THE APPLICATION OF MOLECULAR BASED TOOLS FOR BIOAEROSOL SOURCE TRACKING AND DISINFECTION ASSESSMENT

by

Mari Cristina Rodríguez de Evgrafov

B.S.E. Department of Chemical Engineering, Arizona State University, 1998

M.S. Department of Civil and Environmental Engineering,

Arizona State University, 2002

A thesis submitted to the

Faculty of the Graduate School of the

University of Colorado at Boulder in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

Department of Civil, Environmental, and Architectural Engineering

2009

This thesis entitled:

The Application of Molecular Based Tools for Bioaerosol Source Tracking and Disinfection Assessment

written by Mari Cristina Rodríguez de Evgrafov

has been approved for the Department of Civil, Environmental, and Architectural Engineering

_____

Mark Hernandez, Committee Chair

_____

Norman R. Pace, Committee Member

_____

JoAnn Silverstein, Committee Member

_____

Angela R. Bielefeldt, Committee Member

_____

John Spear, Committee Member

Date: April 9, 2009

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

Rodriguez de Evgrafov, Mari Cristina (Ph.D., Civil, Environmental and Architectural Engineering)

The Application of Molecular Based Tools for Bioaerosol Source Tracking and Disinfection Assessment

Thesis Directed by Professors Mark T. Hernandez and Norman R. Pace

Airborne microorganisms play a pivotal role in public health, national security, economic, and agricultural matters, yet our understanding of their identity, distribution and abundance is limited. Employment of molecular based detection and enumeration methods to the study of aerobiology would improve our understanding of commonly encountered microorganisms, as well as establish baseline information for surveillance efforts. This dissertation uses such methods to examine several environments of public health interest.

The first case investigated was the potential partitioning of waterborne microorganisms into the atmosphere by massive pumping and aeration operations of floodwaters in New Orleans following Hurricanes Katrina and Rita. To determine if remediation efforts significantly impacted airborne microbe populations, or resulted in aerosolization of potentially pathogenic microorganisms, paired air and water samples were collected in the immediate vicinity of turbulent pumping and aeration operations and analyzed. Remediation activities were found not to significantly impact the bioaerosol ecology proximal to large engineering works. No pathogenic

species were detected in the aerosol samples. Airborne ecology results were consistent with phylum level taxonomic patterns emerging from observations of outdoor bioaerosol communities.

The second scenario examined was worker exposure to harmful bioaerosols within concentrated animal feeding operations (CAFOs). The recovered ecology was more diverse than previously reported and dominated by organisms associated with animal gut microbiota. No respiratory pathogens of concern were observed; however, the potentially pathogenic species *Aerococcus viridans* was present in several samples. Fungal species were not recovered from indoor samples.

The third situation reviewed was indoor airborne ecology of flood impacted and subsequently remediated homes in New Orleans. Observed fungal populations were substantially different from those commonly recovered using traditional culture methods from water-damaged homes. *Wallemia sebi* was the only potential respiratory pathogen observed in significant abundance and was found in both indoor and outdoor environments.

Finally quantitative PCR was explored as a tool to assess aerosol disinfection efficiency. Bench scale experiments revealed that UV exposure did not impact recovery of QPCR at doses germane to airborne microbial inactivation.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1 Problem Statement

Biological aerosols (bioaerosols) in the atmosphere and indoor environments have important environmental and health implications. Aerosol transmission is a key mode of transport for some of the world's most contagious diseases, such as tuberculosis (6), SARS (131), and influenza (123), among others. Bioaerosols are also associated with noninfectious diseases, such as hypersensitivity, allergies, and asthma (1, 169). In addition to their negative impact on human health, bioaerosols have significant economic implications in agriculture. Hoof and mouth disease (75), porcine respiratory coronavirus (220), and avian influenza (81) are transmitted via aerosols. Surprisingly, despite their health and economic impact, bioaerosols remain poorly understood (34).

Only recently have researchers started to study bioaerosols using modern capture and analytical tools (9, 11, 28, 161, 228). These studies provide some insight into the types of organisms that occur in the air; however, more longitudinal bioaerosol characterizations are lacking, and there remains a paucity of investigations with respect to the microbial ecology of our atmosphere and different indoor environments (178). Currently we know very little about the types of organisms that occur in the air, their stability, longevity, sources or airborne residence times. The majority of work published in this field has been conducted using culture-based analyses, and

while these techniques are well established, they do not recover the majority of microorganisms present in many environments (9, 10, 171, 178). One consequence of reliance on these methods is underestimation of the total numbers and types of microorganisms sampled, particularly those of public health interest (185, 204), by detection of only those microbes which are both viable and culturable. With the advent of molecular based technologies, more comprehensive assessment of airborne microbe populations in various environments is feasible (9, 178).

Considerable work in this field is needed to help establish health and safety guidelines, as well as to provide guidance for development of cost-effective engineering controls for mitigation of bioaerosol exposures where substantive risks occur. At present there are no threshold exposure levels for bioaerosols. The American Conference of Governmental Industrial Hygienists (ACGIH) has stated that there is not enough information available with which to develop such a standard (2). The application of molecular based technologies to the study of airborne microbial ecology provides a more comprehensive and reliable understanding of the atmospheric environment than currently available, and begins to put in place modern scientific-based criteria and analytical methods for developing bioaerosol exposure standards.

1.2 Research Objectives and Approach

In response to the current limitations for characterizing bioaerosols, the overall goal of this work was to use advanced molecular techniques to comprehensively survey airborne microorganisms, and assess the potential impact that ultraviolet irradiation may have on the recovery of airborne microbial populations. This broad goal was accomplished through the following objectives:

- Characterize bioaerosols in “contaminated” environments using molecular methods and compare the observed ecology to that obtained through conventional culture based methods.
- Develop and validate a polymerase chain reaction (PCR) based assay for the detection and quantification of select bioaerosol pathogens.
- Assess the potential impacts of UV on the quantitative PCR recovery of candidate airborne pathogens with respect to doses currently used to design germicidal UV systems for indoor environments.

1.3 Dissertation Overview

This chapter provides the background and architecture for bioaerosol investigations presented in the subsequent chapters of this dissertation. The organization of this work is such that each chapter can be read independently with a minimal cross-referencing to other chapters. Each chapter contains its own literature review, material and methods, results, and discussion sections germane to their hypothesis; all chapters share a common genetic-based

ecological quantitation and characterization of bioaerosol in unique indoor and outdoor environments. The final chapter synthesizes a salient theme, which emerged from environmental bioaerosol observations, provides recommendations for future research and presents applications to industrial hygiene and engineering practice.

CHAPTER 2

LITERATURE REVIEW

2.1 Background

2.1.1 Definition of Bioaerosol

Bioaerosol is the term referring to airborne particulate matter of biological origin. Included in this definition are microbial toxins and waste products, plant and insect fragments, pollen, dander, bacteria, fungi and their spores, and viruses (106). Depending on their source and type, bioaerosol particles can range in size between 0.01 and 100 mm in mean aerodynamic diameter (106). Particles in the size range between 1 and 10 mm include bacteria and fungal spores; below 3μm, many airborne particles are respirable and therefore of particular interest to researchers and public health advocates (36, 106). High airborne microbe concentrations have been correlated with adverse respiratory symptoms (18). Bioaerosols are ubiquitous and have been reported to account for nearly a quarter of the total particulate matter in the atmosphere (24, 241). Bioaerosols are frequently attached to other particles and their transport is affected by temperature, relative humidity, thermal and electrostatic field effects, gravity, and diffusion (106).

Airborne microbes are classified as either viable or nonviable (103). Viable bioaerosols are generally considered as living or otherwise surviving organisms, capable of reproducing under favorable conditions; aerosols referred to as pathogenic or infectious are viable. Bacteria, fungi, their

respective spores, and viruses can be considered viable, when recovered from an air sample such that they form colonies on solid media, increase turbidity of liquid media or, in the case of viruses, cause plaques or syncitia in host cell cultures. Nonviable bioaerosols consist of biological matter not living or capable of reproducing in standard culture. Microbial fragments, their byproducts, pollen, and dander are considered nonviable bioaerosols. Both viable and non-viable bioaerosols have important impacts on human health through hypersensitivity, toxigenic and allergenic diseases.

2.1.2 Public Health Impact of Bioaerosols

Airborne transmission of viable microorganisms is an important mode of disease transfer. Tuberculosis, acute respiratory diseases, measles, and whooping cough, all transmitted by viable bioaerosols, are the leading causes of death in the developing world (67, 91, 170, 236). Legionnaire's disease (53, 64, 168), pneumonia (245), Valley Fever (125, 223), Hantavirus (152), and gastrointestinal diseases (209) are also transmitted through the air, and comprise some of the most recognized pathogenic bioaerosols. The increased movement of people across borders, for leisure or refuge, will likely accelerate the spread of emerging and re-emerging airborne infectious diseases (6, 26, 41, 86). The 2003 SARS (Severe Acute Respiratory Syndrome) outbreak in East Asia and North America highlights the potential impact of globalization on bioaerosol related disease transmission.

The economic impact of bioaerosol related disease transmission is substantial. A study published in 1985 estimated that direct treatment of

respiratory infections, both upper and lower, cost the US economy $15 billion a year (63). Indirect costs due to lost time and associated diminished productivity were estimated to exceed $9 billion a year. The same study reported that an estimated 1.25 million people are hospitalized annually for community acquired respiratory tract infections. The cost of this hospitalization was estimated to exceed $4 billion. Nosocomial infections reportedly affect approximately 300,000 people a year and treatment of these is estimated to cost approximately $470 million (63).

A recent study estimated the domestic costs of lower respiratory tract infections (LRTI) to be $8.9 billion a year (153). Similar cost studies of community acquired pneumonia (CAP) infections report direct costs ranging from $11 to $14 billion annually (30, 51, 144, 153, 164). Indirect costs for CAP infections due to lost productivity were reported to be $9 billion (30, 144). Direct costs associated with acute exacerbations of chronic bronchitis (AECB) are approximately $2 billion annually (165).

Inhalation of nonviable bioaerosols can cause numerous toxigenic or allergenic diseases. Ailments such as hypersensitivity pneumonitis, humidifier fever, severe asthma, sick building syndrome, organic dust toxic syndrome, and chronic bronchitis are all caused by nonviable bioaerosols (35, 92, 97, 169). Between 10 and 30% of the adult population and 40% of children in the developed world suffer from allergenic diseases. Of these populations 10% are considered severe cases (55, 78, 83, 94, 108). The

number of individuals who suffer from allergenic diseases is predicted to increase in the future (217).

The financial costs associated with allergic diseases rivals those published for pathogen induced respiratory infections. The annual direct costs for treating asthma in the US were estimated in 1998 to be $12.7 billion. Asthma's indirect costs for the same year were approximated to be $2.7 billion (231). Treatment costs associated with rhinitis were slightly lower – between $1.2 and $5 billion annually (195, 231). Indirect costs attributed to rhinitis, estimated in 2003, were between $1.7 and $4.3 billon annually (195). More than 2 million school days are missed each year to do rhinitis (195).

2.1.3 Bioaerosols as Weapons

The deliberate release of pathogenic bioaerosols as an act of terrorism or warfare was a troubling possibility that has become a frightening reality. In Fall 2001, *Bacillus anthracis* spores were mailed in envelopes around the country and resulted in killing 5 people, sickening 17 others, and contaminating several Senate, post, and media offices (122). Smallpox (*Variola* virus) is considered to have the greatest bioweapon potential (100). The World Health Organization (WHO) launched a global campaign to eradicate smallpox in 1967. Eradication was pronounced in 1980 and since that time the global population has not been vaccinated against the virus (235). Other potential bioweapons capable of being dispersed by air include *Francisella tularensis*, *Yersinia pestis*, *Brucella spp.*, *Variola virus*, and *Coxiella burnetii* (15, 122). Bioweapons are predicted to be the weapons of

mass destruction of the future as they are inexpensive with a high probability of delivering considerable devastation and large scale panic (101).

2.1.4 Agricultural Impacts of Bioaerosols

In addition to negatively impacting human health, bioaerosols also adversely affect agriculture, resulting in large economic losses. Airborne microorganisms are responsible for a lengthy catalogue of livestock diseases. Those with the greatest economic impacts include hoof and mouth disease (7, 85), porcine respiratory and reproductive syndrome (PRRS) (159, 220), bovine respiratory disease (82, 132), salmonella transmission in chickens (198) and the potentially pandemic influenza H5N1 (81). The damages to the British agricultural economy associated with a recent outbreak of hoof and mouth disease were on the order of £10 billon (230). Economic losses due to bovine respiratory disease are also reported to be substantial (156).

Only recently has the role of bioaerosols in livestock disease transmission been appreciated. The spread of disease among domesticated animals is now understood to be strongly influenced by industrialized livestock practices of transportation and close quartering of animals (75, 132). These practices, which include high densities of animals being raised within a single facility, result in extended contact between animals, concentrated air and waste emissions, as well as an increased potential for interspecies disease transmission between the animals and workers (121, 159).

Similarly bioaerosols also play an instrumental role in the spread of crop diseases (148, 232). Examples of plant diseases caused by airborne

pathogens include wheat rust, late blight of potato, Cercospora leaf spot on beets, soya bean rust, and rape stem rot (232). The damages caused by plant pathogens are substantial. Rapeseed stem rot damages approximately 20% China's annual crop (149); oilseed rape losses due to phoma stem cankers have been reported to affect between 30% and 50% of Asian crops (233). The effect of climate change will likely increase the threats posed by plant pathogens as new or rare diseases are transported to new regions where they will cause outbreaks that were previously unforeseen (119, 232).

2.2 Bioaerosol Sampling

The following section provides a brief introduction to several of the more common bioaerosol collection methods.

2.2.1 Impactors

Impaction is a popular method of bioaerosol collection for culture based analyses. Multistage or slit impactors collect particles onto agar media which can be incubated to provide a measure of the viable and culturable organisms present in a specific volume of air. An impactor separates particles based on size and density as a composite index of aerodynamic diameter (105). As air is forced through an impactor, it is diverted around impaction plates, which causes both the air and the particles in it to make abrupt 90^{o} bends. Particles with too large an inertia cannot follow the air stream and collide with the flat collection plate surfaces, thereby being removed from the air stream. Particles with smaller inertial values are able to

follow the air streamlines and avoid colliding with the media (105). The smallest particles ultimately pass through the impactor.

The Andersen impactor is perhaps the one most frequently used by the industrial hygiene sector, and reports of its use are common in the literature. The instrument was designed to collect bioaerosols within the range that was relevant to human respiratory system (0.65 to 7 micrometers) (146). There are two models available for viable bioaerosol collection– a two stage and a six-stage device. Both instruments are designed to operate at 28.3 L/min. Collection time is limited to 5 minutes as beyond this agar collection surfaces begin to dry resulting in increased particle bounce (decreased collection efficiency) and reduced cell viability due to desiccation (38). Sampling collection is limited by the agar surface and the upper quantitation limit is 10^4 colony forming units (CFU)/m^3. Beyond this value, multiple cells are likely deposited on top of one another resulting in a high potential for enumeration error (38).

2.2.2 Filters

Filtration is another popular method for bioaerosol collection. Filtration sampling of bioaerosols is performed by passing air through a porous filter material or membrane, where particles are captured via inertial impaction, interception, diffusion, gravitational settling, or electrostatic attraction (104). Filter materials used in bioaerosol ecology studies include polycarbonate, polyvinylchloride, polytetrafluoroethyline (PTFE or Teflon™), and mixed cellulose esters (146, 178). Standard filter sizes are 25, 37 and 47mm

diameter with standard pore sizes being 0.01 to 2 mm. Filters are usually mounted in/on permanent or disposable cassettes (closed faced) or holders (open faced) and are subject to airflow rates of 1 to 50 L/min (38). Samples collected on filters can be eluted or a filter can be dissolved to recover sample for further analysis. Filtration is often favored because it is simple, versatile, and economical. Disadvantages associated with filtration sampling include low sample volumes, high energy demands, desiccation and loss of viability (103). For these reasons filtration is not recommended for culture-based analysis.

2.2.3 Impingers

Liquid impingement is the capture of particles by impaction into a liquid and is becoming the preferred mode of collecting viable bioaerosols. Bioaerosols captured by liquid impingement avoid desiccation and experience less sampling stress (133). Other benefits of liquid capture include greater analytical flexibility as samples can be used for culturing, microscopy, immunoassay, flow cytometry, and molecular methods as well as the ability to dilute or concentrate samples as needed (38).

The Swirling Aerosol Collector (SAC) has been a widely accepted liquid capture bioaerosol device and received substantial attention in the air pollution literature. Introduced in 1998, the SAC improved the existing impinger design by adding inertial separation to the collection mechanism. Particles are injected at an angle into a swirling airflow where they are impacted and pulled into a liquid by impaction and centrifugal forces. Sub-

micrometer particles are also entrained by diffusion (237). The SAC design modified single jet impingers such that it reduced re-aerosolization and increased the viable cell survival rate (133, 237). The result of this improvement was a substantially increased collection efficiency (near 100%) over the particle size range of greatest public health interest (0.2 to 2 mm) (222, 237). SAC instruments can be used for wet or dry capture (133). When used for wet capture, a flow rate of 12.5 L/min is used and samples are collected into a relatively small reservoir (20 mL), which can be aliquoted for concurrent analyses. Liquid impingement is typically used to capture airborne bacteria. Several fungal spores are relatively hydrophobic and are likely to partition into impinger reservoirs at a lower efficiency than their vegetative counterparts (68). Viral particles can be collected using SACs, however the collection efficiency of the instruments falls below 90% for particles less than 0.5mm in diameter (237). To collect enough microbes for an aerosol sample for culture, microscopy and/or genetic analyses, impinger collection times are usually longer (~30 minutes) than those for impaction based counterparts, so they can provide a more composite description of the sampled environment. Disadvantages of impingers include low flow rates and the inability to separate particulate matter into size ranges that are critical with respect to health impacts (2.5 mm and 10mm) (178).

2.2.4 High Volume Samplers

The latest in bioaerosol capture technologies is the high volume sampler, which was developed for monitoring biological terrorism events.

These instruments are capable of sampling more than 100 L/min (178) and yield a collected sample that can be analyzed by culturing and/or molecular analyses. Disadvantages of these instruments include cost (10 to 200 times more than an SAC impinger) and lack of peer reviewed performance data (178). Further, these devices typically recover relatively high masses of inert airborne particulate matter, the nature of which can cause interferences with genetic characterization and associated PCR-based amplification.

2.3 Analytical Methods

The following section briefly describes some of the analytical methods performed on bioaerosol samples to assess microbial ecology.

2.3.1 Culture

Culture of microorganisms historically has been the most widely used analytical method in bioaerosol research. Microbes associated with particulate matter collected from air samples are typically grown on a "nonselective" agar at 28^{o}C for one to seven days (38). Examples of ostensibly nonselective media include trypticase soy agar or R2A agar for bacteria and malt extract agar for fungi (1). To prevent overgrowth, liquid samples are generally diluted (146). Samples collected using gelatin or cellulose membrane filters are directly placed on the agar media. Following a prescribed incubation period, microbial colonies are counted and phenotypically identified, if possible, and then reported as colony forming units (CFU)/m^3 air. Disadvantages of this method will be discussed in Section 2.4.

2.3.2 Microscopy

Microbial number concentrations and identification analyses can also be performed on bioaerosol samples using a variety of microscopy techniques, including light, epifluorescence, and scanning electron microscopy. Direct count analysis provides a measure of microbial number per volume of air. Samples are concentrated onto a filter, stained and visualized under an epifluorescent or transmitted light on a microscope. There are many different types of stains used to enumerate collected airborne microbes. These include Gram's, non-specific, DNA intercalating agents (DAPI and acridine orange), enzyme activity dyes (tetrazolium reduction) and fluorescent protein conjugates (fluorescent and calcofluors) (1, 146). To identify particular organisms more specific dyes that bind to select antibodies and oligonucleotides can be used. Microscopic analyses, in conjunction with select stains, can provide information about both viable and nonviable organisms. Disadvantages of this method include its laborious nature and susceptibility to human optical error and interpretation (9, 117).

2.3.3 Polymerase Chain Reaction

The steady improvement and application of molecular methods over the past 25 years has vastly expanded our understanding of the microbial world by making possible the detection and identification of microbes based on their ribosomal DNA (171). The application of polymerase chain reaction (PCR), the process by which select nucleic acid sequences are exponentially copied, to microbial ecology studies has made this progress possible.

Regrettably, the potential of this assay has not been fully capitalized upon by aerosol researchers.

PCR is sensitive, quantitative, and culture independent so it is unaffected by sampling stress and can analyze microorganisms which cannot be cultured or easily cultured (9, 178). PCR's impervious nature is especially important as some airborne pathogenic species cannot be, or at least are not easily cultured and others require special laboratory safety equipment for public health protection (178). Other benefits of PCR include accuracy, reduced cost and time of analysis.

The assay requires knowledge of a conserved nucleotide sequences of target genes and the creation of short oligonucleotides (primers) that are complementary to this sequence. Primers can be designed for a specific microbe or group of microbes. Following amplification, sequences can be compared to public databases for identification. PCR products are used in microarray and fingerprinting analyses and clone library construction. PCR can be used with genetic data from various sources, but can only be performed with DNA. RNA requires an additional assay whereby it is converted to DNA via reverse transcriptase prior performing PCR.

Real time PCR, also known as quantitative PCR (QPCR), allows for the monitoring of a particular target sequence during amplification. A fluorescent probe, used in the amplification reaction provides an estimate of the initial number of target sequences in the sample based on its excitation response. How quickly a specific PCR amplification (visualized by a

fluorescent signal) reaches a concentration threshold (Ct) can be standardized such that it corresponds to an original amount of target DNA present in a given sample (229). When properly controlled, the amount of target DNA in a sample is determined by comparing the sample Ct with a standard made from known concentrations of the target sequence, for instance a control plasmid or known concentration of cells.

2.4 Current Knowledge Gaps

Despite their significant impact to public health and negative economic potential, airborne microorganisms remain poorly characterized in the atmosphere and in indoor air (178). At present, our knowledge of the identity, distribution and abundance of airborne microorganisms is limited. Reasons for these shortcomings include the dilute concentrations of microbes in air with respect to other matrices, the limitations of aerosol sampling equipment, and the continued reliance on phenotypic analytical methods.

First, bioaerosols are typically present at very dilute concentrations, which makes them difficult to collect in quantities amenable for robust characterization (9). Atmospheric microbial concentrations are estimated to be on the order of 10^4 to 10^6 cells/m^3 air (227). With respect to the aquatic and terrestial biospheres, the atmosphere is a harsh environment that is nutrient deficient and oligotrophic (178, 227). Microorganisms in the atmosphere are susceptible to solar radiation, chemical poisoning, fluctuations in temperature and dessication. Atmospheric concentrations vary according to geography, season and weather events (36).

Second, bioaerosol sampling has traditionally been performed using impaction devices. These methods are fraught with disadvantages that include space limitations, sampling time limitations, enumeration error, poor collection efficiency of small particles, and desiccation (222). Owing to these limitations, sampling times for impaction devices are relatively short with respect to the residence time of most indoor environments allowing for only “snap shot” recovery with respect to the types and quantities of organisms present in a sampled environment (222). Likewise, these collection methods restrict the types of analytical methods that can be performed on the recovered samples.

Finally, bioaerosol researchers often continue to rely on traditional culturing methods for analyses (9, 178, 222). These methods assume that the captured organisms are culturable, will grow in a predetermined time frame and exhibit “classical “ characteristics (9). Previous studies of marine and terrestrial environments have found that the majority of microorganisms (>99%) observed microscopically are not culturable using standard methods (10). A compendium of studies comparing culture-based and direct microscopic recovery of airborne microbes suggest less than 1% of total airborne microbes are represented by conventional culturing (178). As with microbes recovered from other environmental media, reliance on culture based assays has painted a skewed picture of airborne microbial ecology as the ecological bias associated with them is well documented (10, 171, 240). Use of nonselective media and multiple incubation temperatures does not

reduce or remove ecological bias in culture-based assays as the nutrient and growth requirements of microorganisms differ (146). Organisms that do not grow under the selected conditions remain undetected and may be the ones of greatest public health interest (40, 112, 207). These pitfalls are compounded by a loss in culturability due to stresses associated with sample collection (37, 38, 56, 224). All these factors combine to underestimate the number and type of microorganisms present in the air (146). Lastly, culture based methods are labor intensive, slow, potentially hazardous and cost inefficient (9).

2.5 Summary

Bioaerosols potentially play a significant role in public health, national security, economic, and agricultural matters and yet our understanding of them is quite limited. Employing existing enhanced detection and enumeration methods to field of aerobiology would go far in remedying the current knowledge gaps. Established protocols are available for these methods in other biospheres and they can readily be adapted for air samples. Adaptation of these techniques needs to take on a large scale to address developing issues in air quality and to help develop solutions to existing concerns. These techniques are rapid, robust, and unaffected by sampling stress or duration. Likewise, they circumvent issues associated with slow growing, non-culturable or non-viable organisms. This dissertation explores the use of these methods to examine several environments of public health interest and to assess a current control technology.

CHAPTER 3

MOLECULAR SOURCE TRACKING OF BIOAEROSOLS IN THE QUARANTINED KATRINA FLOOD ZONE

Abstract

The clean up response following hurricanes Katrina and Rita included massive pumping and aeration operations to reduce biochemical oxygen demand (BOD) of floodwaters trapped within the city of New Orleans. Such engineering operations aerosolized tremendous quantities of water as microdroplets, which in-turn increased the potential for waterborne microorganisms to partition into the atmosphere. To determine if remediation efforts significantly impacted airborne microbe populations, or resulted in aerosolization of potentially pathogenic microorganisms, we performed direct microscopy, broad spectrum PCR, and DNA sequencing analysis on paired air and water samples collected in the immediate vicinity of turbulent pumping and aeration operations throughout flooded New Orleans.

We report here that remediation activities following Hurricanes Katrina and Rita did not significantly impact bioaerosol ecology proximal to large engineering works which promoted floodwater aerosolization. With exception to the minor representation of species associated with common skin infections, no pathogenic species were detected in this atmospheric sampling campaign. When compared to the growing genetic catalogues of atmospheric molecular ecology surveys, results from this sampling campaign

were consistent with, but limited to, phylum level taxonomic patterns emerging from observations of outdoor bioaerosol communities.

3.1 Introduction

In the weeks following Hurricanes Katrina and Rita, the US Army Corps of Engineers drained nearly a trillion gallons of floodwater from the city of New Orleans by coordinating a massive pumping operation (52). Two water quality studies conducted immediately following Hurricane Katrina found elevated numbers of pathogenic bacteria (or cultured surrogate indicators) in floodwaters distributed throughout the city (174, 190). High biochemical oxygen demands (BOD) (80mg/L average) observed in many flooded locations, prompted emergency response crews to execute *in-situ* water reclamation practices using high-power mechanical aeration and turbulent pumping discharges in many of New Orleans' major canals. Such practices were employed for the express purpose of entraining air into moving floodwaters, and resulted in the aerosolization of tremendous quantities of water. Since significant numbers of microbes have been shown to partition into the atmosphere from relatively quiescent water bodies (12, 19-22, 172), the Katrina floodwater reclamation practices could have significantly impacted local aerobiology by enhancing the partitioning potential of waterborne microbes into aerosols. The microbial concentrations of air bubbles bursting at the air-water interface may be 10 to 1000 times the concentration in the water (20). A comprehensive review of the fundamental mechanics of air water partitioning can be found in the works published by Blanchard (19-22).

Throughout New Orleans, floodwaters containing elevated numbers of potentially pathogenic microbes and high organic carbon levels as reflected by reduced oxygen levels were subject to sustained mechanical energy inputs on an enormous scale. A study performed days after Hurricane Katrina reported total coliform concentrations to range from $2x10^3$ to $8x10^6$ CFU/ml and dissolved oxygen levels to range from 6.95 to 8.83 mg/L (190). In response to these unique environmental conditions, we identified and enumerated microorganisms associated with active water reclamation operations to determine whether (i) the local ecology of airborne microbes was influenced by proximal water sources, and (ii) to ascertain if microbes present in the air posed a potential health risk to emergency response personnel working in the vicinity of water aerosolizing devices. Direct microscopy was used to quantify the microbiological loads present in aerosols and water samples. Broad-spectrum PCR and rRNA sequence analysis were conducted to assess microbial community compositions in the vicinity of outdoor remediation operations in the weeks between Hurricanes Katrina and Rita (September 18 – September 25, 2005), and the week immediately following Rita's landfall.

3.2 Materials and Methods

3.2.1 Aerosol and source water sampling

A total of 55 samples were collected from 22 sites throughout the City of New Orleans between September 18 and 25, 2005. Based on safe accessibility, seven of these 22 were chosen to collect concomitant aerosol

and proximal source water samples in the immediate vicinity of operating aerators and the turbulent terminus of floodwater transmission pipes. Table 3.1 lists their GPS coordinates. Figure 3.1 is a map showing the sampled sites. As a cohort of unperturbed background observations, samples were also collected on the quiescent west shore of Lake Pontchartrain, where no engineering operations were engaged. During the entirety of this sampling campaign, local wind speeds were below measurable level.

Aerosol samples were collected with SKC Biosamplers (BioSampler SKC Inc., Eighty Four, PA), positioned approximately 1 meter above the ground. The collection medium was 20 ml of DNA-free sterile water (USP, Hospira, Inc. Lake Forest, IL). Biosamplers were operated at a flow rate of 12.5 L/min. Two impingers were run in parallel for each sampling site and collected continuously for at least one hour at each sampling site. To maintain appropriate aerosol collection efficiency within the impingers, sterile water was episodically added to the Biosamplers to make up for any evaporative losses. At the end of each sampling event, the collection fluids from each impinger were combined into DNA-free sterile 50ml conical vials and placed on ice until they could be processed, which was less than 8 hours in all cases.

Samples were processed and archived in the laboratory facilities at the Louisiana State University Coastal Ecology Institute. Samples were aliquoted as follows: 10mL for direct microscopy analysis preserved with 2% (v/v) formaldehyde, and 30mL for molecular analysis concentrated on 0.2

micrometer nitrocellulose filters (100mL) (Nalgene Analytical Test Filter Funnel, Nalge Nunc International Rochester, NY). At the end of the sampling campaign all samples were stored on ice or frozen with liquid nitrogen and shipped overnight to the University of Colorado where they were stored at 4^{o}C (direct count samples) or –20^{o}C (DNA samples) until analyzed.

3.2.2 Airborne microbe enumeration

Aerosol samples were stained and enumerated using 4'6-diamidino-2-phenylindole (DAPI) (Sigma Chemicals, St. Louis, MO) in accordance with previously described methods (102). DAPI is a nonspecific fluorescent DNA intercalating agent and provided a suitable background for direct microscopy. A minimum of 10 random fields were counted per slide and only intact, brightly stained cells with obvious bacterial or fungal morphology were counted. Direct counts were reported as the average of all fields counted; in all cases the coefficient of variation observed was less than 20% (102).

3.2.3 Genomic DNA extraction

Concentrated samples and negative controls were extracted from the filters using a bead beating protocol modified from Frank et al (80). Extracted DNA was resuspended using 20 microliters of Tris-Ethylenediamine Tetraacetic Acid (TE). Extracted DNA concentrations were measured using a PicoGreen dsDNA Quantitation Kit (Molecular Probes, Carlsbad, CA) and the NanoDrop® ND-3300 Fluorospectrometer (NanoDrop Technologies, Wilmington, DE).

3.2.4 PCR of rRNA genes

Extracted DNA was assayed using universally conserved (515F/1391Rev) and bacteria specific (8F/1391Rev) primers following the protocol described by Papineau et al (173). PCR controls with no DNA template were included with every assay. Extraction and negative controls never showed amplification.

3.2.5 Clone library construction

One clone library, a total of at least 96 randomly selected rRNA clones, was constructed for each sample using the method described by Papineau et al (173).

3.2.6 DNA sequencing

Sequencing was performed as previously described by Papineau et al (173). Base calling and assembly of raw sequence data were performed with the PHRED and PHRAP software packages (72) using the software XplorSeq (79).

3.2.7 Phylogenetic analysis

The sequence collection was screened for chimeric sequences with the Bellerophon software package (110). The closest known relative of each rRNA clone was determined by comparison of its sequence to all rRNA sequences in GenBank by BLAST (8). Sequences were aligned with the NAST software package (62) and their phylogenetic positions were determined by analysis with the ARB software package (138) and a curated ARB database and tree with ~100,000 rRNA sequences ≥ 1250 nucleotides in

length from the NAST project (62). Sequences were added to the ARB tree by parsimony insertion.

3.2.8 Richness estimates

Estimates of potential true rRNA sequence diversity in each sample were calculated with the Chao1 statistic (46) as previously described (173). Sequences were collected into operational taxonomic units (OTUs), or relatedness groups, based on sequence identity at fixed intervals ranging from 95% to 100% in increments of 1%. OTU data were used to calculate the Chao1 richness estimate using a Python script (J.J. Walker, unpublished software).

3.2.9 Comparative phylogenetic methods

Sequence sets representative of the microbial composition of water and air samples were compared statistically with the online phylogenetic statistics software package UniFrac (136, 137) and the SONS program (212). UniFrac compares sets of related sequences (samples) using a phylogenetic tree and calculates a comparative metric based on the fraction of the total distance in the tree that is unique to a particular sample. Ecological relationships between samples were explored using the distance metric with two complementary methods- hierarchical classification and ordination. Samples were clustered hierarchically with the Unweighted Pair Group Method with Arithmetic means (UPGMA), and statistical support for the resulting phylogenetic tree was assessed by jackknife iterations. Principal Coordinates Analysis (PCA) (135) was used to order the samples by

maximizing the linear correlation between distances from the UniFrac distance matrix in a two dimensional projection. The SONS program compared the membership and structure of the selected (sourced) communities at a specified phylogenetic level. Based on the selected phylogenetic level, the program assigned sequences to operation taxonomic unit (OTU) bins (membership) and then evaluated the abundance in each bin for each sample and associated community content. Community similarity was assessed based on shared and unique memberships and overall extent of structure alikeness.

3.2.10 Potential pathogens

A list of known human pathogens was used to compare the closest match by BLAST for all 2100 sequences catalogued in this study (225). The list of pathogen names and the closest BLAST matches were exported to Microsoft Excel and compared using a series of lookup functions.

3.3 Results

In the weeks between hurricanes Katrina and Rita, we collected 14 paired air and proximal water samples at seven locations throughout innercity New Orleans where pump induced turbulence and/or mechanical aeration practices were purposely aerosolizing floodwater (GPS coordinates are summarized in Table 3.1). Airborne microbes were collected as 2 hour composite samples in liquid impingers with high capture efficiency (> 90%) in the mean aerodynamic diameter (MAD) range between 0.4 - 10μm (237).

Samples were processed for microscopic and molecular analyses to determine the quantities and phylogenetic signatures of the microbes present.

3.3.1 Airborne microbe quantitation

As determined by direct epifluorescent microscopy, the concentrations of bacteria, fungi and their spores in the floodwater and aerosol samples are summarized in Figure 3.2. Floodwater microbe concentrations were ten-fold or more over that observed in western Lake Pontchartrain, the designated background, and were similar to microbial numbers previously reported for partially treated domestic wastewater ($2x10^{11}$cells/L) (172). The bioaerosol loads measured above the Katrina flood zone were in the range between $1x10^5$ and $3x10^6$ cell/m^3; these levels were elevated above those reported for other undisturbed outdoor environments ($2x10^4$ - $1x10^5$cell/m^3) (142, 227) and were similar to those measured in another flood-impacted region (La Junta, CO, $3x10^5$ - $2x10^7$ cell/m^3) (73); these levels however, were markedly less than those reported from a Sonoran desert site flooded with partially treated domestic wastewater (Chihuahua, Mexico, $1x10^7$-$1x10^9$ cell/m^3) (172). Based on the data available from the aforementioned bioaerosol studies using similar sampling equipment and microscopic analyses, the airborne microbe concentrations observed here are in the range of other outdoor bioaerosol concentrations observed in the absence of measurable wind velocity.

3.3.2 Microbial composition and relative representation

DNA extracted from the collected samples was used as templates for PCR with both bacterial (8F/1391R) and universal (515F/1391R) primers.

Resultant PCR products then were processed such that 96 randomly selected clones were sequenced for each sample. 30 rRNA gene libraries were analyzed and 2100 sequences determined. While this sampling and analysis scheme did not have the statistical power to detect all phylotypes, it provided a robust survey of the more dominant taxonomic units. Figure 3.3 summarizes the phylogenetic distribution of rRNA sequences observed in the proximal air and water environments, to which nonparametric Chao1 analyses was applied to estimate richness (46) within the collected samples. Sequences were parsed into relatedness groups based on operational taxonomic units (OTUs) assigned to differentiate species at or above 97% sequence identity. As judged by this level of stringency, between 100 and 400 OTUs populated the air samples, where proximal water samples contained between 150 and 480 distinct OTUs. Chao1 analyses at this OTU level suggested that all but one of the 14 samples analyzed contained a range of bacterial diversity that was beyond the resolution of the clone libraries observed here. However, more than 90% of the sequences resolved were associated with 10 of the currently indexed bacterial phyla, and no sequences were associated known human pathogens.

3.3.3 Atmospheric - aquatic relationships

To assess potential relationships between local airborne and aquatic community structure, we compared their phylogenetic composition using accepted bioinformatics practices leveraging UniFrac analyses with an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) (137). These

were applied to quantify relationships between different OTU sets in a phylogenetic tree built solely from these greater New Orleans samples; an independent principal coordinates analysis (PCA) was then applied to expose salient environmental associations. Results of the UPGMA analysis summarized in Figure 3.4, showed a distinct separation between aerosol and proximal source water sequences, which was statistically supported by Jackknife permutations (c.a. 1000). In all cases, OTU clusters in aerosol samples were more similar to one another than to OTU clusters in proximal floodwaters. PCA converged on a complimentary result (Figure 3.4), which suggested that the phylogenetic variations observed could be explained by source — atmosphere or floodwater — and that some variation could be associated collection date (pre- or post- Hurricane Rita).

UniFrac analyses did not identify any significant correlations between the conglomerate phylogenetic compositions of the paired air and water samples; however, some phyla overlap suggested some sites may have had similar kinds of organisms. In order to assess this, we inspected the OTUs shared among samples using the SONS analysis package (212), which binned these sequence collections into OTUs. Figures 3.5 and 3.6 summarize SONS outcomes, showing that only 73 of the total 974 OTUs observed, were shared between the atmospheric and floodwater samples. Further, five of eight sites had less than 8% of their OTUs present any significant overlap in proximal air and water samples; the maximum overlap at any given site was approximately 18% of the total OTUs recovered. Closer

examination of the shared OTUs revealed that in most instances the shared sequences were not from the same sampling environment.

3.3.4 Regional aerosol communities

The relatedness between air samples, and lack of significant overlap between OTUs recovered from aerosols and nearby aerated/turbulent floodwaters prompted indications of an intraregional aerobiological population with respect to the near-ground level atmosphere. Thus, additional SONS and Unifrac analyses were conducted using genetic data from other rRNA-based outdoor bioaerosol studies in the US and Europe available from GenBank (17, 28, 76, 111, 142, 172). At a phylum level, many atmospheric environments ostensibly had similarities in their composition, with representatives of the Proteobacteria, Firmicutes, and Actinobacteria dominating these sequence databases (see URBA cluster in Figure 3.3 (top)). However, when scrutinized at species level (97% sequence ID), SONS analysis indicated few examples of shared diversity. UniFrac analyses applied to seek order among these atmospheric databases were inconclusive because of variability. Such variance between environments could be the result of many different factors including a relatively small numbers of sequences considered, metrological factors, and sample collection differences.

3.4 Discussion

The history of aerobiology has been dominated by industrial hygiene, and little is known about the kinds of organisms that are common to the

atmosphere on local, regional or continental scales (178). Aerobiology has traditionally focused on health concerns associated with indoor environments, with particular emphasis on airborne fungi and their spores (33, 89, 95, 108, 139), and there is a paucity of outdoor bioaerosol surveys. Until recently, aerobiology investigations have largely relied upon culture-based methods to describe airborne microbial populations, yet it is well established that culture methods are biased and insufficient for capturing the microbial diversity present in any environment (10, 171). Moreover, sampling of bioaerosols is difficult due to low cell densities and consequently large volumes of air must be collected (221), which can induce significant stresses and affect culture recovery. In recent years, reliable culture independent methods have been developed for the characterization and quantification of bioaerosols (9, 178), but these methods have yet to be widely adopted by the industrial hygiene community.

The total bioaerosol load observed in this study were an order of magnitude above those reported for quiescent rural environments (142, 227), but markedly less (100-300x) than those published for a windy agricultural site flooded with domestic wastewater (172). These findings indicate that water-to-air partitioning of microorganisms was not a major contributor to the bacterial bioaerosol loads under the circumstances reported here. Rather, the loads are characteristic of what are emerging to be consistent outdoor background levels in the absence of wind. This study provided the first observations of the greater New Orleans atmosphere and adds to the growing

but limited database available on the molecular ecology of outdoor environments.

As judged by modern phylogenetics, the microbial composition of floodwaters and the associated local atmospheric environments presented relatively limited diversity, where 10 phyla circumscribed more than 90% of the total sequences recovered.

Both the atmospheric environment and some floodwaters appeared to be significantly influenced by the passage of Hurricane Rita. The aerosol contents were markedly different for samples collected between hurricanes Katrina and Rita than those collected immediately after Rita. Prior to Hurricane Rita, representatives of the *Firmicutes* comprised 41% of the overall airborne ecology, mainly spore-forming *Bacillus spp*. and *Clostrium spp*., with α-*proteobacteria* representatives being the next largest group. Following Hurricane Rita, representatives of *Firmicutes* disappeared almost entirely and the α-*proteobacteria* representatives constituted 33% of the total atmospheric sequences. A similar division was also observed in the water samples. ε-*proteobacteria* representatives composed the largest group of organisms in the sampled water bodies prior to Hurricane Rita; however, after the storm event, the dominant phyla in the flood waters shifted to representatives of the *Firmicutes*. Of the ε-*proteobacteria* representatives observed, nearly half were members of the *Sulfurimonaceace* family, perhaps indicating that anoxic conditions dominated the sampled water bodies. *Firmicutes* observed in water samples following Hurricane Rita were primarily

Erysipelothrix spp., related to organisms cultured from a mangrove and an anoxic swine lagoon.

Recent studies suggest that the risk for airborne infectious disease outbreak is low following natural disasters, particularly for developed nations (77, 215). Sequences corresponding to *Propionibacterium acnes* and *Staphylococcus epidermidis* were the two most abundant potentially pathogenic organisms with greater than 97% BLAST ID. These findings are consistent with medical reports from the Katrina disaster: most acute medical cases observed in the first three weeks following Katrina were dermatologic in nature (14, 73). While incidences of upper respiratory infections and pneumonias were also reported, there were no major reported respiratory disease outbreaks (73), and a medical group reported that management of chronic illnesses made up most of their patient visits (59). From an exposure perspective, these findings indicate that floodwater reclamation practices, which aerosolize appreciable amounts of water, do not appear to significantly impact the local aerobiology under high humidity and the absence of wind. Such appurtenances, likely did not pose an elevated health risk to emergency response personnel in this environment.

Richness estimates applied to each sample determined that almost all environments were under sampled given the resolution offered by the clone libraries recovered. These richness findings were not unexpected given the dynamic environmental events and cleanup operations that took place during the sampling campaign, and the numbers of sequences recovered and

analyzed. Even so, the microbial compositions observed provide some understanding of the types and distribution of microorganisms that were present in these unique environments.

Results from community comparisons with UniFrac, that incorporated sequence data from another large aerosol study (28), showed that microbes in the New Orleans near ground atmosphere were more similar to an extra-regional aerosol environment than they were to their proximal source waters. A SONS community membership analysis of the post-Katrina air and water sequences revealed that less than 10% of species-level OTUs were shared between the two environments, and those in common were not from the same environment in most instances. These results suggest that the most abundant airborne microbes collected near operating remediation equipment were not aerosolized by remediation operations.

Disconnect between the dominant microbes found in the local atmosphere and perturbed floodwaters in this setting may indicate that the airborne microbes detected are part of a complex collection of organisms that may pervade near ground-level on a yet-to-be determined environmental scale. In the samples analyzed, any contribution to atmospheric biocomplexity from artificially generated aerosols appears minor, regardless of the tremendous energy inputs from mechanical aerators and pump-induced turbulence. Through a series of additional statistical analyses leveraging sequence data from all known airborne molecular ecology surveys, we observed that membership similarity among these atmospheric environments

was limited to a phylum level. Community structure analyses at more stringent phylogenetic levels reaffirmed that the membership similarity in genetically characterized atmospheric communities observed remains superficial. Variability between atmospheric environments was convoluted and could not be resolved. These conclusions are not surprising considering the dynamic nature of the atmosphere, sampling and analytical differences between this and other atmospheric molecular studies, and the finite data available for analyses. Additional long term molecular based aerosol studies are needed to resolve the environmental scales containing the airborne microbial biosphere.

Sample ID	Descriptive Location	Latitude	Longitude
LPK	Lake Pontchartrain	30.028333	57.501667
OCK	Orleans Canal Aerator	30.026944	59.501389
LAK	London Avenue Canal[a]	30.02175	9.081467
SCK	17th St. Canal at the levee Break	30.020278	89.501944
IHR	Inner Coastal Waterway near Paris Road	30.00475	89.9949683
ILR	Inner Coastal Waterway near Paris Road	30.0051	89.949267
ISR	Inner Coastal Waterway near Paris Road	30.005	40.498889
CGR	Inner Harbor Navigation Canal Coast Guard Station	29.96555	90.025917

Table 3.1 Site identification key and GPS coordinates of sample locations in the flooded City of New Orleans during September, 2005. Three letter acronyms identify each site and timing of sample collection. Sample designations ending with K, signify sampling date between hurricanes Katrina and Rita (September 18 - 22, 2005); sample designation ending with R signify sampling date within a week after Rita's landfall (September 23, 2005).

Figure 3.1 Map of New Orleans showing the sampled sites. Map courtesy of Google Earth. A single point is used to represent sites IHR, ILR, and ISR as they were taken at the same location.

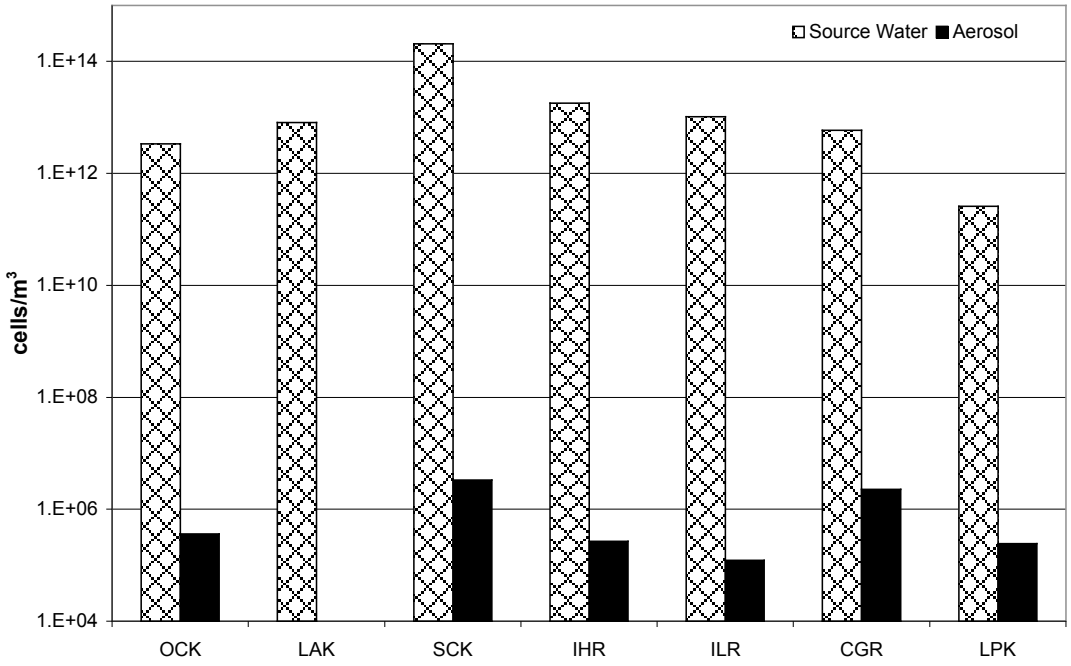


Figure 3.2 Direct microscopic count of bacteria, fungi and their spores in atmospheric samples and nearby aerated/turbulent floodwaters. No counts are available for the aerosol sample taken from LAK, as the entire sample was concentrated for sequencing analysis. Site LPK was designated to represent the background enviroment.

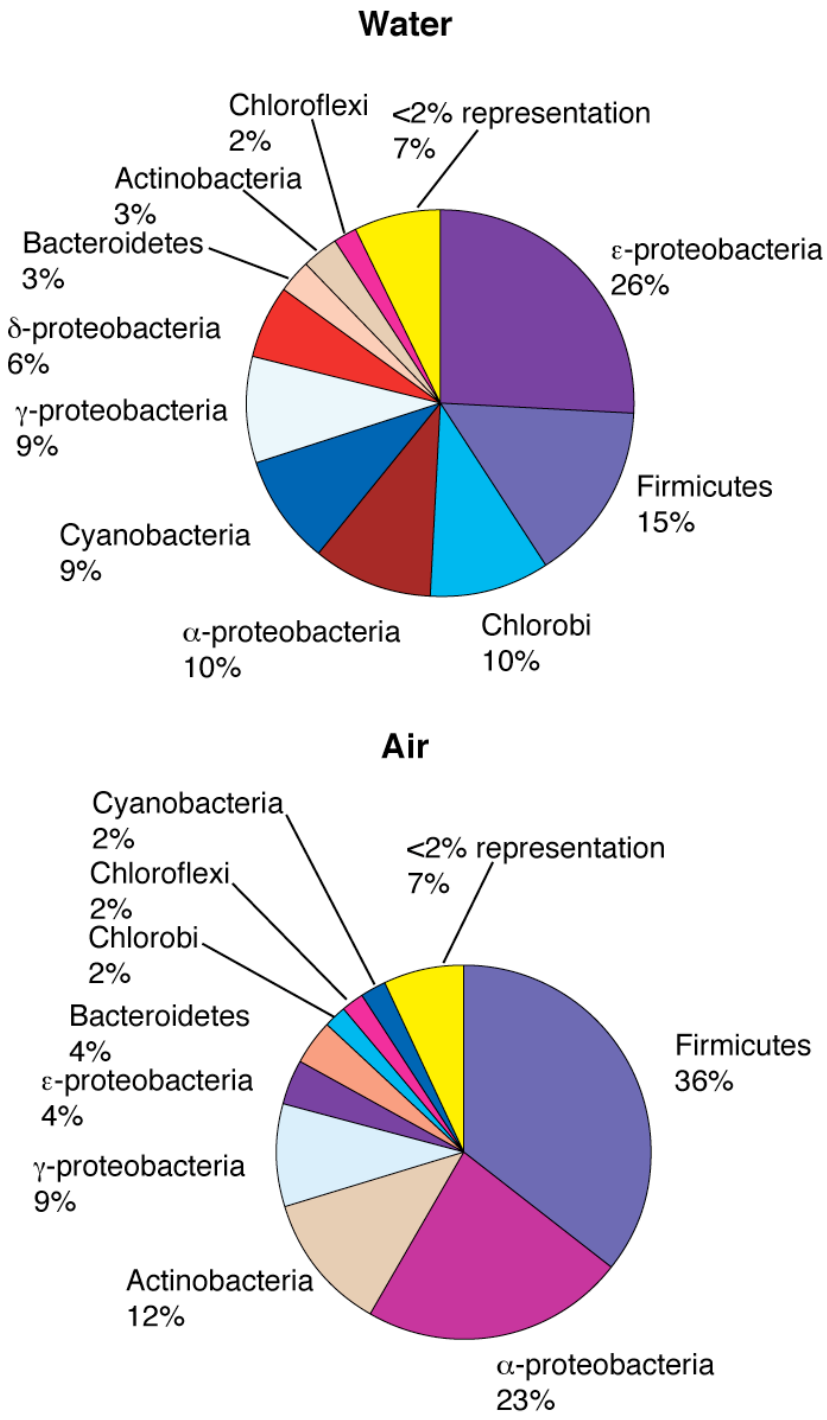


Figure 3.3 Phylogenetic distribution of rRNA sequences observed from pooled atmospheric samples and nearby aerated/turbulent floodwaters.

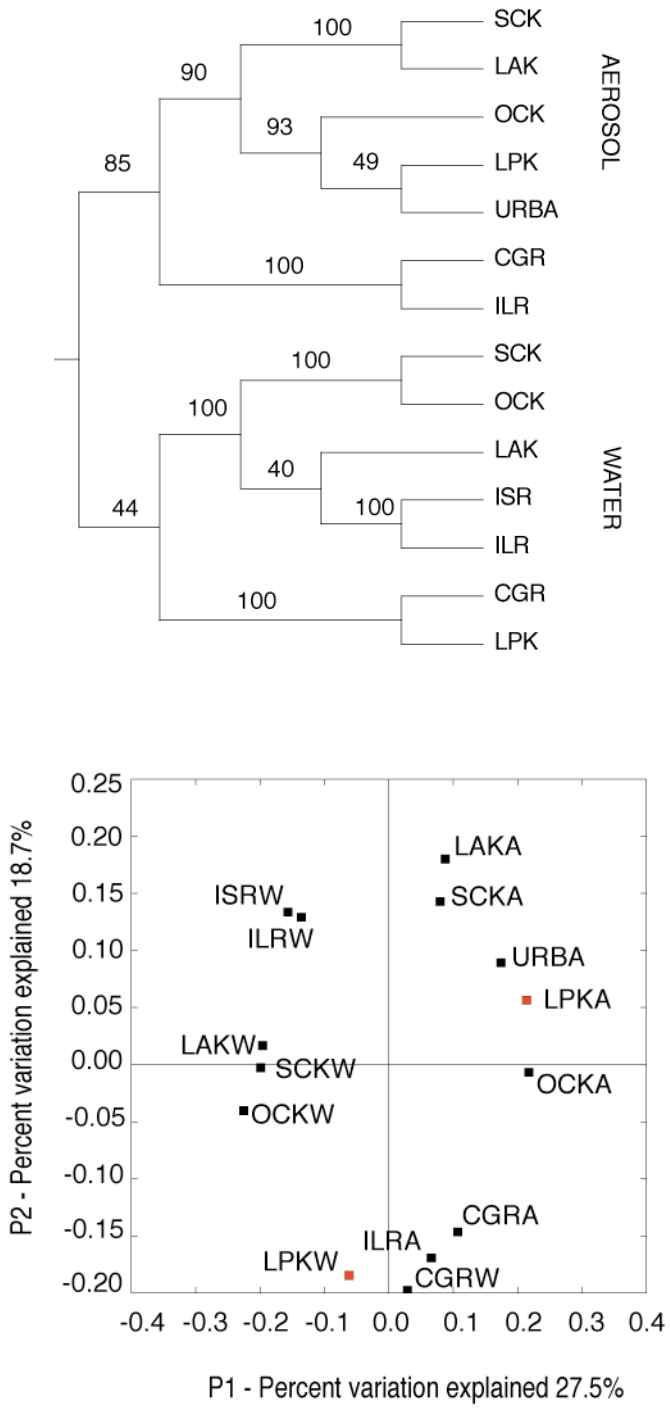


Figure 3.4 Acronyms identify each site and timing of sample collection. Sample designations including K, signify sampling date between hurricanes

Katrina and Rita; sample designation including R signifies sampling date within a week of Rita's landfall.

Final letter signifies sampling environment: designations ending with A, signify atmospheric sample; designations ending with W signify floodwater sample.

(Top) UPGMA distance matrix results for sequenced samples recovered from air and proximal floodwaters environments following 1000 jackknife iterations.

(Bottom) Principal Components Analysis (PCA); Component 1 (sample environment) is on the x-axis. Component 2 (sample date) is on the y-axis.

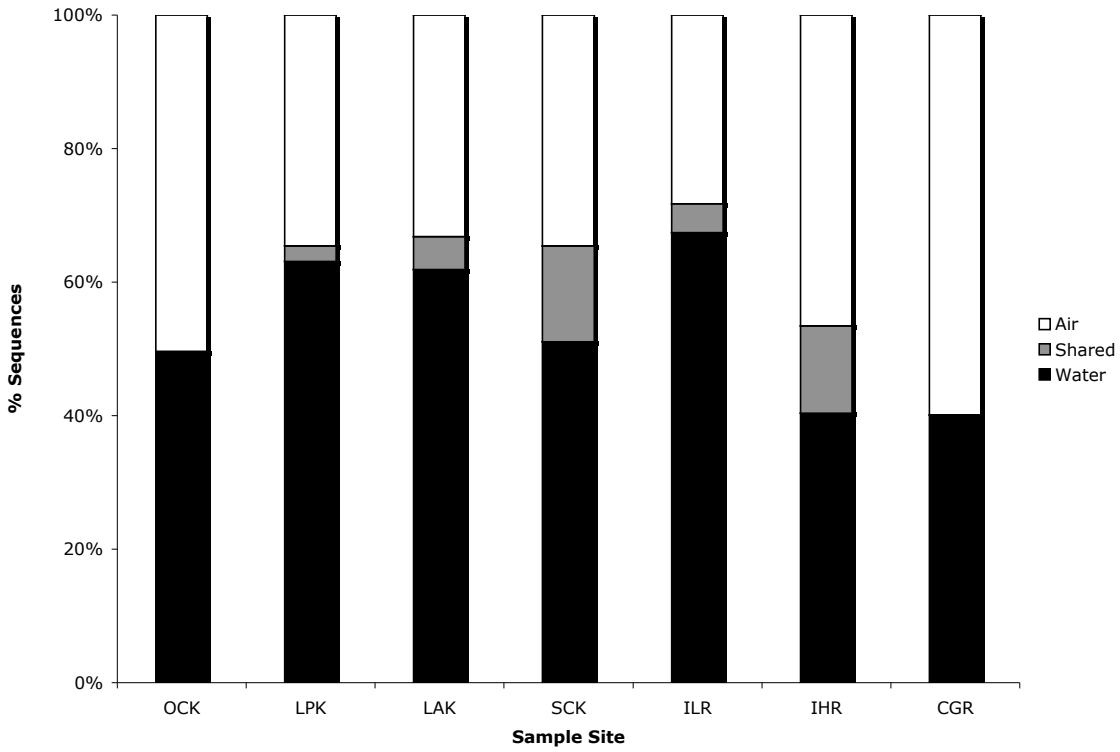


Figure 3.5 Percent distribution of OTUs (≥ 97% sequence identity) as judged by rRNA sequences observed in atmospheric samples and nearby aerated/turbulent floodwaters. A total of 954 distinct OTUs were observed in both the air and water environments. While 73 OTUs were shared between the two environments some sites had almost no overlap; other shared up to 18% overlap.

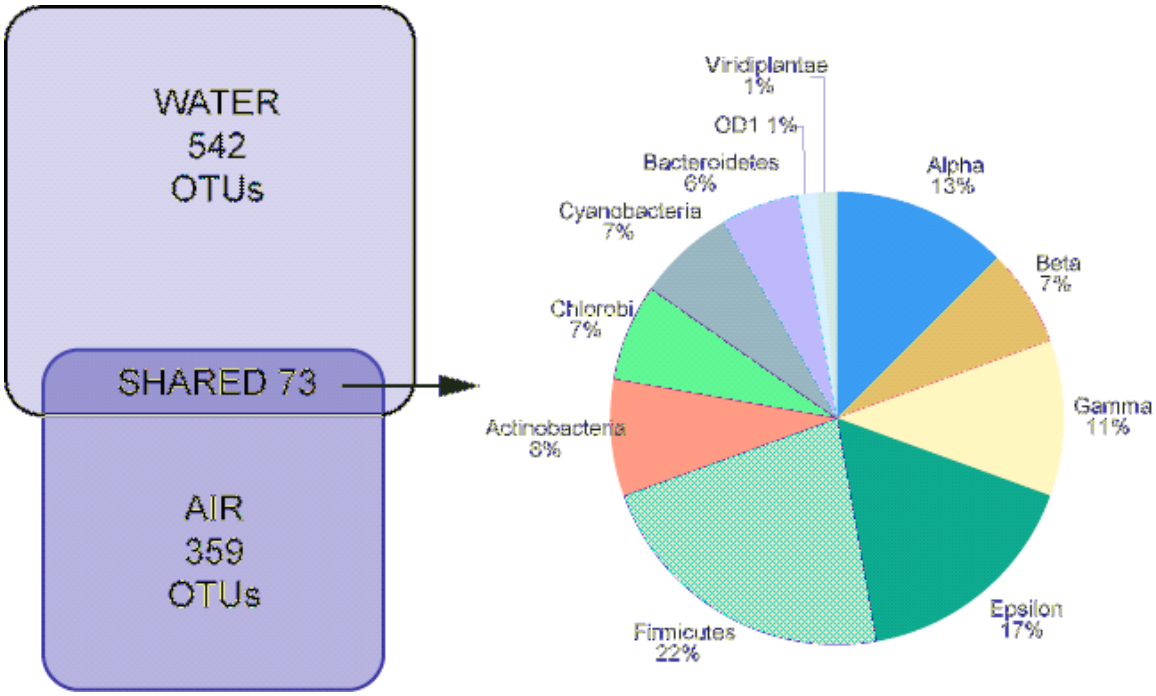


Figure 3.6 Sequence associations of the 73 shared OTUs (≥ 97% sequence identity) in atmospheric samples and nearby aerated/turbulent floodwaters.

CHAPTER 4

CULTURE-INDEPENDENT ANALYSIS OF BIOAEROSOLS IN CONCENTRATED ANIMAL FEEDING OPERATIONS (CAFOS)

Abstract

Respiratory illnesses are a significant cause of morbidity for individuals who work within concentrated animal feeding operations (CAFOs). Contact with high densities of livestock in confined spaces exposes CAFO workers to bioaerosols, which can induce acute and chronic respiratory diseases of largely unknown etiology. In response, we characterized the identity, distribution, and abundance of airborne microorganisms present in CAFOs using direct microscopy, broad-range rRNA PCR, and DNA sequence analysis of air samples collected from within and nearby swine and cattle operations in the western United States. Phylogenetic analyses were used to quantitatively assess the relatedness between indoor and outdoor samples as well as between swine and dairy operations.

We report here that indoor airborne microbial loads were not elevated above those immediately outdoors. Further, the ecology of these indoor environments was highly diverse as judged by Chao, ACE, Shannon and Simpson analyses. Members of phyla associated with animal gut microbiota including *Bacillaceae*, *Clostridiaceae,* and *Lachnospiraceae* appeared most frequently. We detected no respiratory pathogens of concern, but found the potentially pathogenic species *Aerococcus viridans* present in several

samples. Fungal species were not recovered in any of the indoor clone libraries.

4.1 Introduction

Since the end of the Second World War, the commercial livestock industry has undergone increased industrialization. The result of this modernization has been a decrease in the number of animal farms in the United States, but an increase in the numbers of animals being raised (226). Indoor confinement practices are commonly used by this industry to reduce labor costs and ensure consistent growth conditions and product quality. When animal population densities are relatively high, these environments are commonly referred to as CAFOs (concentrated animal feeding operations). As a result of indoor confinement practices, the livestock that are raised and the people who work with them are routinely exposed to relatively high concentrations of airborne microbes, dusts, gases, and endotoxins (98, 191, 213).

Modern CAFO employment is associated with increased incidences of multiple respiratory disorders, including asthma, chronic bronchitis, organic dust toxic syndrome, chronic obstructive pulmonary disease, rhinitis, hypersensitivity, hydrogen sulfide and carbon dioxide poisoning, and acute lower respiratory tract inflammation (49, 61, 211). To better understand the correlation between exposure and respiratory disease, many aerosol studies of swine and avian CAFOs have been conducted to establish dust, gas, and endotoxin exposure levels (16, 57, 163, 186, 188, 213). These studies have

been instrumental in understanding the contribution of these agents to CAFO-associated respiratory diseases. The airborne microbiology within CAFO environments is poorly understood because most observations of the aerobiology in these environments have been based on traditional culture analyses, which are known to isolate only a small fraction of the microbes present in a sample (10, 171). Typically these studies were limited to descriptions of mesophilic aerobic bacteria and/or fungi (45, 54, 57, 115, 120, 189) recovered from these environments.

This study employed culture independent methods to assess the airborne biological load and to describe the airborne microbial ecology within two types of CAFOs. The use of culture independent molecular methods for characterization of microbial populations potentially provides a broader perspective on the microbial diversity of CAFOs, particularly with respect to microbes that cause respiratory diseases. We report here that the indoor CAFO ecology was dominated by species associated with animal gut microbiota, but was highly diverse with 90% of species only being observed once or twice. The results of this study will provide a deeper description of the airborne microbial communities and bioaerosols loads in CAFO environments.

4.2 Materials and Methods

4.2.1 Aerosol sampling.

Aerosol samples were collected from one dairy and two swine facilities located in the Rocky Mountain region of the United States. Background air

samples were taken for all locations at least 5 miles away from each facility and upwind.

The dairy was privately owned family business with approximately 1000 dairy cows on the property. Several studs and many newborn calves were also on the premises in outdoor pens. All animals were the same breed. Cows were held in large outdoor (unenclosed) holding pens until milking time. The milking facility consisted of 24 stalls and was opened at one end for the animals to enter and exit. Average time spent by the cows within the building was 12 minutes. Sample collection took place at the closed end of the building and as near to the milking operations as possible. Following milking, the cows returned to their holding pens until their next milking session. A total of seven samples were collected at this facility and six were selected for sequencing analyses. Samples were collected at this facility during Spring 2007.

One swine facility (Swine A) was a private property with three types of barns - nursery, grower and finisher. Piglets arrived at the facility at 3 weeks of age and were housed in the nursery for five weeks. After this time, the animals were transferred to grower barns (a total of 3 were located on the facility) where they remained for five weeks. At the end of fifth week, the animals were moved to the finisher barns where they remained for 10 weeks. Animals at this facility were all female and all the same breed. Samples were collected from each type of barn. The nursery was divided into six rooms separated by doors and was entirely enclosed. Each room was

approximately 25’ wide by 32’ long. Fan units in each room were operated as needed to maintain a temperature of approximately 25^{o}C. The grower and finisher barns were approximately 50’ wide by 100’ long and contained 40 pens. Each pen held 10 to 12 pigs. These barns were partially enclosed with 0.6-meter gaps between the roof and walls to induce circulation, and a 0.3-meter gap between the roof slats. Manure pits were located under slated concrete floors in both barns. Sample collection took place as close to the center of each barn as was possible. A total of six samples were collected within the facility. All were samples were processed for sequencing analyses. Samples were collected at this facility during Summer 2007.

Another swine facility (Swine B) was owned and operated by a corporation. The sampled facility was one of many pig barns operated by the corporation and located in a 100 square mile area. Samples were collected from an entirely enclosed barn with 18 rooms (approximately 33’ wide by 60’ long) separated by doors. Pigs at this facility were all female and the same breed. The animals were brought to the building after leaving a farrowing barn and remained within the sampled barn for the duration of their lives. At the time of sampling the pigs were at the finishing stage (between 20-30 weeks old). Air circulation within the barn was controlled automatically by motors, which open and closed the barn walls to maintain a temperature of approximately 25^{o}C with each room. Samples were collected at one end of the barn. A total of four samples were collected within the facility. All were

samples were processed for sequencing analyses. Samples were collected at this facility in Fall 2007.

Aerosol samples were concurrently collected with SKC Biosamplers (BioSampler SKC Inc., Eighty Four, PA) and an Omni 3000 Portable Sampler (Sceptor Industries, Inc., Kansas City, MO). SKC Biosamplers were positioned approximately 1 meter above the ground. The Omni was stationed 1.8 meters above ground. The collection medium in the SKC samplers was sterile 1X Tris EDTA (Thermo Fisher Scientific, Pittsburg, PA). SKC Biosamplers were operated at a flow rate of 12.5 L/min. Two impingers were run in parallel for each sampling site. The impingers were allowed to collect continuously for at least 30 minutes at each sampling site. To maintain appropriate fluid levels within the impinger reservoir, sterile water was added to the Biosamplers to compensate for any notable evaporative losses. At the end of each sampling event, the collection fluids from each SKC Biosampler were combined into one sterile 50mL conical vial and then placed on ice until they could be processed, which was typically less than 8 hours. The Omni was run in parallel with the SKC Biosamplers and operated at a flow rate of 270 L/min. The collection medium in the Omni was sterile 1X PBS supplemented with 0.005% Tween (Thermo Fisher Scientific, Pittsburg, PA). Samples were collected in 5-minute intervals with multiple samples being collected during a 30-minute period. To maintain appropriate fluid levels within the Omni collection device, sterile water (Thermo Fisher Scientific, Pittsburg, PA) was added to the system at regular intervals. At the end of

each sampling event, the collection cartridge was removed and placed on ice until the sample could be processed with the SKC samples.

All samples were processed and archived in the laboratory facilities at the University of Colorado, Boulder. Impinger samples were aliquoted as follows: 10mL for direct microscopy analysis preserved with 2% (v/v) formaldehyde, and 30mL for molecular analysis concentrated and dried on 0.2 micron nitrocellulose filters (100mL) (Nalgene Analytical Test Filter Funnel, Nalge Nunc International Rochester, NY). Omni samples were processed in the same manner as impinger samples with the following aliquots: 1mL for direct microscopy and 8mL for molecular analysis. Processed samples were stored at 4^{o}C (direct count samples) or –80^{o}C (DNA samples) until analyzed.

4.2.2 Airborne microbe enumeration

Aerosol samples were stained and enumerated using 4'6-diamidino-2-phenylindole (DAPI) (Sigma Chemicals, St. Louis, MO) in accordance with previously described methods (102). DAPI is a nonspecific fluorescent DNA intercalating agent that provides a suitable background for direct microscopy. Samples were incubated with DAPI (20 mg/mL) for 5 minutes at 4^{o}C and then filtered through a 25mm diameter, 0.22micron (average pore size) black polycarbonate filter (Poretics, Inc. Livermore, CA). Filters were then mounted in low fluorescence immersion oil containing an antifadent (CitiFluor, Leiscester, UK) and examined under 1100x magnification using a Nikon Eclipse E400 epifluorescence microscope fitted with a mercury lamp and

polarizing filters. A minimum of 10 random fields was counted per slide and only intact, brightly stained cells with obvious bacterial or fungal morphology were counted. Direct counts were reported as the average of all fields counted; in all cases the coefficient of variation observed was less than 20% (102).

4.2.3 Genomic DNA extraction

Concentrated samples and negative controls were extracted from filters using a bead beating protocol modified from Frank et al (80). Extracted DNA was resuspended using 20 microliters of Tris-Ethylenediamine Tetraacetic Acid (TE).

4.2.4 PCR of rRNA genes

Extracted DNA was assayed using universally conserved (515F/1391Rev) primers and HotMasterMix® (Eppendorf North America, Inc., Westbury, NY). A typical 20microliter PCR reaction included: 8 microliters 2X HotMasterMix, 0.5 microliters Forward Primer (25ng/ml), 0.5 microliters Reverse Primer (25ng/ml), 8 microliters water, 2 microliters bovine serum albumin (10 microgram/ml), and 1 microliter DNA template. Assays were performed on an Eppendorf Mastercycler® (Eppendorf North America, Inc., Westbury, NY). The amplification protocol was as follows: 94^{o}C for 2 minutes, 94^{o}C for 20 seconds, 52^{o}C for 20 seconds, 65^{o}C for 90 seconds, 30 cycles total. PCR controls with no DNA template were included with every assay. Following PCR, all samples were run on a 1% (w/v) agrose gel for confirmation. Amplified samples were identified and then assayed in

quadruplicate. Extraction and negative controls never showed evidence of amplification.

4.2.5 Clone library construction

One clone library, a total of at least 96 randomly selected rRNA clones, was constructed for each sample. Amplicons from four independent PCR reactions were pooled and purified for each sample using agarose gel electrophoresis and a Montage® DNA Gel Extraction Kit (Milipore, Billerica, MA). Purified product was then ligated into pCR4.0 TOPO plasmid vectors and transformed using Top10 electro competent cells (Invitrogen Corporation, Carlsbad, CA). Transformed cells were plated and grown overnight on Luria-Bertani (LB) agar. For each library, a total of 96 clones were picked at random, individually placed in 10 ml of 1X TE and heated at 85^{o}C for 10 minutes. Following thermal lysis, samples were centrifuged at 4000G for ten 10 minutes to pellet cellular debris.

4.2.6 DNA sequencing

Sequencing was performed as previously described by Papineau et al (173). Briefly, sequencing template was prepared from rRNA clones by PCR amplification with T3 and T7 vector primers, and treated with ExoSAP-IT (GE Healthcare, Piscataway, NJ). Templates were sequenced with the DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare, Piscataway, NJ) on a Mega-BACE 1000 DNA Sequencer using T3 and T7 primers. Base calling and assembly of raw sequence data were performed with the PHRED and PHRAP software packages (72), using the software XplorSeq (79).

4.2.7 Phylogenetic analysis

The sequence collection was screened for chimeric sequences with the Bellerophon software package (110). The closest known relative of each rRNA clone was determined by comparison of its sequence to all rRNA sequences in GenBank by BLAST (8). Sequences were aligned with the NAST software package (62) and their phylogenetic positions were determined by analysis with the ARB software package (138) and a curated ARB database and tree with ~100,000 rRNA sequences (≥ 1250 nucleotides) in length from the NAST project (62). Sequences were added to the ARB tree by parsimony insertion. Sequences were clustered into operational taxonomic units (OTUs) at a 97% sequence identity cutoff using the computer program sortx (79).

4.2.8 Richness estimates

Sample coverage and species richness estimate were calculated using biodiv (79). The program calculates species richness estimates (Chao and ACE) and diversity indicies (Simpson, Goods, Shannon) based on a user defined sequence identity level. Estimates were calculated separately for each environment.

Sequence libraries were compared using UniFrac and SONS (137, 212). UniFrac compares related sets of sequences (samples) using phylogenetic and calculates a distance metric based on the fraction of the total distance in the tree that is unique to a particular sample. The SONS

program compares the membership and structure of two communities at a specified phylogenetic level.

4.3 Results

4.3.1 Aerosol microbial concentrations

Total airborne microorganism concentrations collected during the sampling campaigns are presented in Figure 4.1. Results are organized by environment type (indoor, outdoor, swine, dairy) and sampling location within the CAFO. The airborne microbe concentrations observed within the different CAFOs were in the range between $6x10^5$-$2x10^7$ cells/m^3 and their corresponding outdoor environments were in the range between $2x10^6$-$3x10^6$ cells/m^3. These observations were similar to those previously reported bioaerosol loads ($1x10^6$-$7x10^7$ cells/m^3) from other industrial animal farming environments measured using similar microscopic methods (5, 48, 161).

4.3.2 Phylogenetic diversity of sampled environments

DNAs extracted from the collected samples and used as templates for PCR with universal rRNA primers (515F/1391R) produced PCR products, which were successfully cloned; and at least 96 were randomly selected and sequenced for each sample. A total of 17 rRNA gene libraries were analyzed and 1451 sequences determined.

The majority of sequences had a high similarity to existing sequences in the GenBank database. A total of 157 sequences (11% of total sequences) had less than 95% sequence similarity to any genetic catalogues and these sequences were subsequently checked for chimeras. Of these sequences,

41 were found to be chimeras and were removed from further analyses. Approximately 90% of the sequences had similarities greater than 97% to known species.

Analysis of the sequence data at a level of 97% sequence similarity (87) identified 758 different operational taxonomic units (OTUs). The number and distribution of these OTUs is shown in Figure 4.2. The most frequently occurring OTUs from all environments were from an uncultured rumen *Clostridium sp*. (n=86), *Streptococcus alacloyticus* (n=62), *Lactobacillus acidophilus* (n=41), and an unclassified *Clostridiaceae* from the human gut (n=29). Diversity estimates and indices (Chao, ACE, Shannon, and Simpson) suggest that the dairy parlor samples had the highest species richness of the indoor environments followed by the grower and finisher barns (624-1762 OTUs as predicted by Chao). Outdoor environments presented markedly lower richness (197-592 OTUs as predicted by Chao). Sample coverage, defined as number of OTUs observed divided by number of predicted OTUs, for all environments ranged between 7 and 21%, indicating that much biodiversity remains uncharacterized in these environments. A summary of the clone library characteristics is presented in Table 4.1. Rarefaction curves for pooled samples of the CAFO and outdoor environments are shown in Figure 4.3.

4.3.3 Microbial composition of the CAFO environments

The phylogenetic distribution of sequences recovered from the indoor CAFO air and their corresponding outdoor environments is shown in Figure

4.4. Table 4.2 lists in detail the families present and their abundance within the observed phyla for each environment sampled. Outdoor clone data is presented as a composite. Nearly 80% of all sequences cloned from the CAFO indoor environments matched members of the *Firmicutes* phylum. Twenty one percent of these sequences had a high similarity (>97%) to sequences of animal or human gut clones. An additional 6% of the sequences were closely related to human microbiota species - a subgingival or a vaginal epithelium clone. The remaining *Firmicutes* sequences were diverse with many observed only once. The potentially pathogenic species *Aerrococcoccus viridans* was observed a total of 13 times and in all environments, except the swine nursery.

Other notable phyla observed within the CAFO environments included the *Bacteroidetes*, *Proteobacteria* and *Methanobacteria*. Representatives of the *Bacteroidetes* comprised the second most abundant phylum (9%) observed indoors. Most sequences (67%) within this phylum belonged to the *Prevotellaceae* family and were closely related to microorganisms previously detected in the human gut. *Proteobacteria* composed 5% of the total sequences observed with *Gammaproteobacteria* being the largest phylum. The *Gammaproteobacteria* observed were primarily from indoor dairy air and were related to microbes from a wide variety of environments. Members of the Archaeal class *Methanobacteria* were observed in all three indoor CAFO environments and contributed 3% to the total sequences recovered; these were closely related to rumen gut clones.

The airborne ecology of the respective outdoor environments was collectively more diverse than the CAFOs, with no phylum containing more than 26% of the total sequences. Groups with the largest representation in the outdoor samples were *Actinobacteria* (26%), *Firmicutes* (26%), *Bacteroidetes* (19%), and *Proteobacteria* (15%). The majority of sequences observed in these phyla occurred just once; however there were a few instances where sequences in these phyla were observed in clusters (>10 clones). The *Actinobacteria* phylum had two such groups - the first related to a soil clone from Antarctica, *Anthrobacter sp*. P1 and the second block related to a *Propionibacterium acnes* species isolated from a deep sea. The *Bacteroidetes* phylum was represented by sequence group related to a *Saprospirales sp.* that was recently cloned from the outdoor air of an urban environment. No large sequence groups were observed within the *Firmicutes* and the *Proteobacteria* phyla.

4.3.4 Community Comparisons

We analyzed relationships among sequences recovered from indoor CAFO environments and immediately outdoors using UniFrac (137) and SONS (212) analyses. The UniFrac distance quantifies phylogenetic relationships between sets of taxa in a phylogenetic tree. Pair-wise comparisons between our samples were made using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and the Principal Coordinates Analysis (PCA). Results of these of analyses are shown in Figures 4.5 and 4.6.

The 16S RNA sequences clustered into three distinct groups, which were statistically supported by Jackknife permutations. As judged by UPGMA (Figure 4.5), airborne microbes recovered from indoors were markedly different than those recovered outdoors, regardless of the animals being housed. Airborne microbes were distinctly different inside the dairy and swine houses observed. Within the swine houses, significant differences in airborne microbe populations could be associated with housing practices. When this genetic catalogue was subject to PCA (Figure 4.6), the variation observed was best explained by aerosol sample source – indoor, outdoor, or animal association (56%) – followed by animal housing differences (13%).

A SONS analysis was used to determine the fraction and richness of OTUs shared among the environments sampled. This program bins sequences into collections based on identity and quantifies the OTUs shared between environments as well as provides the frequency with which OTUs were observed in each environment. Our analysis was conducted at the 97% sequence ID level and results are summarized in Figure 4.7. At this level of stringency, the results suggest limited overlap in the types of microbes recovered from these environments. When comparing outdoor and indoor environments less than 10% of OTUs were shared either on a composite or individual site basis. The largest numbers of shared OTUs was observed in the Swine Facility B, which was a single enclosed building where all the animals were nearly at the same age. A comparison of the OTUs observed

indoors at the different swine facilities showed few species in common (<10%).

4.4 Discussion

Our study investigated the airborne biological load and the microbial diversity of three representative animal confinement facilities. Total microbial load was assessed using a nonspecific DNA intercalating stain with direct microscopy. The microbial ecology associated with airborne particulate matter recovered within these buildings was evaluated using broad-spectrum rRNA gene PCR, sequencing, and phylogenetic analyses. To the best of our knowledge, this is the most comprehensive study of the airborne ecology within animal confinement buildings to date.

Total airborne microbe concentrations observed in all three facilities were similar to each other as were their corresponding outdoor environments. Loads within both swine facilities were comparable to those reported in previous studies using similar counting methods (5, 48, 161). Significant differences in total airborne microbial loads measured at Swine Facility A may be attributed to animal density within the sampled barns as well as local weather. The grower barn housed three times as many pigs as the sampled nursery room, which may explain why airborne microbe concentrations in the nursery were markedly lower. Heavy rains fell during the indoor sampling of the finisher barn, which may have decreased the total microbial load in the air, since it was not completely enclosed, such that the rain fell down the center of the barn where the samplers were positioned. With the exception of

the grower barn, the loads measured at Swine Facility A were statistically indistinguishable as those measured for the outdoor environment.

There are no reports of airborne microbial concentrations in dairies other than those based on culture. The total airborne microbial load observed in this study was greater (10-1000x) than those levels previously reported for airborne fungi (3, 129). Overall, there are relatively few published reports available on the total (both viable and nonviable) numbers of airborne microorganisms present within CAFO environments. The present study adds to this limited body of literature.

Until very recently, CAFO aerobiology has been primarily characterized with culture based techniques with particular emphasis on those species that grow best under mesophilic and aerobic conditions (45, 54, 57, 115, 120, 189). The results of this study suggest that the microbial diversity aloft within swine and dairy CAFO environments is substantially greater than that previously recovered by culture. More than 750 OTUs were observed among the 1451 sequences recovered with nearly 90% of these OTUs observed only once or twice. Diversity estimates (ACE and Chao) predicted large numbers of species (between 350-1762) present in the CAFO air. The most diverse environments in this sampling campaign, measured by both Shannon and Simpson diversity indices, was predicted in those environments where some mixing with outdoor air took place, such as the dairy parlor and the swine grower and finisher barns. Based on these catalogue index estimates and the number of OTUs observed, the estimated clone library coverage was only

between 7-21% of what could have been recovered. These findings indicate that the airborne ecology of the aerosols in these CAFO environments remains largely unknown and would be a good candidate for composite, longitudinal sampling campaigns which leverage deep sequencing analyses.

The most frequently observed species in this study were primarily associated with nonpathogenic gut microorganisms, such as rumen *Clostridium sp*. (n=86), *Streptococcus alacloyticus* (n=62), *Lactobacillus acidophilus* (n= 41), and *Clostridiaceae* recovered from humans (n=29). Sequences closely related to the pathogenic species *Aerococcus viridans* were found in all environments (n=13), except the nursery. *A. viridans* is opportunistically pathogenic for humans and swine and has been isolated from aerosol samples in previous studies (118, 238). In humans, this species has been associated with urinary tract infections, arthritis, endocarditis, and meningitis (74, 88, 160, 187). In swine, the species has been associated with arthritis (145). Sequences corresponding to *Propionibacterium acnes*, a common human commensal, also were recovered from all environments (n=17). While *P. acnes* is often considered harmless, it has been associated with corneal ulcers, acne, endocarditis, cholesterol gallstones among other illness (31). The species appears to be ubiquitous and has been observed in other outdoor aerosol studies (Rodriguez de Evgrafov, unpublished data) (29, 228).

Members of the *Firmicutes* phylum dominated the bacterial flora within the CAFO environments. The major phylogenetic lineages that were

observed within this phylum were *Bacilli* (33%), *Lachnospiraceae* (30%), and *Clostridiaceae* (20%). The relative abundance of phylotypes (*Aerococcus sp*., *Lactobacillus sp*., *Streptococcus sp*., and *Clostridium sp*.) observed in both swine facilities was similar to that recovered from swine gastrointestinal tracks (130, 218) and reported in previous culture based bioaerosol studies of swine confinement buildings (54, 57, 161, 189). The lineages observed in the dairy samples was similar to those found in the feces of dairy cows (65) and cultured in a previous bioaerosol study of cattle feedlots (239). Specific species and their proportions however differed among the three dairy studies. Presumably then, the airborne bacteria originated from aerosolization of manure.

There were no significant amounts of fungi recovered in this sampling campaign, while in previous animal confinement bioaerosol studies fungi contributed a sizable portion to the overall observed (via culture) airborne ecology (3, 4, 45, 48, 96, 115, 120, 129). These culture based studies recovered orders of magnitude less fungi than bacteria on broad spectrum media. The universal primers used in this study are able to readily amplify fungal DNA in other environments, so specificity is not an issue. Rather the apparent discrepancy in our study likely was due both to amplification bias of smaller bacterial amplicons and the overabundance of bacteria relative to fungi in these particular environments. Although use of fungi specific primers could resolve this issue, this was not pursued because our primary interest was in surveying the most abundant microorganisms in CAFOs.

Statistical community comparisons showed that the three CAFO environments were distinctly different from one another as well as their corresponding background environments in terms of microbial community composition. Community membership analysis at the species level showed that few OTUs were shared among and within the environments. This finding and the Chao analyses may indicate that sample size is not large enough for overlap to be observed given the resolution potential of the clone libraries built. Because the majority of species observed in this study were related to gut microorganisms, the limited number of OTUs shared between environments probably reflects the complex nature of these ecosystems.

In many published studies of CAFO airborne microbial communities, culture methods have been used to characterize the observed ecology. Our results provide the first in-depth culture independent characterization of the airborne microbial ecology within a CAFO environment. The results of this study illustrate that the potential microbial diversity of CAFO environments is greater than what has been previously reported. To date our knowledge about the types and quantities of microorganisms found within this environment has been limited to a few specific genera. Considering the potential health ramifications associated with CAFO work exposure as well as the potential microbial ecology present, CAFO environments should be considered for pyrosequencing analyses.

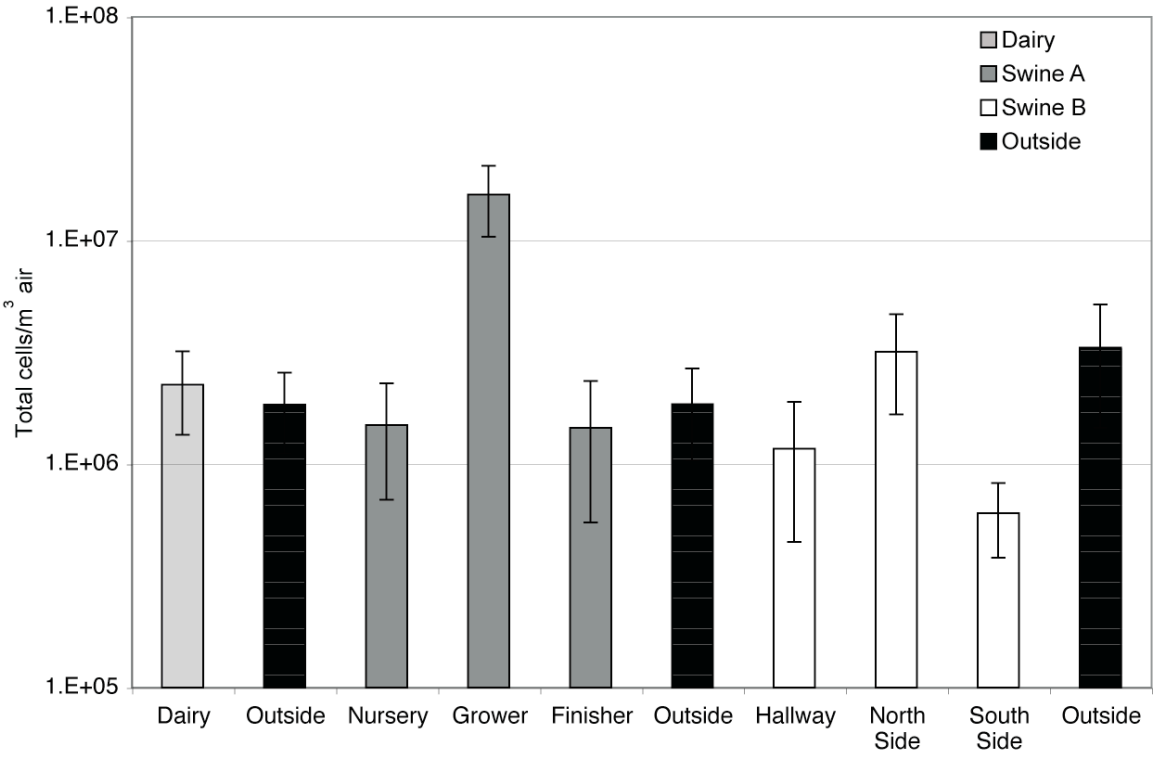


Figure 4.1 Total microbial loads for the CAFOs and the outdoor environments.

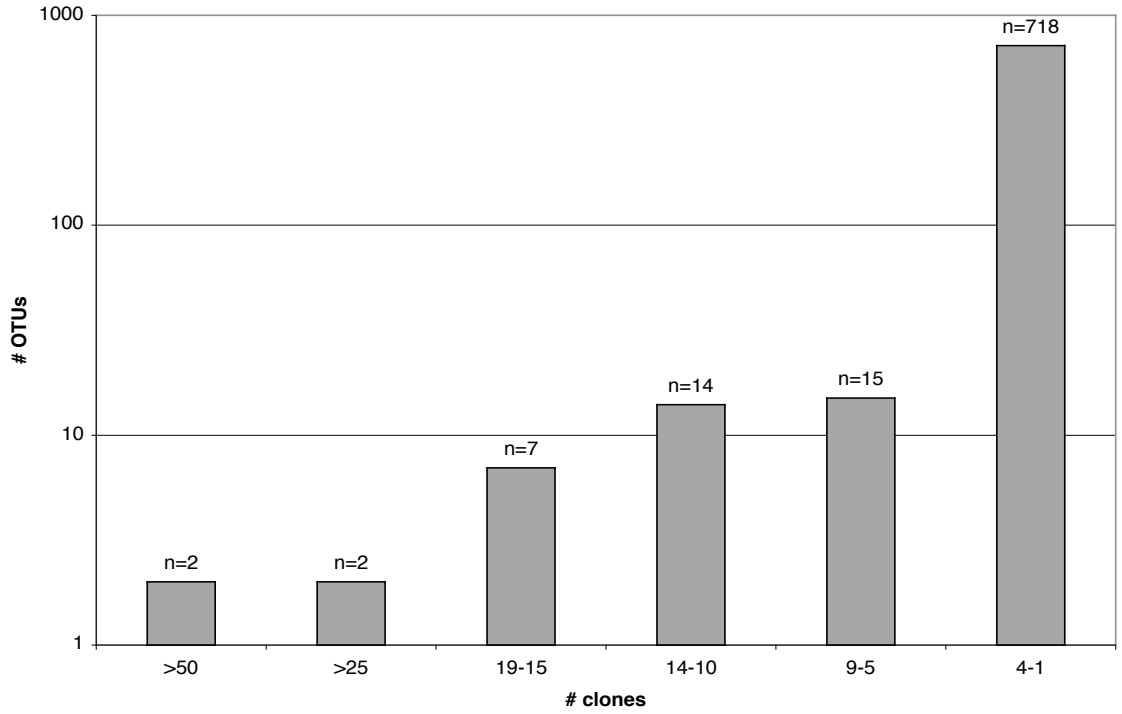


Figure 4.2 Abundance and distribution of OTUs in all clone libraries. Sequences were placed into OTU bins based on the 97% sequence identity similarity criterion. The number atop of each column signifies the number of OTU bins represented in the column.

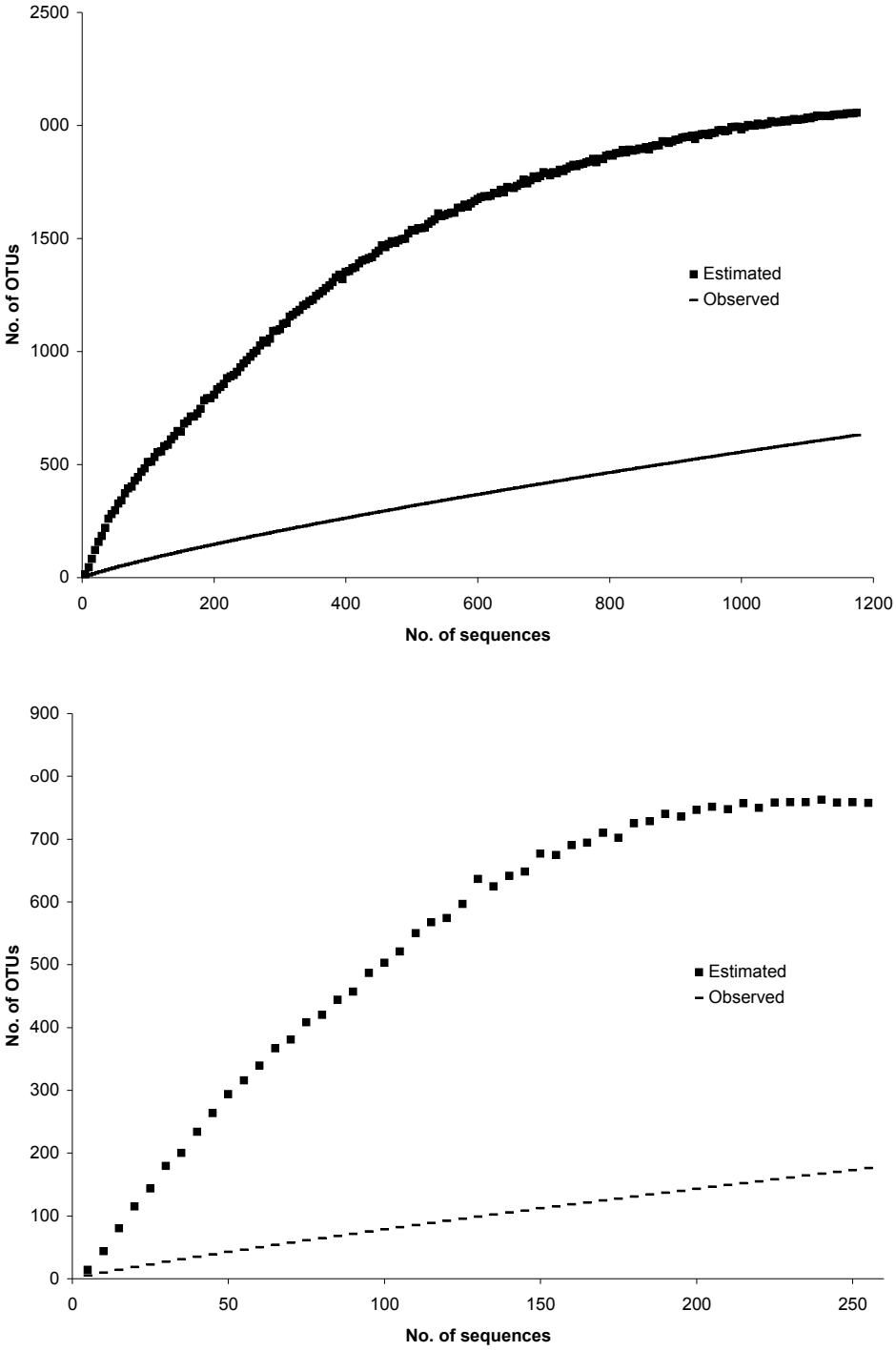


Figure 4.3 Rarefaction estimates for the CAFOs (A) and their corresponding outdoor environments (B). Curves reflect the estimated and observed OTU richness and the number of sequences sampled in pooled datasets at the 97% sequence identity level.

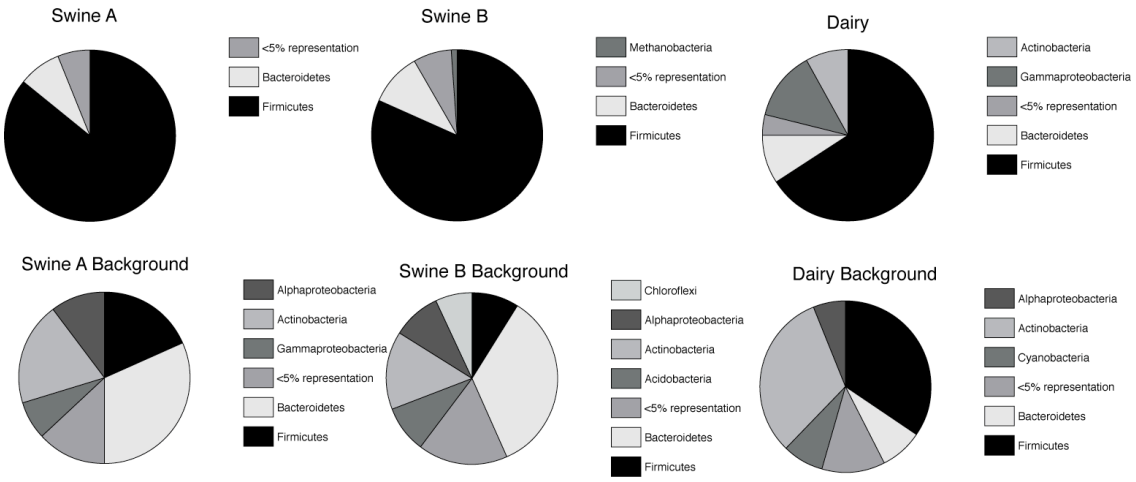


Figure 4.4 The phylogenetic distribution of sequences recovered from the indoor CAFO air and their corresponding outdoor environments.

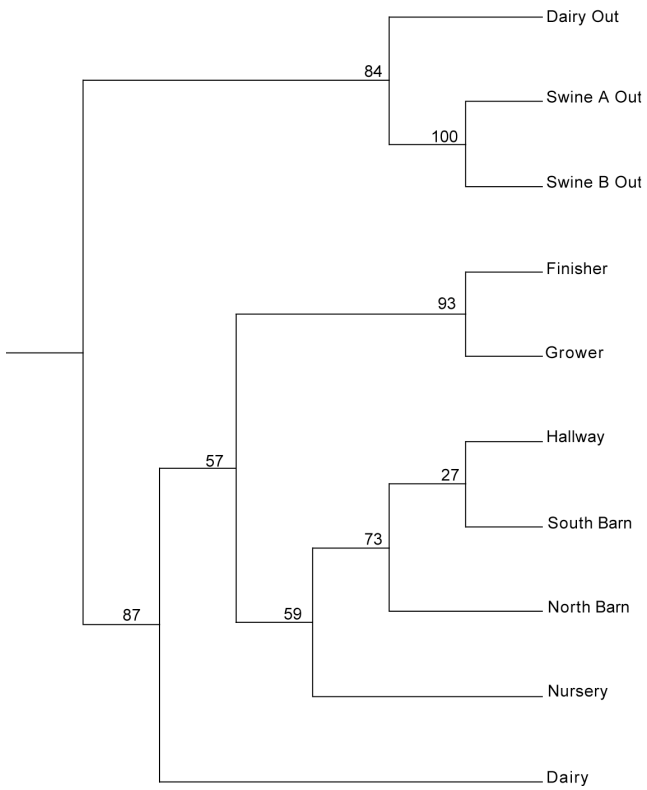


Figure 4.5 UPGMA and Jackknife results for all sampled environments. Jackknife values reflect 100 permutations. Nursery, Grower and Finisher are the sampled sites from Swine Facility A. Hallway, South Barn and North Barn are the sampled sites from Swine Facility B.

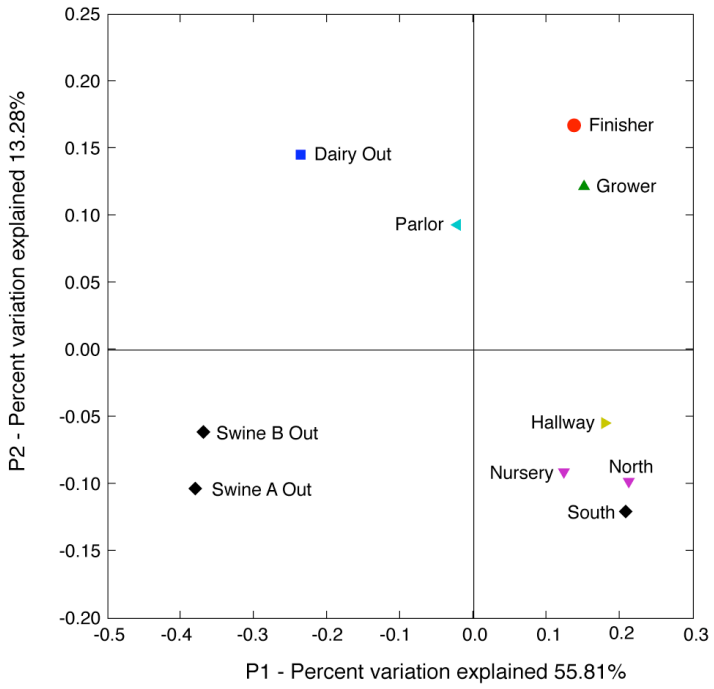


Figure 4.6 Principal coordinates analysis of all sampled environments. The first principal component (PI) represents indoor/outdoor environment. The second component (P2) represents animal housing conditions (complete enclosure vs partial).

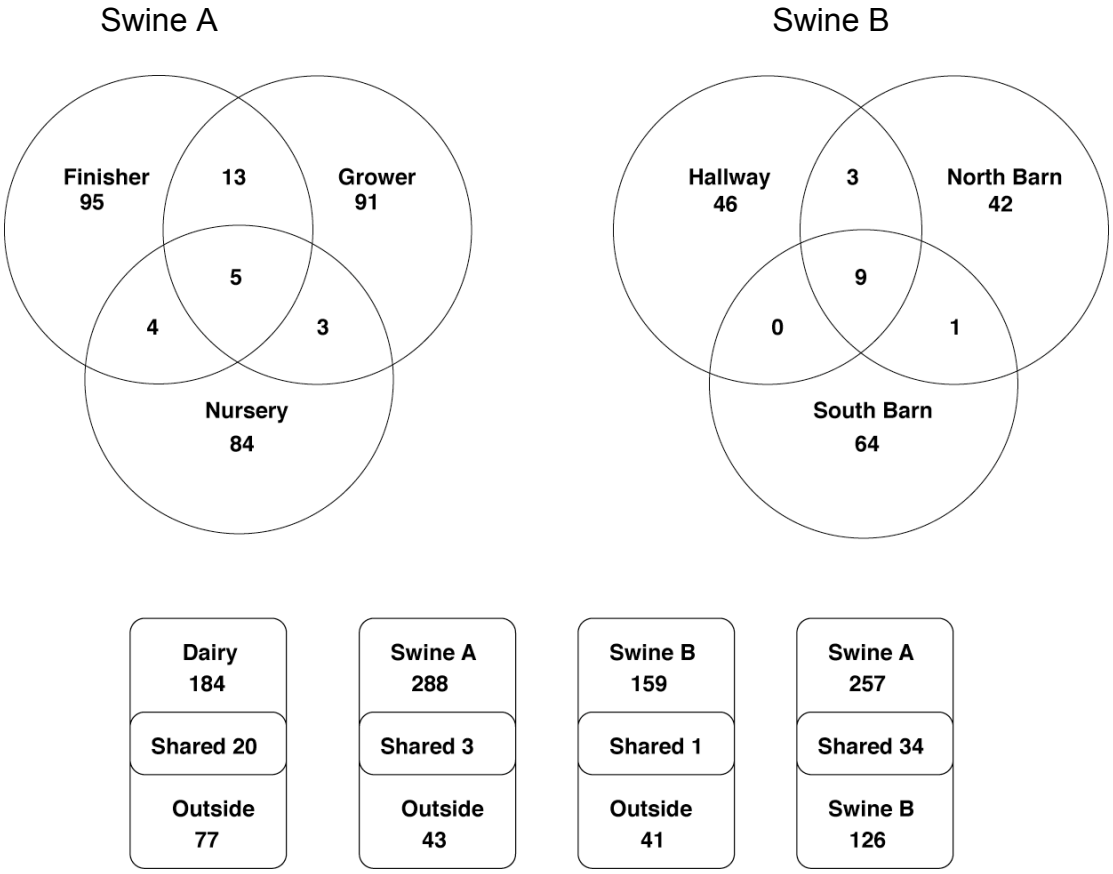


Figure 4.7 Community membership results at 97% sequence identity level.

Environment	No. of Libraries	No. of Sequences	No. of OTUs	Chao1	ACE	Chao Coverage (%)	ACE Coverage (%)	Shannon
Swine A Out	1	67	46	197	318	23	14	5.81
Finisher	2	188	117	624	839	19	14	6.95
Grower	2	188	112	1258	1217	9	9	6.92
Nursery	2	164	96	375	463	26	21	6.91
Swine B Out	1	46	42	313	388	13	11	5.20
Hallway	1	85	58	608	719	10	8	5.81
North Barn	1	94	54	350	451	15	12	5.98
South Barn	1	91	73	469	991	16	7	6.00
Dairy Out	2	144	97	592	708	16	14	6.56
Parlor	4	366	204	1762	1626	12	13	7.75
Total	17	1433						

Table 4.1 Characteristics of all clone libraries.

Bacterial Families	No. of Clones	Dairy	Nursery	Grower	Finisher	Hall	North Barn	South Barn	Outdoors
Firmicutes	953								
Acidaminococcaceae	32	1	8	4	4	6	3	5	1
Aerococcaceae	82	46		6	15	3	2	1	9
Bacillaceae	3	1							2
Carnobacteriaceae	5	3							2
Clostridiaceae	184	17	7	65	68	11	7	7	2
Enterococcaceae	1		1						
Lachnospiraceae	272	93	62	35	26	13	15	24	4
Lactobacillaceae	98	4	35	26	9	4	12	8	
Mollicutes	19	9	2	1	4	2		1	
Paenibacillaceae	3	1							2
Peptostreptococcaceae	104	39	1	17	22	4	5	3	13
Planococcaceae	26	11				1		1	13
Sporolactobacillaceae	1								1
Staphylococcaceae	20	7	3			1			9
Streptococcaceae	88	1	4	6	8	22	29	17	1
Unclassified Lactobacillales	6		3	1	1				1
Uncultured Firmicutes	6	4							2
Unclassified Firmicutes	3	1			1				1
Bacteroidetes	150								
Algoriphagaceae	1								1
Bacteroidaceae	12	11	1						
Flavobacteriaceae	13	2	3	7					1
Flexibacteraceae	7	4							3
Hymenobacteraceae	10	1							9
Pontibacteraceae	1								1
Prevotellaceae	55	9	14	3	4	8	8	9	
Rikenellaceae	1	1							
Sphingobacteriaceae	5			2					3
Unclassified Bacteroidetes	41	3	4		4			1	29
Uncultured Bacteroidetes	4	3							1
Actinobacteria	99								
Bifidobacteriaceae	2	1						1	
Brevibacteriaceae	2			1					1
Cellulomonadaceae	3								3
Corynebacteriaceae	11	9							2
Corynebacterineae	2								2
Geodermatophilaceae	1								1
Intrasporangiaceae	4	2							2
Microbacteriaceae	5	1							4
Micrococcaceae	22	8			1				13
Nocardioidaceae	8	2							6
Propionibacteriaceae	20	1							19
Pseudonocardiaceae	2								2
Streptomycetaceae	3								3
Unclassified Actinobacteria	9	2							7
Uncultured Actinobacteria	5	1		1	1	1	1		
Gammaproteobacteria	65								
Aeromonadaceae	2	2							
Enterobacteriaceae	15	14							1
Moraxellaceae	30	22	1		2				5
Pasteurellaceae	2		1			1			
Pseudomonadaceae	6	6							
Succinivibrionaceae	7	3	4						
Uncultured Gammaproteol	2								2
Xanthomonadaceae	1								1
Deltaproteobacteria	5								
Uncultured Deltaproteoba	5		1						4
Betaproteobacteria	12								
Burkholderiaceae	1								1
Comamonadaceae	4	2							2
Neisseriaceae	1			1					
Nitrosomonadaceae	1	1							
Oxalobacteraceae	5	1							4
Alphaproteobacteria	21								
Acetobacteraceae	1	1							
Beijerinckiaceae	1								1
Bradyrhizobiales	2								2
Rhodobacteraceae	4								4
Rhodoplanaceae	1								1
Sphingomonadaceae	6								6
Unclassified Alphaproteoba	2	1							1
Uncultured Alphaproteoba	4								4
candidate division WYO	8								
Uncultured candidate divis	8								8
Chloroflexi	5								
Anaerolineae	1								1
Uncultured Chloroflexi	4	1							3
Cyanobacteria	4								
Uncultured Cyanobacteria	4			1	1				2
Acidobacteria	5								
Uncultured Acidiobacteria	5								5
Thermi	2								
Trueperaceae	2								2
Gemmatimonadetes	2								
Uncultured Gemmatimona	2								2
Spirochaetes	1								
Spirochaetaceae	1				1				
Fusobacteria	1								
Fusobacteriaceae	1	1							
Archeal Families	42								
Methanobacteriaceae	39	5	3	4	7	4	11	5	
Crenarchaeote	3								3
Eukarya	76								
Davidiellaceae	17								17
Dothioraceae	25								25
Hetermiditae	1								1
Klebsormid	1								1
Mortierellaceae	1								1
Phaeosphaeriaceae	1								1
Trichocomaceae	7								7
Unclassified fungi	2								2
Wallemia	11								11
Viridiplantae	10	1	2		1				6

Table 4.2 Abundance of clones and OTUs. Distribution of the clones and OTUs in taxonomic groups and their abundance in the individual samples are displayed. Number of clones per environment in the largest phyla is indicated in the row with the name of the phylum.

CHAPTER 5

CULTURE-INDEPENDENT ANALYSIS OF BIOAEROSOLS IN POST HURRICANE KATRINA FLOOD RECLAIMED HOMES

Abstract

In the three years since the catastrophic damage caused by Hurricanes Katrina and Rita, residents of New Orleans have continued to return to their city and remediate their homes. Indoor aerosol studies conducted immediately following the storms, reported elevated concentrations of mold, endotoxin and fungal glucans within water-damaged homes. Subsequent indoor air investigations following clean up and remediation activities have not been published. In response, we characterized the identity, distribution and abundance of airborne microorganisms present within flood impacted homes at different stages of remediation and compared them to immediate outdoor environments using direct microscopy, broad range rRNA PCR, and DNA sequencing analysis. Phylogenetic analyses were used to quantitatively assess relatedness among houses at different stages of remediation as well as indoor and outdoor samples. We report here that indoor microbial loads were not elevated above the immediate outdoors and were similar to those previously reported for non-water damaged homes. The fungal populations observed in all three home types were substantially different from those commonly recovered from water-damaged homes - *Alternaria*, *Cladosporidium*, *Penicillium* and *Aspergillus* - were rarely observed in our sequence data. Plant pathogens and non-edible mushrooms

dominated the fungal ecology observed within the home environments. *Wallemia sebi* was the only potential respiratory pathogen observed in significant abundance and was found in both indoor and outdoor environments.

5.1 Introduction

The modern lifestyle is such that people spend most of their time indoors (107); consequently factors affecting this environment become critical to the wellbeing of the inhabitants. Unfortunately, indoor environments are likely to harbor more contaminants than the outdoors, thereby creating the potential for increased exposure (208). Air quality indices and threshold exposure levels have been well established for chemical pollutants and particulate matter; however, no such guidelines have been established for contaminants of microbial origin (34).

Bioaerosols is the term used to describe all particulate matter of biological origin. Included in this definition are all microorganisms – culturable, nonculturable, and dead – their fragments, and associated toxins and waste products (1). Bioaerosols are ubitquitous and can range in size from 0.1 to 100 micrometers. Particles with aerodynamic diameter less than 5 micrometers can penetrate deep into the human lung (36, 89, 197).

Absence of guidelines for bioaerosol contaminants is the result of insufficient environmental and health data upon which to base such regulations (1). Airborne microbial contamination is currently assessed by visual inspection, review of occupant symptoms, comparison of species

recovered on culture plates from indoors and outdoors, and professional judgment (1, 167). NATO and WHO have recommended that the assessment of airborne microbial contamination be performed by more accurate and robust methods (143, 234).

Extensive flooding to the New Orleans area caused by Hurricanes Katrina and Rita resulted in severe mold contamination of more than 100,000 homes (43). Aerosol studies and public health surveys conducted in months following the storms reported high levels of airborne molds and other microbial markers (47, 192, 199, 219) and increased incidences of respiratory ailments (58) in the flood damaged homes. Previous culture based airborne microorganism studies have established biological contaminants can originate from building materials and household goods even after remediation work had been performed (60, 73, 157). Moreover, the occupation of moisture damaged buildings has been correlated with adverse health conditions including respiratory illness (25, 114). Despite acknowledged persistence of microbial contamination in some of the New Orleans remediated homes and a recommendation (219) for further investigation, no long-term surveys of airborne microbial levels and composition have been reported.

In response to the needs for better contamination assessment and long term study of the New Orleans mold damaged homes, we performed a study of partially and completely remediated homes nearly two years after the storms using culture independent methods, which are acknowledged to be more robust and accurate than the currently used culture methods (9, 10,

171, 178). Direct microscopy was used to quantify the microbiological loads present within and immediately outside flood impacted homes. Broad-spectrum PCR and rRNA sequence analyses were conducted to assess microbial community compositions for these environments. The goals of this study were to determine if indoor airborne microbial levels remained elevated and/or if indoor composition remained unchanged following extensive remediation procedures and the passage of two years.

5.2 Materials and Methods

5.2.1 Aerosol sampling.

A total of 12 indoor aerosol samples were collected from 12 flood-damaged homes in New Orleans, Louisiana from January 19-22, 2008. The sampled homes were located in the St. Bernard, Uptown, Littlewoods, Algiers, New Orleans East, 9th Ward, and Gentilly parishes. Samples were collected from two non-remediated, six partially remediated and four completely remediated homes. Table 5.1 lists the samples collected at each site, the home types, and the site location. Figure 5.1 shows the sampled sites on a map. All were samples were processed for sequencing analyses.

Homes with no remediation were mostly left unchanged since the flooding event two years prior. One home contained the entire possessions of its owners prior to the storm. A second home was without household goods. Both homes were closed and were not regularly exposed to ambient air.

Homes categorized as partially remediated had undergone cleaning with detergent, biocide application, and had their water damaged interior walls, carpeting, and household goods removed and disposed. Of these homes, most did not have glass windows, but were boarded up. One home had severe structural damage and was missing an external wall.

Homes classified as completely remediated had undergone cleaning with detergent, biocide application, had their water damaged interiors and goods removed and disposed, were renovated and redecorated, and occupied.

Aerosol samples were concurrently collected with SKC Biosamplers (BioSampler SKC Inc., Eighty Four, PA). SKC Biosamplers were positioned approximately 1 meter above the ground. The collection medium for the samplers was sterile 1X Tris EDTA (Thermo Fisher Scientific, Pittsburg, PA). SKC Biosamplers were operated at a flow rate of 12.5 L/min. Two impingers were run in parallel for each sampling site. The impingers were allowed to collect continuously for at least 30 minutes at each sampling site. To maintain appropriate fluid levels within the impinger reservoir, sterile water was added to the Biosamplers to compensate for evaporative losses. At the end of each sampling event, the collection fluids from each SKC Biosampler were combined into one sterile 50mL conical vial and then placed on ice until they could be processed, which was typically less than 8 hours.

All samples were processed and archived in the laboratory facilities at Dillard University, New Orleans, LA. Impinger samples were aliquoted as

follows: 10mL for direct microscopy analysis preserved with 2% (v/v) formaldehyde, and 30mL for molecular analysis concentrated and dried on 0.2 micrometer nitrocellulose filters (100mL) (Nalgene Analytical Test Filter Funnel, Nalge Nunc International Rochester, NY). Processed samples were stored at 4^0C (direct count samples) or –20^oC (DNA samples) until the end of the sampling campaign when all samples were stored on ice and shipped overnight to the University of Colorado where they were stored at 4^0C (direct count samples) or –80^0C (DNA samples) until analyzed.

5.2.2 Airborne microbe enumeration

Aerosol samples were stained and enumerated using 4'6-diamidino-2-phenylindole (DAPI) (Sigma Chemicals, St. Louis, MO) in accordance with previously described methods (102). DAPI is a nonspecific fluorescent DNA intercalating agent that provides a suitable background for direct microscopy. Samples were incubated with DAPI (20 microgram/mL) for 5 minutes at 4^oC and then filtered through a 25mm diameter, 0.22 micrometer (average pore size) black polycarbonate filter (Poretics, Inc. Livermore, CA). Filters were then mounted in low fluorescence immersion oil containing an antifadent (CitiFluor, Leiscester, UK) and examined under 1100x magnification using a Nikon Eclipse E400 epifluorescence microscope fitted with a mercury lamp and polarizing filters. A minimum of 10 random fields was counted per slide and only intact, brightly stained cells with obvious bacterial or fungal morphology were counted. Direct counts were reported as the average of all

fields counted; in all cases the coefficient of variation observed was less than 20% (102).

5.2.3 Genomic DNA extraction

Concentrated samples and negative controls were extracted from filters using a bead beating protocol modified from Frank et al (80). Extracted DNA was resuspended using 20 microliters of Tris-Ethylenediamine Tetraacetic Acid (TE).

5.2.4 PCR of rRNA genes

Extracted DNA was assayed using universally conserved (515F/1391Rev) primers and HotMasterMix® (Eppendorf North America, Inc., Westbury, NY). A typical 20 microliter PCR reaction included: 8 microliter 2X HotMasterMix, 0.5 microliter Forward Primer (25ng/ml), 0.5 microliter Reverse Primer (25ng/ml), 8 microliter water, 2 microliter bovine serum albumin (10 microgram/ml), and 1 microliter DNA template. Assays were performed on an Eppendorf Mastercycler® (Eppendorf North America, Inc., Westbury, NY). The amplification protocol was as follows: 94^{o}C for 2 minutes, 94^{o}C for 20 seconds, 52^{o}C for 20 seconds, 65^{o}C for 90 seconds, 30 cycles total. PCR controls with no DNA template were included with every assay. Following PCR, all samples were run on a 1% (w/v) agrose gel for confirmation. Amplified samples were identified and then assayed in quadruplicate. Extraction and negative controls never showed amplification.

5.2.5 Clone library construction

One clone library, a total of at least 96 randomly selected rRNA clones, was constructed for each sample. Amplicons from four independent PCR reactions were pooled and purified for each sample using agarose gel electrophoresis and a Montage® DNA Gel Extraction Kit (Milipore, Billerica, MA). Purified product was then ligated into pCR4.0 TOPO plasmid vectors and transformed using Top10 electro competent cells (Invitrogen Corporation, Carlsbad, CA). Transformed cells were plated and grown overnight on Luria-Bertani (LB) agar. For each library, a total of 96 clones were picked at random, individually placed in 10 microliter of 1X TE and heated at 85^{o}C for 10 minutes. Following thermal lysis, samples were centrifuged at 4000G for ten 10 minutes to pellet cellular debris.

5.2.6 DNA sequencing

Sequencing was performed as previously described by Papineau et al (173). Briefly, sequencing template was prepared from rRNA clones by PCR amplification with T3 and T7 vector primers, and treated with ExoSAP-IT (GE Healthcare, Piscataway, NJ). Templates were sequenced with the DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare, Piscataway, NJ) on a Mega-BACE 1000 DNA Sequencer using T3 and T7 primers. Base calling and assembly of raw sequence data were performed with the PHRED and PHRAP software packages (72), using the software XplorSeq (79).

5.2.7 Phylogenetic analysis

The sequence collection was screened for chimeric sequences with the Bellerophon software package (110). The closest known relative of each rRNA clone was determined by comparison of its sequence to all rRNA sequences in GenBank by BLAST (8). Sequences were aligned with the NAST software package (62) and their phylogenetic positions were determined by analysis with the ARB software package (138) and a curated ARB database and tree with ~100,000 rRNA sequences (≥ 1250 nucleotides) in length from the NAST project (62). Sequences were added to the ARB tree by parsimony insertion. Sequences were clustered into operational taxonomic units (OTUs) at a 97% sequence identity cutoff using the computer program sortx (79).

5.2.8 Richness estimates

Sample coverage and species richness estimate were calculated using biodiv (79). The program calculates species richness estimates (Chao and ACE) and diversity indices (Simpson, Goods, Shannon) based on a user defined sequence identity level. Estimates were calculated separately for each environment.

Sequence libraries were compared using UniFrac and SONS (137, 212). UniFrac compares related sets of sequences (samples) using phylogenetic and calculates a distance metric based on the fraction of the total distance in the tree that is unique to a particular sample. The SONS program compares

the membership and structure of two communities at a specified phylogenetic level.

5.3 Results

5.3.1 Airborne microbial concentrations

Total airborne microorganism concentrations collected during the sampling campaign are presented in Figure 5.2. Results are organized by remediation status (least to most remediated) and environment type (indoor vs. outdoor). In general, the concentrations measured within the three residence types ($9x10^4$ – $8x10^5$ cells/m^3) were similar to one another and their corresponding background environments ($7x10^4$ - $1x10^6$ cells/m^3). The indoor airborne concentrations measured in this study were less than those previously reported for remediated flood impacted homes ($8x10^5$-$2x10^7$ cells/m^3) using a similar counting basis (73).

5.3.2 Microbial diversity of the sampled environments

The phylogenetic diversity of the sampled airborne microbial communities was assessed using 16S and 18S rRNA clone sequencing. Table 5.2 provides a summary of the sequence results for each of the sampled sites. Samples from Sites 2, 3, and 9 (indoor only) did not amplify due to low DNA concentration and were removed from further phylogenetic analyses. Outdoor samples from Sites 10 and 12 were not collected because of freezing temperatures (Site 10) and the open nature of the sampled home (Site 12). A total of 24 rRNA libraries (16 16S rRNA and 8 18S rRNA) and 2154 sequences (1085 bacterial and 1069 eukaryal) determined. Table 5.2

lists a total of 2203 sequences. This discrepancy is the due to instances of low sequence quality, which resulted in the removal of 49 sequences from further analyses.

Approximately 95% of these sequences had a high similarity (95% or greater) to existing sequences in the GenBank database. The remaining 5% (116 sequences) were subsequently checked for chimeras. Of these, 24 were found to be chimeras and were removed from further analyses.

Sequences clustered into 723 (570 Bacterial and 153 Eukaryotic) different operational taxonomic units (OTUs) based on the 97% sequence identity similarity criterion (87). Of these clusters, 471 (83% of the total) occurred in only one sample and 399 (70%) occurred only once. The number and distribution of OTUs is shown in Figure 5.3. The most frequently occurring OTUs in both indoor and outdoor samples were eukaryotes: *Phoma sp.* (n = 183), *Trametes versicolor* (n=91), *Cladosporium cladosporioides* (n = 50), *Wallemia sebi* (n = 45), and *Setosphaeria rostrata* (n=43). *Phoma sp.*, *Cladosporium cladosporioides*, and *Setosphaeria rostrata* are common fungal plant pathogens. *Trametes versicolor* is common mushroom species. *Wallemia sebi* is a fungal species typically found in agricultural environments. Bacterial OTUs were observed in much lower numbers (typically <20); the most frequently occurring was *Flavisolibacter sp.* (n=32). The lowest OTU numbers (environments with the least observed diversity) were obtained from the renovated (n=101) and unremediated (n=129) home samples;

approximately three times as many OTUs were observed in the outdoor and partially treated homes samples.

Diversity estimates (Chao and ACE) for the clone libraries ranged between 430 and 1000 OTUs for the bacterial libraries and between 83 and 180 OTUs for the eukaryal libraries. Diversity was lowest in the non-remediated homes and greatest for the outdoor samples. Sample coverage, defined as number of OTUs observed divided by number of predicted OTUs, for all environments ranged from 10 to 29% in the bacterial libraries and 30 to 78% in the eukaryal libraries, indicating that much biodiversity remains uncharacterized in these environments. A summary of the clone library characteristics is presented in Table 5.2. Rarefaction curves for the pooled samples of the homes and outdoor environments are shown in Figure 5.4.

5.3.3 Bacterial composition of the sampled environments

The phylogenetic distribution of the bacterial sequence data from the sampled homes and their corresponding outdoor environments is shown in Figure 5.5. Data are presented as composites because of the varying library sizes and uneven distribution of sequence data (Table 5.2). Within the non-treated homes, members of the *Firmicutes* phylum represented nearly 40% of the total sequence data; however, no single family within the *Firmicutes* was observed to be dominant. Sequences of public health interest found within non-treated homes included *Shigella dynsenteriae* (n=8) and *Clostridium sp.* (n=4). Environmental sequences composed the majority of species observed within the non-treated homes.

Approximately 50% of the partially treated homes sequence data belonged to either the Bacteroidetes or Actinobacteria phyla. *Flavisolibacter ginsengiterrae and Flavisolibacter ginsengisoli* (*Chitinophagaceae* family), two species observed in ginseng cultivating soil, accounted for 70% of the sequence data within the Bacteroidetes phylum. A variety of *Rubrobacter sp*. comprised nearly 50% of the sequences belonging to the *Actinobacteria*. The potentially opportunistic pathogen *Pseudomonas stutzeri* was observed eight times in the partially treated homes sequence data. Incidences of *Pseudomonas stutzeri* related infections include bacteremia and meningitis in persons infected with HIV, community acquired osteomyelitis and pneumonia, and an nosocomially acquired brain abscess (134, 196, 203, 246)

Members of the *Firmicutes* and *Actinobacteria* composed over 60% of the completely remediated homes sequence data. Distribution of sequences within these phyla was such that no one family dominant. Sequences closely related to *Staphylococcus epidermidis* (n=5) and *Shigella dysenteriae* (n=3) were observed in the recovered sequence data. Infections due to *Staphylococcus epidermidis* are rare and usually nosocomial related. *Shigella dysenteriae* is common pathogen responsible for bloody diarrhea, high fever, and abdominal pain. The *Shigella* species are capable of producing endotoxins, cytotoxins, and neurotoxins that can lead to death if left untreated (141).

The outdoor bacterial sequence data were spread across 18 phyla with no phylum containing more than 24% of the total sequence data. Phyla with

the largest representation were *Bacteroidetes* (24%), *a-proteobacteria* (23%), and *Actinobacteria* (19%). Sequences with BLAST ID 97% or higher to the potentially pathogenic *Staphylococcus epidermidis*, *Shigella dysenteriae*, *Propionibacterium acnes* and *Pseudomonas stutzeri* were observed in low numbers (n<6). Although members of the *Nocardioidaceae* family accounted for 24% of the observed *Actinobacteria*, none were related to any pathogenic species. Representatives of *Flavisolibacter sp.* (*Bacteroidetes*) and *Sphingomonas sp.* (a-proteobacteria) each composed more than 40% of the species observed in their respective phyla.

5.3.4 Eukaryal composition of the sampled environments.

Eukaryal data are presented as composites because of the varying library sizes and uneven distribution of sequence data (Table 5.2). Approximately 90% of all eukaryal sequences identified in the sampled environments were members of the Fungi Kingdom and belonged to either the *Ascomycota* or *Basidiomycota* phyla. In non-treated homes, 55% of fungal sequences were closely related to *Wallemia sebi* (*Basidiomycota*), a species found a wide variety of environments from food to agricultural settings (69, 96, 155, 242). Metabolites associated with *Wallemia sebi* are suspected to cause Farmer's Lung disease and bronchial asthma (194, 205).

Plant pathogens *Ceriporia sp.* and *Setosphaeria sp.* were the two most frequently observed species in the partially treated homes. The potentially pathogenic genus *Alternaria sp.* was also frequently (n=17) observed in the recovered sequence data. *Alternaria* are a ubiquitous species commonly

found on rotting plant material (13, 151). The spores of this fungi are known to trigger asthma attacks and other allergic reactions (66, 183). Other species observed in large numbers (n=17) included the inedible mushrooms *Bjerkandera fumosa* and *Trametes versicolor*.

Approximately 60% of fungal sequence data for the completely remediated homes were representatives of *Setosphaeria sp.*, *Trametes versicolor*, and the plant pathogen *Phoma sp.*. *Cladosporium cladosporioides*, a common household mold, accounted for less than 3% of the sequence data. At elevated concentrations, *C. cladosporioides* can elicit allergic reactions.

Outdoor samples contained many of the same species (*Phoma*, *Alternaria*, *Setosphaeria*, *Ceriporia, Bjerkandera fumosa, Trametes versicolor and C. cladosporioides*) observed within the three home categories. *Phoma sp.* had the largest representation at nearly 40% of the total fungal sequence data. *Trametes versicolor* was the next most frequent species and accounted for just over 10% of the fungal sequence data.

5.3.5 Community comparisons

Relationships among sequences recovered from the indoor air samples, the immediate outdoors, and other unrelated studies were analyzed using the Unifrac (136, 137) and SONS (212) programs. The UniFrac distance quantifies phylogenetic relationships between sets of taxa in a phylogenetic tree. Pair-wise comparisons between our samples were made using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and

the Principal Coordinates Analysis (PCA). A SONS analysis was used to determine the fraction and richness of OTUs shared among the environments sampled. The SONS program bins sequences into collections based on identity and quantifies the OTUs shared between environments as well as provides the frequency with which OTUs were observed in each environment.

A comparison of the indoor (composite) and outdoor (composite) sequence data at species level similarity revealed 123 (77 bacterial and 46 eukaryal) shared OTUs out of the total 723 (17%). Contained within these were approximately 40% of the bacterial sequence data and more than 80% of the eukaryal sequence data. Summary of these shared species is shown in Figure 5.6. These findings indicate that the outdoor environment had some influence on the indoor ecology.

The composite indoor and outdoor environments were separated and evaluated on a site-by-site basis. Due to the unequal sequence distribution individual comparisons could not be performed for each site. Comparisons of the bacterial sequence data could be performed for Sites 6, 7, 8 and 11. Fewer than 10 OTUs were shared between the indoor and outdoor environments for each of these sites. The shared OTUs for each of these environments ranged from 21% (Site 8) to 14% (Site 6). A summary of the shared bacterial OTUs for Sites 6, 7, 8, and 11 are shown in Figure 5.7. Similar analyses for the eukaryal sequence data could only be performed for Sites 1, 4, 5, and 6. The number of OTUs shared between the selected indoor and outdoor environments ranged from 5 to 11. These shared OTUs

translated to between 44% (Site 6) and 90% (Site 4) of the total sequence data recovered from the selected sites. Figure 5.8 shows the shared eukaryal OTUs for the selected sites.

Separating the indoor environments into three categories (non, partially, and completely remediated) and comparing their airborne ecologies at species level uncovered 16 OTUs (8 bacterial and 8 eukaryal) common to all housing types. Figure 5.9 illustrates the shared species and their proportions. Sequences within the shared OTUs accounted for less than 9% bacterial and 16% eukaryal sequence data. These findings indicate that the ecology observed in a particular home category was likely distinct.

The PCA results also confirmed the SONS findings that the three home environments were not similar to each other. Results of the analyses also revealed that indoor/outdoor air exchange was responsible for the largest variation in data for both the bacterial (43%) and eukaryal (61%) data sets. The next largest variations in the home sequence data were attributed to structural damage (eukaryal, 25%) and remediation (bacterial, 30%).

Additional SONS and UniFrac analyses were conducted using genetic data from other rRNA based indoor ecology studies of moisture damaged and non-moisture damaged residential buildings, available from GenBank (184, 202), to compare the types of organisms observed. The results of the bacterial SONS comparison revealed several shared species (17 to 26 OTUs), but the contribution of these species to the overall sequence data was limited (<10%). Similar results were observed in the fungal data comparison;

20 shared OTUs, but only 3% of the total sequence data. PCA and UPGMA results revealed no similarities in bacterial or eukaryal community structure among the environments sampled.

5.4 Discussion

We report here the microbial diversity and biological loads present in a cohort of homes flooded by Hurricanes Katrina and Rita. The study was conducted in the city of New Orleans two years after the hurricanes and considered three types of homes – non, partial, and completely remediated. Total microbial load was assessed using a nonspecific DNA intercalating stain and direct microscopy. Microbial ecology associated with the airborne particulate matter recovered was evaluated using broad spectrum PCR, sequencing, and phylogenetic analyses. The results for samples collected within the homes show microbial loads at background levels as well as evidence of fungal blooms.

The airborne microorganisms concentrations measured indoors were similar to each other, and their corresponding outdoor environments. Loads in all indoor environments were an order of magnitude lower than those reported for flood remediated homes in a previous study by Fabian (73). However the climate and building materials were markedly different than those in the US Gulf Coast. Differences in the indoor airborne loads between the two studies may also be attributed to the season when samples were collected. In this study, sample collection took place during winter precipitation events, which may have influenced the microbial load in the air

and limited passive ventilation (open windows and doors). In contrast, the Fabian study was conducted during the summer, when outdoor bioaerosol concentrations have been correlated to or otherwise shown to influence indoor concentrations (124, 158, 162), in homes that relied on passive ventilation for cooling. Bioaerosol studies of homes damaged by Hurricanes Katrina and Rita conducted within 3 months of the storms reported elevated indoor fungal loads ($1x10^4$-$6x10^5$ spores/m^3) (47, 192, 199, 219). These findings would indicate that the total bioaerosol concentrations within these flood impacted homes would also be elevated, as fungi are generally observed in concentrations at least an order of magnitude lower than bacteria as judged by conventional culture. The loads measured in this study indicate that the total indoor microbial concentrations have decreased since the previous studies were conducted most likely as a result of remediation activities.

The observed molecular diversity, as judged by less than 3% sequence homology differences in 16S ribosomal DNA, in the indoor environments ranged from 32 to 83 OTUs for eukarya and 67 to 253 OTUs for bacteria. Fungal diversity predictions were similar to those estimated in another survey of a water-damaged building (184). Predicted bacterial diversity (Chao and ACE) at the species level (>97% sequence identity) within all home types was 1.5 to 6 times greater than previously reported for indoor environments, including water damaged buildings (202, 228). Elevated

diversity predictions may indicate that complete eradication of the microbial flora that colonized many of the homes following the storms did not occur.

Bacterial diversity indices were largest for the partially treated home samples and may be attributed to the open nature of these homes as well as the large number of samples. The partially treated homes were less structurally robust than their renovated and unremediated counterparts. Homes in this category were generally windowless (plywood covered the gaps in some cases), without insulation, and in one case missing part of a wall. Partially treated homes comprised the largest number of sampled homes, however we believe this reflects current condition of New Orleans, which is one of repair despite the passage of two years. Unremediated homes were estimated to have the lowest bacterial diversity, which may indicate that bacterial growth in these environments has subsided as a result of desiccation due to the passing of time.

The indoor airborne ecologies observed in this study were compared to those reported in the literature for similar environments. Culture based bacterial studies (175, 182) of moldy homes reported large numbers of isolates from the *Actinobacteria* (*Nocardia sp., Gordonia sp., Rhodococcus sp., Dietzia sp., Micrococcus sp.*) and *Firmicutes* (*Bacillus sp.* and *Staphylococcus sp.*) phyla. A few of these genera were observed among the indoor sequence data, but their low relative abundance may reflect their occurrence in the sampled environments as well as sample bias associated

with conventional culture analyses. The pathogenic members of these genera were not observed.

Most airborne ecology studies of water-damaged buildings have focused on fungal populations. In previous culture based studies (93, 109, 113, 166, 175, 192, 193, 206), *Penicillium sp.*, *Cladosporium sp*., *Aspergillus sp*. and *Alternaria sp.* are reported as the most frequent species observed. The airborne ecology determined from our molecular based study did not find these species in high relative abundance once again revealing the bias associated with culture-based analyses. Sequences closely related (<3% sequence difference) to *Cladosporium sp.* composed less than 5% of the total indoor sequence data and members of the *Trichocomaceae* family, which includes *Penicillium* and *Aspergillus*, accounted for less than 3%. Rather our indoor fungal sequence data had large representations of plant pathogens (14%), *Phoma sp*. and *Setosphaeria rostrata*, and non-edible mushrooms (16%), *Trametes versicolor*, *Bjerkandera fumosa*, and *Ceriporia lacerata*. In a study of New Orleans flood impacted homes two months after the hurricanes (192), Rao reported *Aspergillus niger*, *Penicillium sp*., *Trichoderma* and *Paecilomyces* as the most common molds found. These species were not observed in the current study perhaps indicating that the conditions, which were once favorable for their growth, have ceased with the passage of time.

In general, few species of public health interest were observed in the sampled environments. Those with the most frequent representation and greatest pathogenic potential were *Wallemia sebi* (n=79), *Shigella*

dysenteriae (n=16) and *Pseudomonas stutzeri* (n=10). The distribution of these species was balanced across the sampled environments indicating their ubiquitous nature.

Community comparisons, performed using the SONS programs, revealed limited (<20%) species overlap between composite in- and outdoor environments; nevertheless, within them was a substantial portion of the total sequence data (40% of the bacterial and 80% of the eukaryal). Partially treated homes, due to their open nature, were responsible for the majority of species shared with the outdoor samples. Their influence was substantial on the bacterial data set. Excluding the data from the partially treated homes and reviewing the SONS results again revealed fewer species and sequences shared between the two remaining home types and the outdoors. Remediated homes shared 29 OTUs, containing only 5% of the total sequence data, with the outdoor environment. The number of shared OTUs and sequences in the non-remediated homes was slightly higher, but not significant. This finding indicates that the airborne bacterial ecology present within completely remediated home was likely due to interior activities and sources. A similar conclusion can be drawn for the non-remediated homes; the observed ecology most likely arose from flood damaged building and home materials which remained in these environments since the storm with disturbances or energy inputs to aerosolize their contents.

Analysis of the fungal sequence data in a similar composite fashion revealed the strong influence of the outdoor environment on the ecologies of

the non and completely remediated homes. Approximately 95% of the sequence data from the completely remediated home samples was contained in the 25 OTUs that were shared with the outdoor data. In non-remediated homes, the number of shared species was less (n=18), but contained more than 80% of the sequence data. These results perhaps point to a background outdoor fungal ecology for New Orleans present during the sampling period.

Indoor-outdoor comparisons for each sampled site were also performed. Unequal sequence distribution (i.e. greater eukaryal representation than bacterial) did not permit shared OTUs analyses to be performed for each site. Bacterial comparisons for four sites revealed few (<10) OTUs shared between the indoor and outdoor environments. These shared OTUs accounted for less than 20% of the sequence data for compared environment. These findings indicate that the observed indoor bacterial ecology is independent of the immediate outdoors and most likely originates from the inhabitants and their actions. Comparisons of bacterial sequence data were performed for four sites. Eukaryal comparisons yielded similar limited OTUs sharing, but a greater percentage of shared sequence data (44-90%). Results of the eukaryal comparisons indicate that the observed indoor eukaryal ecology most likely originated from the immediate outdoors.

Similar analyses of the ecologies present in the three home types revealed few species common to all three home environments. Total sequence data contained within the shared OTUs was small: 10% of the

bacterial and 28% of the eukaryal. Between any two environments the number of OTUs shared was larger (1 to 9 more OTUs). However, usually half of the shared OTUs were also found to be shared with the outdoor sequence data. This finding perhaps indicates that the shared OTU was omnipresent rather than an artifact of flood damage. Overall the findings from these analyses indicate that time, remediation work and indoor activities have aided in changing the ecology present within the different home types.

This study investigated the changes in indoor air quality of Katrina impacted homes as a result of time and remediation efforts. Results of this work reveal that the indoor fungal ecologies have undergone significant change since previous sampling in the Fall 2005 (47, 192, 199, 219). Species previously reported to be abundant in flooded impacted homes were either absent or detected in very small numbers in this study. The fungal populations observed in this study were dominated by plant pathogens and organisms associated with wood rot. Bacterial populations recovered from the home environments appear to arise from indoor activities and/or sources such as the residents and dust. Previous airborne ecology studies of Katrina impacted homes did not report on the bacterial ecology present in these environments. Rather these studies concentrated their efforts on fungal ecology and microbial biomarkers. Likewise, the airborne ecologies observed in this study differed from those reported for water damaged buildings in several other published studies. There are many reasons for these differences; some include the use of culture-based methods, sampling

methodologies (impactors vs. impingers, air vs. dust), the extent and duration of water damage, climate and amount of time passed before sampling. Most published studies report on moisture-damaged buildings, where perhaps a single room was water damaged from a potable water pipe or leaky room whereas homes in this study were severely flooded for weeks. This study provides a follow up to the initial studies conducted immediately following the storms. The findings presented here indicate that the severe mold growth reported in earlier studies of Katrina impacted homes appears to have lessened and changed with the passage of time and remediation efforts.

5.5 Acknowledgements

The author wishes to thank the individuals who so kindly allowed her to sample in their homes. The author would also like to thank B. Carter, L. Mullen, V. Alexander, M. Miller, S. Corbett, D. Minor, G. High, K. Guilford, and A. Evgrafov for helping with sample collection.

Site No.	Samples Collected	House type	Parish	Latitude	Longitude
1	In/Out	Completely renovated	St. Bernard	89 57'44.2"	29 56'08.2"
2	In[1]	Completely renovated	Uptown	90 06'05.9"	29 56'05.3"
3	In/Out	Completely renovated	St. Bernard	89 57'43.38"	29 56'09.86"
4	In/Out	Completely renovated	Algiers	90 00'46.8"	29 56'00.8"
5	In/Out	Partial	9th Ward	90 02'27.98"	29 58'28.46"
6	In/Out	No treatment	New Orleans East	89 59'51.25"	30 02'29.49"
7	In/Out	Partial	New Orleans East	89,59'09.06"	30 01'45.75"
8	In/Out	Partial	New Orleans East	89 56'58.28"	30 03'20.58"
9	In/Out	Partial	New Orleans East	89 59'51.9"	30 01'36.8"
10	In[2]	Partial	Littlewoods	89 56'46.9"	30 04'15.8"
11	In/Out	No treatment	9th Ward	90 01'48.9"	29 58'39.8"
12	In/Out	Partial	Gentilly	90 03'33.9"	29 59'34.1"

[1] No outdoor sample collected because collection fluid froze.

[2] No outdoor sample collected because home had no windows and all rooms were open to outside air.

Table 5.1 Site descriptions and GPS coordinates of sample locations in New Orleans during January 2008.

Figure 5.1 Map of New Orleans, LA showing the sampled sites. Map courtesy of Google Earth. A single pin represents Sites 1 and 3 as they are at the same location.

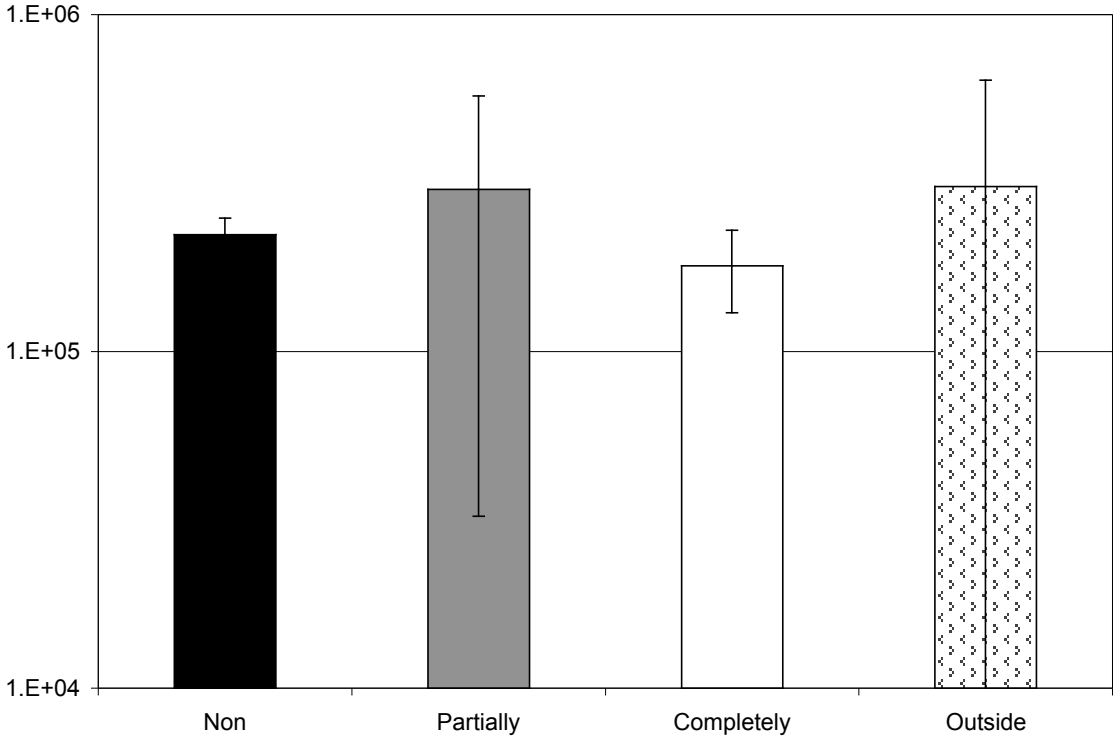


Figure 5.2 Total microbial counts for both the indoor and outdoor environments. Solid bars represent the concentrations within the homes. Solids bars: black, non-treated homes; grey, partially treated homes; white, completely treated homes. Stippled bar represent loads measured for the outdoor environments.

		PCR Amplification		Sequence Distribution				
				Bacterial		Eukaryal/Archeal		
Site No.	**House type**	In	Out	In	Out	In	Out	**Total Sequences/Site**
1	Completely renovated	+	+	90	2	93	93	278
2	Completely renovated	-	-	-	-	-	-	-
3	Completely renovated	-	-	-	-	-	-	-
4	Completely renovated	+	+	5	2	85	83	175
5	Partial	+	+	92	12	95	81	280
6	No treatment	+	+	82	86	103	103	374
7	Partial	+	+	142	83	43	10	278
8	Partial	+	+	151	110	36	74	371
9	Partial	-	+	-	84	-	6	90
10	Partial	+	N/A	28	-	66	-	94
11	No treatment	+	+	61	71	30	22	184
12	Partial	+	N/A	0	-	79	-	79
						Total No. Sequences		2203

Table 5.2 Clone sequencing results for each of the sampled sites.

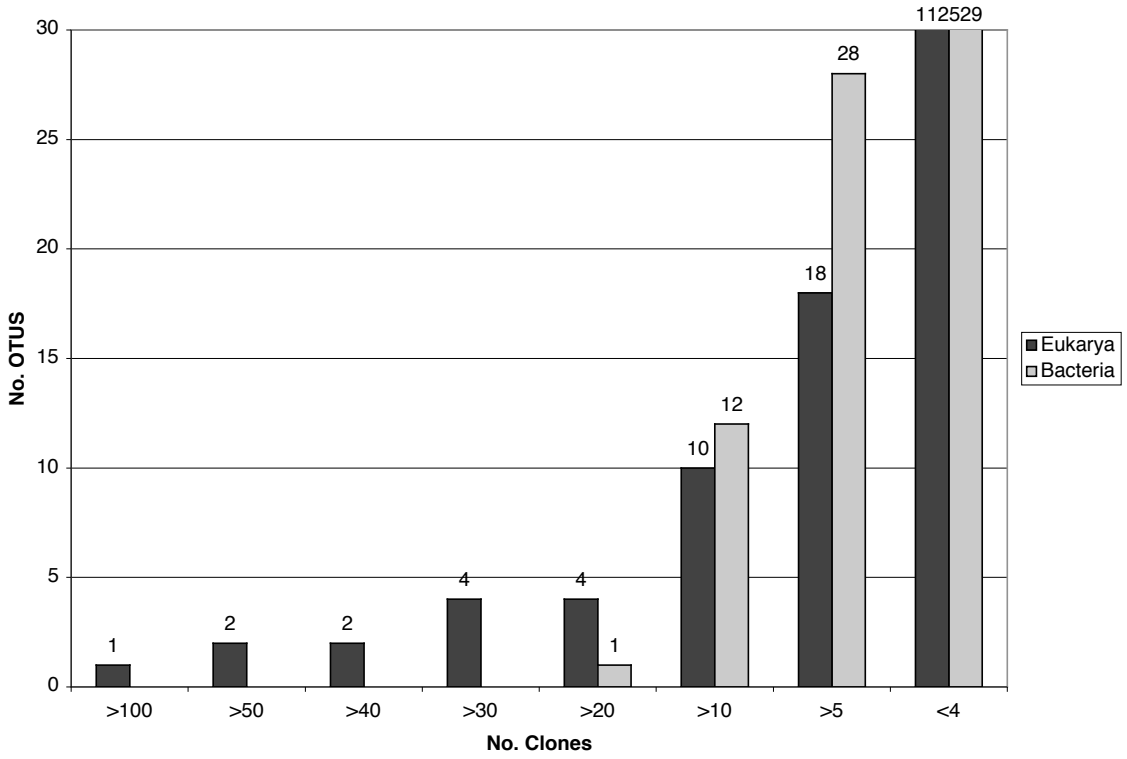


Figure 5.3 Abundance and distribution of OTUs in all clone libraries. Sequences were placed into OTU bins based on the 97% sequence identity similarity. The number atop of each column signifies the number of OTU bins represented in the column.

Bacterial

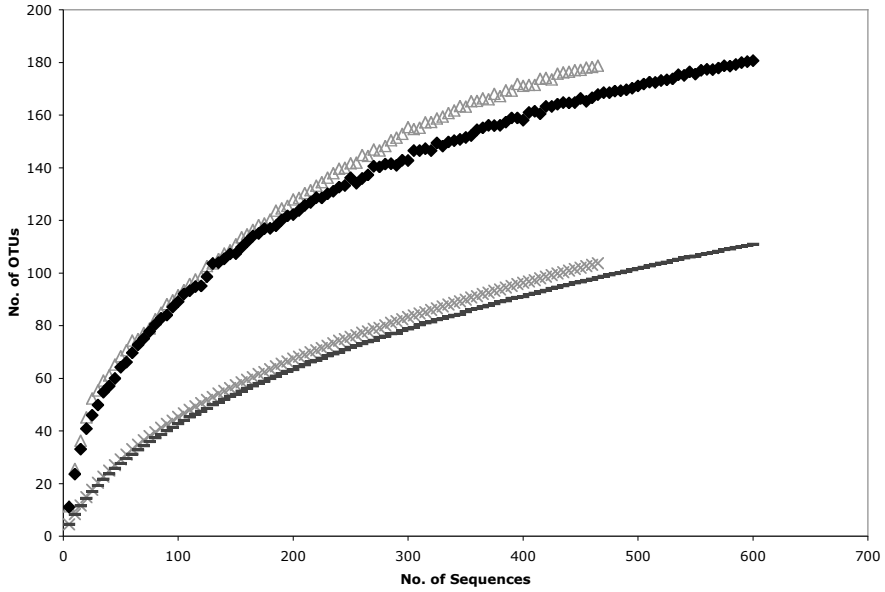
Environment	No. of Samples	No. of Clones	No. of OTUs	Chao1 Estimate	ACE Estimate	Chao Coverage (%)	ACE Coverage (%)	Simpson
Non treated	2	131	97	431	429	23	23	244
Partially treated	5	412	253	872	1032	29	25	178
Completely treated	2	93	67	553	685	12	10	722
Outside	8	449	277	939	1019	29	27	305
Total	17	1085						

Eukaryal

Environment	No. of Samples	No. of Clones	No. of OTUs	Chao1 Estimate	ACE Estimate	Chao Coverage (%)	ACE Coverage (%)	Simpson
Non treated	2	132	32	83	105	38	30	20
Partially treated	5	295	83	106	114	78	73	19
Completely treated	2	174	34	107	120	32	28	23
Outside	8	468	95	179	182	53	52	21
Total	17	1069						

Table 5.3 Characteristics of the clone libraries.

Bacteria



Eukarya

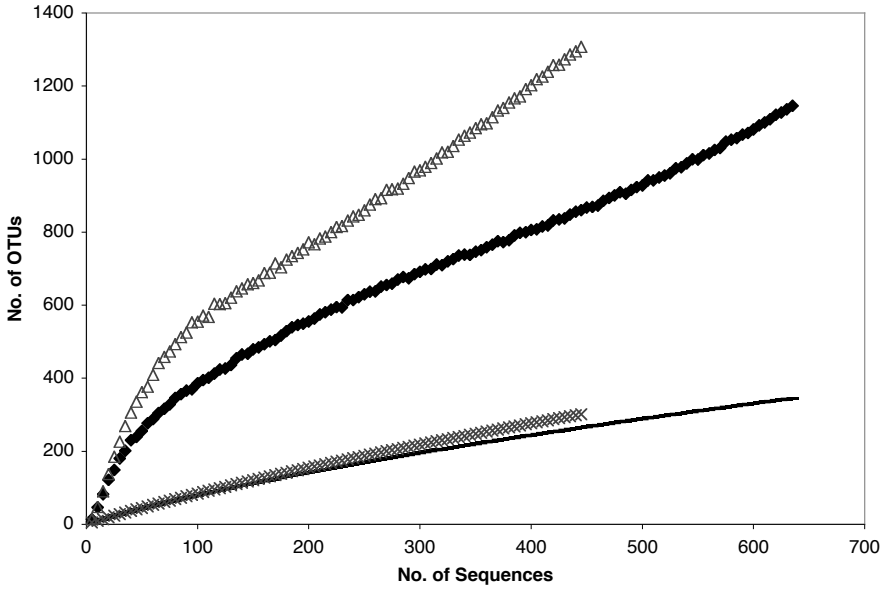


Figure 5.4 Rarefaction curves for the homes and the outdoor environments. The curves reflect the estimated and observed OTU richness and the number of sequences sampled in pooled datasets at the 97% sequence identity level. Symbols: diamonds and open triangles, estimated number of OTUs; line and cross, observed number of OTUs.

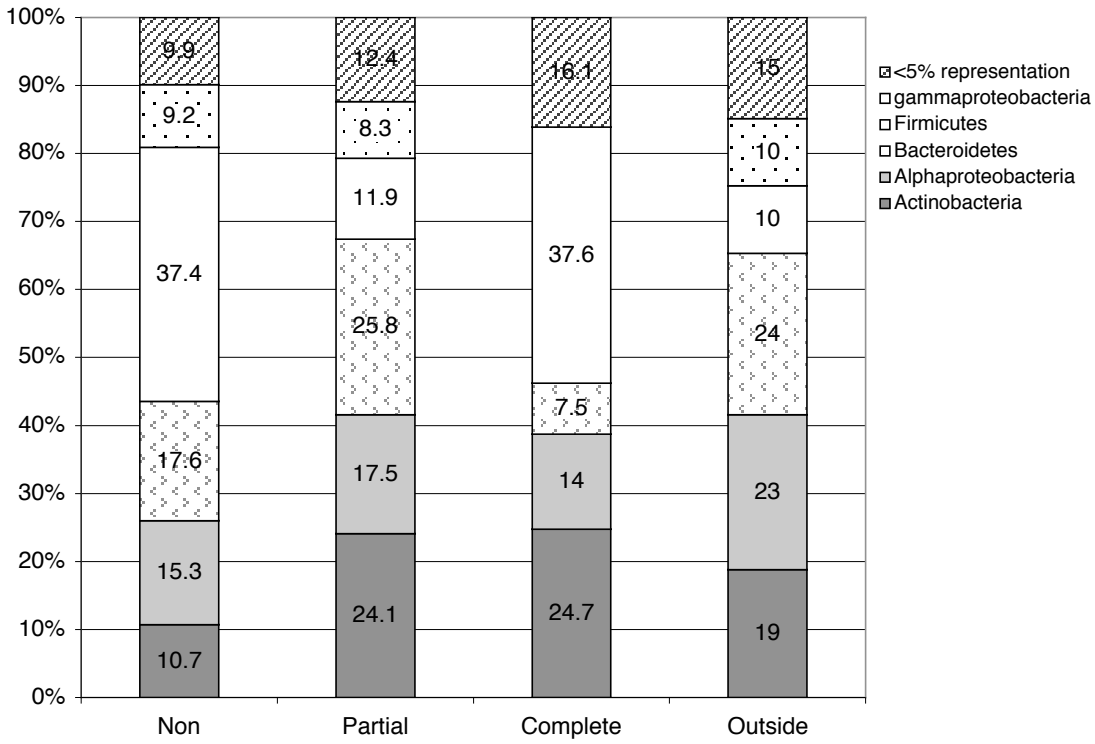
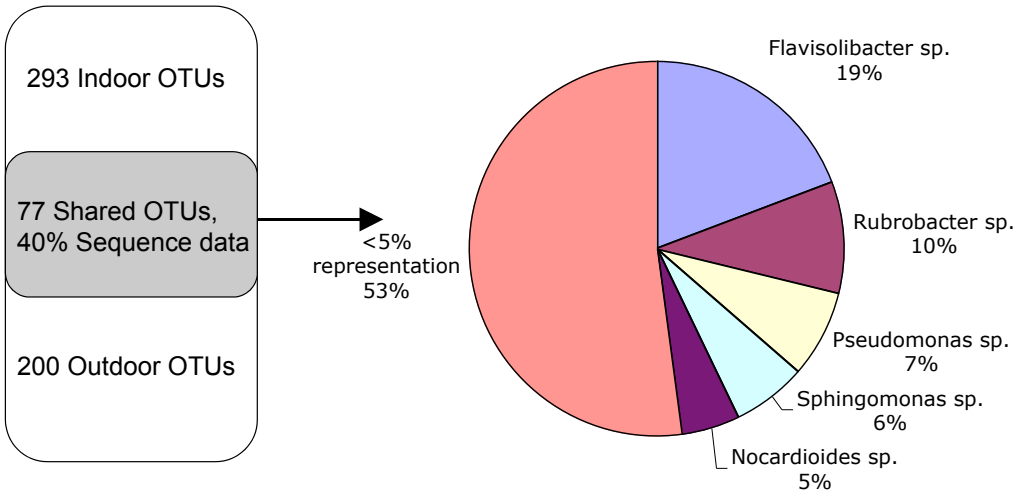


Figure 5.5 Phylogenetic distribution of bacterial sequence data for the sampled homes and the outdoors.

Bacteria



Fungi

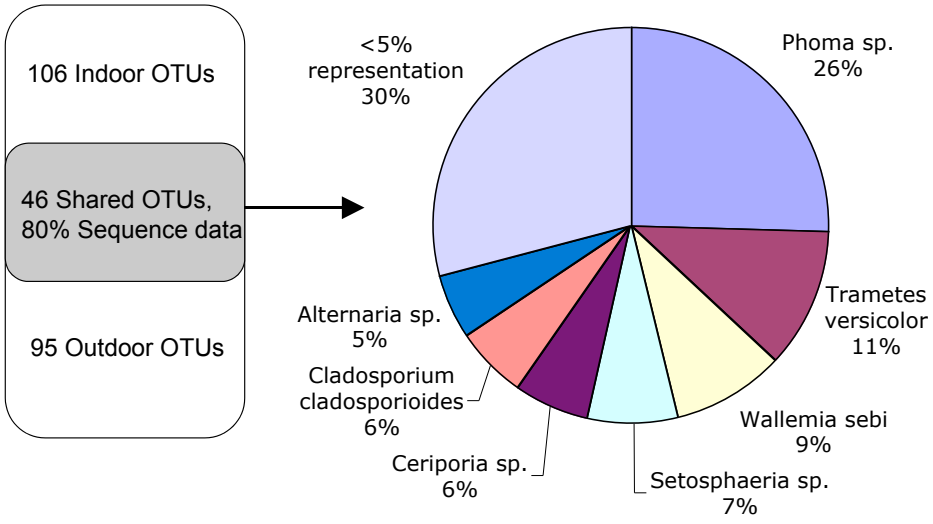


Figure 5.6 Community membership results at 97% sequence identity level for indoor - outdoor comparison. Pie chart illustrates the species and their distribution shared between the two environments.

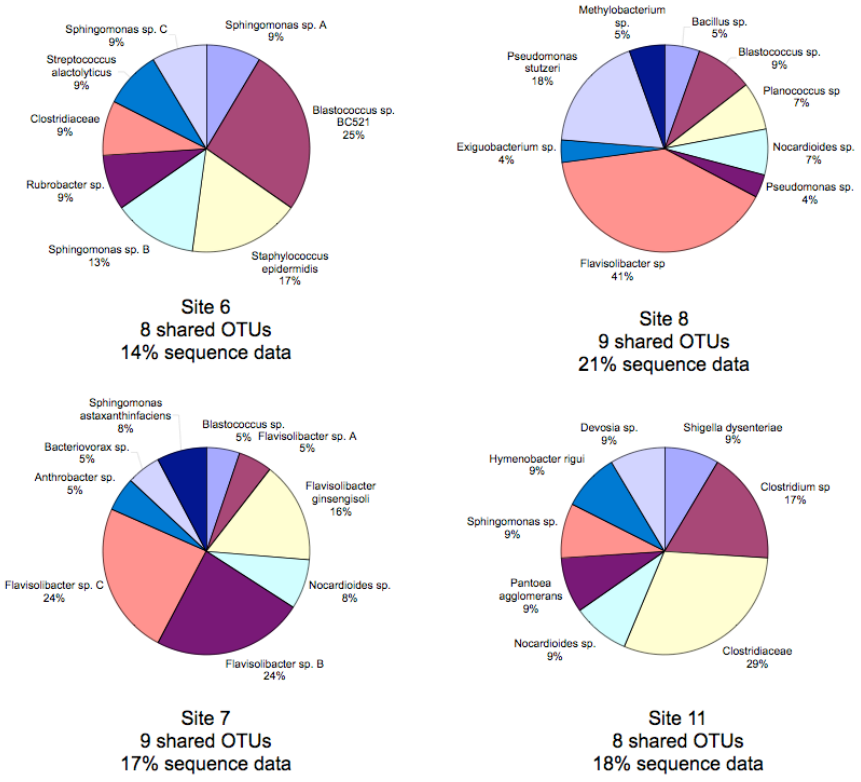


Figure 5.7 Identity and distribution of the shared bacterial OTUs for Sites 6, 7, 8 , and 11.

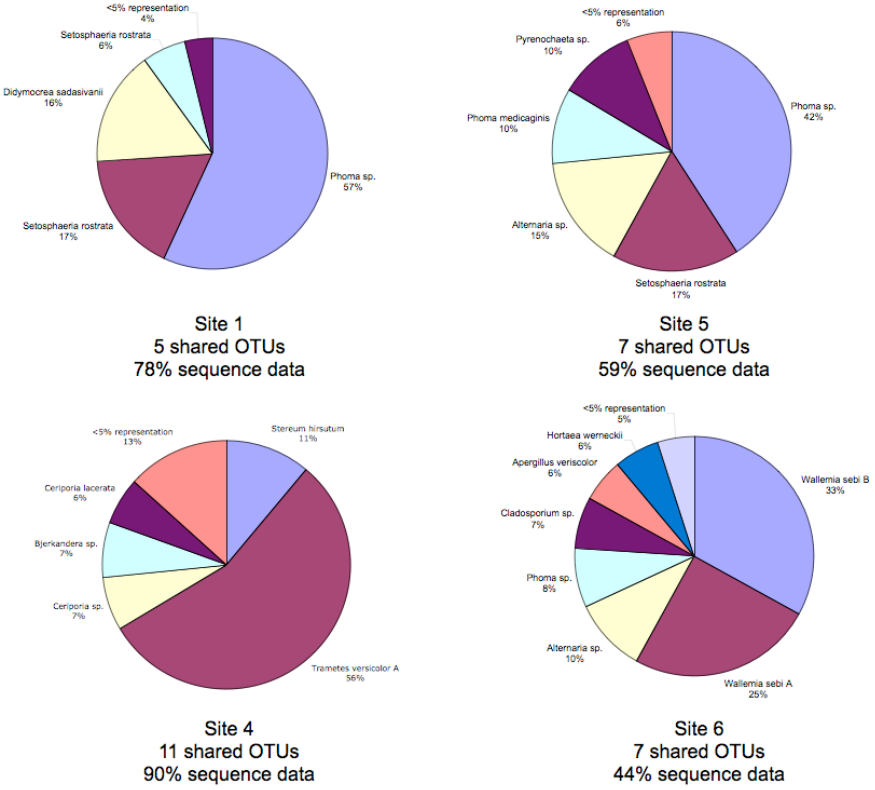
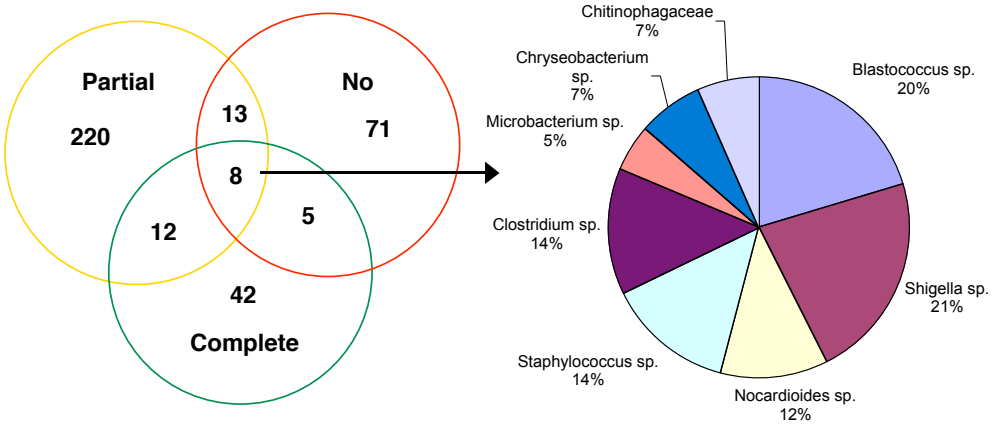


Figure 5.8 Identity and distribution of the shared eukaryal OTUs for Sites 1, 4, 5, and 6.

Bacteria



Fungi

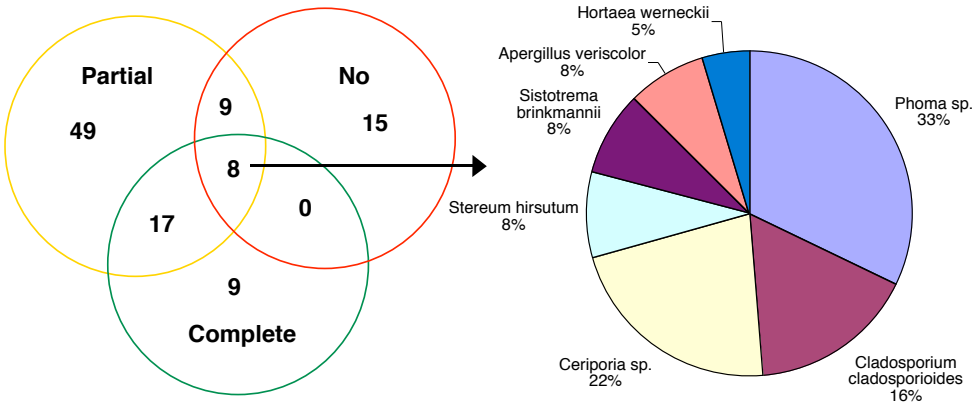


Figure 5.9 Community membership results in the three home types at 97% sequence identity. Pie charts illustrate the species and their distribution shared among the three housing types.

CHAPTER 6

DETECTION OF *MYCOBACTERIUM PARAFORTUITUM* BY QUANTITATIVE PCR FOLLOWING UV DISINFECTION

Abstract

The efficacy of airborne microbial inactivation during ultraviolet irradiation continues to be assessed using conventional culture-based assays. Such methods limit the types of organisms that can be studied, as many microbes cannot be recovered from environmental samples by culture. Quantitative polymerase chain reaction (QPCR) has been proposed as a tool for assessing aerosol disinfection efficiency as it is sensitive, fast, inexpensive, and can identify difficult to culture organisms. To establish QPCR as a reliable and efficient means of monitoring total airborne microorganism numbers through a broad range of germicidal UV doses, bench scale experiments using *Mycobacterium parafortuitum* were performed where inactivation was assessed using QPCR in tandem with traditional culture assays. Direct microscopy was used to quantify the total microbial load present within the reactor and compared to the recovery of QPCR on air samples subject to the same UV dose. The literature suggests that UV exposures much greater than those needed to induce inactivation in air are theoretically required to inhibit PCR amplification; however, such inhibition is template specific. We report here that UV doses did not impact the recovery of QPCR at doses which induced 3-log kill.

6.1 Introduction

Airborne microorganisms are ubiquitous in the atmosphere. Their identities and concentrations are not consistent as they fluctuate according to geographical location, climate events, seasons, and human activities (214). This variability poses a challenge to public health engineers concerned with air quality. Real time monitoring of large volumes of air for specific airborne pathogens and their selective removal is not practical from a cost or engineering perspective. Many microorganisms can have negative health impacts at numbers below reliable sampling detection limits (39). Engineers must therefore devise and use comprehensive control methods to prevent dispersion and reduce concentrations of infectious airborne agents. Current engineering controls include using mechanical ventilation for dilution (and removal) of contaminated air, filtration, negative pressure zones, personal protective (respiratory) equipment and ultraviolet germicidal irradiation (42).

Upper room air ultraviolet germicidal irradiation (UVGI) has been established as an effective means inactivating airborne microorganisms in indoor environments (27, 127, 128, 150, 177, 243, 244). UV light exposure near 260 nm wavelength causes photochemical damage to the nucleic acids in the form of photoproducts that are disruptive to the structural integrity and replication of DNA (176). There are at least four major photoproducts associated with UV induced DNA damage - cyclobutane thymine dimers, spore photoproducts, 6-4 photoproduct, and DNA-protein crosslinks. Of these four, cyclobutane thymine dimers (CTD's) generally have the highest

yields. CTDs occur between adjacent thymine bases and disrupt cell replication process by increasing the frequency that DNA polymerase inserts incorrect nucleotides during replication.

To date, most aerosol disinfection studies have assessed inactivation using only culture assays and while these methods are well established, less than 1% of microbes are able to be cultured (10, 171) and total microbial loads are not enumerated. This is especially important with respect to public health as some airborne pathogenic organisms, such as Mycobacterium tuberculosis, are not easily culturable or not culturable at all (210). Reliance on culture assays has limited the types of organisms that can be studied to those which can be easily grown and thereby hindered the progress of aerosol disinfection work.

Recent water UV disinfection studies have moved toward a molecular based assessment of inactivation. Some results have demonstrated polymerase chain reaction (PCR)-based assessment methods to be successful (23, 70, 71). PCR is a better tool for assessing airborne microorganisms as it is sensitive, quantitative, and culture independent so it is unaffected by sampling stress and can analyze hard to culture and unculturable microorganisms (178). Additional benefits of PCR include accuracy, reduced cost and speed of analysis (hours versus days) (9).

Real time PCR, also known as quantitative PCR (QPCR), allows for the monitoring of a particular target sequence during amplification. A fluorescent probe used in the amplification reaction provides an estimate of

the initial number of target sequences in the sample based on its excitation response. How quickly the signal reaches a concentration threshold value (Ct) corresponds to the original amount of target DNA present in the sample (229). The amount of target DNA in a sample is determined by comparing the sample Ct with a standard curve made from known concentrations of the target sequence, for instance a purified plasmid or known concentration of cells.

The objective of this work was to establish quantitative PCR as a reliable and efficient means of monitoring UV induced inactivation. The basis for this work is that the UV induced DNA lesions, which ultimately result in loss of culturability, may also impede the PCR amplification process by interfering with the DNA polymerase, used for PCR (44, 154). In principal, increased UV exposure will result in more DNA lesions, thereby decreasing the amplification of the damaged DNA segment as compared to an undamaged template. To test this hypothesis we conducted bench scale UVGI experiments using *Mycobacterium parafortuitum*, a well-characterized bioaerosol. Direct microscopy was concomitantly used to quantify the total microbial load airborne within the reactor. Traditional culture assays and quantitative PCR using *Mycobacteria*-specific primers were performed to assess inactivation.

6.2 Materials and Methods

6.2.1 Bench scale aerosol reactor

All UVGI studies were conducted using a previously characterized 0.8m^3 plastic reactor (126, 177). The reactor was cubic and constructed of 1.27cm thick clear Lucite plastic (Lucite International Ltd, Southhampton, UK). The chamber could be operated in batch or continuous mixed flow (CMF) mode. All experiments in this study were performed in the CMF mode. Compressed outdoor air was used as supply air in the CMF experiments. Supply air was passed through a 20cm x 3.8cm (length x inner diameter) polyvinyl chloride tube containing equal volumes of size 6-14 mesh activated carbon (Fisher Scientific, Pittsburgh, PA) and indicating desiccant (W.A. Hammond Drierite Company, Ltd., Xenia, Ohio). The filtered supply air was passed through a rotameter (Gilmon Instruments, Barrington, IL) via two Nalgene (0.635cm ID) tubes. Prior to entering the reactor the filtered supply air passed through two bacterial air vents (Pall Corp., Ann Arbor, MI) to prevent contamination by outdoor microbes. The chamber was mixed using a 42 W fan (Model 707; Caframo Ltd, Wiarton, Ontario, Canada).

The reactor had four low-pressure 30W mercury vapor UV lamps (G30T8, Osram-Sylvania, Hanover, MA), which were installed in the corners of the reactor and ran the entire height of the chamber. The lamps were wrapped in eight layers of aluminum air-conditioning filter mesh (Research Products Corporation, Madison, WI) to maintain the desired irradiance levels.

Irradiance levels were reevaluated using a radiometer outfitted with a narrow band filter (245nm) and determined to be similar to previously reported levels (126, 177). Prior to conducting inactivation experiments the lamps were allowed to run for 15 minutes to achieve a constant irradiance level. During inactivation studies all four lamps were used.

6.2.2 Bacterial cultures and growth conditions

Experiments were conducted using *Mycobacterium parafortuitum* (ATCC 19689). *M. parafortuitum* is a fast growing a rod shaped Gram positive bacteria 2-4 mm in length that forms colonies. The GC content of *M. parafortuitum* is 62-70 mole % (32). *M. parafortuitum* is closely related to *M. tuberculosis* and has previously been used as a surrogate in UV inactivation studies (179, 181, 243, 244).

6.2.3 Bioaerosol Generation

Bioaerosols were generated using a six-jet Collison (BGI Inc.) nebulizer operated at 20psi. All air used in the experiments (aerosol generation and ventilation) was carbon filtered and desiccated prior to use; RH was adjusted using filter sterilized water. The nebulizer was directly connected to the front of the pilot scale reactor via a Swage lock fitting. Aerosols were dispersed into the chamber through the aid of a four fans. An average of 8 minutes of aerosol generation time was used to achieve a sufficiently high bioaerosol concentration (~ 10^8 cells/m^3) within the pilot scale chamber (126, 177).

6.2.4 Bioaerosol Sampling

Bioaerosols were collected in the pilot scale reactor via SKC Biosampler impingers. SKC Biosamplers are the most efficient collection devices (greater than 80% collection efficiency) for particles in the range between 0.2-2.0 mm (237). A pump located outside of the reactor supplied the 12.5L/min vacuum needed for operating the Biosamplers. A DNA-free 1X Tris EDTA (TE) solution was used as the collection fluid.

6.2.5 Control and Experimental Protocol

Experiments were conducted in two phases – natural and engineered inactivation. The first set of experiments were based on a natural decay scenario and observed the deposition and removal of microorganisms using PCR. Bioaerosols were generated to a sufficiently high experimental concentration and allowed to decrease using a predetermined ventilation rate. Samples were collected at predetermined time intervals during the decay period based on parameters reported by Peccia (177). Findings from this set of experiments provided a benchmark from which to evaluate the potential impact that UVGI might have on QPCR. A ventilation rate of 3.5 h^{-1} was used in all experiments after the desired initial concentration of microorganisms was achieved.

A second set of experiments was based on an engineered decay scenario and assessed the inactivation of DNA by UVGI using PCR. After a sufficient bioaerosol concentration was achieved within the chamber, the UV lamps were turned on and samples collected at equally spaced time intervals

(177). Findings from this set of experiments provided an understanding of the impact that UVGI and relative humidity have on QPCR. Collected samples were aliquoted appropriately for all three assays.

6.2.6 Culture Assay

A modified plate method was used to enumerate culturable bacteria (177). Briefly, diluted impinger samples were plated using a spiral dispersing method (Spiral Biotech, Inc. Bethesda, MD) onto nutrient rich Soybean-Casein Digest Agar (SCDA) and incubated at 37^0C. Colonies were counted at the end of three days' incubation. Previous work compared spiral with standard spread plate counts and found no difference in culturing recovery between the two methods. The spiral method was found to have significantly less plating variability than the standard method (177).

6.2.7 Direct Count Enumeration

Aerosol samples were stained and enumerated using 4'6-diamidino-2-pheunulindole (DAPI) (Sigma Chemicals, St. Louis, MO). DAPI is a DNA binding fluorescent stain. Samples were incubated with DAPI for 5 minutes at 4^0C and then filtered through a 25mm diameter, 0.22 micrometer (average pore size) black polycarbonate filter (Poretics, Inc. Livermore, CA). Filters were mounted onto microscope slides using low fluorescence immersion oil containing an antifadent and examined under 1000x magnification using a Nikon Eclipse E400 epifluorescence microscope fitted with a mercury lamp and polarizing filters. A minimum of 10 random fields were counted per slide

and only intact brightly stained cells with obvious bacterial morphology were counted. Direct counts were reported as the average of all fields counted.

6.2.8 Genomic Extraction

Aerosol samples were concentrated onto 47mm diameter 0.2 micrometer nitrocellulose filters (100mL) (Nalgene Analytical Test Filter Funnel, Nalge Nunc International Rochester, NY) prior to genomic analysis. DNA was extracted from the filters using a bead beating protocol modified from Frank et al (80).

6.2.9 QPCR Assay

Extracted DNA was assayed using *Mycobacterium* specific SSU rDNA primers 8F/199Rev and SYBR® Green PCR Master Mix (Applied Biosystems, Forster City, CA). A typical 30 microliter PCR reaction included: 15 microliters SYBR mix, 1 microliter 8F, 1 microliter 199Rev, 11 microliter water, 2 microliter DNA template. Assays were performed on a MJ Research DNA Engine Opticon 2. The amplification protocol was as follows: 95^0C for 10 minutes, 95^0C for 15 seconds, 60^0C for 45 seconds, 80^0C for 1 second, 30 cycles total. All QPCR were performed in triplicate. Experimental samples were evaluated against a plasmid bearing the *M. parafortuitum* target gene. Standard curves were run for each experiment. Following assay all samples were run on 1.8% (w/v) agrose gel for confirmation.

6.2.10 QPCR Calibration

A plasmid bearing the *M. parafortuitum* target gene was generated and quantified by spectrophotometer measurements. Subsequent dilutions of the

plasmid were made to achieve a range of concentrations. Calibration assays were run in triplicate over the range of 10^1 to 10^7 target copies/microliter.

6.3 Results

6.3.1 Inactivation assessed by culture and QPCR

Inactivation assessment by UVGI as observed by standard culture and QPCR assessment are presented in Figure 6.1. A three-log inactivation was observed through the culture assessment, while the corresponding QPCR results show only a half log loss. Total airborne microorganism concentrations collected during the experiment show no cell loss over the duration of the experiment. Additionally, results from the quantitative PCR assay suggests total counts underestimate the concentration of airborne microorganisms by an order of magnitude. The QPCR assay was performed using *Mycobacterium parafortuitum* specific primers 8F/199R. A total of five potential thymine dimer sites were present in the amplified region.

Three additional QPCR reactions using *Mycobacterium* specific primers (199F/Myco2R, 515F/Myco2R, and 805F/Myco2R) were conducted to assess the effect of UV on PCR recovery. Additional reactions were performed to increase the number of potential thymine dimer sites in the amplified regions. All three reactions did not appear to be significantly affected by UV exposure and showed positive amplification.

6.4 Discussion

The goal of the study was to assess the applicability of quantitative PCR as a means for assessing specific microbe populations during and

following the UV inactivation of airborne bacteria. Quantitative PCR results suggest that DNA amplification within the *Mycobacterium parafortuitum* specific region (8F/199R) was not adversely impacted over the selected range of UV doses. In contrast, inactivation as measured by plate counts showed greater than a three-log loss in culturability for the same UV exposures. Additional QPCR assays using longer *Mycobacterium* specific primers (199F/Myco2R, 515F/Myco2R, and 805F/Myco2R) were performed on the same UV exposed airborne cultures. Longer primers were used to increase the number of potential thymine dimmer sites (28, 18, and 11 respectively) encountered by the DNA polymerase. Results of these complimentary amplifications showed no significant change in Ct response.

The positive amplification results from the four assays were not wholly unexpected. The *Mycobacterium parafortuitum* genome is approximately 4 x 10^6 nucleotides (nt) long and has an adenine + thymine (A + T) content of about 35% (50). The primers used in our QPCR assay amplified rRNA segments ranging in size from 191 nt to 762 nt. Contained within these selected regions were 5 to 28 potential thymine dimer sites. The probability of observing thymine dimer damage within the amplified segments was quite small based on estimates using data from previous work (177). The number of lesions per 10J/m^2 per 10Kbp is $7.3x10^{-3}$ at 40% RH and $1.0x10^{-2}$ at 95%RH for *M. parafortuitum*. Changing these parameters to those used in the experiments reported here, 40 J/m^2 dose and 200 nucleotides, the estimated number of lesions to have occurred in the investigated template

were 5.8 $x10^{-4}$ at 40% RH and $8.0x10^{-4}$ at 95% RH. These values correspond to 6 lesions in 10^4 cells and 60 lesions in 10^5 cells; these values are either below or at the limit of detection for the QPCR assay used here. Increasing the UV dose to 80 J/m^2, twice the amount needed for a 3-log loss in cultivation, would still result in too few lesions to be measured by the assay. The estimated number of lesions for *M. parafortuitum* for a given amount of dose and nucleotides is quite low when compared to estimates for other DNAs. Probability (quantum yield) values for several UV-PCR studies are listed in Table 6.1. Based on these estimated probabilities it is reasonable to presume that the QPCR assays were uninhibited because little or no thymine damage occurred in the targeted rRNA regions.

Previous work in the literature supports these findings although none have been reported in whole bacterial cells or in aerosols. A study conducted on single stranded (ss) RNA poliovirus reported that following a UV dose of ~300 J/m^2 four-logs loss of infectivity were observed in plaque assays, however PCR amplification was not adversely impacted (140). UV exposure was then increased to nearly 4000 J/m^2 and PCR amplification was still observed. The amplified regions in this study were approximately 313 and 396 nt in length. In a similar study design, a suspension of human tonisllar lymphocytes were irradiated and analyzed in small segments (<500bp) using PCR (90). Results of this work found that a UV dose of 2000 J/m^2 was needed for before PCR amplification was reduced by 10%. At a dose of 6000 J/m^2, thymine dimer lesions were determined to be present in half of the

susceptible sites. Both experiments were performed in liquid medium. Findings from these two experiments indicate that for shorter DNA segments (<500bp) the UV exposure required to obstruct PCR amplification far exceeds that needed loss of infectivity as measured by cell culture.

The UV exposures used in the short segment studies far exceed those needed for inactivation of several surrogate pathogens in air. Table 6.2 lists the UV exposure as calculated from their published Z values for several airborne species of interest. The dose range for *Mycobacterium parafortuitum* is 6.25 to 8.33 J/m^2 at 50 and 95% relative humidity (RH) (181). These reported values are 200 to 600 times smaller than those used in the poliovirus and lymphocytes studies. The literature also states that inactivation doses in air at 50% RH are much greater than inactivation rates determined in liquid medium (181). In general, the inactivation of airborne pathogenic surrogates occurs at UV exposures much less than that needed to interfere with the PCR amplification.

In studies where UV exposure impeded PCR amplification, the genomic segments analyzed were greater than 2000 nt in length (99, 116, 147). The UV doses (0 to 120 J/m^2) reported in these studies were two to three times larger than those used in the current experiment. Amplification diminished by as much as 75 to 85% at the highest exposures in all three studies. Two of the experiments were performed on cell media, while the third (99) was conducted in buffer solution. Findings from these experiments

indicate that large DNA segments (>2000 nt) and greater UV doses are needed to observe any interference with amplification.

Finally, a recent study has shown that PCR amplification of damaged template depends on the type of damage as well as its location within the template (216). A single thymine dimer was found to have a profound impact on amplification efficiency. Likewise, damage in tandem hindered amplification more so than two damage sites spaced apart by nucleotides.

In summary the use of quantitative PCR to measure airborne microbes during UV induced inactivation was stable. Analysis of earlier work in this area has shown that large segments of template, on the order of 2000 nt, are needed to observe amplification obstruction due to thymine dimer damage. The ramification of this is that the 16S rRNA genome, which is used for microbial community analyses and species identification, does not appear to be long enough (its only 1542 nt) for significant inhibition to occur at common UVGI doses. The UV doses needed to observe small changes in amplification efficiency far exceed those needed to achieve inactivation in air. Additionally, lengthy templates may adversely impact QPCR amplification efficiency (216). Therefore, current methods of assessing UV induced inactivation and thymine dimer concentrations, which include culture, immunoassays and endonuclease sensitive site assays, remain the best tools available for these measurements.

Organism	Lesions $10J/m^2/10$ Kbp	Reference
S. marcescens	1.6E-03	(177)
M. parafortuitum	7.3E-03	(177)
	1.0E-02	(177)
N-ras proto-oncogene	1.1E-02	(90)
T7 bacteriophage	1.49E-01	(99)
Adenovirus	4.00E-01	(71)
mitochondrial DNA	5.80E-01	(116)
hGH gene	6.52E-01	(147)

Table 6.1 Calculated quantum yields for select DNAs from previously published work.

Organism	Z value x 10^4 (cm^2/mW-sec)	UV Dose (J/m^2)	Reference
M. tuberculosis	23-42	2.4-4.3	(201)
	44-55	1.8-2.3	(201)
M. bovis BCG	33-39	2.6-3.0	(201)
	23-28	3.6-4.3	(201)
	5.3	18.9	(201)
	19.1	5.2	(180)
	12	8.3	(244)
M. pheli	2-5.3	18.9-50	(201)
	14	7.1	(84)
M. smegmatis	19	5.3	(84)
M. parafortuitum	12-16.3	6.1-8.3	(181)
	10	10	(180)
B. subtilis veg.	6.2-6.6	15.1-15.9	(181)
B. subtilis spores	3	33.3	(179)
S.marcescens	183-245	0.4-0.5	(200)
	35-45	2.2-2.9	(181)

Table 6.2 Calculated UV dose for select bacteria maintained in air at 50%RH from previously published Z values.

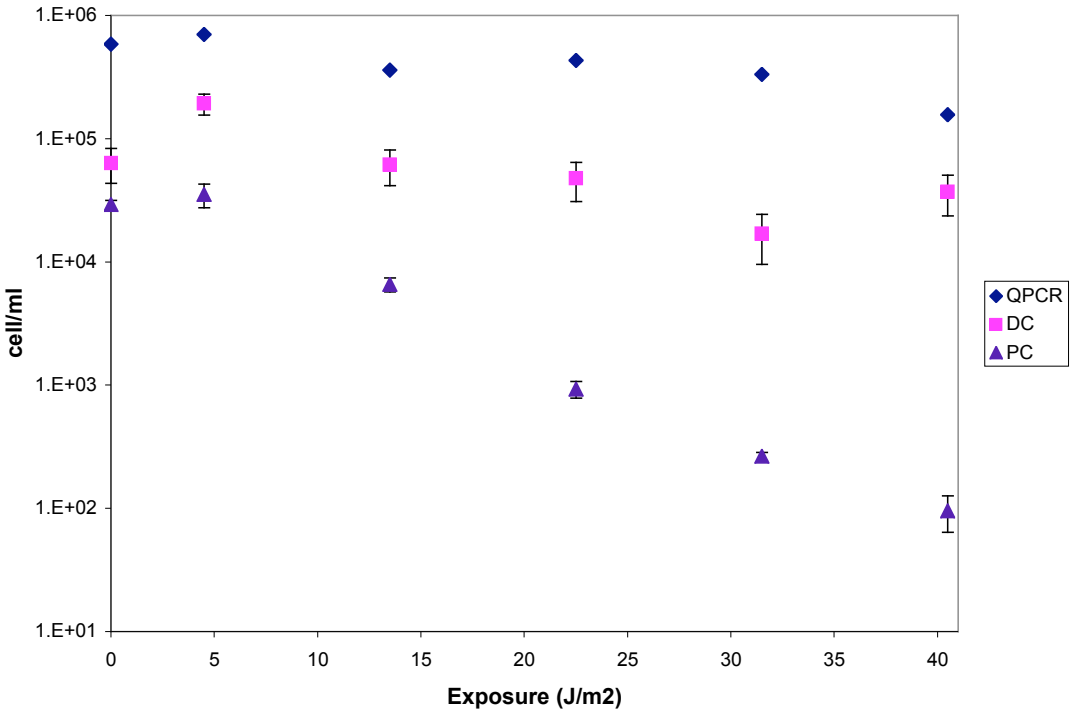


Figure 6.1 Inactivation of aerosolized M. parafortuitum as measured by culture and QPCR. Total counts provide an estimate of total bacteria present in the chamber during the experiment.

CHAPTER 7

CONCLUSIONS AND FUTURE RESEARCH RECOMMENDATIONS

7.1 Conclusions

The objective of this work was to use advanced molecular methods to (1) comprehensively survey airborne microorganisms in selected high exposure indoor and outdoor environments and (2) to observe the effects that typical doses of ultraviolet irradiation applied to aerosols may have on the recovery and amplification of target genes in airborne bacteria. Broad spectrum PCR, DNA sequence analyses and comparative phylogenetic tools were used to investigate three different settings, which have been conventionally considered as high exposure by the industrial hygiene community with respect to airborne microbes or other disease-causing bioaerosols. Quantitative PCR was employed to assess total airborne microorganism numbers over a broad range of germicidal UV doses in laboratory settings. QPCR and UV experiments were performed using pure cultures of *Mycobacterium parafortuitum*, a well-characterized strain often used to model the disinfection behavior of *Mycobacterium tuberculosis*. Results from these investigations led to the following conclusions:

- The microbial diversity recovered for each of the atmospheres sampled was much greater than had previously been observed by culture-based airborne ecology studies in similar settings.
- In all the atmospheric environments observed, potentially pathogenic respiratory agents were not observed in significant amounts as judged by

the relative abundance of their 16S rRNA genomes to the summative recovery of all 16S rRNA.

- Key bioinformatic indices leveraging clone library statistics from each of the investigated environments suggest that they were under sampled with respect to projections of total diversity of 16S genes that are likely present. In general, these findings indicate that airborne microbial diversity is much more complex than has been reported by culture based studies, and that sampling campaigns and the resolution of clone must be extended to more adequately describe the populations of airborne microbes.
- Comparative phylogenetic analyses of indoor and outdoor samples suggest that the ecology of airborne microbes observed indoors is less influenced by the immediate outdoor ecology than was previously thought. These findings also indicate that indoor bioaerosols are far more complex than their outdoor counterparts and that grab sample analysis of immediate outdoor ecology may serve as a poor baseline for industrial hygiene regulations
- Measurement of UV damage using quantitative PCR and 16S ribosomal RNA gene targets was found to be ineffective. The dose required to observe a change in common PCR amplification targets used for microbial identification far exceeds the amount required to observe inactivation by culture assay.

7.2 Applications to Practice and Future Research Recommendations

This research has demonstrated the utility and need for employing molecular based techniques to bioaerosol characterization. Results of the airborne ecology and inactivation studies provide critical information for researchers and engineers to consider when investigating airborne microorganisms in different environments. Based on these findings I present below recommendations for future research, which may help the industrial hygiene and environmental engineering community better understand the true identity, distribution and abundance of airborne microorganisms in a host of different indoor environments.

- Continued longitudinal studies of atmospheric microorganisms are clearly warranted. Consideration should be given to the analysis of other genomes for assessing inactivation response on model organisms, and gene expression profiling may provide insight into how airborne microbes respond to environmental stresses while airborne, and potential pathways toward microbial inactivation in an atmosphere (whether engineered or natural).
- Revision of industrial hygiene standards and practices to decrease regulatory reliance on culture-based analyses and increase the use of molecular based assays.
- Consider conducting long-term longitudinal aerobiology studies of flood-impacted residences. Ideally these studies would begin immediately after floodwaters have been removed, would continue through remediation work, and extend through reoccupation. Additional studies would provide a better

picture of the community changes that take place during remediation and reoccupation, with a reasonable baseline.

- Application of germicidal UV irradiation in real world environments with high particulate matter concentrations. These studies would help determine the limits of this technology. These experiments should be performed in parallel with airborne ecology studies to correlate inactivation with identity.
- Endonuclease-sensitive site (ESS) assay should be investigated for applicability to bioaerosol UV inactivation experiments in real time. The ESS assay sensitively quantifies pyrimidine dimer formation.
- Surveillance and characterization of the airborne microorganisms within poultry facilities should be performed for a more complete picture of the CAFO environment. Likewise, additional surveys of dairies and swine facilities should be considered to build upon the characterization presented here and to provide statistical robustness. Surveys of these healthy environments will provide a baseline upon which compare zoonotic outbreaks.

REFERENCES

1. **ACGIH.** 1999. Bioaerosols: Assessment and Control. American Conference of Governmental Industrial Hygienists, Cincinnati, OH.
2. **ACGIH.** 1998. Biologically Derived Airborne Contaminants, p. 11-14. *In* ACGIH (ed.), 1998 TLVs and BEIs. Threshold Limit Values for Chemical Substances and Physical Agents, BIological Exposure Indices. American Conference of Governmental Industrial Hygienists, Cincinnati, OH.
3. **Adhikari, A., T. Reponen, S. A. Lee, and S. A. Grinshpun.** 2004. Assessment of human exposure to airborne fungi in agricultural confinements: Personal inhalable sampling versus stationary sampling. Annals of Agricultural and Environmental Medicine **11:**269-277.
4. **Adhikari, A., M. M. Sen, S. Gupta-Bhattacharya, and S. Chanda.** 2004. Volumetric assessment of airborne fungi in two sections of a rural indoor dairy cattle shed. Environment International **29:**1071-1078.
5. **Agranovski, V., Z. Ristovski, P. J. Blackall, and L. Morawska.** 2004. Size-selective assessment of airborne particles in swine confinement building with the UVAPS. Atmospheric Environment **38:**3893-3901.
6. **Al-Jahdali, H., Z. A. Memish, and D. Menzies.** 2003. Tuberculosis in association with travel. International Journal of Antimicrobial Agents **21:**125-130.
7. **Alexandersen, S., Z. Zhang, A. I. Donaldson, and A. J. M. Garland.** 2003. The pathogenesis and diagnosis of foot-and-mouth disease. Journal of Comparative Pathology **129:**1-36.
8. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic Local Alignment Search Tool. Journal of Molecular Biology **215:**403-410.
9. **Alvarez, A. J., M. P. Buttner, and L. D. Stetzenbach.** 1995. PCR for Bioaerosol Monitoring - Sensitivity and Environmental Interference. Applied and Environmental Microbiology **61:**3639-3644.
10. **Amann, R. I., W. Ludwig, and K. H. Schleifer.** 1995. Phylogenetic Identification and in-Situ Detection of Individual Microbial-Cells without Cultivation. Microbiological Reviews **59:**143-169.
11. **An, H. R., G. Mainelis, and L. White.** 2006. Development and calibration of real-time PCR for quantification of airborne microorganisms in air samples. Atmospheric Environment **40:**7924-7939.
12. **Angenent, L. T., S. T. Kelley, A. St Amand, N. R. Pace, and M. T. Hernandez.** 2005. Molecular identification of potential pathogens in water and air of a hospital therapy pool. Proceedings of the National Academy of Sciences of the United States of America **102:**4860-4865.
13. **Angulo-Romero, J., A. Mediavilla-Molina, and E. Dominguez-Vilches.** 1999. Conidia of Alternaria in the atmosphere of the city of Cordoba, Spain in relation to meteorological parameters. International Journal of Biometeorology **43:**45-49.

14. **Anonymous.** 2005. Infectious Disease and Dermatologic Conditions in Evacuees and Rescue Workers After Hurricane Katrina--Multiple States, August-September, 2005. JAMA **294:**2158-2160.
15. **Atlas, R. M.** 2002. Bioterrorism: From threat to reality. Annual Review of Microbiology **56:**167-185.
16. **Avery, R. C., S. Wing, S. W. Marshall, and S. S. Schiffman.** 2004. Odor from industrial hog farming operations and mucosal immune function in neighbors. Archives of Environmental Health **59:**101-108.
17. **Baertsch, C., T. Paez-Rubio, E. Viau, and J. Peccia.** 2007. Source tracking aerosols released from land-applied class B biosolids during high-wind events. Applied and Environmental Microbiology **73:**4522-4531.
18. **Bjornsson, E., D. Norback, C. Janson, J. Widstrom, U. Palmgren, G. Strom, and G. Boman.** 1995. Asthmatic Symptoms and Indoor Levels of Microorganisms and House-Dust Mites. Clinical and Experimental Allergy **25:**423-431.
19. **Blanchard, D., and L. Syzdek.** 1971. Bubbles and Water-to Air Transfer of Bacteria. Bulletin of the American Meteorological Society **52:**1136-&.
20. **Blanchard, D., and L. Syzdek.** 1970. Mechanism for Water-to-Air Transfer and Concentration of Bacteria. Science **170:**626-&.
21. **Blanchard, D., and L. D. Syzdek.** 1972. Concentration of Bacteria in Jet Drops from Bursting Bubbles. Journal of Geophysical Research **77:**5087-&.
22. **Blanchard, D. C., and L. D. Syzdek.** 1982. Water-to-Air Transfer and Enrichment of Bacteria in Drops from Bursting Bubbles. Applied and Environmental Microbiology **43:**1001-1005.
23. **Bohrerova, Z., and K. G. Linden.** 2006. Assessment of DNA damage and repair in Mycobacterium terrae after exposure to UV irradiation. Journal of Applied Microbiology **101:**995-1001.
24. **Boreson, J., A. M. Dillner, and J. Peccia.** 2004. Correlating bioaerosol load with PM2.5 and PM10cf concentrations: a comparison between natural desert and urban-fringe aerosols. Atmospheric Environment **38:**6029-6041.
25. **Bornehag, C. G., J. Sundell, S. Bonini, A. Custovic, P. Malmberg, S. Skerfving, T. Sigsgaard, and A. Verhoeff.** 2004. Dampness in buildings as a risk factor for health effects, EUROEXPO: a multidisciplinary review of the literature (1998-2000) on dampness and mite exposure in buildings and health effects. Indoor Air **14:**243-257.
26. **Breugelmans, J. G., P. Zucs, K. Porten, S. Broll, M. Niedrig, A. Ammon, and G. Krause.** 2004. SARS transmission and commercial aircraft. Emerging Infectious Diseases **10:**1502-1503.
27. **Brickner, P. W., R. L. Vincent, M. First, E. Nardell, M. Murray, and W. Kaufman.** 2003. The application of ultraviolet germicidal irradiation to control transmission of airborne disease: Bioterrorism countermeasure. Public Health Reports **118:**99-114.

28. **Brodie, E. L., T. Z. DeSantis, J. P. M. Parker, I. X. Zubietta, Y. M. Piceno, and G. L. Andersen.** 2007. Urban aerosols harbor diverse and dynamic bacterial populations. PNAS **104:**299-304.
29. **Brooks, J., C. Gerba, and I. Pepper.** 2007. Diversity of aerosolized bacteria during land application of biosolids. Journal of Applied Microbiology **103:**1779-1790.
30. **Brown, P. D., and S. A. Lerner.** 1998. Community-acquired pneumonia. Lancet **352:**1295-1302.
31. **Bruggemann, H., A. Henne, F. Hoster, H. Liesegang, A. Wiezer, A. Strittmatter, S. Hujer, P. Durre, and G. Gottschalk.** 2004. The complete genome sequence of Propionibacterium acnes, a commensal of human skin. Science **305:**671-673.
32. **Buchanan, R. E., and N. E. Gibbons.** 1986. Bergey's Manual of Systematic Bacteriology. Williams and Wilkins, Baltimore.
33. **Burge, H.** 1990. Bioaerosols - Prevalence and Health-Effects in the Indoor Environment. Journal of Allergy and Clinical Immunology **86:**687-701.
34. **Burge, H.** 1990. Bioaerosols: Prevalence and health effects in the indoor environment. Journal of Allergy and Clinical Immunology **86:**687-701.
35. **Burger, H.** 1990. Bioaerosols: Prevalence and health effects in the indoor environment. Journal of Allergy and Clinical Immunology **86:**687-701.
36. **Burrell, R.** 1991. Microbiological Agents as Health Risks in Indoor Air. Environmental Health Perspectives **95:**29-34.
37. **Buttner, M. P., and L. D. Stetzenbach.** 1991. Evaluation of 4 Aerobiological Sampling Methods for the Retrieval of Aerosolized Pseudomonas-Syringae. Applied and Environmental Microbiology **57:**1268-1270.
38. **Buttner, M. P., K. Willeke, and S. A. Grinshpun.** 2002. Sampling and analysis of airborne microorganisms, p. 814-826. *In* C. Hurst, R. Crawford, G. Knudsen, M. McInerney, and L. D. Stetzenbach (ed.), Manual of Environmental Microbiology, 2 ed. ASM Press, Washington DC.
39. **Buttner, M. P., K. Willeke, and S. A. Grishpun.** 1997. Sampling and Analysis of Airborne MIcroorganisms p. 629-640. *In* C. J. Hurst (ed.), Manual of Environmental Microbiology. American Society for Microbiology, Washington D.C.
40. **Byrd, J. J., H. S. Xu, and R. R. Colwell.** 1991. Viable but Nonculturable Bacteria in Drinking-Water. Applied and Environmental Microbiology **57:**875-878.
41. **Cairns, L., D. Blythe, A. Kao, D. Pappagianis, L. Kaufman, J. Kobayashi, and R. Hajjeh.** 2000. Outbreak of coccidioidomycosis in Washington State residents returning from Mexico, p. 61-64. Univ Chicago Press.

42. **CDC.** 1994. Guidelines for preventing the transmission of Mycobacterium tuberculosis in health-care facilities. MMWR **43:**1-132.
43. **CDC.** 2006. Health concerns associated with mold in water-damaged homes after Hurricanes Katrina and Rita - New Orleans area, Louisiana, October 2005. Morbidity and Morality Weekly Report **55:**41-44.
44. **Chan, G. L., P. W. Doetsch, and W. A. Haseltine.** 1985. Cyclobutane Pyrimidine Dimers and (6-4) Photoproducts Block Polymerization by DNA-Polymerase-I. Biochemistry **24:**5723-5728.
45. **Chang, C. W., H. Chung, C. F. Huang, and H. J. J. Su.** 2001. Exposure of workers to airborne microorganisms in open-air swine houses. Applied and Environmental Microbiology **67:**155-161.
46. **Chao, A.** 1984. Nonparametric-Estimation of the Number of Classes in a Population. Scandinavian Journal of Statistics **11:**265-270.
47. **Chew, G. L., J. Wilson, F. A. Rabito, F. Grimsley, S. Iqbal, T. Reponen, M. L. Muilenberg, P. S. Thorne, D. G. Dearborn, and R. L. Morley.** 2006. Mold and endotoxin levels in the aftermath of Hurricane Katrina: A pilot project of homes in New Orleans undergoing renovation. Environmental Health Perspectives **114:**1883-1889.
48. **Chi, M. C., and C. S. Li.** 2005. Fluorochrome and fluorescent in situ hybridization to monitor bioaerosols in swine buildings. Aerosol Science and Technology **39:**1101-1110.
49. **Cole, D., L. Todd, and S. Wing.** 2000. Concentrated swine feeding operations and public health: A review of occupational and community health effects. Environmental Health Perspectives **108:**685-699.
50. **Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, A. Krogh, J. McLean, S. Moule, L. Murphy, K. Oliver, J. Osborne, M. A. Quail, M. A. Rajandream, J. Rogers, S. Rutter, K. Seeger, J. Skelton, R. Squares, S. Squares, J. E. Sulston, K. Taylor, S. Whitehead, and B. G. Barrell.** 1998. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature **393:**537-544.
51. **Colice, G. L., M. A. Morley, C. Asche, and H. G. Birnbaum.** 2004. Treatment costs of community-acquired pneumonia in an employed population. Chest **125:**2140-2145.
52. **Conant, J. M.** 2005, posting date. Corps prepares for Hurricane Katrina. [Online.]
53. **Cordes, L. G., D. W. Fraser, P. Skaliy, C. A. Perlino, W. R. Elsea, G. F. Mallison, and P. S. Hayes.** 1980. Legionnaires' Disease Outbreak at an Atlanta, Georgia, Country Club: Evidence for Spread from an Evaporative Condenser Am. J. Epidemiol. **111:**425-431.
54. **Cormier, Y., G. Tremblay, A. Meriaux, G. Brochu, and J. Lavoie.** 1990. Airbrone microbial contents in 2 types of swine confinement

buildings in Quebec. American Industrial Hygiene Association Journal **51:**304-309.

55. **Corren, J.** 2000. Allergic rhinitis: Treating the adult. The Journal of allergy and clinical immunology **105:**S610-S615.
56. **Cox, C. S.** 1989. Airborne bacteria and viruses. Science Prog Oxford **73:**469-500.
57. **Crook, B., J. Robertson, S. Glass, E. Botheroyd, J. Lacey, and M. Topping.** 1991. Airborne dust, ammonia, microorganisms, and antigens in pig confinement houses and the respiratory health of exposed farm-workers. American Industrial Hygiene Association Journal **52:**271-279.
58. **Cummings, K. J., J. Cox-Ganser, M. A. Riggs, N. Edwards, G. R. Hobbs, and K. Kreiss.** 2008. Health effects of exposure to water-damaged New Orleans homes six months after hurricanes Katrina and Rita. American Journal of Public Health **98:**869-875.
59. **Currier, M., D. S. King, M. R. Wofford, B. J. Daniel, and R. deShazo.** 2006. A katrina experience: Lessons learned. American Journal of Medicine **119:**986-992.
60. **Curtis, L., M. Ross, V. Persky, P. Scheff, R. Wadden, V. Ramakrisnan, and D. Hryhorczuk.** 2000. Bioaerosol Concentrations in the Quad Cities 1 Year after the 1993 Mississippi River Floods. Indoor and Built Environment **9:**35-43.
61. **Danuser, B., C. Weber, N. Kunzli, C. Schindler, and D. Nowak.** 2001. Respiratory symptoms in Swiss farmers: An epidemiological study of risk factors. American Journal of Industrial Medicine **39:**410-418.
62. **DeSantis, T. Z., P. Hugenholtz, K. Keller, E. L. Brodie, N. Larsen, Y. M. Piceno, and e. al.** 2006. NAST: a multiple sequence alignment server for comparative analysis of 16S rRNA genes. Nucleic Acids Reserach **34:**W394-W399.
63. **Dixon, R. E.** 1985. Economic Costs of Respiratory-Tract Infections in the United-States. American Journal of Medicine **78:**45-51.
64. **Dondero, T. J., R. C. Rendtorff, G. F. Mallison, R. M. Weeks, J. S. Levy, E. W. Wong, and W. Schaffner.** 1980. Outbreak of Legionnaires-Disease Associated with a Contaminated Air-Conditioning Cooling-Tower. New England Journal of Medicine **302:**365-370.
65. **Dowd, S. E., T. R. Callaway, R. D. Wolcott, Y. Sun, T. McKeehan, R. G. Hagevoort, and T. S. Edrington.** 2008. Evaluation of the bacterial diversity in the feces of cattle using 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP). Bmc Microbiology **8:**8.
66. **Downs, S. H., T. Z. Mitakakis, G. B. Marks, N. G. Car, E. G. Belousova, J. D. Leuppi, W. Xuan, S. R. Downie, A. Tobias, and J. K. Peat.** 2001. Clinical importance of Alternaria exposure in children. American Journal of Respiratory and Critical Care Medicine **164:**455-459.

67. **Dye, C.** 2006. Global epidemiology of tuberculosis. Lancet **367:**938-940.
68. **Eduard, W., and D. Heederik.** 1998. Methods for quantitative assessment of airborne levels of noninfectious microorganisms in highly contaminated work environments. American Industrial Hygiene Association Journal **59:**113-127.
69. **Eduard, W., J. Lacey, K. Karlsson, U. Palmgren, G. Strom, and G. Blomquist.** 1990. Evaluation of Methods for Enumerating Microorganisms in Filter Samples from Highly Contaminated Occupational Environments. American Industrial Hygiene Association Journal **51:**427-436.
70. **Eischeid, A. C., and K. G. Linden.** 2007. Efficiency of pyrimidine dimer formation in Escherichia coli across UV wavelengths. Journal of Applied Microbiology **103:**1650-1656.
71. **Eischeid, A. C., J. N. Meyer, and K. G. Linden.** 2009. UV Disinfection of Adenoviruses: Molecular Indications of DNA Damage Efficiency. Applied and Environmental Microbiology **75:**23-28.
72. **Ewing, B., L. Hillier, M. C. Wendl, and P. Green.** 1998. Base-calling of automated sequencer traces using phred. I. Accuracy assessment. Genome Research **8:**175-185.
73. **Fabian, M. P., S. L. Miller, T. Reponen, and M. T. Hernandez.** 2005. Ambient bioaerosol indices for indoor air quality assessments of flood reclamation. Journal of Aerosol Science **36:**763-783.
74. **Facklam, R., and J. Elliott.** 1995. Identification, Classification, and Clinical Relevance of Catalase-Negative, Gram-Postive Cocci, Excluding the Streptococci and Enterococci. Clinical Microbiology Reviews **8:**479-495.
75. **Ferguson, N. M., C. A. Donnelly, and R. M. Anderson.** 2001. The Foot-and-Mouth Epidemic in Great Britain: Pattern of Spread and Impact of Interventions. Science **292:**1155-1160.
76. **Fierer, N., Z. Z. Liu, M. Rodriguez-Hernandez, R. Knight, M. Henn, and M. T. Hernandez.** 2008. Short-term temporal variability in airborne bacterial and fungal populations. Applied and Environmental Microbiology **74:**200-207.
77. **Floret, N., J. F. Viel, F. Mauny, B. Hoen, and R. Piarroux.** 2006. Negligible risk for epidemics after geophysical disasters. Emerging Infectious Diseases **12:**543-548.
78. **Foresi, A.** 2000. A comparison of the clinical efficacy and safety of intranasal fluticasone propionate and antihistamines in the treatment of rhinitis, p. 12-14. Munksgaard Int Publ Ltd.
79. **Frank, D. N.** 2008. XplorSeq: A software environment for integrated management and phylogenetic analysis of metagenomic sequence data. BMC Bioinformatics **In press**.
80. **Frank, D. N., G. B. Spiegelman, W. Davis, E. Wagner, E. Lyons, and N. R. Pace.** 2003. Culture-Independent Molecular Analysis of

Microbial Constituents of the Healthy Human Outer Ear. J. Clin. Microbiol. **41:**295-303.

81. **Gambotto, A., S. M. Barratt-Boyes, M. D. de Jong, G. Neumann, and Y. Kawaoka.** 2008. Human infection with highly pathogenic H5N1 influenza virus. Lancet **371:**1464-1475.
82. **Gannon, B. W., C. M. Hayes, and J. M. Roe.** 2007. Survival rate of airborne Mycobacterium bovis. Research in Veterinary Science **82:**169-172.
83. **Gergen, P. J., P. C. Turkeltaub, and M. G. Kovar.** 1987. The Prevalence of Allergic Skin-Test Reactivity to 8 Common Aeroallergens in the United-States Population - Results from the 2nd National-Health and Nutrition Examination Survey. Journal of Allergy and Clinical Immunology **80:**669-679.
84. **Gillis, H. L.** 1974. Photoreactivation and Ultraviolet Inactivation of Mycobacteria in Air. Georgia Institute of Technology, Atlanta.
85. **Gloster, J., and S. Alexandersen.** 2004. New directions: Airborne transmission of foot-and-mouth disease virus. Atmospheric Environment **38:**503-505.
86. **Gluckman, S. J.** 2008. Acute respiratory infections in a recently arrived traveler to your part oft he world. Chest **134:**163-171.
87. **Goebel, B. M., and E. Stackebrandt.** 1994. Cultural and phylogenetic analysis of mixed microbial populations found in natural and commercial bioleaching environments. Appl. Environ. Microbiol. **60:**1614-1621.
88. **Gopalachar, A., R. L. Akins, W. R. Davis, and A. A. Siddiqui.** 2004. Urinary tract infection caused by Aerococcus viridans, a case report. Medical Science Monitor **10:**CS73-CS75.
89. **Gorny, R. L., T. Reponen, K. Willeke, D. Schmechel, E. Robine, M. Boissier, and S. A. Grinshpun.** 2002. Fungal fragments as indoor air biocontaminants. Applied and Environmental Microbiology **68:**3522-3531.
90. **Govan, H. L., Y. Vallesayoub, and J. Braun.** 1990. Fine-Mapping of DNA Damage and Repair in Specific Genomic Segments. Nucleic Acids Research **18:**3823-3830.
91. **Gregory, D. S.** 2006. Pertussis: A disease affecting all ages. American Family Physician **74:**420-426.
92. **Gyntelberg, F., P. Suadicani, J. W. Nielsen, P. Skov, O. Valbjorn, P. A. Nielsen, T. Schneider, O. Jorgensen, P. Wolkoff, C. K. Wilkins, S. Gravesen, and S. Norn.** 1994. Dust and the Sick Building Syndrome. Indoor Air-International Journal of Indoor Air Quality and Climate **4:**223-238.
93. **Haas, D., J. Habib, H. Galler, W. Buzina, R. Schlacher, E. Marth, and F. F. Reinthaler.** 2007. Assessment of indoor air in Austrian apartments with and without visible mold growth. Atmospheric Environment **41:**5192-5201.

94. **Hagy, G. W., and Settipan.Ga.** 1969. Bronchial Asthma, Allergic Rhinitis, and Allergy Skin Tests among College Students. Journal of Allergy **44:**323-&.
95. **Hambraeus, A.** 1988. Aerobiology in the Operating-Room - a Review. Journal of Hospital Infection **11:**68-76.
96. **Hanhela, R., K. Louhelainen, and A. L. Pasanen.** 1995. Prevalence of Microfungi in Finnish Cow Barns and Some Aspects of the Occurrence of Wallemia-Sebi and Fusaria. Scandinavian Journal of Work Environment & Health **21:**223-228.
97. **Heederik, D., and J. Douwes.** 1997. Towards an occupational exposure limit for endotoxins ? Annals of Agriculture and Environmental Medicine **4:**17-19.
98. **Heederik, D., T. Sigsgaard, P. S. Thorne, J. N. Kline, R. Avery, J. H. Bonlokke, E. A. Chrischilles, J. A. Dosman, C. Duchaine, S. R. Kirkhorn, K. Kulhankova, and J. A. Merchant.** 2007. Health effects of airborne exposures from concentrated animal feeding operations. Environmental Health Perspectives **115:**298-302.
99. **Hegedus, M., K. Modos, G. Ronto, and A. Fekete.** 2003. Validation of phage T7 biological dosimeter by quantitative polymerase chain reaction using short and long segments of phage T7 DNA. Photochemistry and Photobiology **78:**213-219.
100. **Henderson, D. A.** 1999. Smallpox: Clinical and epidemiologic features. Emerging Infectious Diseases **5:**537-539.
101. **Henderson, D. A.** 1999. Weapons for the future. Lancet **354:**64-64.
102. **Hernandez, M., S. L. Miller, D. W. Landfear, and J. M. Macher.** 1999. A combined fluorochrome method for quantitation of metabolically active and inactive airborne bacteria. Aerosol Science and Technology **30:**145-160.
103. **Hinds, W. C.** 1999. Bioaerosols, Aerosol Technology: Properties, Behavoir, and Measurement of Airborne Particles. Wiley-Interscience, New York.
104. **Hinds, W. C.** 1999. Filtration, p. 182-205, Aerosol Technology: Properties, Behavior, and Measurement of Airborne Particles. Wiley-Interscience, Washington DC.
105. **Hinds, W. C.** 1999. Straight-Line Acceleration and Curvilinear Particle Motion, p. 111-140, Aerosol Technology: Properties, Behavior and Measurement of Airborne Particles. Wiley-Interscience.
106. **Hirst, J. M.** 1995. Bioaerosols: introduction, retrospect and prospect., p. 1-10. *In* C. S. Cox and C. M. Wathes (ed.), Bioaerosols Handbook. Lewis Publisher.
107. **Hoppe, P., and I. Martinac.** 1998. Indoor climate and air quality - Review of current and future topics in the field of ISB study group 10. International Journal of Biometeorology **42:**1-7.
108. **Horner, W. E., A. Helbling, J. E. Salvaggio, and S. B. Lehrer.** 1995. Fungal Allergens. Clinical Microbiology Reviews **8:**161-179.

109. **Horner, W. E., A. G. Worthan, and P. R. Morey.** 2004. Air- and Dustborne Mycoflora in Houses Free of Water Damage and Fungal Growth. Appl. Environ. Microbiol. **70:**6394-6400.
110. **Huber, T., G. Faulkner, and P. Hugenholtz.** 2004. Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics **20:**2317-2319.
111. **Hughes, K. A., H. A. McCartney, T. A. Lachlan-Cope, and D. A. Pearce.** 2004. A preliminary study of airborne microbial biodiversity over peninsular Antarctica. Cellular and Molecular Biology **50:**537-542.
112. **Hussong, D., R. R. Colwell, M. Obrien, E. Weiss, A. D. Pearson, R. M. Weiner, and W. D. Burge.** 1987. Viable Legionella-Pneumophila Not Detectable by Culture on Agar Media. Bio-Technology **5:**947-950.
113. **Hyvarinen, A., T. Meklin, A. Vepsalainen, and A. Nevalainen.** 2002. Fungi and actinobacteria in moisture-damaged building materials -- concentrations and diversity. International Biodeterioration & Biodegradation **49:**27-37.
114. **IOM.** 2004. Damp Indoors and Health. Institute of Medicine of the National Academies, Washington D.C.
115. **Jo, W. K., and J. H. Kang.** 2005. Exposure levels of airborne bacteria and fungi in Korean swine and poultry sheds. Archives of Environmental & Occupational Health **60:**140-146.
116. **Kalinowski, D. P., S. Illenye, and B. Vanhouten.** 1992. Analysis of DNA Damage and Repair in Murine Leukemia-L1210 Cells Using a Quantitative Polymerase Chain-Reaction Assay. Nucleic Acids Research **20:**3485-3494.
117. **Kepner, R., and J. Pratt.** 1994. Use of Fluorochromes for Direct Enumeration of Total Bacteria in Environmental Samples: Past and Present. Microbiological Reviews **58:**603-615.
118. **Kerbaugh, M., and J. Evans.** 1968. Aerococcus viridans in the hospital environment. Applied Microbiology **16:**519-523.
119. **Khan, J., L. E. del Rio, R. Nelson, V. Rivera-Varas, G. A. Secor, and M. F. R. Khan.** 2008. Survival, dispersal, and primary infection site for Cercospora beticola in sugar beet. Plant Disease **92:**741-745.
120. **Kim, K. Y., H. J. Ko, H. T. Kim, C. N. Kim, and Y. S. Kim.** 2008. Assessment of airborne bacteria and fungi in pig buildings in Korea. Biosystems Engineering **99:**565-572.
121. **Kimura, K., A. Adlakha, and P. M. Simon.** 1998. Fatal case of swine influenza virus in an immunocompetent host. Mayo Clinic Proceedings **73:**243-245.
122. **Klietmann, W. F., and K. L. Ruoff.** 2001. Bioterrorism: Implications for the Clinical Microbiologist. Clin. Microbiol. Rev. **14:**364-381.
123. **Klontz, K. C., N. A. Hynes, R. A. Gunn, M. H. Wilder, M. W. Harmon, and A. P. Kendal.** 1989. An Outbreak of Influenza a/Taiwan/1/86 (H1n1) Infections at a Naval Base and Its Association with Airplane Travel. American Journal of Epidemiology **129:**341-348.

124. **Koch, A., K. J. Heilemann, W. Bischof, J. Heinrich, and H. E. Wichmann.** 2000. Indoor viable mold spores - a comparison between two cities, Erfurt (eastern Germany) and Hamburg (western Germany). Allergy **55:**176-180.
125. **Kohn, G. J., S. R. Linne, C. M. Smith, and P. D. Hoeprich.** 1992. Acquisition of Coccidioidomycosis at Necropsy by Inhalation of Coccidioidal Endospores. Diagnostic Microbiology and Infectious Disease **15:**527-530.
126. **Kujundzic, E.** 2005. Engineering Control Technologies for Removing or Inactivating Biologically-Derived Particles in the Indoor Environment. University of Colorado, Boulder.
127. **Kujundzic, E., M. Hernandez, and S. L. Miller.** 2007. Ultraviolet germicidal irradiation inactivation of airborne fungal spores and bacteria in upper-room air and HVAC in-duct configurations. Journal of Environmental Engineering and Science **6:**1-9.
128. **Kujundzic, E., D. A. Zander, M. Hernandez, L. T. Angenent, D. E. Henderson, and S. L. Miller.** 2005. Effects of ceiling-mounted HEPA-UV air filters on airborne bacteria concentrations in an indoor therapy pool building. Journal of the Air & Waste Management Association **55:**210-218.
129. **Lee, S. A., A. Adhikari, S. A. Grinshpun, R. McKay, R. Shukla, and T. Reponen.** 2006. Personal exposure to airborne dust and microorganisms in agricultural environments. Journal of Occupational and Environmental Hygiene **3:**118-130.
130. **Leser, T. D., J. Z. Amenuvor, T. K. Jensen, R. H. Lindecrona, M. Boye, and K. Moller.** 2002. Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. Applied and Environmental Microbiology **68:**673-690.
131. **Li, Y., S. Duan, I. T. S. Yu, and T. W. Wong.** 2005. Multi-zone modeling of probable SARS virus transmission by airflow between flats in Block E, Amoy Gardens. Indoor Air **15:**96-111.
132. **Lillie, L. E.** 1974. Bovine Respiratory-Disease Complex. Canadian Veterinary Journal-Revue Veterinaire Canadienne **15:**233-242.
133. **Lin, X. J., T. Reponen, K. Willeke, Z. Wang, S. A. Grinshpun, and M. Trunov.** 2000. Survival of airborne microorganisms during swirling aerosol collection. Aerosol Science and Technology **32:**184-196.
134. **Loyse, A., R. A. Storring, and M. Melzer.** 2006. Pseudomonas stutzeri pneumonia in an HIV seropositive patient. Journal of Infection **53:**75-76.
135. **Lozupone, C., M. Hamady, S. T. Kelley, and R. Knight.** 2007. Quantitative and qualitative {beta} diversity measures lead to different insights into factors that structure microbial communities. Appl. Environ. Microbiol.
136. **Lozupone, C., M. Hamady, and R. Knight.** 2006. UniFrac - An online tool for comparing microbial community diversity in a phylogenetic context. Bmc Bioinformatics **7**.

137. **Lozupone, C., and R. Knight.** 2005. UniFrac: a New Phylogenetic Method for Comparing Microbial Communities. Appl. Environ. Microbiol. **71:**8228-8235.
138. **Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Forster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. Konig, T. Liss, R. Lussmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, and K. H. Schleifer.** 2004. ARB: a software environment for sequence data. Nucleic Acids Research **32:**1363-1371.
139. **Madelin, T. M.** 1994. Fungal Aerosols - a Review. Journal of Aerosol Science **25:**1405-1412.
140. **Maier, A., D. Tougianidou, A. Wiedenmann, and K. Botzenhart.** 1995. Detection of Poliovirus by Cell-Culture and by PCR after UV Disinfection, p. 141-145. Pergamon-Elsevier Science Ltd.
141. **Mandell, Douglas, and Bennett.** 2005. Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases, 6th ed, vol. 1 and 2. Elsevier.
142. **Maron, P. A., D. P. H. Lejon, E. Carvalho, K. Bizet, P. Lemanceau, L. Ranjard, and C. Mougel.** 2005. Assessing genetic structure and diversity of airborne bacterial communities by DNA fingerprinting and 16S rDNA clone library. Atmospheric Environment **39:**3687-3695.
143. **Maroni, M., R. Axelrad, and A. Bacaloni.** 1995. Nato Efforts to Set Indoor Atr Quality Guidelines and Standards. American Industrial Hygiene Association Journal **56:**499-508.
144. **Marrie, T. J.** 1994. Community-Acquired Pneumonia. Clinical Infectious Diseases **18:**501-515.
145. **Martin, V., A. I. Vela, M. Gilbert, J. Cebolla, J. Goyache, L. Dominguez, and J. F. Fernandez-Garayzabal.** 2007. Characterization of Aerococcus viridans isolates from swine clinical specimens. Journal of Clinical Microbiology **45:**3053-3057.
146. **Martinez, K. F., C. Y. Rao, and N. C. Burton.** 2004. Exposure assessment and analysis for biological agents. Grana **43:**193-208.
147. **McCarthy, M. J., J. I. Rosenblatt, and R. S. Lloyd.** 1996. A modified quantitative polymerase chain reaction assay for measuring gene-specific repair of UV photoproducts in human cells. Mutation Research-DNA Repair **363:**57-66.
148. **McCartney, H. A., S. J. Foster, B. A. Fraaije, and E. Ward.** 2003. Molecular diagnostics for fungal plant pathogen. Pest Management Science **59:**129-142.
149. **McCartney, H. A., M. E. Lacey, Q. Li, and A. Heran.** 1999. Airborne ascospore concentration and the infection of oilseed rape and sunflowers by Sclerotinia sclerotiorum, 10th International Rapeseed Congress, Canberra, Australia.

150. **Miller, S. L., and J. M. Macher.** 2000. Evaluation of a methodology for quantifying the effect of room air ultraviolet germicidal irradiation on airborne bacteria. Aerosol Science and Technology **33:**274-295.
151. **Mitakakis, T., E. Ong, A. Stevens, D. Guest, and R. Knox.** 1997. Incidence of Cladosporium, alternaria and total fungal spores in the atmosphere of melbourne (Australia) over three years. Aerobiologia **13:**83-90.
152. **Mojica, B.** 1998. Airborne pathogens, p. 506-507. Oxford Univ Press Inc.
153. **Monte, S. V., N. M. Paolini, E. M. Slazak, J. J. Schentag, and J. A. Paladino.** 2008. Costs of treating lower respiratory tract infections in outpatients. American Journal of Managed Care **14:**190-196.
154. **Moore, P., and B. S. Strauss.** 1979. Sites of Inhibition of Invitro DNA-Synthesis in Carcinogen-Treated and Uv-Treated Phi-X174 DNA. Nature **278:**664-666.
155. **Moore, R. T.** 1986. A Note on Wallemia-Sebi. Antonie Van Leeuwenhoek Journal of Microbiology **52:**183-187.
156. **Moreno-Lopez, J.** 1990. Acute respiratory disease in cattle, p. 551-554. *In* Z. Dinter and B. Morein (ed.), Virus infections in ruminants. Elsevier publishers, Amsterdam.
157. **Morey, P. R., M. C. Hull, and M. Andrew.** 2003. El Nino water leaks identify rooms with concealed mould growth and degraded indoor air quality. International Biodeterioration & Biodegradation **52:**197-202.
158. **Moschandreas, D. J., K. R. Pagilla, and L. V. Storino.** 2003. Time and space uniformity of indoor bacteria concentrations in Chicago area residences. Aerosol Science and Technology **37:**899-906.
159. **Myers, K. P., C. W. Olsen, S. F. Setterquist, A. W. Capuano, K. J. Donham, E. L. Thacker, J. A. Merchant, and G. C. Gray.** 2006. Are swine workers in the United States at increased risk of infection with zoonotic influenza virus? Clinical Infectious Diseases **42:**14-20.
160. **Nathavitharana, K. A., S. N. Arseculeratne, H. A. Aponso, R. Vijeratnam, L. Jayasena, and C. Navaratnam.** 1983. Acute Meningitis in Early-Childhood Caused by Aerococcus-Viridans. British Medical Journal **286:**1248-1248.
161. **Nehme, B., V. Letourneau, R. J. Forster, M. Veillette, and C. Duchaine.** 2008. Culture-independent approach of the bacterial bioaerosol diversity in the standard swine confinement buildings, and assessment of the seasonal effect. Environmental Microbiology **10:**665-675.
162. **Nevalainen, A., A. L. Pasanen, M. Niininen, T. Reponen, P. Kalliokoski, and M. J. Jantunen.** 1991. The Indoor Air-Quality in Finnish Homes with Mold Problems. Environment International **17:**299-302.
163. **Ni, J. Q., and A. J. Heber.** 2008. Sampling and measurement of ammonia at animal facilities, p. 201-269, Advances in Agronomy, Vol 98, vol. 98. Elsevier Academic Press Inc, San Diego.

164. **Niederman, M. S., J. S. McCombs, A. N. Unger, A. Kumar, and R. Popovian.** 1998. The cost of treating community-acquired pneumonia. Clinical Therapeutics **20:**820-837.
165. **Niederman, M. S., J. S. McCombs, A. N. Unger, A. Kumar, and R. Popovian.** 1999. Treatment cost of acute exacerbations of chronic bronchitis. Clinical Therapeutics **21:**576-591.
166. **Niemeier, R. T., S. K. Sivasubramani, T. Reponen, and S. A. Grinshpun.** 2006. Assessment of Fungal Contamination in Moldy Homes: Comparison of Different Methods. Journal of Occupational and Environmental Hygiene **3:**262 - 273.
167. **NYC-DOH.** 1994. Guidelines on assessment and redediation of fungi in indoor environments. New York City Department of Health & Mental Hygiene, Bureau of Environmental & Occupational Disease Epiddemiology.
168. **O'Loughlin, R. E., L. Kightlinger, M. C. Werpy, E. Brown, V. Stevens, C. Hepper, T. Keane, R. F. Benson, B. S. Fields, and M. R. Moore.** 2007. Restaurant outbreak of Legionnaires' disease associated with a decorative fountain: an environmental and case-control study. Bmc Infectious Diseases **7:**9.
169. **Olenchock, S. A.** 1994. Health effects of biological agents: The role of endotoxins. Applied Occupational and Environmental Hygiene **9:**62-64.
170. **Onyebujoh, P., A. Zumla, I. Ribeiro, R. Rustomjee, P. Mwaba, M. Gomes, and J. M. Grange.** 2005. Treatment of tuberculosis: present status and future prospects. Bulletin of the World Health Organization **83:**857-865.
171. **Pace, N. R.** 1997. A molecular view of microbial diversity and the biosphere. Science **276:**734-740.
172. **Paez-Rubio, T., E. Viau, S. Romero-Hernandez, and J. Peccia.** 2005. Source bioaerosol concentration and rRNA gene-based identification of microorganisms aerosolized at a flood irrigation wastewater reuse site. Applied and Environmental Microbiology **71:**804-810.
173. **Papineau, D., J. J. Walker, S. J. Mojzsis, and N. R. Pace.** 2005. Composition and structure of microbial communities from stromatolites of Hamelin Pool in Shark Bay, Western Australia. Applied and Environmental Microbiology **71:**4822-4832.
174. **Pardue, J. H., W. M. Moe, D. McInnis, L. J. Thibodeaux, K. T. Valsaraj, E. Maciasz, I. van Heerden, N. Korevec, and Q. Z. Yuan.** 2005. Chemical and microbiological parameters in New Orleans floodwater following Hurricane Katrina. Environmental Science & Technology **39:**8591-8599.
175. **Pastuszka, J. S., U. Kyaw Tha Paw, D. O. Lis, A. Wlazlo, and K. Ulfig.** 2000. Bacterial and fungal aerosol in indoor environment in Upper Silesia, Poland. Atmospheric Environment **34:**3833-3842.
176. **Patrick, M., and R. Rahn.** 1976. Photochemistry of DNA and Polynucleotides: Photoproducts. *In* W. SY (ed.), Photochemistry and

Photobiology of Nucleic Acids, vol. II Biology. Academic Press, New York.

177. **Peccia, J.** 2000. The Response of Airborne Bacteria to Ultraviolet Germicidal Radiation. University of Colorado at Boulder.
178. **Peccia, J., and M. Hernandez.** 2006. Incorporating polymerase chain reaction-based identification, population characterization, and quantification of microorganisms into aerosol science: A review. Atmospheric Environment **40:**3941-3961.
179. **Peccia, J., and M. Hernandez.** 2001. Photoreactivation in airborne Mycobacterium parafortuitum. Applied and Environmental Microbiology **67:**4225-4232.
180. **Peccia, J., and M. Hernandez.** 2004. UV-induced inactivation rates for airborne Mycobacterium bovis BCG. Journal of Occupational and Environmental Hygiene **1:**430-435.
181. **Peccia, J., H. M. Werth, S. Miller, and M. Hernandez.** 2001. Effects of relative humidity on the ultraviolet induced inactivation of airborne bacteria. Aerosol Science and Technology **35:**728-740.
182. **Peltola, J., M. A. Andersson, T. Haahtela, H. Mussalo-Rauhamaa, F. A. Rainey, R. M. Kroppenstedt, R. A. Samson, and M. S. Salkinoja-Salonen.** 2001. Toxic-metabolite-producing bacteria and fungus in an indoor environment. Applied and Environmental Microbiology **67:**3269-3274.
183. **PHD-NSW.** 2000. The health of the people of New South Wales: report of the Chief Health Officer, 2000. NSW Health Department.
184. **Pitkaranta, M., T. Meklin, A. Hyvarinen, L. Paulin, P. Auvinen, A. Nevalainen, and H. Rintala.** 2008. Analysis of fungal flora in indoor dust by ribosomal DNA sequence analysis, quantitative PCR, and culture. Applied and Environmental Microbiology **74:**233-244.
185. **Pommepuy, M., M. Butin, A. Derrien, M. Gourmelon, R. R. Colwell, and M. Cormier.** 1996. Retention of enteropathogenicity by viable but nonculturable Escherichia coli exposed to seawater and sunlight. Applied and Environmental Microbiology **62:**4621-4626.
186. **Pomorska, D., L. Larsson, C. Skorska, J. Sitkowska, and J. Dutkiewicz.** 2007. Levels of bacterial endotoxin in air of animal houses determined with the use of gas chromatography - Mass spectrometry and Limulus test. Annals of Agricultural and Environmental Medicine **14:**291-298.
187. **Popescu, G. A., E. Benea, E. Mitache, C. Piper, and D. Horstkotte.** 2005. An unusual bacterium, Aerococcus viridans, and four cases of infective endocarditis. Journal of Heart Valve Disease **14:**317-319.
188. **Predicala, B. Z., R. G. Maghirang, S. B. Jerez, J. E. Urban, and R. D. Goodband.** 2001. Dust and bioaerosol concentrations in two swine-finishing buildings in Kansas. Transactions of the Asae **44:**1291-1298.
189. **Predicala, B. Z., J. E. Urban, R. G. Maghirang, S. B. Jerez, and R. D. Goodband.** 2002. Assessment of bioaerosols in swine barns by filtration and impaction. Current Microbiology **44:**136-140.

190. **Presley, S. M., T. R. Rainwater, G. P. Austin, S. G. Platt, J. C. Zak, G. P. Cobb, E. J. Marsland, K. Tian, B. H. Zhang, T. A. Anderson, S. B. Cox, M. T. Abel, B. D. Leftwich, J. R. Huddleston, R. M. Jeter, and R. J. Kendall.** 2006. Assessment of pathogens and toxicants in New Orleans, LA following Hurricane Katrina. Environmental Science & Technology **40:**468-474.
191. **Radon, K., B. Danuser, M. Iversen, E. Monso, C. Weber, J. Hartung, K. J. Donham, U. Palmgren, and D. Nowak.** 2002. Air contaminants in different European farming environments. Annals of Agricultural and Environmental Medicine **9:**41-48.
192. **Rao, C. Y., M. A. Riggs, G. L. Chew, M. L. Muilenberg, P. S. Thorne, D. V. Sickle, K. H. Dunn, and C. Brown.** 2007. Characterizing airborne molds, endotoxins and glucans in homes in New Orleans after Hurricanes Katrina and Rita. Appl. Environ. Microbiol.**:**AEM.01973-06.
193. **Rautiala, S., T. Reponen, A. Hyvarinen, A. Nevalainen, T. Husman, A. Vehvilainen, and P. Kalliokoski.** 1996. Exposure to airborne microbes during the repair of moldy buildings. American Industrial Hygiene Association Journal **57:**279-284.
194. **Reboux, G., R. Piarroux, F. Mauny, A. Madroszyk, L. Millon, K. Bardonnet, and J. C. Dalphin.** 2001. Role of molds in farmer's lung disease in eastern France. American Journal of Respiratory and Critical Care Medicine **163:**1534-1539.
195. **Reed, S. D., T. A. Lee, and D. C. McCrory.** 2004. The economic burden of allergic rhinitis - A critical evaluation of the literature. Pharmacoeconomics **22:**345-361.
196. **Reisler, R. B., and H. Blumberg.** 1999. Community-acquired Pseudomonas stutzeri vertebral osteomyelitis in a previously healthy patient: Case report and review. Clinical Infectious Diseases **29:**667-669.
197. **Reponen, T., S. A. Grinshpun, K. L. Conwell, J. Wiest, and W. J. Anderson.** 2001. Aerodynamic versus physical size of spores: Measurement and implication for respiratory deposition. Grana **40:**119-125.
198. **Richardson, L. J., C. L. Hofacre, B. W. Mitchell, and J. L. Wilson.** 2003. Effect of electrostatic space charge on reduction of airborne transmission of Salmonella and other bacteria in broiler breeders in production and their progeny. Avian Diseases **47:**1352-1361.
199. **Riggs, M. A., C. Y. Rao, C. M. Brown, D. Van Sickle, K. J. Cummings, K. H. Dunn, J. A. Deddens, J. Ferdinands, D. Callahan, R. L. Moolenaar, and L. E. Pinkerton.** 2008. Resident cleanup activities, characteristics of flood-damaged homes and airborne microbial concentrations in New Orleans, Louisiana, October 2005. Environmental Research **106:**401-409.
200. **Riley, R. L., and J. E. Kaufman.** 1972. Effect of Relative Humidity on Inactivation of Airborne Serratia-Marcescens by Ultraviolet-Radiation. Applied Microbiology **23:**1113-&.

201. **Riley, R. L., M. Knight, and G. Middlebrook.** 1976. Ultraviolet Susceptibility of Bcg and Virulent Tubercle-Bacilli. American Review of Respiratory Disease **113:**413-418.
202. **Rintala, H., M. Pitkaeranta, M. Toivola, L. Paulin, and A. Nevalainen.** 2008. Diversity and seasonal dynamics of bacterial community in indoor environment. Bmc Microbiology **8:**13.
203. **Roig, P., A. Orti, and V. Navarro.** 1996. Meningitis due to Pseudomonas stutzeri in a patient infected with human immunodeficiency virus. Clinical Infectious Diseases **22:**587-588.
204. **Roszak, D. B., D. J. Grimes, and R. R. Colwell.** 1984. Viable but Nonrecoverable Stage of Salmonella-Enteritidis in Aquatic Systems. Canadian Journal of Microbiology **30:**334-338.
205. **Sakamoto, T., A. Urisu, M. Yamada, Y. Matsuda, K. Tanaka, and S. Torii.** 1989. Studies on the Osmophilic Fungus Wallemia-Sebi as an Allergen Evaluated by Skin Prick Test and Radioallergosorbent Test. International Archives of Allergy and Applied Immunology **90:**368-372.
206. **Salonen, H., S. Lappalainen, O. Lindroos, R. Harju, and K. Reijula.** 2007. Fungi and bacteria in mould-damaged and non-damaged office environments in a subarctic climate. Atmospheric Environment **41:**6797-6807.
207. **Samet, J. M.** 2004. How Do We Catch Colds? Am. J. Respir. Crit. Care Med. **169:**1175-1176.
208. **Samet, J. M., M. C. Marbury, and J. D. Spengler.** 1987. Respiratory efects of indoor air pollution. Journal of Allergy and Clinical Immunology **79:**685-700.
209. **Sawyer, L. A., J. J. Murphy, J. E. Kaplan, P. F. Pinsky, D. Chacon, S. Walmsley, L. B. Schonberger, A. Phillips, K. Forward, C. Goldman, J. Brunton, R. A. Fralick, A. O. Carter, W. G. Gary, R. I. Glass, and D. E. Low.** 1988. 25-Nm to 30-Nm Virus Particle Associated with a Hospital Outbreak of Acute Gastroenteritis with Evidence for Airborne Transmission. American Journal of Epidemiology **127:**1261-1271.
210. **Schafer, M. P., J. E. Fernback, and M. K. Ernst.** 1999. Detection and characterization of airborne Mycobacterium tuberculosis H37Ra particles, a surrogate for airborne pathogenic M-tuberculosis. Aerosol Science and Technology **30:**161-173.
211. **Schenker, M. B., D. Christiani, Y. Cormier, H. Dimich-Ward, G. Doekes, J. Dosman, J. Douwes, K. Dowling, D. Enarson, F. Green, D. Heederik, K. Husman, S. Kennedy, G. Kullman, Y. Lacasse, B. Lawson, P. Malmberg, J. May, S. McCurdy, J. Merchant, J. Myers, M. Nieuwenhuijsen, S. Olenchock, C. Saiki, D. Schwartz, J. Seiber, P. Thorne, G. Wagner, N. White, X. P. Xu, and M. Chan-Yeung.** 1998. Respiratory health hazards in agriculture. American Journal of Respiratory and Critical Care Medicine **158:**S1-S76.
212. **Schloss, P. D., and J. Handelsman.** 2006. Introducing SONS, a tool for operational taxonomic unit-based comparisons of microbial

community memberships and structures. Applied and Environmental Microbiology **72:**6773-6779.

213. **Seedorf, J., J. Hartung, M. Schroder, K. H. Linkert, V. R. Phillips, M. R. Holden, R. W. Sneath, J. L. Short, R. P. White, S. Pedersen, H. Takai, J. O. Johnsen, J. H. M. Metz, P. Koerkamp, G. H. Uenk, and C. M. Wathes.** 1998. Concentrations and emissions of airborne endotoxins and microorganisms in livestock buildings in Northern Europe. Journal of Agricultural Engineering Research **70:**97-109.
214. **Shaughnessy, R. J., P. R. Morey, and E. C. Cole.** 1999. Prevention and Control of Microbial Contamination, p. 10-1:10-13. *In* J. Macher (ed.), Bioaerosols: Assessment and Control. American Conference of Governmental Industrial Hygienists, Cincinnati, OH.
215. **Shultz, J. M., J. Russell, and Z. Espinel.** 2005. Epidemiology of tropical cyclones: The dynamics of disaster, disease, and development. Epidemiologic Reviews **27:**21-35.
216. **Sikorsky, J. A., D. A. Primerano, T. W. Fenger, and J. Denvir.** 2004. Effect of DNA damage on PCR amplification efficiency with the relative threshold cycle method. Biochemical and Biophysical Research Communications **323:**823-830.
217. **Sly, R. M.** 1999. Changing prevalence of allergic rhinitis and asthma. Annals of Allergy Asthma & Immunology **82:**233-248.
218. **Snell-Castro, R., J.-J. Godon, J.-P. Delgenes, and P. Dabert.** 2005. Characterisation of the microbial diversity in a pig manure storage pit using small subunit rDNA sequence analysis. FEMS Microbiology Ecology **52:**229-242.
219. **Solomon, G. M., M. Hjelmroos-Koski, M. Rotkin-Ellman, and S. K. Hammond.** 2006. Airborne mold and endotoxin concentrations in New Orleans, Louisiana, after flooding, October through November 2005. Environmental Health Perspectives **114:**1381-1386.
220. **Stark, K. D. C.** 1999. The role of infectious aerosols in disease transmission pigs. Veterinary Journal **158:**164-181.
221. **Stetzenbach, L. D.** 2002. Introduction to aerobioogy *In* C. J. Hurst, R. Crawford, G. Knudsen, M. McInerney, and L. D. Stezenbach (ed.), Manual of Environmental Microbiology. ASM Press, Washington, D.C.
222. **Stetzenbach, L. D., M. P. Buttner, and P. Cruz.** 2004. Detection and enumeration of airborne biocontaminants. Current Opinion in Biotechnology **15:**170-174.
223. **Stevens, D. A.** 1995. Coccidioidomycosis. New England Journal of Medicine **332:**1077-1082.
224. **Stewart, S. L., S. A. Grinshpun, K. Willeke, S. Terzieva, V. Ulevicius, and J. Donnelly.** 1995. Effect of Impact Stress on Microbial Recovery on an Agar Surface. Applied and Environmental Microbiology **61:**1232-1239.
225. **Taylor, L. H., S. M. Latham, and M. E. J. Woolhouse.** 2001. Risk factors for human disease emergence. Philosophical Transactions of

the Royal Society of London Series B-Biological Sciences **356:**983-989.

226. **Tellier, R.** 2006. Review of aerosol transmission of influenza A virus. Emerging Infectious Diseases **12:**1657-1662.
227. **Tong, Y. Y., and B. Lighthart.** 1999. Diurnal distribution of total and culturable atmospheric bacteria at a rural site. Aerosol Science and Technology **30:**246-254.
228. **Tringe, S. G., T. Zhang, X. Liu, Y. Yu, W. H. Lee, J. Yap, F. Yao, S. T. Suan, S. K. Ing, M. Haynes, F. Rohwer, C. L. Wei, P. Tan, J. Bristow, E. M. Rubin, and Y. Ruan.** 2008. The Airborne Metagenome in an Indoor Urban Environment. PLoS ONE **3:**e1862.
229. **Valasek, M. A., and J. J. Repa.** 2005. The power of real-time PCR. Advances in Physiology Education **29:**151-159.
230. **Ward, N., A. Donaldson, and P. Lowe.** 2004. Policy framing and learning the lessons from the UVs foot and mouth disease crisis. Environment and Planning C-Government and Policy **22:**291-306.
231. **Weiss, K. B., and S. D. Sullivan.** 2001. The health economics of asthma and rhinitis. I. Assessing the economic impact. Journal of Allergy and Clinical Immunology **107:**3-8.
232. **West, J. S., S. D. Atkins, J. Emberlin, and B. D. L. Fitt.** 2008. PCR to predict risk of airborne disease. Trends in Microbiology **16:**380-387.
233. **West, J. S., P. Kharbanda, M. Barbetti, and B. Fitt.** 2001. Epidemiology and management of Leptosphaeria maculans (phoma stem canker) on oilseed rape in Austrialia, Canada, and Europe. Plant Pathology **50:**10-27.
234. **WHO.** 1990. Indoor air quality: biological contaminants: report on a WHO Meeting. WHO. Regional Office for Europe, Rautavaara.
235. **WHO.** Smallpox, http://www.who.int/mediacentre/factsheets/smallpox/en/.
236. **WHO.** 2004. The world health report 2004 - changing history. *In* B. Campanini and L. Vita-Finzi (ed.). World Health Organization.
237. **Willeke, K., X. J. Lin, and S. A. Grinshpun.** 1998. Improved aerosol collection by combined impaction and centrifugal motion. Aerosol Science and Technology **28:**439-456.
238. **Williams, R., A. Hirsch, and S. Cowan.** 1953. Aerococcus, a new bacterial genus. Journal of General Microbiology **8:**475-480.
239. **Wilson, S. C., J. Morrow-Tesch, D. C. Straus, J. D. Cooley, W. C. Wong, F. M. Mitlohner, and J. J. McGlone.** 2002. Airborne microbial flora in a cattle feedlot. Applied and Environmental Microbiology **68:**3238-3242.
240. **Woese, C. R.** 1987. Bacterial Evolution. Microbiological Reviews **51:**221-271.
241. **Womiloju, T. O., J. D. Miller, P. M. Mayer, and J. R. Brook.** 2003. Methods to determine the biological composition of particulate matter collected from outdoor air. Atmospheric Environment **37:**4335-4344.

242. **Wood, G. M., P. J. Mann, D. F. Lewis, W. J. Reid, and M. O. Moss.** 1990. Studies on a Toxic Metabolite from the Mold Wallemia. Food Additives and Contaminants **7:**69-77.
243. **Xu, P., E. Kujundzic, J. Peccia, M. P. Schafer, G. Moss, M. Hernandez, and S. L. Miller.** 2005. Impact of environmental factors on efficacy of upper-room air ultraviolet germicidal irradiation for inactivating airborne mycobacteria. Environmental Science & Technology **39:**9656-9664.
244. **Xu, P., J. Peccia, P. Fabian, J. W. Martyny, K. P. Fennelly, M. Hernandez, and S. L. Miller.** 2003. Efficacy of ultraviolet germicidal irradiation of upper-room air in inactivating airborne bacterial spores and mycobacteria in full-scale studies. Atmospheric Environment **37:**405-419.
245. **Ye, X., V. Sikirica, J. R. Schein, R. Grant, V. Zarotsky, D. Doshi, C. J. Benson, and A. A. Riedel.** 2008. Treatment failure rates and health care utilization and costs among patients with community-acquired pneumonia treated with levofloxacin or macrolides in an outpatient setting: A retrospective claims database analysis. Clinical Therapeutics **30:**358-371.
246. **Yee-Guardino, S., L. Danziger-Isakov, M. Knouse, W. Bingaman, C. Sabella, and J. Goldfarb.** 2006. Nosocomially acquired Pseudomonas stutzeri brain abscess in a child: Case report and review. Infection Control and Hospital Epidemiology **27:**630-632.