm water filter to remove intense near-infrared (NIR) photons above 1100 nm (see Schuerger et al., 2003), and then focused on surfaces where bacterial coupons were exposed. The focus lenses in both systems were located 36 cm above the coupon surfaces. In order to match the target UV fluence rates, the light beams were passed

Table 5.1 UV Fluence Rates for Experiments

Spectral Ranges (nm)	**Modeled UV Baseline: *Target* Values (W/m^2)**	**UV Control (+UV): *Actual* Values (W/m^2)**	**Stratosphere (+UV): *Actual* Values (W/m^2)**
UVA (315-400)	82.35	45.83	54.64
UVB (280-315)	4.16	4.16	5.11
UVC (200-280)	0.00550	0.00379	0.00421
Total UV	86.52	49.99	59.75

Table 5.2 Description of Experimental Groups in Stratosphere Simulation

Name	**Location**	**T (°C)**	**P (mb)**	**RH (%)**	**UV (W/m^2)**	**Purpose**
Stratosphere (+UV)	Chamber	-70 (+/- 3)	56	10-12	UVA: 54.64 UVB: 5.11 UVC: 0.00421	Full stratosphere simulation with all environmental variables applied
Stratosphere (-UV)	Chamber	-70 (+/- 3)	56	10-12	Absent	No UV in order to measure effect of stratospheric T, P, and RH
UV Control (+UV)	Benchtop	24	1013	45	UVA: 45.83 UVB: 4.16 UVC: 0.00379	Standard T and P experiment to measure effect of stratospheric UV, alone
Temperature Control (-UV)	Freezer	-70	1013	10-20	Absent	Standard P experiment with no UV to measure effect of stratospheric T and RH
Ground Control (-UV)	Benchtop	24	1013	45	Absent	Control experiment with no UV, standard T, P, and RH

through two opaque materials [a plastic petri dish lid (cat. no. 08-757-11A, Fisher Scientific, Pittsburgh, PA, USA) and a glass petri dish lid (glass-type 7740, Pyrex®Vista™, Corning, Lowell, MA, USA)] chosen to differentially attenuate UVC while allowing a large flux of UVB and UVA to pass. The conditioned UV irradiation was measured and calibrated with two different spectrometers. High-resolution spectral scans were measured with a model OL754 UV spectrometer from Optronic Laboratories, Inc. (Orlando, FL, USA). A handheld UV measuring system for UVB and UVA fluence rates (model IL1400A, International Light, Newburyport, WA, USA) was cross-calibrated with the OL754 unit and used for routine calibration during individual experiments.

Stratosphere Simulation

Simultaneous experiments were conducted to understand the independent contribution of various stratospheric conditions (high UV irradiation, low temperature, extreme desiccation, and low pressure) on the viability of *B. subtilis* spores. Time-steps of 6, 12, 24, 48, and 96 hrs were used. Each experiment had coupons prepared with strain NASA8 or WN696. Coupons were further divided into two separate sets hereafter referred to as "dust" and "dust-free" treatments. In total, there were five distinct experimental groups (see Table 5.2), each containing triplicate coupon samples: "Stratosphere (+UV)", "Stratosphere (-UV)", "UV Control (+UV)", "Temperature Control (-UV)", and "Ground Control (-UV)". The Stratosphere (+UV) coupons were inside the hypobaric environmental chamber and exposed directly to UV irradiation; the Stratosphere (-UV) coupons were also inside the hypobaric chamber but completely shielded from UV light; the

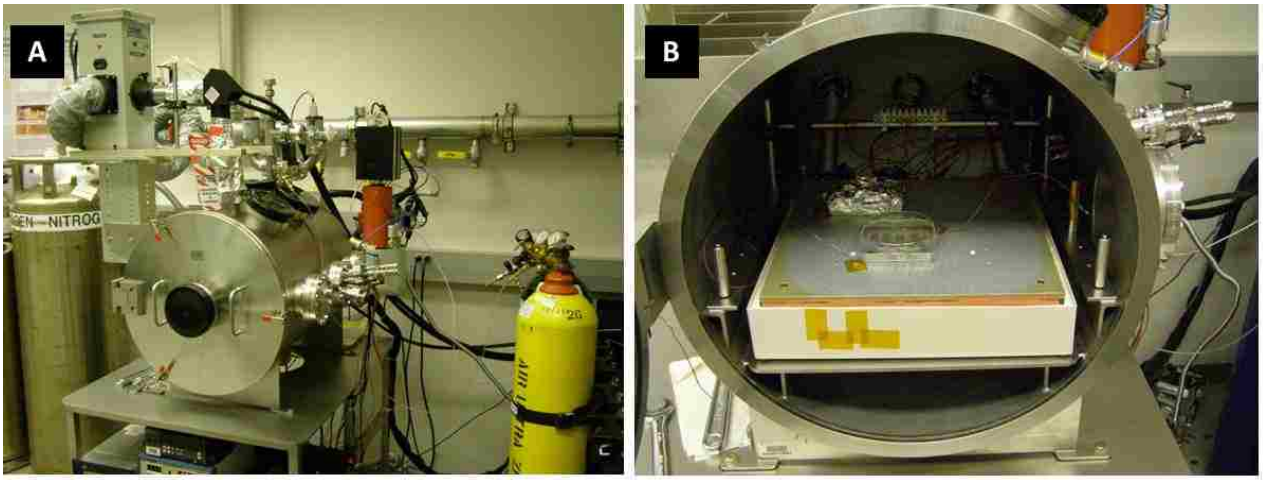

Figure 5.1 Hypobaric environmental chamber used for stratosphere simulation experiments. (A) Temperature, UV light, and gas composition were regulated by liquid-nitrogen (LN_2), xenon-arc lamp source, and a mass control unit, respectively (pictured from left to right). (B) Chamber door ajar, revealing Stratosphere (+UV) and Stratosphere (-UV) treatments on LN_2 cold plate. Stratosphere (+UV) coupons are located in foreground underneath materials that attenuated UV light close to target fluence rates. Stratosphere (-UV) coupons are in background, completely shielded from UV by aluminum foil. Wiring for thermocouples and RH sensors can be seen.

UV Control (+UV) coupons were irradiated like Stratosphere (+UV) coupons, but remained on the lab bench at STP conditions (1013 mb, 24 °C, and 45% RH); the Temperature Control (-UV) coupons were placed in a -70 °C freezer in the laboratory (RH levels were 10-20%); and the Ground Control (-UV) coupons were located on the lab bench, wrapped with aluminum foil and kept at STP conditions. Each experiment was repeated under identical conditions to provide a total of six replicates for both NASA8 and WN696 treatments.

A hypobaric chamber described by Schuerger et al. (2008) (Fig. 5.1) was used to create conditions of -70 °C (+/- 3 °C), 10-12% relative humidity (RH), atmospheric pressure of 56 mb, and a gas composition of N_2 (78.08%), O_2 (20.95%), Ar (0.93%), and CO_2 (0.038%) (Boggs Gases, Titusville, FL). Bacterial coupons were loaded into the hypobaric chamber in plastic petri

dishes that sat directly on the upper surface of a liquid nitrogen (LN_2) thermal control plate that was used to regulated the temperature of the samples. Instantaneous UV fluence rates for the coupon surface were averaged prior to experimental runs. Stratosphere (+UV) coupons were not exposed to UV light until the hypobaric chamber had equilibrated at the desired set points for temperature and pressure (typically 15-20 min after closure and pump-down). Bacteria-doped coupons were placed randomly within the UV light beam.

Immediately following the environmental simulations, the numbers of viable spores per coupon were enumerated using the Most Probable Number (MPN) method described by Mancinelli & Klovstad (2000) and Schuerger et al. (2003; 2006). In brief, bacterial spores were re-suspended in sterile plastic tubes with 10 ml SDIW and vortexed with 1 g of heat-sterilized (24 hrs at 130 °C) silica sand for 2 min. Next, 1 ml of vortexed spore suspension was processed through 10-fold serial dilutions with SDIW. Sixteen separate 20-µl volumes from the six dilution steps (10^{-2} through 10^{-7}) were added to 180 µl of TSB and arranged in a 96-well microtitre plate. The 96-well microtitre plates were incubated at 30 °C for 36 hrs in an InnovaC230 incubator (New Brunswick Scientific, Edison, NJ, USA), and individual wells were visually scored for either positive or negative growth, providing an estimated number of viable cells (see Mancinelli & Klovstad, 2000). The minimum detection limit of the assay was 90 spores per coupon.

Statistical Analysis

The MPN data were log-transformed and analyzed with the statistical program R version 2.3.1 (The R Foundation for Statistical Computing, Vienna, Austria, 2006) at a 95% confidence level.

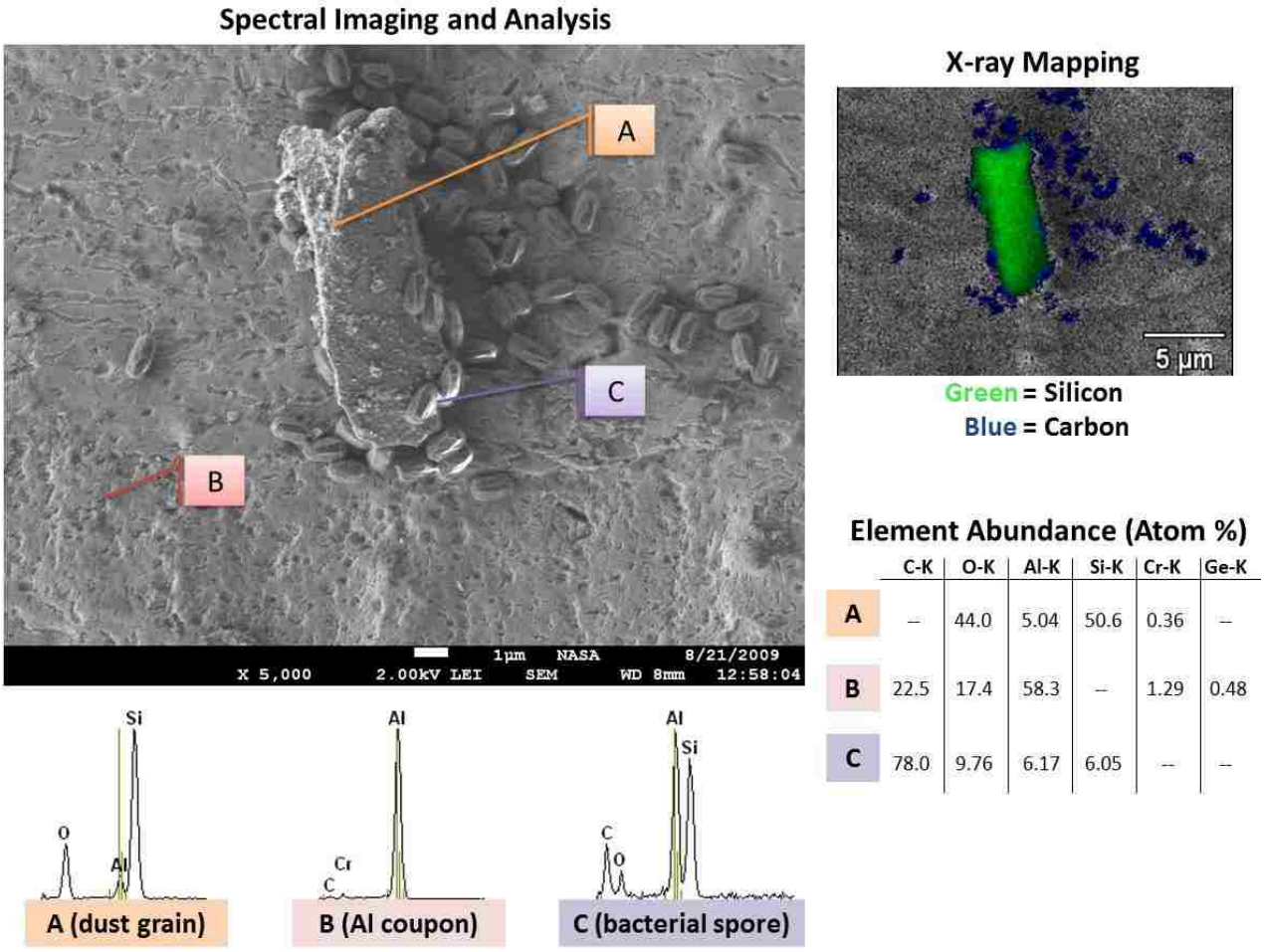

Figure 5.2 Energy-dispersive x-ray spectrometry analysis performed on bacterial coupons. Spectra and elemental abundance of dust (point A), coupons surfaces (point B) and spores (point C) were measured. X-ray mapping highlights dominance of silicon and carbon in palagonite dust analog and bacterial spores, respectively.

Data were subjected to one-way permutations (Kruskal-Wallis rank-sum test) to compare differences in means across time among individual treatment groups. Wilcoxon rank-sum permutation tests (with a continuity correction) were used to measure differences across species- and dust-specific treatment groups.

5.4 Results

Energy-dispersive x-ray spectrometry and scanning electron microscopy (SEM) (Figs. 5.2 and 5.3) were used to characterize bacterial coupons. Spectral analysis of the palagonite dust yielded an elemental composition 50.6% silicon, 44.0% oxygen, 5.04% aluminum, and 0.36% chromium (see point A; Fig. 5.2). Baseline spectra were taken on the Iridite-treated aluminum coupon (point B) and bacterial spores (point C), calculating a relative abundance of aluminum (58.3%) and carbon (78.0%), respectively. X-ray mapping showed the distribution of spores on dust coupons by highlighting the silicon in dust and carbon in spores as two distinct colors. Scanning electron microscopy confirmed that the bacterial spores were dehydrated (Fig. 5.3A) from the coupon preparation method and generally measured 1 μm lengthwise. Dust particles (Fig. 5.3B) on palagonite-treated samples were largely diluted on the coupon surface and stood out as prominent features compared to bacterial spores. Particle shape and size varied, but no dust grain was found to be larger than 20 μm in diameter. Spores attached readily to the surface of dust grains but did not appear fully embedded within the microstructures. Still, SEM images suggest some spores could have been shaded from UV light on the underside of the dust grain. Clumping of spores was minimal and seemed to be more prevalent on the dust-treated coupons than on the dust-free coupons. Where clumping did occur, it was observed to be horizontal (i.e., no vertical layering of cells).

The above sections explained how the target UV fluence rates for environmental simulations were calculated from a modified Beers law equation with data input from Nimbus-7 (SBUV) (McPeters et al., 1984; McPeters et al., 1993) and the 20 km sampling site of Smith et al. (2010). That *modeled* UV baseline is compared to *actual* UV fluence rates in Fig. 5.4. Data for the latter were taken with the high-resolution OL754 UV spectrometer at 1-nm intervals across four random locations within the beam area. The UV Control (+UV) experiment values were

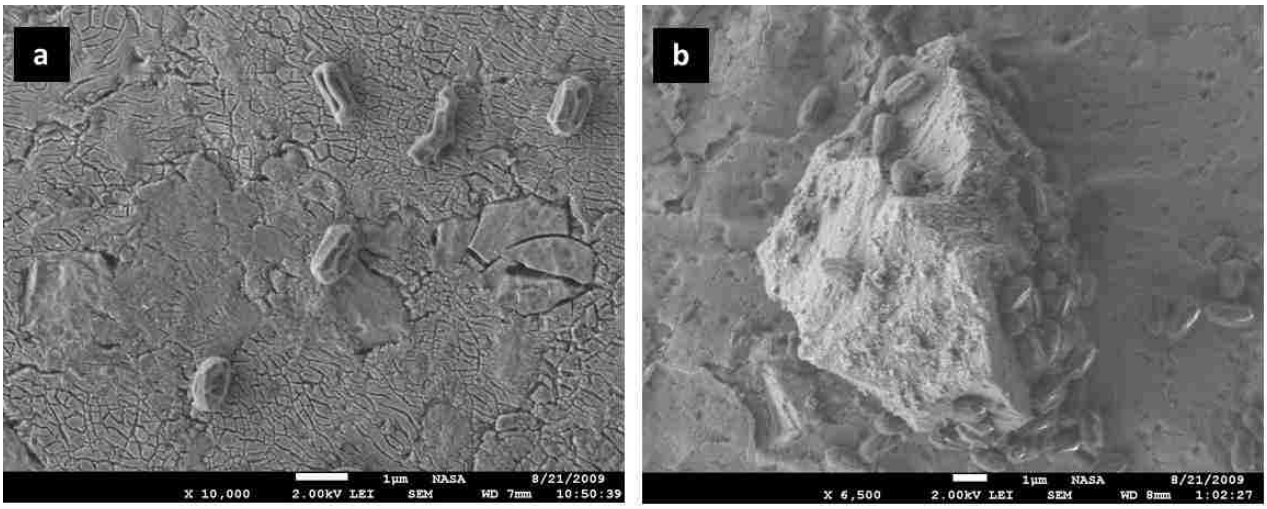

Figure 5.3 Scanning electron microscopy. Monolayers of dehydrated *Bacillus subtilis* spores (ovoid shaped, 1 µm) are visible on coupons used for experiments. Number of spores per coupon was estimated at 1 x 10^6. (A) Typical distribution of spores on dust-free coupon. (B) Example of dust treated coupon where spores can be seen attached to a prominent particle.

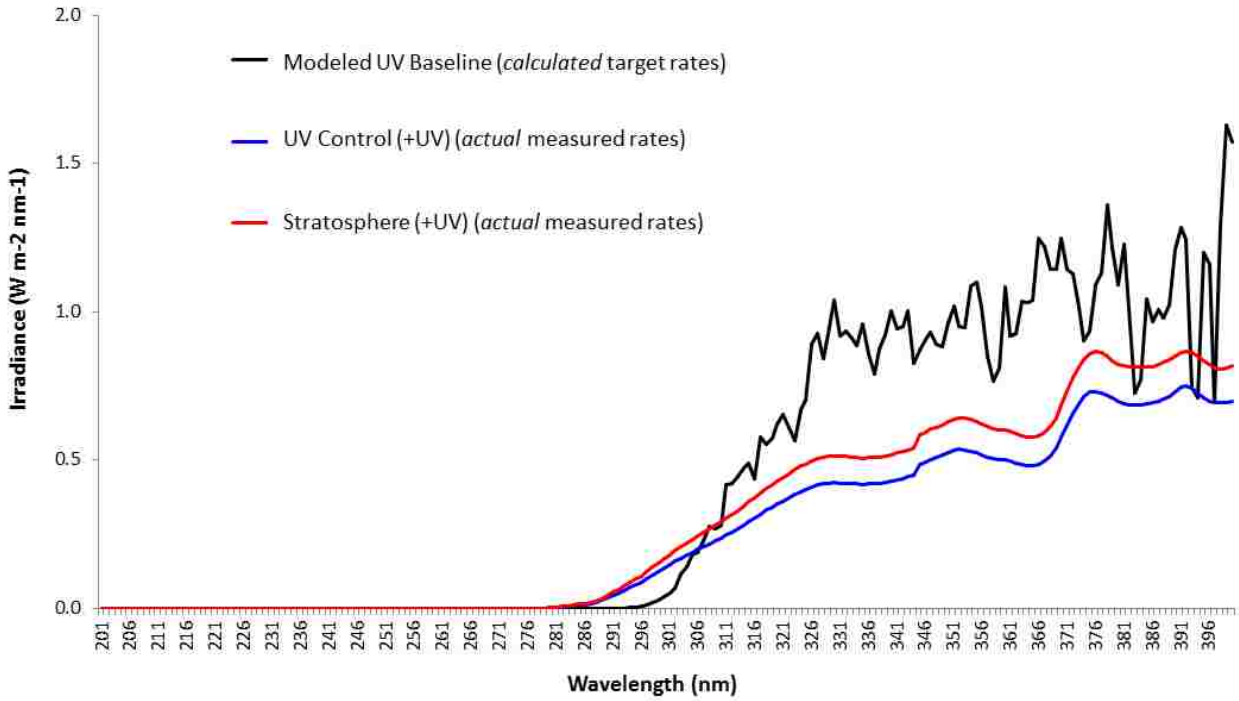

Figure 5.4 Ultraviolet light values. Compilation of modeled (*target*) and measured (*actual*) UV fluence rates from 200-400 nm. Baseline values from Nimbus-7 SBUV are depicted in black; UV Control (+UV) values are depicted in blue; Stratosphere (+UV) values are depicted in red.

0.00379, 4.16, and 45.83W/m^2 in UVC, UVB, and UVA, respectively. Ultraviolet fluence rates for the Stratosphere (+UV) experiment were nearly identical: UVC, UVB, and UVA totaled 0.00421, 5.11, and 54.64 W/m^2, respectively. In both (+UV) experimental setups, the total UV was slightly lower than the modeled values because the light attenuation disproportionately lowered the UVA flux in order to achieve closer-to-target UVC and UVB values.

Bacillus subtilis strains NASA8 and WN696 exhibited similar responses to the stratospheric simulations (Fig. 5.5). The drop in viable spores over time for UV-exposed (+UV) samples was approximately four orders of magnitude (99.9%), without regard to isolate or dust treatment [Stratosphere (+UV) coupons $P < 0.001$; UV Control (+UV) coupons $P < 0.001$]. While this was an extreme decline from a starting value of ~1 x 10^6 cells, the spore die-off was actually incomplete: both NASA8 and WN696 coupons maintained hundreds to thousands of viable spores through the last time step of 96 hrs. Even though the difference between the number of viable NASA8 and WN696 spores in the UV Control (+UV) experiment was generally higher than in the Stratosphere (+UV) experiment (except for WN969 dust-treated samples), most of the means had overlapping standard errors. The number of viable spores in Stratosphere (+UV) coupons compared to UV Control (+UV) coupons was calculated as non-statistically significant in each case ($P = 0.0749$ to 0.714).

Rapid loss in viability for both isolates occurred in the first 6 hrs of experimental exposure and was typically three orders of magnitude. Compared to the non-UV irradiated treatments [Stratosphere (-UV), Temperature Control (-UV), and Ground Control (-UV)], this difference was significant ($P < 0.001$). During subsequent time-steps (12-96 hrs), there was an occasional

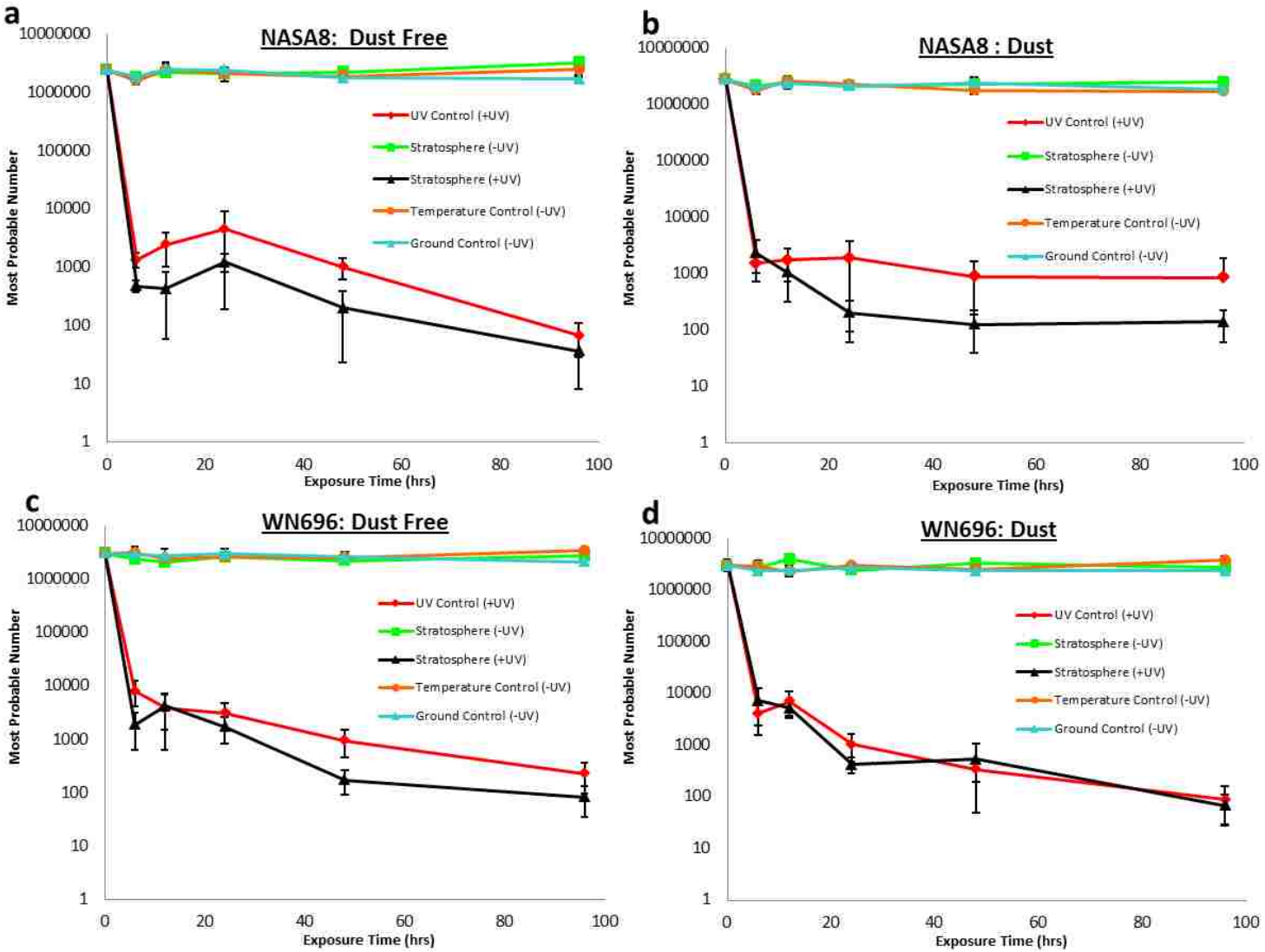

Figure 5.5 Simulated stratosphere experiments. Viability results (MPN) from t = 0-96 hrs. Minimum detection limit of enumeration assay was 90 spores per coupon. Values are means of six replicates; *bars* represent standard errors of the means. (A) NASA8 dust-free coupons. (B) NASA8 dust coupons. (C) WN696 dust-free coupons. (D) WN696 dust coupons.

increase in the number of viable spores, but the data were within (or close to) standard errors of the means. Also between 12-96 hrs, the decrease in spore viability was more moderate compared to the first 6 hrs of exposure, changing only 1-2 orders of magnitude. Interestingly, dust did not significantly affect spore viability (NASA8 coupons P = 0.737 to 0.817; WN696 coupons P = 0.558 to 0.901). In every environmental-treatment group and with both isolates, spores on dust coupons declined as described earlier. Controls (-UV) for both dust and dust-free samples indicated that pressure, temperature, and desiccation had no effect on the survival of NASA8 or WN696 ($P > 0.05$); all cell counts were within one-half order of magnitude relative to the starting points of the assay.

5.5 Discussion

Motivation for this study was to evaluate the tolerance of microorganisms to stratospheric environmental conditions. We used microbes collected during a trans-Pacific sampling flight at 20 km (Smith et al., 2010) and simulated the stratosphere environment in a hypobaric chamber to address whether the cells were actually capable of a long-term stay in the upper atmosphere. Stratosphere-isolated NASA8 was compared with the ground-isolate WN696 to look for evidence of natural selection and to understand what physical factors were limiting cell viability in the upper atmosphere. Surprisingly, NASA8 and WN 696 had almost identical responses to the simulation: dying rapidly when exposed to UV irradiation and ending with significantly fewer (99.9%) viable cells after 96 hrs. Running simultaneous experiments with one or more stratospheric factors allowed the effect of individual environmental variables to be measured. For example, Stratosphere (+UV) and Stratosphere (-UV) coupons differed only in the UV-light treatment and shared identical temperature, pressure, and desiccation parameters. But nearly all

Stratosphere (+UV) cells were killed, while spores in the Stratosphere (-UV) experimental group did not decline. This finding demonstrates UV irradiation was the primary biocidal component in the simulation, as predicted prior to experimentation. A similar drop in viability for UV Control (+UV) coupons at STP conditions supports the idea that UV irradiation alone, and not simulated stratospheric temperature, pressure, or desiccation influenced the survival of bacteria. The correlation between Stratosphere (+UV) and UV Control (+UV) coupons implies that combining UV with other extreme environmental conditions did not create a synergistic stress effect on microbes. However, these experiments were conducted only with endospores of two *B. subtilis* strains, which are known to resist many common environmental stress factors. It is plausible that interactive effects of stratospheric UV, pressure, temperature, and desiccation might impact non-spore forming species to a much greater extent.

Our results suggest that NASA8 could have been among the 0.1% of surviving spores recovered from the stratosphere by Smith et al. (2010); perhaps representing "persister" cells that tend to exist microbial populations subjected to in high-stress environments (Kussell et al., 2008). Why, then, did NASA8 not outlast its ground-derived counterpart (WN696) in stratosphere simulation experiments? Several studies (Nicholson & Law. 1999; Nicholson et al., 2002; Bernadini et al., 2003; Fajardo-Cavazos & Nicholson, 2006; Osman et al., 2008) have compared the UV tolerance between natural populations and reference microbes and may provide insight when comparing NASA8 and WN696. One possibility is that NASA8 spore characteristics may have been substantially different in a laboratory than in the stratosphere. Nicholson & Law (1999) observed that the UV resistance of a natural *B. subtilis* strain was lost when spores were cultivated in the laboratory, even after one round of growth and sporulation. In other words, spore resistance may depend on the conditions in which cells most recently

sporulated (e.g., nutrient high, ground-normal conditions in laboratory incubators), with no resistance legacy carried over from the extreme environment of the in situ niche (Nicholson et al., 2000). The fact that the spore preparation method used in this study was identical for both NASA8 and WN696 would then explain the similar survival patterns. In this scenario, it is possible that the same variance in spore construction (e.g. thicker, higher protein ratio, ability to repair biomolecules, etc.) was equally probable for both strains.

Another possibility is that spore survival was not based on genetic resistance but instead physical circumstances. For example, microniches on the aluminum coupon surface could have shaded a small fraction of spores from UV (Schuerger et al., 2005; Osman et al., 2008). Dust was expected to have the same type of protective effect on microbes, yet it did not, possibly because the concentration of the dust was too low. Also, spores that were associated with dust grains were generally stuck to rather than embedded within the particle, meaning that only cells on the underside of large dust grains (> 1 μm) could have received some irradiation protection. Yet even that protection may have been incomplete since UV photons could scatter around the base of a dust particle. After using the same coupons Schuerger et al. (2003) noted that complete protection was only achieved when spores were encased in pits or scratches on the material surface. It is worth noting that our decrease in spore viability from 12 to 96 hrs (1-2 orders of magnitude) implies that the shading effect from dust and/or coupon microniches cannot account for *all* surviving spores. Following the rapid inactivation of non-shaded spores, one would expect no change in the viability of cells protected by microniches since low temperature, high desiccation, and low pressure did not have a measurable effect on the bacteria.

In this study, UV rates were based on actual measurements from Nimbus-7 (McPeters et al., 1984; McPeters et al., 1993). Instead of trying to simulate diurnal cycles and scattering effects,

our bacterial coupons were exposed continuously to UV irradiation at a fluence rate similar to a solar zenith angle of 30°. Obviously this situation would not occur in nature, but the decision to maintain a constant UV flux was because the light equipment did not allow for ramping of diurnal cycles. Also, spore viability depends on cumulative UV dosage, not the rate of UV exposure. Even if simulations included a dark period, the relief would have coincided with a temperature of -70 °C, which is likely too cold for DNA self-repair or cellular activity (Junge et al., 2006). The UV simulation was a close match to the stratosphere at 20 km, with the exception of slightly higher UVB and slightly lower UVA fluence rates (Table 5.1). Most spore inactivation in our study was due to UVB and UVA irradiation, not to UVC. For stratospheric altitudes considerably higher than 20 km (i.e., above the ozone layer), one would expect a faster kill of cells due to the action of more potent UVC wavelengths. Despite some of the limitations to our UV parameters described above, our simulation improved upon previous resistance studies (Imshenetsky et al., 1977; Yang et al., 2008a, 2008b; Shivaji et al., 2006, 2009) because: (1) the UV model was specific to the sampling site of the stratosphere isolate; (2) UV was combined with other environmental factors (temperature, pressure and desiccation) to weigh the relative effects of each variable; and (3) species-level comparisons of microbial resistance were possible.

By measuring the resistance of *B. subtilis* we addressed the hypothesis that the stratosphere can act as a population bottleneck to airborne microbes transported globally. While the resistance of stratosphere-isolated NASA8 was not unusual compared to the ground strain WN696, the fact that overall numbers were substantially reduced reveals the enormous potential for directed or random selection in the stratosphere. The amount of biological cells in just1 g of soil is staggering – perhaps 10^9 – and it has been estimated that as many as 10^{21}-10^{24} cells are lifted into the atmosphere annually (Griffin et al., 2011). In many regards, spore-forming

microbes are ideally adapted to endure the physical threats associated with airborne transport. Atmospheric mixing events between the troposphere and stratosphere (Griffin, 2004; Dehel et al., 2008; Randel et al., 2010) are particularly intriguing because injecting microbes into the upper atmosphere would disperse species globally. Even if 99.9% are inactivated in the stratosphere by UV irradiation, the surviving 10^{18}-10^{21} cells still represents a huge amount of biomass dispersed. A proxy for predicting microbial dispersal in the stratosphere could be Mt. Pinatubo aerosols, where global percolation throughout both hemispheres occurred months after the mixing event (Wallace & Hobbs, 2006). The potential for that kind of global dispersal, combined with the fact that microbial populations are enormous and fast-reproducing, might help explain why distinct biogeography ranges seem absent with many bacteria (Martiny et al., 2006). Another possibility is that microbes surviving stratosphere transport could represent a substantial change from soil-derived gene pools; landing in distant environments with new niches could activate previously suppressed genes (see Wainwright et al., 2006). In both regards, the upper atmosphere could play a major role in the distribution and composition of common microbial species (see Papke & Ward, 2004; Kellogg & Griffin, 2006; Martiny et al., 2006).

Future studies should consider extending the duration of UV experiments to test whether longer stratospheric survival may be possible (weeks or months). The oxidative effects of ozone (Broadwater et al., 1973; Komanapalli & Lau, 1998; Deguillaume et al., 2008) — one biocidal factor not included in this study — should also be measured in an atmospheric context. Embedding microbes inside aerosolized dust would be a useful way of establishing a more dynamic and realistic stratospheric environment. In our study, spreading the cells in monolayers provided interesting information about the ability of individual spores to resist UV, but perhaps it did not simulate the variety of arrangements cells can have while airborne in the stratosphere.

For instance, Harris et al. (2002) and Narlikar et al. (2003) noted that microbes collected from 20 to 41 km were clumped together, which implies that the ability to aggregate could be an important aspect of UV resistance at altitude (Wainwright et al., 2006). Additional stratosphere isolates should also be tested (Imshenetsky et al., 1978; Lysenko, 1980; Harris et al., 2002; Wainwright et al., 2002; Narlikar et al., 2003; Griffin, 2004; Shivaji et al., 2006; Griffin, 2008; Yang et al., 2008a, 2008b; Shivaji et al., 2009; Smith et al., 2010), since conclusions from this study most accurately represent the resistance of *B. subtilis* and similar spore-forming bacteria. When using microorganisms for experiments, it would be beneficial to extract spores directly from the environmental samples, in order to ensure that the character of the isolate has not changed because of laboratory germination. Overall, there seems to be a need for standardizing future work so that the relative resistance of various microbes can be more readily compared. Unfortunately, the upper atmosphere remains mostly inaccessible to microbiologists due to the cost and difficulty associated with direct sampling, but future investigations can benefit greatly from laboratory simulations.

5.6 Acknowledgements

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Any use of trade names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

Chapter 6

CONCLUSIONS

6.1 Synthesis

The upper altitude boundary of the biosphere has not been established. The atmosphere is, arguably, the last major unexplored biological environment on the planet. In the preceding chapters, we tested the hypothesis that (1) transpacific plumes routinely deliver rich microbial populations to North America and (2) the upper atmosphere can be a critical barrier for the dispersal of surface life. Our primary goal was to develop a framework for analyzing global patterns of microbial biogeography. By combining airborne microbial observations with atmospheric chemistry measurements, we closed a spatial/temporal gap acknowledged in aerobiology literature. Focusing on transpacific plumes with distinct chemical compositions and coherent transport histories allowed us to eliminate local emission sources. We have demonstrated synergies between microbiology and atmospheric science: mutually-informative, independent approaches for understanding emission sources and global transport.

Using aircraft and mountaintop sampling methods, we discovered viable species in the lower stratosphere and surprising levels of abundance and species richness (spanning all three major biological domains) in the upper troposphere. The detection of viable cells and many gram-positive, endospore-forming families (e.g., Actinobacteria and Firmicutes) in long range transported air demonstrates the potential for new genes (or diseases) to move between distant continents. Rapid selection in the upper atmosphere, due mostly to UV irradiation, can impose a bottleneck on airborne populations and play a major role in the distribution and composition of microbial species.

6.2 Future Work

The atmosphere has been considered a conduit for life and not an ecosystem because airborne microorganisms originate from the surface and must eventually return to it. Future studies should consider measuring mRNA transcripts and other short-lived molecules in order to infer metabolic activity – such a discovery would certainly settle the debate of whether or not the atmosphere can be considered an ecosystem in its own right. Moving forward, filter membranes are not an ideal material for capturing airborne cells. Removing DNA from the filters imposes challenges and potentially inflates species richness values from non-specific hybridizations generated from aggressive extraction methods required to remove cells from filter membranes. Liquid impingement and high throughput sequencing could revolutionize the field of aerobiology, providing more accurate species identifications and the ability to detect novel species (unlike microarrays which are limited to known microbial taxa), respectively. Also, molecular methods sensitive to the detection of fungi and viruses should also be employed, since these groups of microorganisms probably outnumber airborne archaea and bacteria.

Some of these advances will be integrated into a series of stratospheric balloon missions called MIST (Microorganisms in the Stratosphere), currently being developed by my team at NASA Kennedy Space Center. Balloons offer several advantages compared to other high-altitude carriers. Rockets were used in early aerobiology missions to reach into the mesosphere, but the platform makes it is difficult to capture air on accent/descent and the sampling window is

Figure 6.1 Microorganisms in the Stratosphere (MIST), a NASA/KSC balloon mission under development that will measure microbial populations at extreme altitudes (Principal Investigator: David J. Smith).

short. Piloted aircraft cannot achieve altitudes above the lower stratosphere (approximately 20 km) and sampling devices are inefficient to minimize aerodynamic interference. In contrast, balloons can: (1) fly higher and stay aloft longer (therefore collecting larger volumes of air); (2) carry heavier and more sophisticated payloads for higher efficiency sampling devices; and (3) provide more opportunities for experimental controls during and after flight activities.

MIST has two major scientific goals: sampling microorganisms and measuring survival rates. A customized air sampler controlled remotely from ground commands will trap microorganisms once the balloon reaches the stratosphere. To prevent contamination, the hardware will be capable of auto-sterilization before/after sampling and air will be pumped-in using an extension line away from the balloon gondola. If contamination occurs, we will be able to trace the source by sputter-coating fluorescent beads onto flight-hardware elements prior to launch. On separate flights, known quantities of bacteria will mounted on the exterior of the balloon gondola exterior in order to measure inactivation rates in the stratosphere. The number of surviving cells and UV-induced mutations will be measured post-flight and compared to results from Chapter 5. Overall, MIST should help establish the boundary of the biosphere, while developing sterile sampling instruments and procedures that could prove useful for planetary protection and the reduction of false-positives in NASA missions with astrobiology flight objectives.

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VITA

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EDUCATION

Princeton University Princeton, NJ	Ecological & Evolutionary Biology	B.A. (2007)
University of Washington Seattle, WA	Biology and Astrobiology	Ph.D. (2012)

APPOINTMENTS

2008-2012 University of Washington, Research Associate and Teaching Assistant, Biology and Astronomy Department

2007-pres NASA Kennedy Space Center, Student Engineer, Co-op Program

2007 NASA Ames, Research Associate, NASA Academy for Space Exploration

AWARDS/HONORS

2012 National Science Foundation, US Antarctic Program Field Service Award

2012 Washington NASA Space Grant Consortium, Graduate Fellowship

2011 NASA Astrobiology Institute, Director's Discretionary Fund Award

2011 National Geographic Society Waitt Grant Program, Grantee

2011 Department of Biology (UW), Sargent Award

2011 NASA Patent, Conductive Carbon Nanotube, Case No. KSC-13343

2010 NASA KSC, Group Achievement Award: Smart Materials Development Team

2009 American Philosophical Society, Lewis and Clark Field Scholar in Astrobiology

2008-11 National Science Foundation, IGERT Research Fellowship

2008 NASA KSC, ECO System Award: Launch of STS-122 and STS-123

2008 NASA Astrobiology Institute, Santander Summer School, Scholar

2008 NASA KSC, On the Spot Award: MIR SDS Life Cycle Cost Analysis

2008 National Outdoor Leadership School, Graduate

2008 NASA JPL, 20th Annual Planetary Science Summer School, Graduate

2007 Princeton Univ. Dept. of Ecological & Evolutionary Biology, Honors Graduate

2006-7 Princeton Astrobiology Club, Co-Founder and President

PEER-REVIEWED PUBLICATIONS

1. **Smith DJ** et al. (2012) Intercontinental dispersal of bacteria and archaea in transpacific winds. Appl. Environ. Microbiol. (in press November 2012)
2. **Smith DJ** et al. (2012) Free tropospheric transport of microorganisms from Asia to North America. Microb. Ecol. 64(4):973-985
3. Birmele MN, **Smith DJ**, Morford MA, Roberson LB, Roberts MS (2012) Evaluation of an ATP assay to quantify bacterial attachment to wetted surface in variable gravity conditions. 42nd ICES (AIAA 2012):3508
4. **Smith DJ**, Griffin DW, Jaffe DA (2011) The high life: Transport of microbes in the atmosphere. Eos 92(30): 249-250
5. **Smith DJ**, Griffin DW, McPeters RD, Ward PD, Schuerger AC (2011) Microbial survival in the stratosphere and implications for global dispersal. Aerobiologia 27:319-332
6. **Smith DJ**, Griffin DW, Schuerger AC (2010) Stratospheric microbiology at 20 km over the Pacific Ocean. Aerobiologia 26:35-46
7. **Smith DJ** et al. (2009) Survivability of *Psychrobacter cryohalolentis* K5 under simulated martian surface conditions. Astrobiology 9(2): 221-228

ORAL PRESENTATIONS (CONFERENCE AND INVITATIONS)

1. Griffin DW, **Smith, DJ**, Schuerger AC (2013) "Desert Dust Dispersion, Microbiology, and Impacts", American Meteorological Society, January 2013
2. (Invited) "The High Life" – University of Oregon, Ecology and Evolution Department seminar, October 2012
3. **Smith DJ** (2012) "Microorganisms in the Upper Atmosphere", Astrobiology Graduate Student Conference, August 2012
4. **Smith DJ**, Griffin DW, Schuerger AC (2010) "Life on the Edge of Space", Astrobiology Science Conference, April 2010

5. (Invited) “The Moon and Antarctica: Lessons from Life in a Remote Antarctic Field Camp”, Space Life Science Lab Seminar Series – Kennedy Space Center, July 2009
6. **Smith DJ**, Roberson L, Mueller R, Metzger P (2008) “Rapidly Deployable Blast Barriers for Lunar Surface Operations”, Lunar Exploration Analysis Group Conference, October 2008
7. (Invited) “This is your Future – Why the Moon?” Next Gen Panel Discussion at Lunar Science Conference – NASA Ames Research Center, July 2008
8. **Smith DJ** et al. (2008) “Survivability of *Psychrobacter cryohalolentis* K5 in Simulated Martian Surface Conditions”, Astrobiology Science Conference, April 2008
9. Heldmann JL, Williams K, McKay CP, Toon OB, **Smith DJ** (2007) “Study of Terrestrial and Martian Snowpacks: Correlation of Ambient Environmental Conditions, Liquid Water Melt, and Biological Activity”, Geological Society of America Annual Meeting, October 2007

PRESENTATIONS (POSTER)

1. Kirschvink JL, Tobin T, Ward PD, **Smith DJ** et al. (2010) “Integrated, High-Resolution Magneto-, Bio-, and Chemo-stratigraphy of the Cretaceous/Tertiary Sediments of the James Ross Basin, Antarctica”, AGU Meeting of the Americas, August 2010
2. Chen E, **Smith DJ** et al. (2009) “Argus: A New Frontiers mission to observe Io”, Lunar and Planetary Science Conference, March 2009
3. Schuerger AC, **Smith DJ** (2008) “The Moon’s Surface may be a Self-Sterilizing Environment for Terrestrial Microorganisms”, Lunar Exploration Analysis Group Conference, October 2008
4. Chen E, **Smith DJ** et al. (2008) “Argus: A New Frontiers mission to observe Io”, Division of Planetary Sciences Annual Meeting, October 2008
5. **Smith DJ** et al. (2007) “Novel Techniques for Stratospheric Spore Collection”, SEDS National Conference, November 2007