THESIS

EVALUATION OF BACTERIAL SAMPLING METHODS FOR USE WITH THE BACTERIAL TAG-ENCODED FLEXIBLE (FLX) AMPLICON PYROSEQUENCING (bTEFAP) TECHNIQUE

Submitted by

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ABSTRACT

EVALUATION OF BACTERIAL SAMPLING METHODS FOR USE WITH THE BACTERIAL TAG-ENCODED FLEXIBLE (FLX) AMPLICON PYROSEQUENCING (bTEFAP) TECHNIQUE: A STANDARDIZED APPRAOCH.

Background: The need to enumerate airborne microorganisms during infectious disease outbreaks, indoor air quality evaluations, and agricultural health studies has identified limitations in culture-based or viable sampling and characterization of bioaerosols. Pyrosequencing promises to be a novel, molecular-based technology that is exceptionally sensitive, low-cost, and provides a reasonable turnaround in the identification, distribution and concentration of aerosolized microorganisms. However, bioaerosol sampling methods for use with pyrosequencing have not been thoroughly evaluated. The intent of this project was to investigate a standardized sampling protocol for use with bTEFAP that would ultimately provide occupational scientists a novel and effective tool in the quest to characterize bioaerosol exposure and its subsequent relationship to worker health

Methods: Four filter types (Millipore Durapore® Membrane Filter, SKC water-soluble gelatin filter, SKC PTFE, SKC PVC) were prescreened for low-background DNA content using Pyrosequencing. Studies comparing the performance of the SKC Polyvinylchloride (PVC) and SKC gelatin filters in IOM samplers to an impinger - the SKC biosampler - were conducted in a previously characterized bioaerosol chamber using a Collision nebulizer. The challenge organism was a spore former, *Bacillus atrophaeus*. Tag-encoded FLX amplicon pyrosequencing analyses utilized Roche 454 FLX instrument with DNA extraction, massively parallel bTEFAP and

bacterial identification data analysis was performed at the Research and Testing Laboratory (Lubbock, TX).

Results**:** From an initial filter analysis, both the SKC PVC and SKC gelatin filters were selected for use in this project based on low-background DNA content, ease of use and cost. The two filter types and the SKC biosampler were challenged against *B. atrophaeus* for 30 minute sampling times in a series of six trials. Post pyrosequencing of detectable samples, it was demonstrated that the biosampler performed less effectively when compared to the PVC ($p=0.0002$) and gelatin filter ($p=0.0006$) based on an alpha value of 0.05. No significant difference was demonstrated between the two filter types ($p=0.8$). Of the original n=66 samples analyzed through pyrosequencing, only n=15 were reported to have counts for the challenge organism. In comparison to the pyrosequencing data, the cultured count demonstrated a significant difference when compared to the filters and biosampler media ($p=0.003$) in countable spores.

Conclusions: The results indicate that with the model used in this study, the biosampler performed significantly different when compared to two filter types, the SKC PVC and the SKC gelatin, when challenged with *B. atrophaeus*. In addition, the microbial results suggest that there is possible significant contamination in the pyrosequencing methods used and or in the handling methods prior to analysis. Method analysis needs to be completed before further studies are completed.

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DEDICTATION

Without the unrelenting love and support of my family, I could not have accomplished what I have, nor have become the young man that I am. I dedicate this work to my mother, who has taught me that to love, is our greatest gift. I also dedicate this work to my sister Connie, my youngest brother Geoff, my oldest brother Jim, his wife Kristine and Poppy, Jackie, Dave, Rod, Jan, Gram and Kiki. Your continuous encouragement and support has extended from childhood and I am forever grateful for your love. “La Famiglia!”

To the Wolf Pack: Thank you for the constant reminder to focus on the big picture, but to never lose sight of those “tiny little bubbles”.

"*I am the vine; you are the branches. If a man remains in me and I in him, he will bear much fruit; apart from me you can do nothing*” John 15:5

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LIST OF ACRONYMS

ACGIH	American Conference of Governmental Industrial Hygienists
AFO	Animal Feeding Operation
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
CAFO	Concentrated Animal Feeding Operation
CD14	Cluster of Differentiation 14
CFU/m^3	Colony Forming Unit per Cubic Meter
CMD	Count Median Diameter
COPD	Chronic Obstructive Pulmonary Disorder
DAPI	4', 6-Diamino-2-Phenylindole
DECOS	Dutch Expert Committee on Occupational Safety
DGGE	Denaturing Gradient Gel Electrophoresis
dNTP	Deoxyribonucleotide Triphosphate
FEV_1	Forced Expiratory Volume in One Second
FEF	Forced Expiratory Flow
FISH	Fluorescence Staining or Fluorescence In Situ Hybridization
FLX bTEFAP	Bacterial Tag-Encoded Flexible Amplicon Pyrosequencing
EPA	Environmental Protection Agency
GSD	Geometric Standard Deviation
HICAHS	High Plains Intermountain Center for Agriculture Health and Safety
HP	Hypersensitivity Pneumonitis
IL-6	Interleukin-6
LAL	Limulus Amebocyte Lysate Endotoxin Assay

LBP	Lipopolysaccharide Binding Protein
LOD	Limit of Detection
LPS	Lipopolysaccharide
MDII	Lymphocyte Antigen 96
Mg/m^3	Milligram per Cubic Meter
MMD	Mass Median Diameter
Ng/m^3	Nanogram per Cubic Meter
NIOSH	National Institute for Occupational Safety and Health
OEL	Occupational Exposure Limit
ODTS	Organic Dust Toxic Syndrome
OSHA	Occupational Safety and Health Administration
PCR	Polymerase Chain Reaction
PEL	Permissible Exposure Limit
PGN	Peptidoglycan
$PM_{2.5}$	Particulate Matter < 2.5 µm
PM_{10}	Particulate Matter < 10 µm
PM	Particulate Matter
PNOS	Particles Not Otherwise Specified
PPi	Pyrophosphates
QPCR	Quantitative Real Time Polymerase Chain Reaction
REL	Recommended Exposure Limits
rFC	Recombinant Factor C
SPM	Suspended Particulate Matter
STEL	Short Term Exposure Limit

TLR4	Toll-like Receptor 4
TLV	Threshold Limit Value
TWA	Time-Weighted Average
TNF	Tumor Necrosis Factor
USDA	United States Department of Agriculture
µm	Micrometer

CHAPTER 1

INTRODUCTION

Respiratory diseases associated with agriculture were recognized as early as 1555 when Olaus Magnus (Schenker 1998) commented on the dangers of inhaling grain dusts. Yet, despite this recognition of early respiratory hazards associated with agriculture, it has only been recently, within the 20th century, that this issue has been researched and characterized (Schenker 1998). Today, agriculture is ranked as one of the most hazardous occupations in the world (Kirkhorn, et al. 2000, Rautiainen, et al., 2002). In the United States there are over 5 million individuals involved in agricultural production and it has been suggested that over 70% of a developing country's population is devoted to working in the agricultural trade (Schenker 1998). Therefore, agriculture has become an important public health concern, as the potential for disease is tremendous. Even since Olaus Magnus's observations, respiratory disease is at the heart of concern within the agriculture profession.

The inhalation of organic and inorganic dusts has been associated with a significant increase risk of morbidity and mortality among farmers and farm workers with injurious consequences well documented (Schenker 1998). The populations at greatest risk for respiratory distress are those workers in the animal production industry as exposure to poor air quality is greatest with high density confinement of animals within small spaces inside enclosed buildings (Millner, 2009). More than one million farm workers in the United States alone are at increased risk for occupational lung disease from enclosed livestock operations (Merchant 1995). Animal confinement operations allow for aerosol production that includes silica, clay, and constituents of manure, animal dander, feed, spores, antigens, pollen grains, chemicals,

urine, endotoxins and a plethora of microorganisms including bacteria and fungi (Beard 1994; Popendorf 1997; Schenker 1981).

Exposure to inhalable bioaerosols, as documented through previous studies, is associated with a myriad of acute and chronic deleterious health consequences when compared to similar unexposed workers (Becklake 1980; Chen-Yeung 1981, 1992). Respiratory symptoms include, but are not limited to: chest tightness, wheezing, bronchitis, rhinorrhea, hypersensitivity pneumonitis, organic dust toxic syndrome (ODTS), as well as acute and chronic airway inflammation with well documented decreases in normal lung function as illustrated through forced expiratory volume in one second (FEV_1) and forced expiratory flow (FEF) (Corey 1982; Cormier 1991; Choudat 1994; Donham 1995; Donham 2000; Rylander 2006; Von Essen 1999).

Unfortunately, most guidelines regarding agricultural dust pertain to grain dust and the current American Conference of Industrial Hygienists (ACGIH) threshold limit value (TLV) for organic dust is incorporated under 'particles not otherwise specified' (PNOS) with a suggestion to keep airborne concentrations under $3mg/m^3$ (respirable particles) and $10mg/m^3$ (inhalable particles) (ACGIH 2010). The Occupational Health and Safety Administration (OHSA) have similar, however more liberal, permissible exposure limits (PEL). Reynolds, Donham and colleagues have demonstrated through dose-response studies in swine and poultry operations that a suggested exposure guideline of 2.4 mg/m^3 of organic dust be adopted. This occupational exposure limit (OEL) has also been supported through the studies of Burch 2010 and Reynolds 2012.

As previously mentioned, bacteria and fungi are typical constituents of organic dusts. Aerosolized bacteria have been shown to influence some of the adverse health effects (asthma, chronic bronchitis, chronic obstructive disease etc.) associated with organic dusts (Poole 2010; Von Essen 1999). Gram-positive bacteria (peptidoglycan; PGN) and Gram-negative bacteria (endotoxins, lipopolysaccharide, LPS) are habitually measured as a link to inflammatory outcomes. Specifically, endotoxins from Gram-negative bacteria (components of the cell wall) have been vigorously investigated in regards to their association with disease in exposed workers (Poole 2010). Again, due to conflicting studies, an agreed upon dose-response relationship has yet to be developed (Rask-Anderson 1989). Another hurdle that has prevented a uniform standard has been the highly diversified echelon of endotoxins and bacteria present as indicated by Reynolds (2005). Not only is the diversification dependent upon location and climate, but also industry and means of cultivation and sampling methods (Nonnenmann 2010; Poole 2010, Saito 2009, Duchaine 2000).

Historically, bacterial sampling has been analyzed indirectly via the Limulus Amebocyte Lysate Endotoxin Assay (LAL) and the Recombinant Factor C (rFC) endotoxins assay. The culture method has been a long-standing gold standard for direct enumeration. However, the culture method is restricted to viable cultivation; the researcher is limited to selected media and is unable to culture the majority of microorganisms (Nonnenmann 2010). Therefore, traditional means of microbial quantification are limited and non-culturable methods have been recommended (Nonnenmann 2010; Poole 2010).

Recent developments in non-culture analytical tools have greatly expanded bacterial quantification and identification. Of those molecular based methods, pyrosequencing has

proven a novel non-culturable technology that could be used to not only measure the biodiversity of microorganisms, but also to characterize exposure to these microorganisms in occupational settings (Nonnenmann 2010). The bacterial tag-encoded flexible (FLX) amplicon pyrosequencing (bTEFAP) approach utilizes the ribosomal DNA 16s gene for phylogenetic analysis. These highly conserved structures can be used to identify individual genera or species of bacteria from varied and diverse samples. In addition to being an exceptionally sensitive method, pyrosequencing is relatively low in cost and allows for a relatively quick turnaround in the identification, distribution and concentration of bioaerosols (Nonnenmann 2010). However, reliable sampling methods in use with pyrosequencing have yet to be investigated. A standardized sampling protocol for use with bTEFAP would be highly valuable in providing occupational scientists a novel and effective tool in the quest to characterize bioaerosol exposure and its subsequent relationship to worker health.

CHAPTER 2

LITERATURE REVIEW

History

Respiratory diseases associated with agriculture were recognized as early as 1555 when Olaus Magnus (Schenker 1998) commented on the dangers of inhaling grain dusts. The risks were again noted in 1556 by Georgius Agricola in 'De Re Metalica' that certain kinds of dusts produced breathing difficulties (Agricola 1950). Bernardino Ramazzini's (1700) influential piece, '*De Morbis Artificum'* made similar interpretations indicating that the inhalation of specific materials resulted in recurrent and predictable manifestation of disease (Wright 1940, Dinardi 1997). For the next 100 years, Ramazzini's observations and therapeutic, curative control recommendations provided insight into agriculture and health, specifically, the observed association between farmers and an increase in respiratory distress. After Turner Thackrah's book, which is considered to be the second all-inclusive monograph on occupational diseases (following Ramazzini's work), an American named Benjamin W. McCready elaborated on occupational disease in his prize winning essay, *On the Influence of Trades, Professions, and Occupations in the United States, in the Production of Disease.* McCready's 1837 piece exemplified a developed and representative approach in evaluating conditions underlying specific diseases associated with 19th century American occupations including the hazards of respiratory disease in agriculture. Just as Magnus, Agricola, Thackrah and McCready all had observed and suggested during their own time, agriculture continues to be ranked among the most hazardous occupations in the world (Kirkhorn 2000, Rautiainen 2002). In the United States there are over 5 million individuals involved in agricultural production (Schenker 1998).

Workers at most risk for respiratory distress are those in the animal production/confinement operations as exposure to bioaerosols, organic and inorganic dusts are often significantly increased (Millner 2009). Therefore, the potential for disease is also increased making respiratory illness in agriculture an important public health concern.

Agricultural Workforce

Agriculture provides over 2.5 million jobs to Americans and is one of Colorado's leading industries in both work force and economic value (Schenker 1998). As of the census in 2007, there were 2.2 million farms in the United States, covering an area of 922 million acres (3,730,000 km^2), with an average farm size of 418 acres (1.7 km^2) (USDA 2007). Over the past 40 years, Americans living on farms has declined by 40% with the number of farms in the United States decreasing by 30% in the same time period (Schenker 1998). However, and as suggested by Schenker (1998) "…the traditional U.S. agricultural workforce has declined from 6.8 million to 2.8 million, this decline has been nearly matched by the increase in migrant and seasonal farm workers". In Colorado, over 53% of farm work is now completed by migrant labor (USDA 2007). Despite the changing portrait of agricultural in the United States, the industry increased the national Market Value of Products Sold in 2007 by 48%, $201 billion to $297 billion, from 2002 and continues to remain one of the largest industries in the United States and in Colorado (USDA 2007).

The predominant farm owner in Colorado is a Caucasian male with an average age of 57 (USDA 2007), while the most common farm workers are younger Hispanic males (Burch 2010). In Colorado there are 37, 054 farms covering 31,604,911 acres (127,900 km^2) with an average farm size of 853 acres (3.5 km^2) (USDA 2007). The Market Value of Products Sold in Colorado in

2007 increased by 34%, similar to the national trend, with $6 billion gross income. However, 67% of all agricultural sales came from the livestock industry. In 2007, Colorado's top agricultural livestock commodities included cattle (5th in the U.S.), dairy (16th), Hogs (16th), and sheep/goats (2nd) (USDA 2007). Colorado's livestock industry makes a significant contribution to the state's agricultural economy and animal production has changed dramatically over the past 30 years. The industry has shifted from small-scale; family owned operations to massive corporately run businesses that utilize animal confinement holdings as the primary means of livestock production. Working with animals presents an inherent hazard and movement towards animal confinement operations has only increased the concern for deleterious health consequences, especially concerning respiratory diseases.

Animal Feeding Operations

The number of livestock operations in Colorado is on the decline statewide; however, operations continue to increase in size and become more specialized and integrated. These facilities are described by the Environmental Protection Agency (EPA) as Concentrated Animal Feeding Operations (CAFO) based on potential pollution profiles. Specifically, a CAFO is an Animal Feeding Operation (AFO), but has a much narrower criteria and is defined by the EPA as a) confines and maintains animals for a total of 45 days or more in any 12 month period, and b) crops, vegetation, forage growth, or post-harvest residues are not sustained in the normal growing season over any portion of the lot or facility. The EPA has outlined three categories of CAFO's, ordered in terms of the facilities capacity: small, medium or large (EPA 2012). Categorization is dependent on the number and size of animals calculated using specie specific

animal units. For example, a 1,000 pound beef cow is a standard measurement of an animal unit (EPA 2012).

There has been a significant amount of research characterizing swine and poultry CAFO's and thus AFO's, with the highest concentrations of dust and endotoxin documented in poultry houses (Valerie 2010, O'Shaughnessy 2010, Donham 1989, 1990, Iverson 2000, Cormier 1991, Kirychuk 2008, 2010). Previous studies, including a review completed by Iverson et al. in 2000, suggests that in regards to a human health perspective dust exposure in pig farming is a significant issue due to the large number of workers needed along with increased number of working hours. A dose-response relationship has been shown to exist between symptoms and number of working hours in both swine and poultry houses (Iverson 2000, O'Shaughnessy 2010). From limited studies, it has been suggested that dust exposure in swine and poultry confinement operations offer the most significant exposure to workers compared to cattle and dairy operations (Iverson 2000). One such longitudinal study indicates that swine farmers had an accelerated decline in FEV_1, whereas dairy farmers did not (Iverson 2000). However, even though swine and poultry operations have been repeatedly characterized, there is significantly less documented knowledge about the airborne exposures and characteristics within dairy facilities (Donham 1986). So much less is understood about the hazards to dairy operators that the High Plains Intermountain Center for Agriculture Health and Safety (HICAHS) has prioritized the characterization and reduction of injury and illness among dairy producers within its region of oversight (Colorado, Wyoming, North Dakota, South Dakota, Utah, and Montana).

The Dairy Industry

Dairy products are one of the top five commodities in Colorado adding $456,076, 000 to the state's agricultural economy. According to the 2007 census of Agriculture, Colorado's dairy industry ranked 16th in the nation producing 2,547,050,000 pounds of milk from 170 licensed dairy herds with an average of 647 cows per dairy (Lester 2008). Colorado has been following the national trend in which the dairy industry has condensed from many small operations into larger, regional facilities (USDA 2010). From 2001 to 2009, dairy herds with 500 head or more increased by 20% with an average decrease of 35% in dairy herds with 500 head or less (USDA 2010). In the United States' during 2006 9,112,000 cows produced 181,798,000,000 pounds of milk. Production of milk, along with herd sizes, continues to increase every year. This trend of increased milk production and herd sizes is only expected to continue to grow (Cooley 2007).

With an increase in dairy herd sizes, productivity has also increased due to advances in technology. More animals are raised in less time and with less effort, just as more milk is produced in less time with less effort. The industrialization of the dairy industry has increased environmental, public, personal and animal health concerns. Due to an increase in animal capacity, feed and water are served continuously, which produces an increased amount of manure slurry. The slurry is made up of a mixture of cow feces and urine. The slurry remains stagnant until moved into holding areas, large storage containers or lagoons, until later used in field applications. The condensing of dairy operations has led to a general increase in feeding, and feed handling, application of bedding materials, barn cleaning and maintenance, manure handling and milking. With an increase in activity, personnel are at a greater risk to hazards from aerosolized dusts and byproducts. It has been noted that due to increased work-loads, 12-

14 hour shifts and up to 6 working days a week, workers in dairy barns are at an increased exposure risk to complex organic dusts containing toxic and immunogenic constituents (Kullman 1998).

Evidence for Human Health Effects

The transition towards intensive livestock production has increased confinement of animals and therefore has increased the concern for indoor air quality inside the confinement facility (Barber 1992). Workers in the animal production industry are often at an even greater risk of exposure due to the confinement of a high density of animals within small spaces inside enclosed buildings (Millner 2009). Animal confinement operations allow for aerosol production that includes silica, clay, and constituents of manure, animal dander, spores, antigens, pollen grains, ammonia, carbon monoxide, carbon dioxide, urine, endotoxins and a plethora of microorganisms including bacteria and fungi (Schenker 1981, Kullman 1998, Iverson 2000, Popendorf 2007). Some contaminants emit strong odors that can be a nuisance to workers and to residents living in proximity to the AFO's (Donham 1985, Cooley 2007). Due to continued close contact between workers and animals, workers are continuously exposed to a myriad of chemical, biological and the more obvious physical hazards (Kullman 1998). It is well documented that working in swine and poultry confinement buildings doubles the rate of respiratory symptoms (Valerie 2010). Marked decreases in lung function over a single work shift have been associated with dust concentrations in confinement buildings (O'Shaughnessy 2010, Kirychuck 1998, Iverson 2000). It has also been shown that employees working in confinement operations are exposed to elevated levels of carbon monoxide, carbon dioxide, ammonia and hydrogen sulfide (Donham 1990; Kullman 1998). These gases are able to cause acute effects

such as loss of consciousness, headaches, irritation and cough, chemical burns, chronic lung inflammation and even death (Donham 1990, Latenser 2000, Reynolds 1996).

Fewer studies have been completed in the dairy industry; however, similar exposures have been recognized (Kullman 1998, Cathomas 2002). Routine exposures to endotoxin in dairy production facilities have been documented (Burch 2010, Reynolds 2009). Studies completed by Poole and Lange have indicated that dairy parlors are well suited to bacterial growth due to humid and moist environments (Poole 2010, Lange 1997). In the most comprehensive study completed assessing workers exposures in dairy barns to environmental agents, it was documented that ammonia was present at elevated levels and endotoxins, mite antigens, cow urine antigen, fungi (mold and yeasts), mesophilic bacteria, thermophilic bacteria and respirable dusts were also present in quantifiable levels (Kullman 1998). Exposures to these organic dust constituents and gases are believed to be risk factors for respiratory disease (Kullman 1998, Tripp 1999).

Work in a dairy barn exposes workers to complex organic dusts that are often cited as etiology of respiratory disease (Kullman 1998, Lecours 2010). Although Kullman et al. cites that dairy farmers are most at risk for rhinitis, hypersensitivity pneumonitis (HP) and ODTS; dairy workers are also at risk for: cough, phlegm production, wheezing, reduced respiratory function, chronic bronchitis, asthma, as well as chronic obstructive pulmonary disease (COPD) (Valerie 2010, Rylander 1983, Heedrick 1991). Even though clinical symptoms have been cited in dairy barn workers, how these workers are exposed and how exposures impact respiratory health is poorly understood (Lecours 2010).

It is worth noting that like most agricultural activities, dairy farming is subject to cyclic exposure patterns that are related to season. For example, it has been shown that dairy farmer exposures to dust increases during the winter months because most activities take place indoors and also because ventilation rates are reduced to conserve energy costs (Schenker 1998). However, even though it is well documented that there is a substantial burden to workers health from exposures in animal confinement operations, exposure limits do not exist for most agents, especially biologic agents. The reasons for this are partially due to the art of occupational hygiene in that exposure response relationships must be established before standards are set; but also because aerosols are complex and methods of evaluation are non-standardized (Schenker 1998). The research within this paper primarily focuses on biological agents and will therefore restrict information to organic dusts, endotoxins, bioaerosols and their measurement in industry.

Particulates

Particulates are tiny subdivisions of solid matter suspended in a gas or liquid and are also known as particulate matter (PM) or suspended particulate matter (SPM). This is in contrast to an aerosol, which is classified as a colloid suspension of particles or liquid droplets in a gas and an aerosol includes both the particle and the suspending gas (Hinds 1999). The composition of particles and aerosols depends primarily on their source. While some particulates occur naturally, such as those created from wildfires, dust storms or volcanos, others are residuals from manufacturing processes, such as coal combustion (Lippman 2003). There are chiefly two classifications for particulates, those that are composed of inorganic material and those composed of organic material. Inorganic particulate, or inorganic dust, has

been associated with respiratory disease, specifically, the pathologic potential of soil silicates (Guthrie 1993). Clays in farm soil dusts have also shown to have a pathogenic potential in their ability to adsorb and carry hazardous organics such as pesticide residues (Schenker 1998). There have only been a limited number of studies focusing on inorganic dust exposure during farming operations and Schenker et al. have commented that those studies that have been completed have 'serious limitations'. However, dust within animal confinement operations is predominantly composed of organic compounds and is therefore the greatest exposure risk for workers with in AFO's including dairy farmers (Schenker 1998, Lester 2008).

Organic dust is a broad term that refers to all airborne and settled particulate material that is of biologic origin (Schenker 1998). These complex mixtures are composed of animal dander, urine and fecal material, insect, rodent and avian parts, microorganisms (bacteria, fungi etc.), pollen grains, flour and tobacco dusts and endotoxins (Donham 1986, Rylander 1985, Schenker 1998). Organic dust particles have significantly variable size distributions that allow deposition with in the respiratory system to occur at varying levels. It is the actual aerodynamic size of the particle that is the main determinate of where in the respiratory tract a particle will come to rest when inhaled. Particles larger than 10 micrometers (μm), generally filtered by the nose and throat cilia, can be deposited in the upper respiratory tract. Particles ranging in size from 2 to 10 μm (referred to as PM_{10}) are often deposited in the lower respiratory tract from the branched bronchi all the way into the lungs. Particles smaller than 2 μm (referred to as $PM_{2.5}$) tend to penetrate the deepest part of the lungs such as the alveoli (Figure 1.1) (Donham 1986, Schenker 1998, Tripp 1999).

Symptoms associated with organic dust exposure in confinement workers include wheeze, cough, and shortness of breath, ODTS and chronic bronchitis (Donham 1989, Kullman 1998, Schenker 1998, Rylander 2006). Workers exposed to organic dusts in swine and dairy confinement operations have been shown to have a decrease in FEV_1 and FEF (Choudat 1994, Donham 2000, Iverson 2000, Burch 2010, Reynolds 2012).

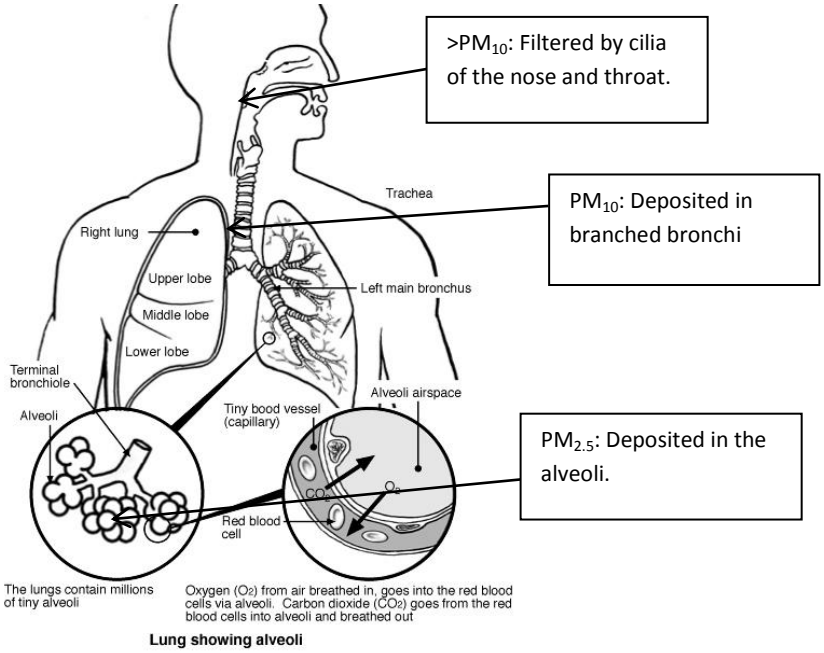

Figure 2.1.: Depiction of particulate matter (PM) deposition within the lung system (Modified from EMIS 2012, United Kingdom).

Proposed standards and legal requirements depend on the type of dust workers are exposed to and multiple agencies have varying recommended exposure limits. The American Conference of Governmental Industrial Hygienists (ACGIH) and the National Institute for Occupational Safety and Health (NIOSH) recommend 8-hour time weighted averages (TWA) and Short Term Exposure Limit (STEL) standards called Threshold Limit Values (TLVs) and Recommended Exposure Limits (RELs), respectively. The Occupational Safety and Health

Administration (OSHA) also have 8-hour TWA standards called Permissible Exposure Limits (PELs). There are currently no standards for organic dusts found in ACO's and guidelines that do exist regarding agricultural dust pertain specifically to grain dust (Schenker 1998). In the United States, hygienists must rely on OHSA's standards for PNOS. PNOS limits for dust are listed in the (29 CFR 1910.1000) at 15 mg/m^3 (total dust) and 5 mg/m^3 (respirable dust) (OSHA 1989). OSHA, ACGIH and NIOSH have recommended PELs and RELs for grain dust, cotton dust and wood dust (Table 1.1). However, all agencies acknowledge that the current exposure limits may not be entirely protective if dust is contaminated with microorganisms (Schenker 1998).

Table 2.1.: Current 8-hour dust exposure recommendations.

Hazard	ACGIH TLV	OSHA PEL	NIOSH REL
Grain Dust	4 mg/m^3	10 mg/m^3	4 mg/m^3
Wood Dust	1 to 5 mg/m^3	5 mg/m^3	1 mg/m^3
Organic Dust	NS	NS	NS
Endotoxin	NS	NS	NS
PNOS Total	NS	15 mg/m^3	NS
PNOS Inhalable	10 mg/m^3	NS	NS
PNOS Respirable	3 mg/m^3	5 mg/m^3	NS

*PNOS = "Particulates not otherwise specified", NS = Not specified
Sources: 29 CFR 1910.1000, NIOSH and ACGIH and Schenker 2008.

Reynolds, Donham and colleagues (1996) have demonstrated through dose-response studies in swine and poultry operations that a suggested OEL of 2.4 mg/m^3 for total dust and 0.23 mg/m^3 for respirable dust be adopted as the proportion of disease increases in workers above these levels (Reynolds 1996, Donham 1995, 2000). This OEL has also been supported through the studies of Burch et al. 2010 and Reynolds 2012. The National Health Council of The

Netherlands has suggested an 8-hour occupational exposure limit of 1 mg/m^3 for total dust and the Canadian Thoracic Society Standards Committee has recommended a personal exposure limit of 5 mg/m^3 (Schenker 1998, DECOS 1997). Inhalation of organic dust has been illustrated to cause adverse health effects, including ODTS, chronic bronchitis and other deleterious respiratory illnesses in workers. The current legal regulations do not adequately protect animal confinement workers, including dairy barn farmers, from over exposures and thus manifestation of acute and chronic disease. The establishment of dust and endotoxin thresholds for exposure in animal confinement buildings should be a short-term goal for the industry (Iverson 2000, Kullman 1998).

Endotoxins

Endotoxins are heat-stable lipopolysaccharides (LPS) that contribute greatly to the structural integrity of outer cell walls of gram-negative bacteria. LPS are amphipathic macromolecules that contain an inner component called Lipid A (Figure 2.2).

Lipid A is hydrophobic in nature and allows the anchoring of LPS to Gram-negative bacteria outer membranes (Travers 2008, Basinas 2011). In humans, when LPS is bound to lipopolysaccharide-binding protein (LBP), the LPS structure binds to CD14 receptors on inflammatory signal producing cell membranes.

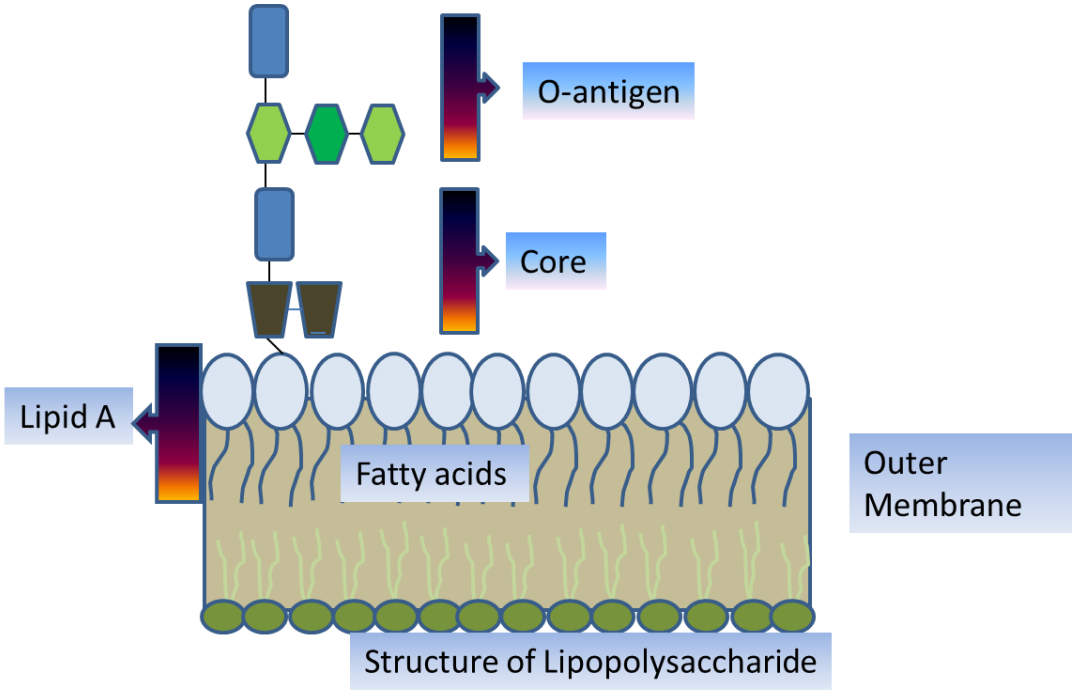

Figure 2.2: Gram-negative bacterial lipopolysaccharide (LPS).

The CD14 receptor also binds to a non-anchored protein called MDII, which dimerizes Toll-like receptor 4 (TLR4) (Travers 2008). Figure 1.3 illustrates how CD14 and TLR4 activate the immune system by up regulating monocytes and macrophages by release of pro-inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-6 (IL-6) (Travers 2008, Zhiping 1996).

The exodus of cytokines such as interleukins and chemokines may lead to the observed clinical effects of bronchoconstriction due to inflammation, toxic pneumonitis, and ODTS (Rylander, 2002). LPS can elicit a response at picogram per milliliter quantities and is therefore a potent inflammatory agent that produces systemic effects at low levels of exposure (Heederik 1991, Lester 2008, Travers 2008).

Previous studies have indicated that endotoxins are highly prevalent in animal confinement operations in which organic dust is generated or handled (Reynolds 2009, Schenker 1998).

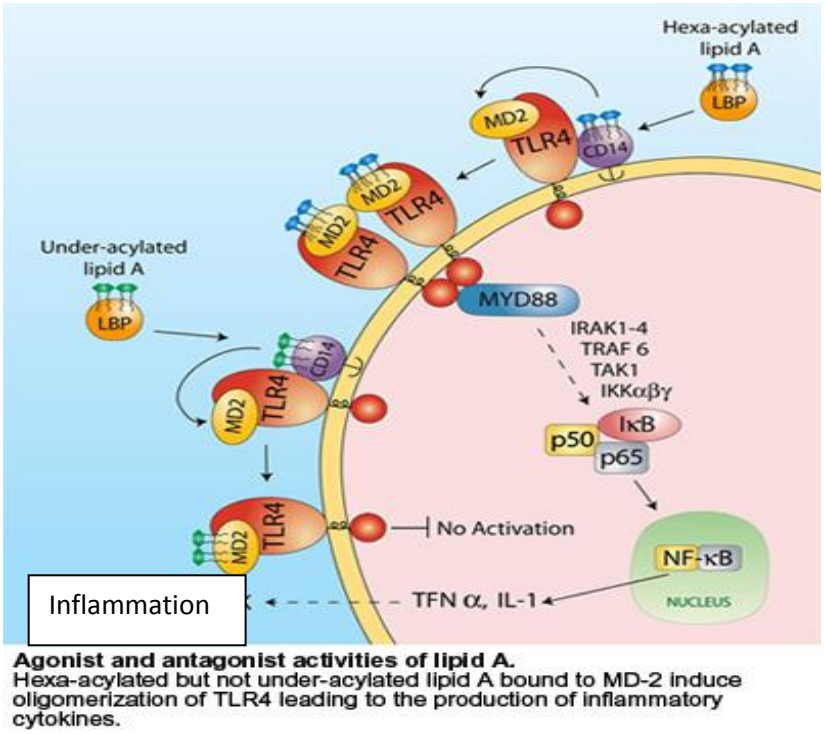

Figure 2.3: Activity of lipid A in the dimerization of Toll-like receptor 4 (TLR4) during the release of pro-inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-6 (IL-6) (From InvivoGen, www.invivogen.com, San Diego, California).

Animal feces and dander contaminated with bacteria are cited as the major contributors of endotoxin in organic dust as LPS is often liberated and released into the environment during cell lysis and or damage (Schenker 1998). Gram-positive bacteria and fungi have not been found to produce LPS. However, in a study assessing thirty-eight swine workers in Sweden, it was found that Gram-negative and Gram-positive bacteria as well as fungi play a role in the

induction of airway inflammation when exposed to dust compared to just LPS alone (Zhiping 1996, Poole 2010).

Endotoxin has been recognized as an important factor in the cause of occupational lung diseases caused by organic dust exposure (Schenker 1998, Basinas 2011). Studies examining inhalation and intravenous administration of LPS showed clinical effects such as fever, malaise, cough, chest tightness and dose-dependent acute lung function impairment with acute bronchial obstruction (Schenker 1998). However, there are no established occupational exposure limits or standards for endotoxin within the United States. The International Commission on Occupational Health (ICOH) has reported that ODTS is elicited at an endotoxin concentration between 1,000 and 2,000 ng/m^3 and mucous membrane irritation occurred at levels between 20 and 50 ng/m^3 (Rylander 1998). In the Netherlands, the National Health Council has suggested an occupational exposure limit of 90 ng/m^3 for an eight-hour TWA (DECOS 2010) and Donham (1995) has suggested guidelines of 600 ng/m^3. A study performed by Reynolds (2012) found that geometric mean endotoxin concentrations exceeded both of the previous standard suggestions in a pulmonary function assessment of agricultural workers in Colorado and Nebraska. In addition, in a review completed by Basinas (2011) of 28 publications involving dust and endotoxin exposure in animal farming operations, inhalable endotoxin ranged between 400 and 6,600 EU/m^3. Although guidelines have been suggested for swine and poultry facilities (Donham 1995, Reynolds 1996), there are current no recommendations for dairy operations. The large variation in reported concentrations can partly be attributed to the different sampling and analytical methods. Longitudinal studies are gravely needed to gain further insight into acute and chronic exposure to endotoxin and the accompanying symptoms.

In addition, standardization of endotoxin sampling and analysis from dust samples, such as those performed by Reynolds et al. (2005 and 2002) are needed in order to characterize not only endotoxin concentrations but also worker exposure.

Gram-positive Organisms

Although LPS and gram-negative organisms have been highlighted throughout the literature as potential health concerns, less is known about gram-positive microorganisms. Most pathogens to humans are gram-positive species and exposure to them may lead to adverse chronic health effects such as inflammatory outcomes compared to non-exposed workers (Poole 2010). Peptidoglycan (PGN) is a murein, which is a polymer of sugars and amino-acids that forms the outer membrane of the cell wall. Muramic acid is the chemical marker for PGN and has been shown to correlate with inflammatory outcomes in exposed European swine workers (Zhiping 1996). It has been predominantly located in large animal feeding operations, such as in swine production facilities and dairy barns (Poole 2010). Even though a possible PGN dose-response relationship has been illuminated, very little work has been done to investigate PGN in US agriculture environments (Poole 2010). Guidelines have been suggested for endotoxin exposure, but muramic acids have not been well studied and therefore little knowledge is available. It was shown that bronchial epithelial cells displayed cytokine responsiveness to agricultural dusts with high levels of muramic acid and is the probable cause of the inflammatory response in large animal production facilities (Poole 2010). It is therefore pertinent to investigate a sampling method that will be able to be used for sampling both gram-negative and gram-positive microorganisms.

Bioaerosols

Suspended microorganisms are a major component of the toxicants released into the air during animal confinement operations. These microorganisms may include bacteria, fungi, fungal and bacterial spores, viruses, cell debris, pollens and aeroallergens (Douwes 2002, Heedrick 2002, Lester 2008). Bioaerosols typically range in size from 0.01 to 100 µm; however, fungal spores range between 5 to 100 µm and bacteria range from 0.3 to 5 µm (Thorne 2000, Schenker 1998, Lester 2008). Whether or not bioaerosols exist as a single particle or as a conglomerate of particles, inhalation is the primary method of exposure.

Bioaerosols may be classified as infectious or non-infectious, although non-infectious bioaerosols have been documented as the more frequent cause of chronic adverse health effects (Schenker 1998). Infectious bioaerosols are always a concern for worker and public safety; however, although more serious acute symptoms are the result of exposure, infectious microorganisms are typically less common and more isolated. Non-infectious microorganisms are pervasive within the agriculture environment and are responsible for many different dust-induced pulmonary conditions, such as ODTS, hypersensitivity pneumonitis, dyspnea and allergies. Specific farming activities have been shown to subject workers to higher than normal exposures. It has been documented that manual cleaning of enclosed grain bins and silo uncapping are highly hazardous activities (Schenker 1998). In regards to AFO's, any work with straw (stacking, throwing, chopping and distribution) or spreading of hay, especially spoiled hay, for bedding or feed significantly increases exposure to bioaerosols (Valerie 2010).

There have been some studies investigating the concentrations and varieties of bacteria in organic dust for animal confinement industries. Dutkiewicz and Lacey (1994) have been the

only ones to publish fairly in depth listings of microorganisms found within agricultural facilities. Microorganisms within animal production operations typically range from bacterial genera such as *Pseudomonas*, *Enterobacter, Flavobacterium, Bacillus,* and *Corynebacterium* (Dutkiewicz 1994, Lacey 1994). In dairy farms specifically, *Saccharopolyspora rectivirgula* has been the main bacterial identified between 10^4 to 10^5 CFU/m^3. Fungal equivalents were also reported, however fungi and the corresponding mycotoxins are not a focus of this paper. Mean concentrations for total bacteria were reported as 10^6 colony forming units per meter cubed (CFU/m^3) for poultry, 10^5 CFU/m^3 for swine, and 10^3 CFU/m^3 in dairies (Basinas 2011, Duchaine 2000, Karwowska 2005, Dutkiewicz 1994, Lacey 1994). There are very few studies characterizing represented organisms and their quantity in dairy barns. However, in most instances, identification of organisms may be more important than actual microbial quantification (Schenker 1998).

Culture-Based Methods

Historically, bioaerosol bacterial analysis relied heavily upon culturable methods. These methods entail using appropriate growth media (solid, liquid, or agar) in addition to sampling methods that physically separate particles. Direct impaction, such as Anderson samplers, uses inertia forces of particles to separate them from the airflow entering a sampling device. Once the particles have entered the device, they are forced to deposit onto surfaces of agar medium. Filtration is a method that collects airborne bacteria by passing air through a porous membrane filter. Inertial forces, diffusion, and electrostatic attraction are the forces that are responsible for particle collection (Gilbert and Duchaine 2009). Many different filter types and sizes are available that work with an array of airflow rates. Typically, filters are dissolved or vortexed to

collect microorganisms for culture-based analyses. Impingers are similar to impaction in that they rely on the particles inertial for collection (Gilbert and Duchaine 2009). Air is drawn into the device and the particles impact a liquid, which is generally a diluted saline buffer or oil. The impinger allows for direct serial dilutions. Aliquots from the impinger and extracted filters (suspended) are used to spike agar plates in an effort to culture sampled bioaerosols. Agar is then incubated for 2-7 days for bacterial enumeration either manually or with the aid of image analysis techniques (Douwes 2002). Other samplers that have been used to collect bioaerosols include cyclones, vertical elutriators, and electrostatic precipitators (Milner 2009).

There are some serious drawbacks to counting of culturable microorganisms including poor reproducibility, species selection is based on media and temperature, dead microorganisms and cell debris cannot be detected and personal air sampling for culturable microorganisms is not accurate due to short sampling times (Douwes 2002). Long sampling durations substantially decreases the viability of microorganisms. In addition, damaged or nonviable microorganisms and their byproducts are not culturable, but can still have significant effects on human health as for a number of bioaerosol-induced diseases; dead organisms are as potent contributing agents as living ones (Schenker 1998, Gilbert and Duchaine 2001, Douwes 2002, Eduard 1997, Milner 2009). It has been shown that non-culturable organisms could represent more than 95% of the total bioaerosol load, both viable and nonviable (Schenker 1998). This presents major issues in bioaerosol characterization not only within dairy parlors, but the entire animal confinement operation industry. Due to lack of standardization between air samplers, sampling techniques and culture based drawbacks; the characterization of agricultural bioaerosols has been severely inhibited. Milner states that validation and thus the

standardization of sampling protocols are vital in order to further bioaerosol investigations. Additional research is desperately needed beyond the traditional culture-based methods.

Non-Culture Methods

Traditionally used culture methods have proven to be of limited use for exposure assessments (Douwes 2002). Recent studies have begun to enumerate organisms without regard to viability via non-culture-based methods. These molecular approaches have significantly revealed a 100- to 1000-fold difference in microorganism diversity in bioaerosol composition compared to culture-dependent methods (Nehme 2008, Milner 2009). Although microscopy has been used with fluorescent stains to quantify microorganisms, different bacterial genera, or live or dead cells, cannot be discriminated without immunospecific fluorescence staining or fluorescence in situ hybridization (FISH) and or 4', 6-diamino-2-phenylindole (DAPI) staining. These methods are based on antibodies labeled with a specific fluorescent molecule that targets specific proteins or DNA/RNA sequences (Gilbert and Duchaine 2009). Fluorescence staining, in addition to microscopy, can be used to quantify and identify airborne microorganisms with the use of flow cytometry (Lange 1997, Gilbert and Duchaine 2009). Other ways to characterize bioaerosols include polymerase chain reaction (PCR), or quantitative real time polymerase chain reaction (QPCR). These methods rely on the amplification, and in the case of QPCR, quantification of a targeted DNA molecule. These methods are very rapid and highly sensitive, enough to detect specific and single microorganisms. The PCR method has been successfully employed in varies studies (Sharma 2007, Nehme 2008). 16S rRNA gene-targeted PCR denaturing gradient gel electrophoresis (DGGE) analysis has also been indicating promising potential (Nehme 2008). However,

pyrosequencing, one 16s rRNA method, has proven a novel non-culturable technology that could be used to not only measure the biodiversity of microorganisms, but also to characterize exposure to these microorganisms in occupational settings (Nonnenmann 2010).

Pyrosequencing

The bacterial tag-encoded flexible (FLX) amplicon pyrosequencing (bTEFAP) approach has been utilized in bacterial identification and quantification of airborne bioaerosols in poultry houses and in the characterization of bacteria in cattle rumen (Nonnenmann 2010, Dowd 2008). Environmental studies have used pyrosequencing to describe the seasonal dynamics of bacterioplankton within the Baltic Sea (Andersson 2010) and has also been used in the rapid detection of *Mycobacterium tuberculosis* antibiotic resistance (Bravo 2009) and in the rapid screening of clarithromycin resistance in *Helicobacter pylori* (Moder et al. 2007). Pyrosequencing is a method of DNA sequencing based on the "sequencing by synthesis" principle. It is arguably the most successful non-Sanger method developed (Metzker 2005). The pyrosequencing technique is based on the detection of released pyrophosphates (PPi) during DNA synthesis. In short, inorganic PPi is released during the single addition of a deoxyribonucleotide triphosphate (dNTP) onto the complementary strand template (the sequence to be determined). The released PPi is subsequently converted to adenosine triphosphate (ATP) by ATP sulfurylase, which in turn provides the energy to luciferase to oxidize luciferin. This mechanism generates light and is recorded as a series of peaks called a pyrogram, which corresponds to the order dNTPs are incorporated (Ronaghi 2001, Metzker 2005). By performing hundreds of thousands of these reactions in parallel, the sequence of the template can be determined because the added nucleotides are known and therefore the sequence is

known. The bacterial ribosomal DNA (16s) is a universal molecule that is a part of the bacterial ribosome and has areas of evolutionary conservation (Stackebrandt 1994, Nonnenmann 2010). These are the genes sequenced during pyrosequencing and have become the standard technique of genetic analysis in bacterial phylogenetic studies.

Pyrosequencing has the advantage of accuracy, parallel processing, greatly improved turn-around time and can be automated (Ronaghi 2001, 2008, Holt 2008, Nonnenmann 2010). Compared to conventional methods, various studies have documented sensitivities of 97.4% with no false positives during rifampin and isoniazid resistance study (Bravo 2009) and greater than 95% precision levels when describing genera and species as percentages and counts (Nonnenmann 2010). Microorganism diversity is dynamic and fluctuates during seasonal changes and pyrosequencing of 16S DNA genes allows for the continuous monitoring of community structure with a high degree of taxonomic resolution and allows for the collection of thousands of sequences from multiple samples with increased sample throughput with use of indexing (Andersson 2010, Bartram 2011). In addition, both culturable and non-culturable microorganisms can be determined simultaneously. The use of this method for the identification of bacterial populations has obvious applications in epidemiological, pathogen detection and microbial diversity studies (Dowd 2008a, 2008b). The use of this novel method for the characterization of inhalable bioaerosols in animal confinement operations presents a relatively low-cost approach that is sensitive and quantitative. Unfortunately, there is very few bioaerosol application studies, with the exception of poultry and swine, which have been completed with use of bacterial tag-encoded FLX focused on microbial biodiversity in animal confinement operations. Whether in dairy operations or within the animal confinement

industry as a whole, there is a severe lack of standardization for exposure assessment purposes (Lecours 2012). With the advent of novel molecular sequencing approaches, it has become more important, and possible, to normalize sampling protocols. Standardization would allow for streamline study comparison and would further increase the knowledge of the microbial biodiversity in dairy parlors and thus the characterization of disease among workers.

Measurement

Within the agricultural industry, there is very little consensus on the methods and devices for use in measurement of occupational and environmental contaminants (Lester 2008). Literature reports a variety of sampling devices as well as a broad range of contaminants within animal confinement operations. For example, the average concentration of airborne bacteria found across five different types of swine houses was $3.3x10^5$ CFU/m^3 (Chang 2001); whereas, in 2009, Letourneau et al. found an average airborne bacterial concentration in swine houses to be $2.29x10^6$ CFU/m^3. Dairy farm bacterial exposure was documented at $1.7x10^7$ CFU/m^3 in one study completed by Milner et al. (2009) and from as low as 10^6 and as high as 10^8 CFU/m^3 in a 13 dairy farm investigation (Lecours et al. 2012). Poultry and beef operations also suggest significant variations in airborne bacterial concentration among similar studies.

Issues that may cause such vast differences in bacterial concentration could result from dissimilarities in exposure assessment between laboratories (Thorne 1997, Reynolds 2005). In addition, variances in media use, transportation and storage have been shown to affect the activity of biological agents, e.g. endotoxin, during culture practices (Thorne 1997). As mentioned, most papers investigating microbial biodiversity in air rely on culture for the quantification and identification of airborne bacteria (Gilbert and Duchaine 2009). Culture

methods are dependent upon exacting media use, incubation temperatures and times, aseptic techniques and spreading practices, all of which differ among studies as there are no standardized procedures. This notable absence of standardized and validated methods for enumeration of microorganisms could describe the wide range in prevalence and concentrations among diverse types of animal operations (Milner 2009). It has been noted that no one sampling technique, due to the deficiencies with culturing, is suitable for all groups of microbes (Milner 2009). However, with the use of molecular techniques such as bTEFAP, the development of standard practices for regulatory compliance is possible with a single sampling approach. The question remains as to which air sampling methods are most appropriate with use of the Bacterial Tag-Encoded Flexible (FLX) Amplicon Pyrosequencing (bTEFAP) Technique.

Review

There is a vast number of sampling devices available on the market for use in air monitoring. Although actual methods differ significantly between studies, the personal samplers vary very little. In industry, the IOM (Institute for Occupational Medicine) and the SKC Button Aerosol Sampler are considered standards for personal inhalable particulate matter (PM) monitoring. However, the button sampler has shown to deviate from the inhalable performance curves when challenged with agricultural dust samples (Reynolds 2009). The IOM sampler capable of using multi-dust foam disks allowing sampling of both inhalable and respirable fractions. Kenny 1999, found that the IOM sampler better reflected human inhalability of an inhalable fraction aerosol. The unique design of the IOM allows for both the insert and filter to be analyzed which eliminated the issue of internal loss that plagued the 37-mm cassette. However, it was demonstrated that the IOM Sampler oversamples particles larger

than 20 µm (Li 2000)., but is still closest to the inhalability curve (Aizenberg 2010). In addition, the IOM is prone to contamination that could affect both the inhalable and respirable mass fraction. The Button sampler is a newer means of accurately collecting the inhalable fraction. The Button is designed with a rounded multi-perforated stainless steel cap that reduces airflow turbulence that allows for even distribution of an aerosol onto the filter. The Button, like the IOM, doesn't have the issue of internal loss. Another advantage of the Button is that sampling efficiency is not related to orientation (Aizenberg 2010). However, the Button falls behind the IOM sampler in terms of matching the inhalability curve for inhalable aerosols (Aizenberg 2010). Both closely follow the ACGIH/ISO sampling criteria for inhalable particulate mass. However, the IOM has shown to have the lowest coefficient of variation (best precision), when compared to the button in both field and wind tunnel studies (Reynolds 2009). The IOM performs at 2 L/min (liters per minute) while the Button Sampler runs at 4 L/min. Both are used for collection of bioaerosols for viable or non-viable analysis (SKC 2011). The stainless steel Button Sampler uses a porous curved-surface inlet designed to improve the collection characteristics of inhalable dust (including bioaerosol) by reducing electrostatic effects and reducing sensitivity to wind direction and velocity (SKC 2011). The IOM is a conductive plastic sampling head that features a closed cassette that allows for single unit weighing for gravimetric analysis. Both samplers have been widely used in studies using culture based methods and in more recent studies that utilize molecular based methods. The concerning issue for this study is the identification of filters that are best suited for bioaerosol sampling with the use of bTEFAP and with which personal sampler.

A study performed by Nehme et al. (2008) in Quebec, Canada compared the use of an All Glass Impinger (AGI-30, Ace Glass Incorporated, Vineland, NJ, USA) to 25 mm gelatin membrane filters (SKC, Ancaster, ON, Canada) housed in IOM cassettes. The study indicated that quantitative real-time PCR and DGGE could be applied to Swine Confinement Buildings (SCB) and that the 25 mm gelatin filter was highly suited to molecular approaches as it performed with a high degree of accuracy and reproducibility (Nehme 2008). At the Broiler Research Center at the Walter C. Todd Agricultural Research Center in Austin, Texas, Nonnenmann et al. (2010) investigated culture-independent characterization of bacteria and fungi in a poultry bioaerosol. IOM's (SKC Inc., Eighty Four, Pa.) were used as the inhalable samplers and were loaded with 25 mm, sterile, gelatin membrane filters with a pore size of 3 µm (SKC Inc.). Massively parallel bTEFAP was performed and the inhalable fraction of bacteria and fungi were estimated to be 7503 cells/m^3 and 1810 cells/m^3 (Nonnenmann 2010, Funk 2011). The use of gelatin filters to assist in the identification and quantification of bioaerosols is an exciting strength for use with pyrosequencing.

Liquid impingment has become a commonly used method for measuring airborne microorganisms. The liquid collection media of an impinger allows for immediate culturing ability and also one-step dilution capabilities. One problem when using tradition impingers is that the airflow produces bubbling in the liquid reservoir and this causes already collected particles to become re-aerosolized into the effluent airflow (Lin 1997). Another issue concerning traditional impingers such as the AGI-30 or the AGI-4 (Ace Glass Incorporated, Vineland, NJ, USA) is that fluids readily evaporate during long sampling trials and this reduces physical collection efficiency for particles due to liquid evaporation and particle re-

aerosolization (Lin 1997). The development of the SKC biosampler (SKC Inc., Eighty Four, PA) has improved the collection efficiency and prolonged the sampling time for microorganisms (Willeke 1998). The biosampler has three tangential nozzles that act as critical orifices, each allowing 4.2 L/min of air to pass through for a total inflow of about 12.5 L/min (SKC 2011). The nozzles are angled toward a curved surface and this allows the particles to be collected by the combined forces of impaction and centrifugation (Lin 2000). This design produces a swirling motion that reduces re-aerosolization and is gentler on the collection of microorganisms (Lin 2000). Multiple groups have investigated the suitability of the biosampler against conventional impingers and have found the biosampler to have several advantages over the widely used AGI-30 (Willeke 1998, Lin 2000). The SKC biosampler has been shown to provide a greater sampling efficiency over a longer sampling time that preserves microorganism integrity and viability (SKC Inc., Willeke 1998, Lin 2000, Li 2011). The biosampler also allows collected bioaerosols to be analyzed by a variety of methods including molecular approaches, specifically pyrosequencing.

Summary and Research Needs

It is well documented that exposure to inhalable bioaerosols potentiates a myriad of acute and chronic deleterious health consequences when compared to similar unexposed workers (Donham 1995; Popendorf 1997, Kullman 1998, Schenker 2000, Donham 2000, Iverson 2000, Rylander 2006). Respiratory symptoms include, but not limited to: chest tightness, wheezing, bronchitis, rhinorrhea, hypersensitivity pneumonitis, organic dust toxic syndrome (ODTS), as well as acute and chronic airway inflammation with well documented decreases in normal lung function as illustrated through FEV_1 and FEF (Cormier 1991, Choudat 1994, Donham 1995, Donham 2000, Rylander 2006). Studies have also been specifically completed

investigating respiratory illnesses among employees in the dairy industry (Reynolds 2009, Burch 2010). Studies within the dairy industry have also reported high exposures to organic dust and bioaerosols (Donham et al. 1986, Heederik 1991, Schenker 1998).

In order to protect workers from over exposures, protective guidelines must be established. In order to develop these standards, the bioaerosol environment within animal confinement operations and thus, dairy operations must first be characterized. To characterize exposures within this industry, validation of collection and sample analysis protocols must be resolved.

Pyrosequencing has proven a novel non-culturable technology that could be used to not only measure the biodiversity of microorganisms, but also to characterize exposure to these microorganisms in occupational settings (Nonnenmann 2010). However, sampling methods in use with pyrosequencing have yet to be investigated. A standardized sampling protocol for use with bTEFAP would be highly valuable in providing occupational scientists a novel and effective tool in the quest to characterize bioaerosol exposure and its subsequent relationship to worker health.

The specific aims for this project are presented below:

Aim one: Investigate if pyrosequencing is a feasible option for testing with *B. atrophaeus* in terms of detection. In addition, characterize how well the culture results of *B. atrophaeus* from the biosampler compare to the results from pyrosequencing.

Aim two: Investigate which sampling method (filter or biosampler media) is most effective for the recovery of *B. atrophaeus* and for use with pyrosequencing.

CHAPTER 3

PURPOSE AND SCOPE

Four filter types, (Millipore Durapore® Membrane Filter, SKC water-soluble gelatin filter, SKC PTFE, SKC PVC) were evaluated for low-background DNA content using Pyrosequencing. From this prescreen, two filter types were selected and compared against the liquid media (PBS) in the SKC biosampler. All media types were challenged with aerosolized *Bacillus atrophaeus* using a Collision nebulizer in a previously characterized bioaerosol chamber. The purpose of this study was to standardize a sampling protocol that is accurate and reproducible so as to exploit the novel technology (bTEFAP) in order to reliably evaluate and quantify occupational exposure to bioaerosols. Following collection, media was processed and shipped to a pyrosequencing laboratory (Research and Testing, Lubbock, TX) for genetic analysis and quantification.

CHAPTER 4

MATERIALS AND METHODS

4.1 Filter Background Evaluation

Filters

A preliminary study using bTEFAP had significant background filter contamination during the sampling of a dairy farm in Colorado. Therefore, all four initial filters were tested for background endotoxin (Appendix 1) and bacterial 16s rRNA contamination before filter selection was made. Based on these results and cost of filter type, the SKC Gelatin and the Millipore Durapore® filters were initially selected for comparison within the study. Due to irreconcilable sampling errors due to excessive pressure drops during data collection, the Millipore Durapore® filters were abandoned for the SKC PVC filters. The pressure drops from Millipore Durapore® filter usage shut down AirChek XR5000 (SKC Inc.) pumps when used with both the IOM and Button samplers (SKC Inc.) at and below recommended sampling flow rates.

The four initial filters were originally selected based on cost, previous use in bioaerosol studies and ease of use with study samplers. The four filter types chosen for comparison were: A SKC Gelatin filter that has been previously sterilized with gamma radiation, has a nominal pore size of 3.0 µm, a thickness of 250 µm and a diameter of 25 mm (SKC Inc., Eighty Four, PA.); A SKC polytetrafluoroethylene (PTFE) filter that is hydrophobic, has a pore size of 3.0 µm, and is 25 mm in diameter (SKC Inc., Eighty Four, PA); A SKC Polyvinylchloride (PVC) filter that has a pore size of 5.0 µm and a diameter of 25 mm (SKC Inc., Eighty Four, PA); A Millipore Durapore® membrane filter that is hydrophobic, has a pore size of 5.0 µm, a thickness of 125 µm and a

diameter of 25 mm (EMD Millipore Corporation, Billerica, MD.). The Gelatin filters were used throughout the study and the PVC filters replaced the Millipore Durapore® membrane filter

4.2 Aerosol Approach

A 6-jet Collison nebulizer (BGI Incorporated, Waltham, MA) was used during the aerosolization process. 100 µl of *Bacillus atrophaeus* (ATCC# 9372) was aseptically added to 20 ml of Phosphate Buffered Saline with Tween 20 (PBS-Tween20) (Fischer Scientific, Fair Lawn, NJ), which was then pipetted into the nebulizer. The nebulizer was sealed and transported to the mixing element. Contaminant free air was delivered at 20 psi to the nebulizer which was controlled by a Dwyer® Rate-Master® Flowmeter RMB-52 (Dwyer Instruments Inc., Michigan City, IN). A second stream of controlled air, dilution air, was measured at 50 Lpm using Dwyer ® Rate-Master® Flowmeter RMC-103 (Dwyer Instruments Inc., Michigan City, IN). Both the aerosol and dilution air were neutralized using a Kr-85 neutralizer (TSI Inc. Shoreview, MN) before entering the chamber in order to discharge the aerosol to the Boltzmann equilibrium charge distribution. Before aerosolization, dilution air ran for 10 minutes to remove any debris within the chamber. Times were recorded when the dilution air and aerosol air were started and stopped. Aerosolization ran for 4 minutes before sampling pumps were initiated.

Post the 30 minute aerosol generation time, pumps were shut off along with the aerosolization air. Using the glove ports, the tubing was removed from all of the samplers and capped. The biosamplers were disassembled and the vial trap was plugged and sealed in a personal Whirlpak bag. The IOMs were also sealed in Whirlpak bags. The chamber was than sprayed with 15% bleach and the pump-down phase was initiated. During the pump down phase the chamber was actively filtered by a Pall Life Sciences HEPA Capsule via a Maxima C

Pump (Fischer Scientific, Fair Lawn, NJ) for 65 minutes, allowing a 3x air turnover within the chamber. The biosampler media was serial diluted for viable quantification and both the biosampler media and filters were transferred to Research and Testing Laboratories for analysis.

4.3: Aerosol Test Chamber

Chamber Design

The bioaerosol test chamber used was previously characterized (Frazey 2012) and is depicted in (Figure 4.3.1 and figure 4.3.2). The chamber is constructed from one-quarter inch aluminum plating and was fabricated by Design Metal Manufacturing of Fort Collins, Colorado. The chambers' overall interior dimensions are 1.49 meter in length, 1.22 meter in height, and 0.86 meter in width. The total volume of the chamber is 1.4 cubic meters and has a surface area of 1.18 square meters. A HEPA filter (Air Handler ®, Dayton Electric Manufacturing Company, SD) was embedded within a plate wall along with a Dwyer Magnehelic ® (Dwyer Instruments Inc., Michigan City, IN 46360) differential pressure gauge, Model 2301. The test chamber was designed with two glove port openings, each with a diameter of 0.254 meters (TerraUniversal ® Fullerton, CA). A permanent viewing window directly above the glove ports has dimensions of 0.75 meter by 0.150 meter. A working area opening was placed opposite to the glove port side and was 0.85 meter by 0.5 meter. An additional working and viewing window was installed on the top of the chamber with dimensions of 0.8 meter by 0.5 meter. Each of these openings were sealed with Plexiglas® cut to overlap the openings by 50 mm. Pure silicone rubber gasket, 1/16" inch thickness (DieCutTech, Denver, CO), was used to seal each opening.

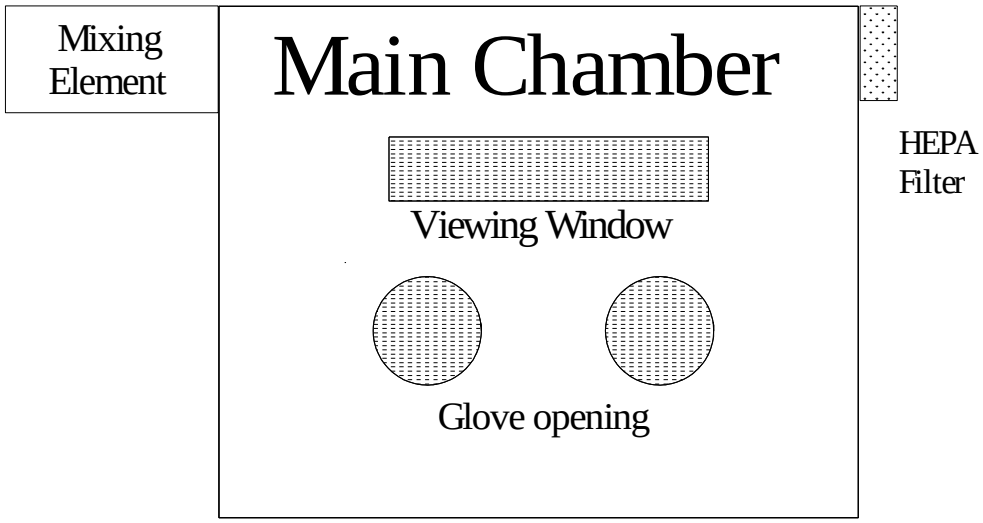

Figure 4.3.1: Schematic of the aerosol chamber and a picture of the aerosol chamber; adopted from John S. Frazey, Ph.D. Candidate.

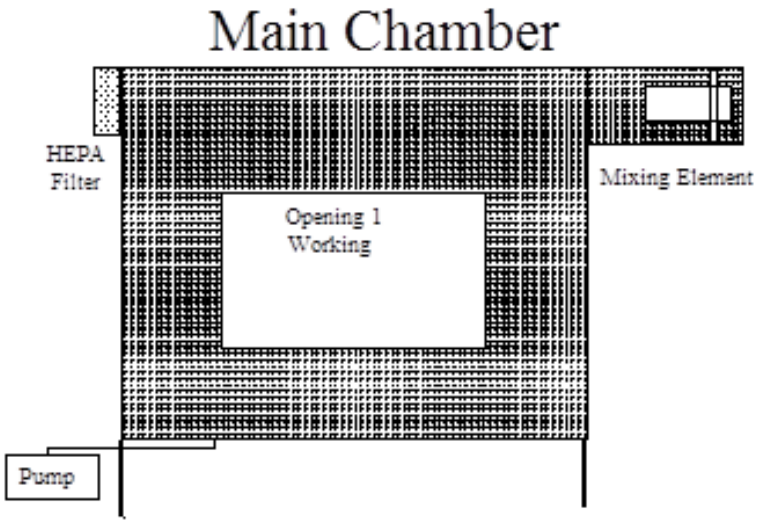

Figure 4.3.2: Schematic of aerosol chamber and a picture of the aerosol chamber; adopted from John S. Frazey, Ph.D. Candidate.

Mixing Element

The mixing element delivers contaminant free air into a Collison nebulizer for delivery into the test chamber. The mixing element was 0.5 meter in length, 0.25 meter in height, and 0.25 meter in width. It had a working opening of 0.28 meter by 0.11 meter and was sealed with Plexiglas® and rubber gaskets. The mixing element housed both a Collison nebulizer and a Kr-85 neutralizer. Figure 4.3.3 illustrates the mixing element design.

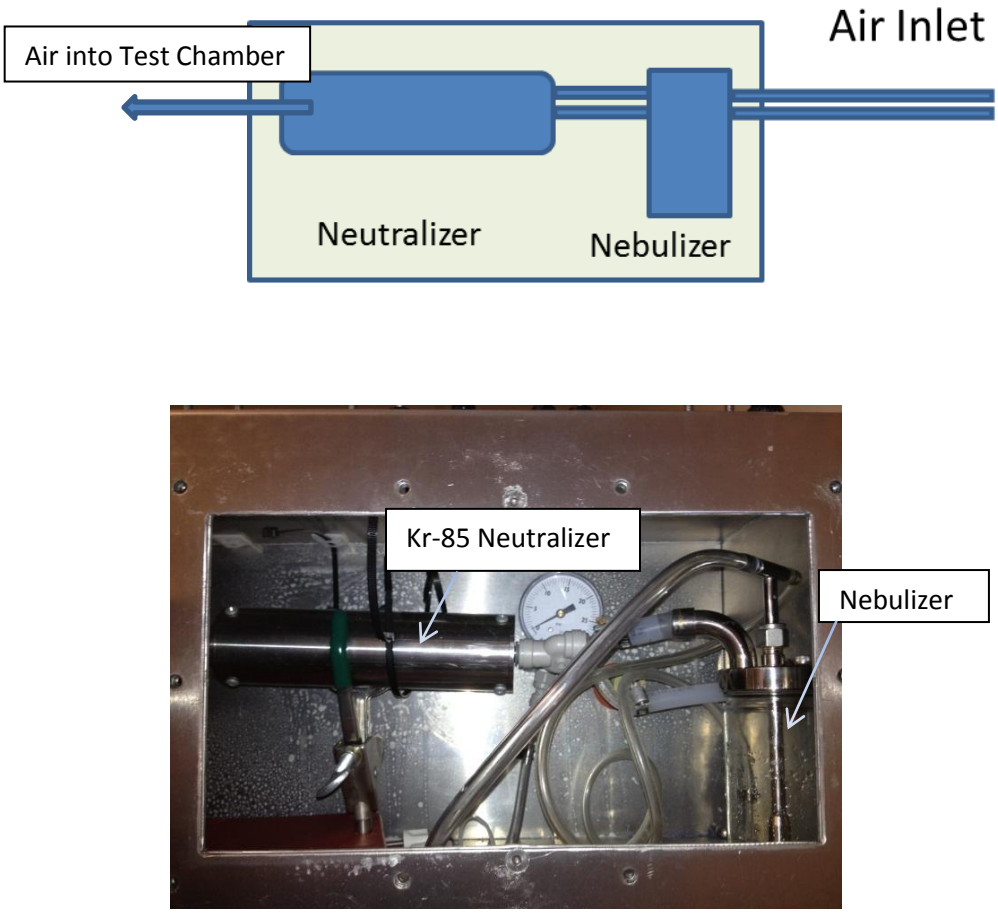

Figure 4.3.3: Schematic of the mixing element that illustrates the direction of flow as well as both the nebulizer and the Kr-85 neutralizer.

4.4: Microbial Methods

Bacillus atrophaeus spores were purchased from Yakibou, Inc (Apex, NC). The spores were acquired in two concentrations—3.1 x 10^8 spores/mL and 3.1 x 10^9 spores/ml. Spores were stored in sealed containers under refrigeration at 37^0F. For nebulization, the spores were diluted in sterile Phosphate Buffered Saline with 0.05% Tween 20 (Fisher Scientific, Waltham, MA).

4.5: Sampling Trials

Temperature and Humidity

Ambient temperature and relative humidity was measured using a Fisher Scientific Traceable Monitor (Fischer Scientific, Denver, CO). The monitor has an LCD screen that reports temperature with accuracy to ±1°C and resolution to 0.1°C/°F. The monitor was placed in a standardized spot to the right of the right glove port and ten inches towards center. The monitor screen was facing the large working window, and therefore the center of the chamber. Data was recorded at time zero, 15 minutes and 30 minutes and were calculated as average values.

SKC Biosampler

Total culturable organisms were sampled using two SKC biosamplers (SKC Inc.). The samplers were positioned in SP1 or SP2 locations with a control biosampler placed in SP3 location as seen in Figure 4.4.1. Air was sampled directly into collection media at a flow rate of 12.5 L/min for 30 minutes by Vac-U-Go sonic flow sampling pumps that were positioned outside of chamber (SKC Inc). A 30 minute sampling period was decided upon due to the evaporation potential of the collection media. Samplers were dissembled and autoclaved

before every trial and were only opened and reassembled inside the aerosol chamber. The collection media were prepared by aseptically pipetting 20 ml of diluted sterile Phosphate Buffered Saline 10x (PBS) (Fischer Scientific, Fair Lawn, NJ) into sterile 50 ml conical tubes (Corning® Inc., Corning, NY) for transfer to the chamber which was located in an adjacent building. Biosamplers were assembled and the media was added just prior to sealing of the chamber.

IOM Sampler

Inhalable organisms were sampled for using six IOM samplers (SKC Inc.) with three of the six IOMs housing the gelatin filter and the other three IOMs housing the PVC filter. All six IOMs were connected to AirChek XR5000 (SKC Inc.) pumps that were located outside of the chamber on a sampling stand. Tygon® tubing, with an OD of 3/8 (McMaster-Carr #: 55485K57) was used to connect the external pumps to the samplers inside the chamber with Through-Wall Acetal Push-Connect couplings (McMaster-Carr # 51055K5). Sampling was conducted for 30 minutes at 2 L/min. Prior to all trials, the IOM samplers were soaked in diluted bleach for 30 minutes, rinsed three times with filtered water and dried in a Biosafety cabinet overnight. IOMs were randomly selected for filter type and the filters were aseptically transferred to the IOMs from sealed packages. The samplers were than sealed in personal Whirlpak bags (Nasco, 4oz) for transfer to aerosol chamber. Samplers were selected at random for positioning within the chamber along with one control for each filter type as indicated in Figure 4.4.1.

Controls

Experimental controls were co-located within the chamber but were not connected to a pump during sampling. A control was used for both filter types and for the SKC biosamplers;

three experimental controls in total. Positioning of controls remained constant throughout all trials and controls were handled and processed in a similar matter to non-controls.

4.6: Culture Methods

Culture media were prepared by aseptically by pouring 25 ml of Trypticase Soy Modified Agar (TSA II, Becton, Dickinson and Company, Sparks, MD) for control and dilution plates. All media was cooked according to manufactures instructions, stored at 4^0C and used within 7 days.

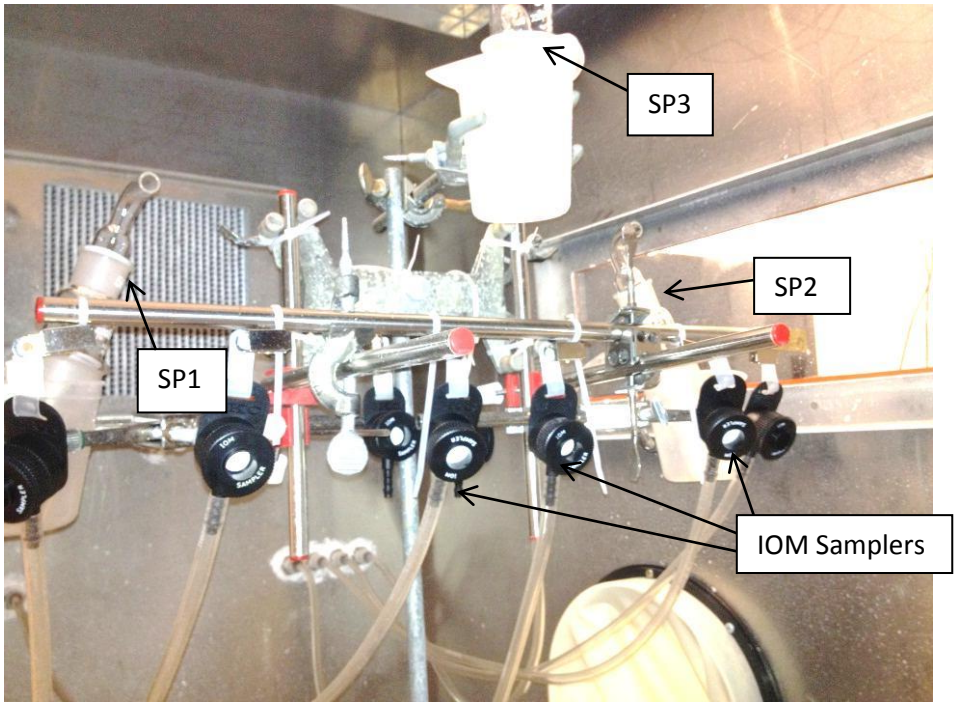

Figure 4.6.1: Sampling set-up with sampler positioning. Shown are both the SKC biosampler in SP1, SP2 and SP3 and the IOM sampler.

Post sampling, the biosampler media (PBS) were prepared for plating using 10-fold serial dilutions. One hundred microliters (µl) from the bulk 20 ml (biosampler) media was pipetted into 900 µl of sterile PBS and vortexed to reach a 10^{-1} dilution. From the 10^{-1} dilution 100 µl was pipetted into 900 µl of sterile PBS and vortexed to reach a 10^{-2} dilution. This process was

repeated until a 10^{-9} dilution was reached. 100 µl of each dilution was plated in duplicate on TSA II agar plates. Inoculated plates were incubated for 78 hours at 37.2^{o}C. Colonies were counted for each duplicate, averaged and reported as colony forming units per cubic meter of air (CFU/m^3). Dilution plates were selected for counting based on a counting range between 30 and 300 CFU's.

Controls

Control plates were made by spread plating TSA II agar plates with 100 µl of concentrated (2.2 x 10^9) *Bacillus atrophaeus* spores. Pre- and post-nebulizer control plates were also spiked with the tip of the transfer pipette after transfer of spores to nebulizer and after transfer to dilution vials. In addition, positive and negative controls were placed inside the chamber during trials along with a lab negative control.

4.7: Pyrosequencing

DNA extraction

Samples were sealed in 50 ml conical tubes (Corning Inc., Corning, NY), parafilmed and shipped to the Research and Testing Laboratory in Lubbock, TX, for pyrosequencing as previously described by Dowd 2008a and Nonnenmann 2010. Filters and biosampler PBS media were shipped without any reagents added. Samples were homogenized and 200 mg aseptically suspended in 500 µl RLT buffer (Qiagen, Valencia, CA) (with β- mercaptoethanol). A sterile 5 mm steel bead (Qiagen, Valencia, CA) and 500 µl volume of sterile 0.1 mm glass beads (Scientific Industries, Inc., NY, USA) was added for complete bacterial lyses in a Qiagen TissueLyser (Qiagen, Valencia, CA), run at 30 Hz for 5 min. Samples were centrifuged and 100 µl of 100% ethanol added to a 100 µl aliquot of the sample supernatant. This mixture was added

to a DNA spin column, and DNA recovery protocols were followed as instructed in the Qiagen DNA Stool Kit (Qiagen, Valencia, CA) starting at step 5 of the Protocol. DNA was eluted from the column with 50 µl water and samples were diluted accordingly to a final nominal concentration of 20 ng/µl. DNA samples were quantified using a Nanodrop spectrophotometer (Nyxor Biotech, Paris, France).

Massively parallel bTEFAP

Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) was performed as described previously using Gray28F 5'TTTGATCNTGGCTCAG and Gray519r 5' GTNTTACNGCGGCKGCTG (Callaway 2010, Guerrero 2009). Initial generation of the sequencing library utilized a one-step PCR with a total of 30 cycles, a mixture of Hot Start and HotStar high fidelity taq polymerases, and amplicons originating and extending from the 28F for bacterial diversity. Tag-encoded FLX amplicon pyrosequencing analyses utilized Roche 454 FLX instrument with Titanium reagents, titanium procedures performed at the Research and Testing Laboratory (Lubbock, TX) based upon RTL protocols (www.researchandtesting.com).

Bacterial diversity data analysis

Following sequencing, all failed sequence reads, low quality sequence ends and tags and primers were removed and sequences' collections depleted of any non-bacterial ribosome sequences and chimeras using B2C2 as has been described previously (Callaway 2010, Guerrero 2009, Gontcharova 2010). To curate the data short reads (<150bp), sequences with ambiguous base calls, sequences with homopolymers > 6bp were removed. To determine the identity of bacteria in the remaining sequences, sequences were denoised, assembled into OUT clusters at 96.5% identity, and queried using a distributed .NET algorithm that utilizes Blastn+

(KrakenBLAST www.krakenblast.com) against a database of high quality 16s bacterial sequences. Using a .NET and C# analysis pipeline the resulting BLASTn+ outputs were compiled and data reduction analysis performed (Research and Testing Laboratories, LLC, Lubbock, Texas).

Bacterial identification

Based upon the above BLASTn+ derived sequence identity (percent of total length query sequence which aligns with a given database sequence) the bacteria were classified at the appropriate taxonomic levels based upon the following criteria. Sequences with identity scores, to known or well characterized 16S sequences, greater than 97% identity (<3% divergence) were resolved at the species level, between 95% and 97% at the genus level, between 90% and 95% at the family and between 85% and 90% at the order level , 80 and 85% at the class and 77% to 80% at phyla. After resolving based upon these parameters, the percentage of each bacterial ID were individually analyzed for each sample providing relative abundance information within and among the individual samples based upon relative numbers of reads within each. Evaluations presented at each taxonomic level, including percentage compilations represent all sequences resolved to their primary identification (Research and Testing Laboratories, LLC, Lubbock, Texas).

4.8: Optical Particle Counter

Particle size distributions and concentrations were measured using a Grimm portable aerosol spectrometer (Grimm Industries, PAS 1:108, S/N 8F0020008, Douglasville, GA). Data was logged per minute of sampling time for 15 data points per trial. Grimm Dust Monitor Software (v3.1) was used to obtain aerosol size and concentration distributions.

4.9: Statistical Analysis

Excel databases were combined and analyzed using SAS Version 9.1 (SAS Institute, Cary, NC). Statistical inferences were based on a $P<0.05$ level of significance. The original power calculations suggested eight samples per media type for a power of 90%.The normality and variance of the data was tested using Shapiro-Wilks test and Levene's test respectively. For data where the residuals did not appear normal, a log transformation was used. Comparisons of sampler location, trial and media type were made using analysis of variance with two and one way models. Correlation coefficients were calculated to evaluate associations between media types. The mean mass diameter and geometric standard deviation for aerosol size distribution was calculated per trial and compared.

CHAPTER 5

RESULTS

5.1 Results for Quality Control Study

As a part of the initial quality control study, selected filter types and conical tubes, used for storage and transportation, were assessed for background bacterial contamination and endotoxin presence. The four filter types chosen for comparison and analyzed were an SKC Gelatin filter (SKC.), an SKC polytetrafluoroethylene (PTFE) filter (SKC Inc., Eighty Four, PA), an SKC Polyvinylchloride (PVC) filter (SKC Inc., Eighty Four, PA) and a Millipore Durapore® membrane filter (EMD Millipore Corporation, Billerica, MD.).

The gel picture in figure 5.1.1 illustrates the results from an initial PCR were the filters and conical tubes were analyzed. The lower portion of the gel picture is a control dataset for signal strength. C1 through G5 are identification tags for the conical tubes and filters. The bands above the identification tags are the signals produced by the conical tubes and filters. It is evident through this gel picture that the signals are almost as weak as they could be before the signal is lost entirely. Pyrosequencing is a sensitive method of molecular evaluation and even though the density of organisms may be low, i.e. low signal strength, the method has the ability to enumerate low signal strength into counts for specific bacteria types. The results for the most common genera from a background investigation are illustrated in Figure 5.1.2.

The results of the endotoxin analysis performed for the quality control study are depicted in table 5.1.1. The background concentration is presented in EU/ml, endotoxin units per ml and the spiked results are presented in percent recovery.

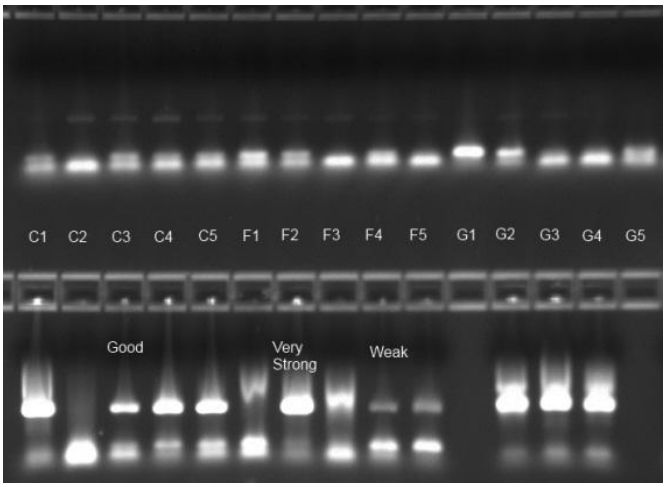

Figure 5.1.1: PCR gel-picture for initial background contamination study from non-inoculated filters

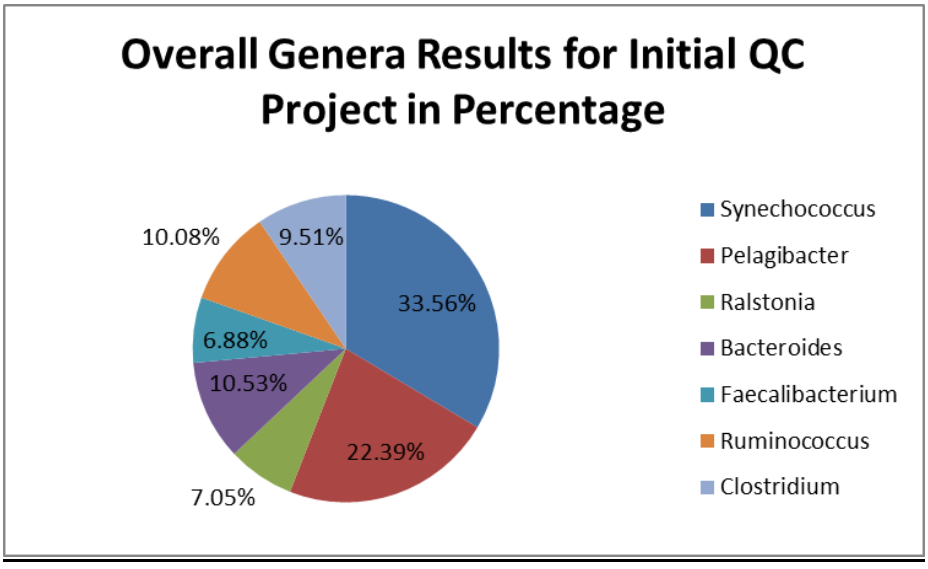

Figure 5.1.2: Results of the most common genera from the quality control study.

Table 5.1.1: Results for all filters from endotoxin quality control study.

Endotoxin Spikes in EU/ml (n=3 for each filter type)				
FILTER	Average	Std Dev	CV (%)	% Recovery
MILL	0.343	0.010	2.855	34.321
GEL	0.013	0.002	16.502	1.285
PTFE	1.210	0.076	6.266	121.037
PVC	0.231	0.111	47.839	23.101
Background Endotoxin EU/ml (n=3 for each filter type)				
FILTER	Average	Std Dev	CV (%)	Normalized***
MILL	0.053	0.010	19.485	0.160
GEL	0.058	0.012	20.271	4.839
PTFE	0.055	0.007	11.790	0.046
PVC	0.052	0.004	11.790	0.222
Mill=Millipore, Gel=gelatin, PTFE=polytetrafluoroethylene, PVC=Polyvinylchloride (n=3 for each filter type)				
***Adjusted for background recovery				

5.2 Aerosol Size Distribution

Particle size distributions and concentrations were measured using a Grimm portable aerosol spectrometer (Grimm Industries, PAS 1:108, S/N 8F0020008, Douglasville, GA). Grimm Dust Monitor Software (v3.1) was used to obtain aerosol count and to calculate concentration distributions. The specific time period at 22 minutes was chosen for all trials and values for all size bins were plotted on log-probability plot revealing CMD (Count Median Diameter) and GSD (Geometric Standard Deviation) then converted to MMD (Mass Median Diameter). Results for all trials are illustrated in table 5.2.1.

The limit of detection (LOD) was calculated for both the filters individually and for the biosamplers based on the standard deviation of the blanks. Both the PVC and biosampler blanks were all found to be zero. The Gel filter blanks LOD (10.66 counts/m^3) was calculated by

multiplying the Gel blanks combined standard deviation by three. All Gel filters were above the LOD.

Table 5.2.1: Individual trial results for aerosol size distribution.

Trial one		***Trial 2		**Trial Three**	
GSD	1.384	GSD	0	GSD	1.41
CMD	0.26	CMD	0	CMD	0.26
MMD	0.652	MMD	0	MMD	0.62
d16	0.18	d16	0	d16	0.18
d84	0.36	d84	0	d84	0.36
Trial Four		**Trial Five**		**Trial Six**	
GSD	1.43	GSD	1.41	GSD	1.39
CMD	0.265	CMD	0.26	CMD	0.28
MMD	0.601	MMD	0.62	MMD	0.653
d16	0.18	d16	0.18	d16	0.2
d84	0.37	d84	0.36	d84	0.39

GSD=Geometric Standard Deviation (unitless), CMD=Count Median Diameter (micrometers), MMD=Mass Median Diameter (micrometers), d16=16th percentile, d84=84th percentile. ***Trial 2 error, no results.

5.3 Summary Statistics

If a sample was listed a non-detect, no amplification was possible even after double PCR. A sample listed as zero could not be analyzed for *B. atrophaeus*, no *B. atrophaeus* was present on media, but was detected for other species. For statistical purposes, all non-detects for *B. atrophaeus* and zeros were treated as true zeros and were removed from the analysis. The predominant contaminating genus types were *Flavobacterium, Pseudomonas* and *Ralstonia* (Figure 5.3.1).

Six sampling trials were completed resulting in a total of 11 samples per trial and 66 samples total for the six trials. Each sampling trial involved two biosamplers (plus one control),

three IOM samplers with gel filters (plus one control), and three IOM samplers with PVC filters (plus one control). Of the 66 samples sent for analysis 18 were controls and were non-detectable except for one Gel filter control that had a count of four for B. atrophaeus. With the control blanks removed, 48 samples remained, 36 were filter samples (both Gel and PVC) and 12 were biosamplers. Of the 48 samples remaining 24 could not be detected (seven IOM gel, 10 IOM PVC, and seven biosamplers). Of the original 36 filter samples (18 gel and 18 PVC), only 11 Gel and eight PVC samples (total = 19) were returned with detectable results. Of these 19 samples, only six Gel filters and six PVC filters were detectable for B. atrophaeus. From the original 12 biosampler samples, only five were returned with detectable results and of these five, only three were detectable for B. atrophaeus. Table 5.3.1 shows the detectable results. The blank rows and the variance in rows and trials is a result of samples that were non-detectable for the challenge organism. Table 5.3.2 depicts all media means and standard deviations for counts/m^3. Temperature and humidity stayed relatively linear throughout all trials and those results are illustrated in table 5.3.3 and table 5.3.4.

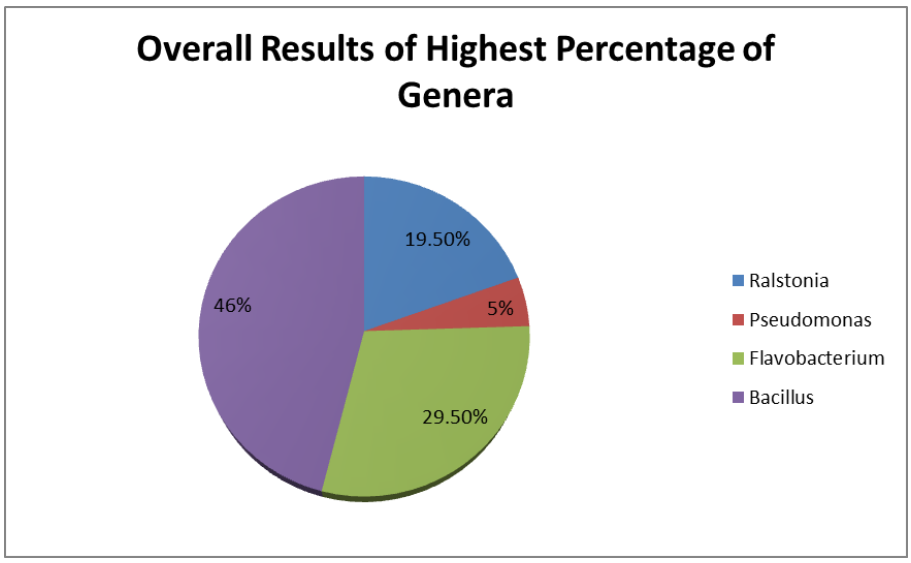

Figure 5.3.1: Overall percentage of the most common genera from pyrosequencing results.

Table 5.3.1: Results in counts/m^3 for trial and media.
***Variance in trial is a result of samples that were non-detectable for challenge organism**

Trial	PVC	GEL	Biosampler	Culturable Count Average
1				70,933
2	20,933	1,183	11	39,200
	57,200	4,200		
		1,983		
3	67	13,317	5	10,200
	85,550			
4				63,733
5	2,333	1,533		31,733
6	5,917	4,083	11	29,912

Table 5.3.2: Summary results for all media types in total counts/m^3.

Media	Total (counts/m3)*	Mean	STDEV
PVC	172,000	28,667	35,039
GEL	26,300	4,383	4,562
Biosampler	27	9	3
Culturable	245,712	40,952	22,687
*Total for all trials combined			

Table 5.3.3: Temperature and humidity averages over trials.

Temperature (0C)	0 Min	15 Min	30 Min	Average	STDEV
Trial 2	25.90	25.80	25.60	25.77	0.15
Trial 3	24.80	25.00	24.90	24.90	0.10
Trial 5	26.00	26.00	26.10	26.03	0.06
Trial 6	24.10	24.20	24.30	24.20	0.10
Humidity (%RH)	0 Min	15 Min	30 Min	Average	STDEV
Trial 2	0.21	0.21	0.41	0.28	0.12
Trial 3	0.21	0.23	0.38	0.27	0.09
Trial 5	0.19	0.22	0.27	0.23	0.04
Trial 6	0.22	0.22	0.22	0.22	0.01

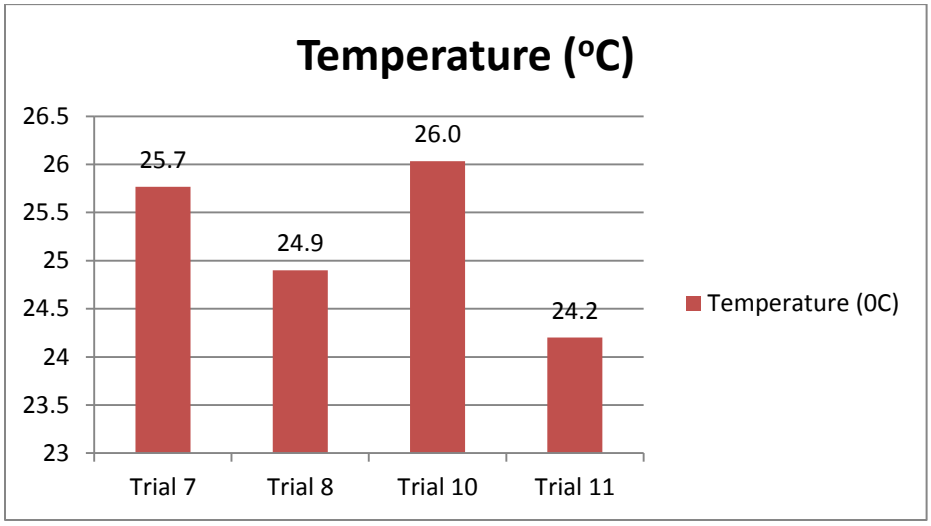

Figure 5.3.2: Summary of temperature over all four trials.

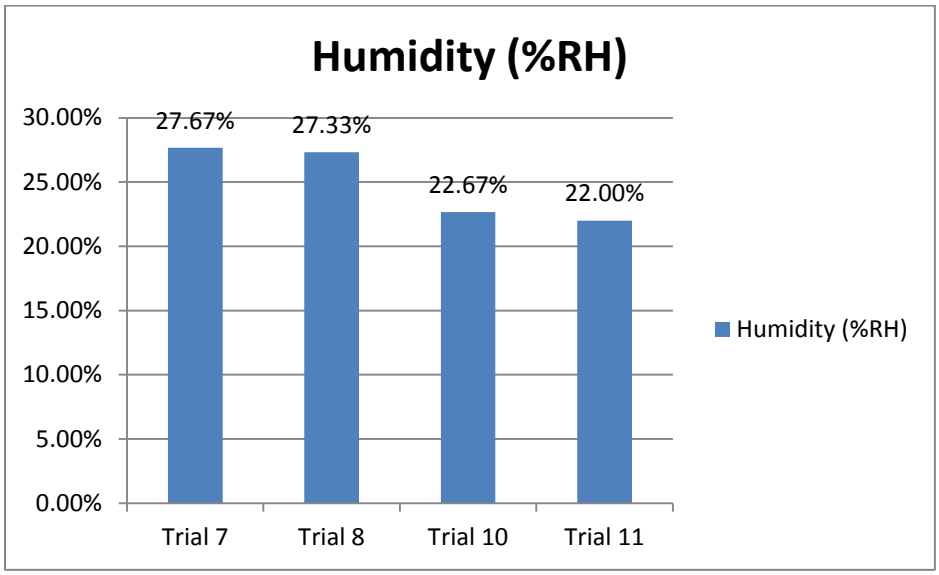

Figure 5.3.3: Summary of humidity over all four trials.

5.4 Location Results

An analysis of effect of location on results was performed on location A, B and C; illustrated in Figure 5.4.1. Location A and B were used with PVC and Gel placement so that each filter type was sandwiched between two of the other filter type. Location C was established for the sole use of Biosamplers. Test assumptions, i.e. normality and equal variance, for location B and C were met, however, it was necessary to use the Wilcoxon Scores (Rank Sums) non-parametric test for location C in order to achieve assumptions of location C test. After an analysis of variance was completed, it was demonstrated that no significant difference occurred by location, A (p=0.34,) B (p=0.85), C (p=0.67). These results prompted the removal of location as a significant effect.

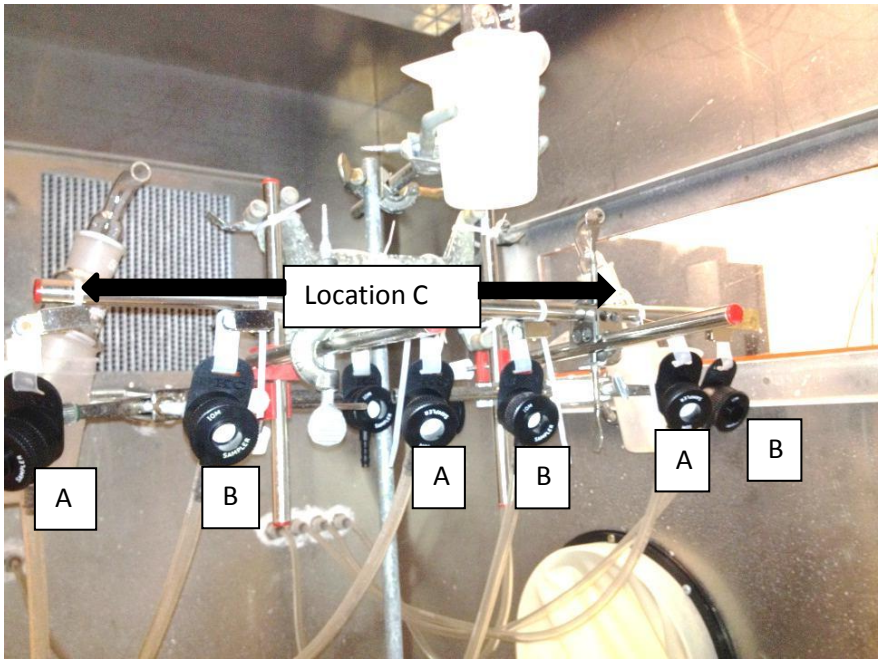

Figure 5.4.1: Illustration of sampling location within the aerosol chamber.

5.5: Media and Trial Results

During analysis, the culturable results appeared to be considerably larger in terms of counts/m^3 compared to the PVC filters, Gel filters and Biosampler media that was analyzed using pyrosequencing. Table 5.5.1 depicts all culturable results based on biosampler. An unbalanced 2-way Anova was completed comparing the effect of media and trials and any possible interactions between media and trails. Assumptions for the test were met. The initial analysis indicated that there was significant difference in means among the varying types of media, as shown in Figure 5.5.1. Specifically, the culturable media were significantly lower when compared to the biosampler (p=0.0033) and the Gel filters (0.0029) in pairwise comparison using the least square means (Table 5.5.2). The model indicated that there is a potential for a difference in means between the culturable media and PVC filters (p=0.09), however, this was not indicated in this model.

Table 5.5.1: Biosampler culturable results with estimated CFUs/m^3.

Trial	Sampler ID	Plate Average	CFU's	CFU's/m^3	Trial Average CFU's/m3
1	SKC 1	128	25,600	68,267	70,933
	SKC 2	138	27,600	73,600	
2	SKC 3	11	2,200	5,867	39,200
	SKC 4	136	27,200	72,533	
3	SKC 5	33	3,250	8,667	10,200
	SKC 6	44	4,400	11,733	
4	SKC 7	140	27,900	74,400	63,733
	SKC 8	100	19,900	53,067	
5	SKC 9	56	11,100	29,600	31,733
	SKC 10	64	12,700	33,867	
6	SKC 11	112	22,400	59,733	29,912
	SKC 12	90	18,000	90	

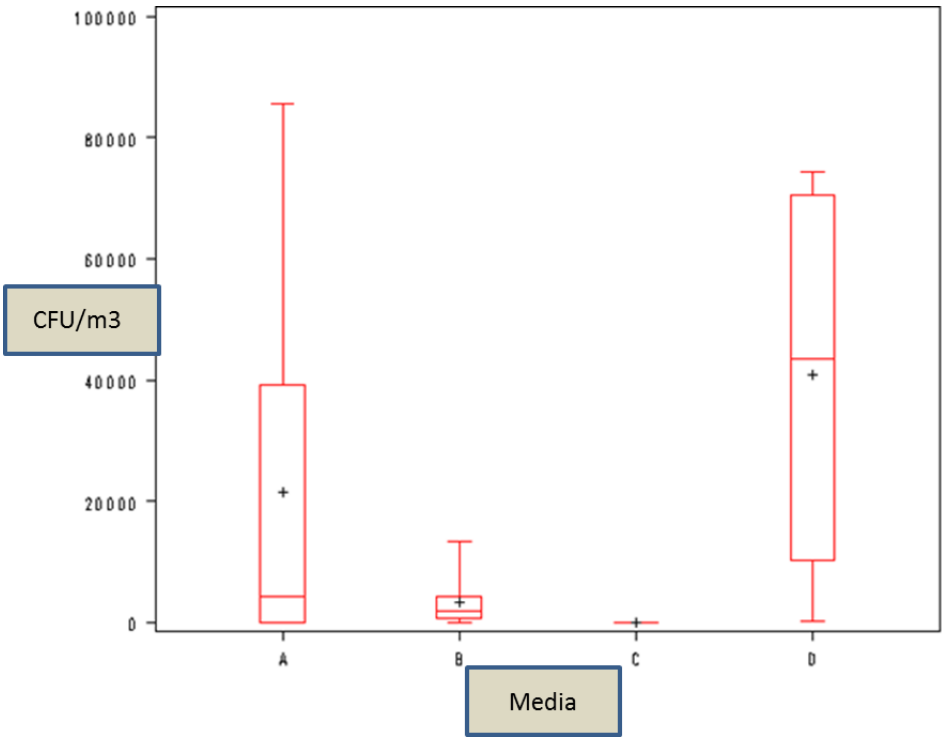

Figure 5.5.1: Histogram Illustrating Culturable Media Significant Difference in Means.

*** A=PVC, B= Gel, C=Biosampler, D=Culturable biosampler**

Table 5.5.2: Results for Least Square Means for Effect of Media.

Least Square Means For Effect Media				
Media	**PVC**	**GEL**	**Biosampler**	**Culturable**
PVC		1.48	1.54	-1.71
		p=0.15	p=0.13	p=0.09
GEL	-1.48		0.16	-3.31
	p=.15		p=.87	0.002
Biosampler	-1.54	-0.16		-3.25
	p=.13	0.87		0.003
Culturable	1.7	3.3	3.2	
	p=.09	p=0.002	p=.003	

The results also indicated that there was no interaction present between trial and media (p=0.8) and that there was not a significant difference between in the pairwise comparison in trials using the least square means (p=0.9), as illustrated in table 5.5.3.

Table 5.5.3: Results for Least Square Means for Effect of Trial.

Least Square Means For Effect Trial				
Trial	**2**	**3**	**5**	**6**
2		0.21	0.64	0.57
		p=0.84	p=0.54	p=0.58
3	-0.21		0.41	0.35
	p=0.84		p=0.69	p=0.73
5	-0.63	-0.41		-0.06
	p=0.5	p=0.69		p=0.96
6	-0.58	-0.35	0.056	
	p=0.57	p=0.73	p=0.96	

Once culturable media was removed from the analysis to test any effect or interaction among the remaining media types and trials, it was found that there was again no effect between trials (p=0.8) or between media types (p=0.5) and that there was no interaction between media and trial (p=0.90). Trial had no apparent significant effect and was therefore removed from the analysis in order to investigate an effect or interaction purely based on media types. An unbalanced one-way Anova was completed between media type and counts (in CFU/m^3) and the resulting data did not meet the assumptions of normality and equal variance. The data was than log transformed, assumptions were met and the results are

indicated in figure 5.5.2 and table 5.5.4. Statistical analysis for correlations was completed, however, due to low samples numbers, no correlation was suggested from the results.

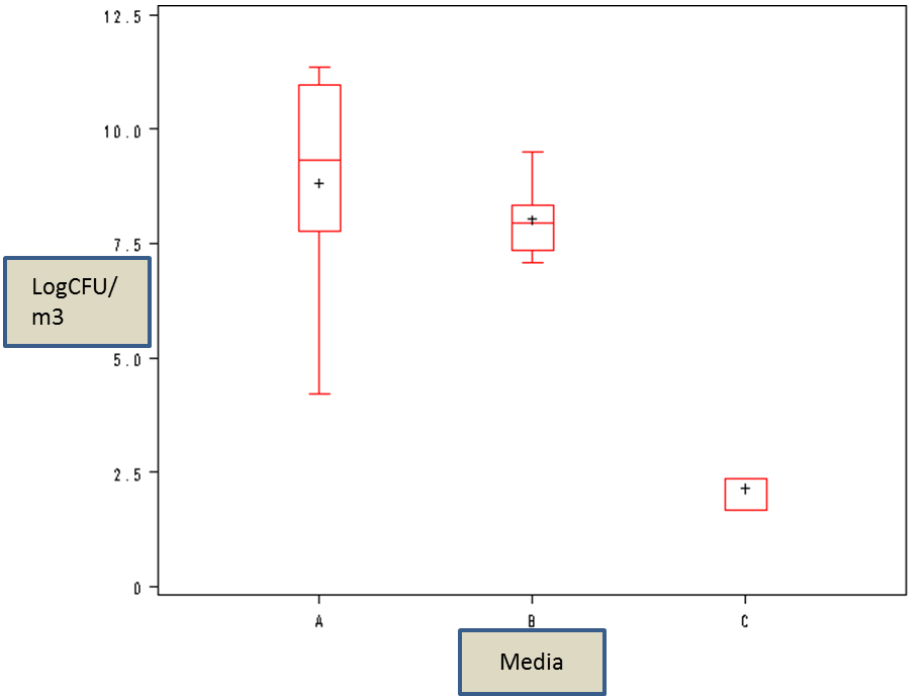

Figure 5.5.2: Log-Transformed Comparison of Media Type
*** A=PVC, B= Gel, C=Biosampler, D=Culturable biosampler**

Table 5.5.4: Least Square Means Effect for Media Post Log-Transformation

Least Squares Means for Effect media			
Media	**PVC**	**Gel**	**Biosampler**
PVC		0.759403	5.240783
		p=0.4623	p=0.0002
Gel	-0.7594		4.620734
	p=0.4623		p=0.0006
Biosampler	-5.24078	-4.62073	
	p=0.0002	p=0.0006	

CHAPTER 6

DISCUSSION

Overview

Exposure to inhalable bioaerosols, as documented through previous studies, is associated with a myriad of acute and chronic deleterious health consequences when compared to similar unexposed workers (Becklake 1980; Chen-Yeung 1981, 1992). Most guidelines regarding occupational dust pertain to grain dust and the current ACGIH TLV for organic dust is incorporated under PNOS with a suggestion to keep airborne concentrations under 3mg/m^3 (respirable particles) and 10mg/m^3 (inhalable particles) (ACGIH 2010). OHSA has similar PELs. Reynolds, Donham and colleagues have demonstrated through dose-response studies in swine and poultry operations that a suggested exposure guideline of 2.4 mg/m^3 of organic dust be adopted. This OEL has also been supported through the studies of Burch 2010 and Reynolds 2012. However, uniform standards within the United States have yet to come to fruition when considering bioaerosols, especially exposure to bacteria, due to conflicting studies and the difficulty in characterizing workplace exposure because of such a rich diversity of bacterial species. It has been noted that the traditional culture methods are inherently flawed as they are restricted to viable cultivation and that non-viable microorganisms can still have significant effects on human health (Schenker 1998, Gilbert and Duchaine 2001, Douwes 2002, Eduard 1997, Milner 2009, Nonnenmann 2010).

Recent developments in non-culture analytical tools have greatly expanded bacterial quantification and identification. Of those molecular based methods, pyrosequencing has proven a novel non-culturable technology that could be used to not only measure the

biodiversity of microorganisms, but also to characterize exposure to these microorganisms in occupational settings (Nonnenmann 2010). However, reliable sampling methods in use with pyrosequencing have yet to be investigated. The intent of this research project was to investigate a standardized sampling protocol for use with bTEFAP that would ultimately provide occupational scientists a novel and effective tool in the quest to characterize bioaerosol exposure and its subsequent relationship to worker health.

Quality Control Study

The bTEFAP method is relatively new and very little analysis for accuracy and reliability has been completed for its use in air sampling. Previous studies have attempted to use the bTEFAP method but have demonstrated that there is concern for background contamination when using various filter types. It was therefore important to elucidate the background contamination on the filters chosen for this study. In addition to the filters used, the conical tubes used in storage and transport were also investigated for background contamination and these came back with negligible results. The filters selected for this project were based on a set of criteria that included cost, ease of use and availability within the industry. As previously noted, the four filter types chosen were an SKC Gelatin filter (SKC.), an SKC polytetrafluoroethylene (PTFE) filter (SKC Inc., Eighty Four, PA), an SKC Polyvinylchloride (PVC) filter (SKC Inc., Eighty Four, PA) and a Millipore Durapore® membrane filter (EMD Millipore Corporation, Billerica, MD.).

The quality control PCR results demonstrated that the signal background contamination was low in the samples, or the density or quantity, was low compared to a control dataset. However, because pyrosequencing is a sensitive method of molecular evaluation, low signals or

counts can still be identified. Therefore, a variety of bacteria genus types were enumerated from the initial quality control study during full pyrosequencing. Of those identified the genera *Synechococcus* and *Pelagibacter* were most common. *Synechococcus* is a unicellular cyanobacterium that is widespread in marine environments, but has also been described in freshwater. *Pelagibacter* has single species called *Pelagibacter ubique* and is a member of the SAR 11 clade that is highly prevalent in both salt and fresh water. *Ralstonia* is a genus of proteobacteria that used to be classified within *Pseudomonas* and has been an issue of contamination for the Research & Testing Laboratory.

During the background endotoxin analysis, the percent recovery during an endotoxin spike was highest for the PTFE filter (121%) and lowest for the Gel filter (1.3%). The Millipore Durapore® membrane filter (34.3%) and the PVC filter (23.1%) were relatively equal. The background endotoxin average measured in EU/m^3 was largest for the Gel filter (4.8), lowest for the PTFE (0.05) and both the PVC and the Mill had similar values with (0.22) and 0.16) respectively. However, the coefficient of variance was lowest in the PVC filter (11.8%) and highest in the Gel filter (20.3%). The PTFE had the highest recovery rate and one of the lowest background endotoxin values, however, was the most expensive filter and because of cost was dropped from the study. The Millipore filter had the second best recovery rate and was used in initial trials with both the Button and IOM sampler. However, the Millipore filter was designed for liquid sampling and caused pump failures due to pressure drops and was removed as a potential filter type. The Gel filter, even though it had a low recovery rate during spiked trials, has been manufactured specifically for use with bioaerosols as it is pre-sterilized with gamma radiation, is suitable for isokinetic sampling and has been designed for the collection and

analysis of airborne microbes. In addition, Nehme (2008) found that the 25 mm gelatin filter was highly suited to molecular approaches as it performed with a high degree of accuracy and reproducibility. The PVC filter is cheap, well defined with use of either the Button or IOM sampler and is used within industry. Therefore, the PVC and Gel filter were selected as filter types for this project.

Aerosol Size distribution

Bacillus atrophaeus is a gram-positive, aerobic, endospore-forming, rod-shaped bacterium that is nearly identical to that of *Bacillus subtilis* excluding production of pigments on specific media types (Lore 2012). Therefore, *B. atrophaeus* bacteria are frequently used as simulants for *B. anthracis* in aerosol testing (Lore 2012). The vegetative state of *B. atrophaeus* usually occurs as a single rod-like cell 0.7-0.8 µm in diameter and 2-3 µm in length. The aerodynamic diameter has been reported with a fairly wide size distribution (0.5-1.5 µm) but has been noted to change due to biological variability and conditions (Tobias 2006). The *B. atrophaeus* spores approximately average 0.7 µm in diameter and 1.8 µm in length with an average aerodynamic diameter of 0.8 µm and range between 0.5-1.5 µm (Lore 2012, Tobias 2006).

The Grimm PAS data values demonstrated consistency throughout all sampling trials, as indicated in table 5.2.1. The CMD average was 0.265 the GSD average was 1.4 and the MMD average was .63 µm. Although the mass median diameter was lower than the average aerodynamic diameter presented by Lore (2012), the MMD is defined as the diameter for which half the mass is contributed by particles larger than the MMD and half by particles smaller than the MMD (Hinds 1999). That being noted, the particle range would include 0.3-1.2 µm

indicating that the about half of the data was more than likely measuring evaporated PBS-Tween20 droplets (salts from the PBS Tween mixture) and about half was measuring individual *B. atrophaeus* spores or aggregates of PBS-Tween20 droplets and aggregates of spores.

Media and Pyrosequencing

This study was designed to test the null hypothesis that there was no difference between the PVC, Gel and biosampler media (both cultured and molecularly analyzed). No other studies have compared these media types with the use of bTEFAP in a controlled aerosol chamber. The SKC biosampler has been compared to other sampling devices, such as in the Fabian et al. (2005) study, and has shown to provide greater sampling efficiency over a longer period of time that also preserves microorganism integrity (Willeke 1998, Lin 2000, Li 2011). The Gel filter has been shown to function well in molecular studies and the PVC filter is a gold standard when it comes to occupational air sampling.

An original total of n=122 samples, including lab and chamber blanks, were analyzed via pyrosequencing. This data included the Millipore filters and which were disregarded due to the inability to calculate with a degree of certainty actual sampled air volumes. The issue occurred from filter pumps failing and stopping, so a true sampling time was unknown. After removing the Millipore sets and blanks, n=66 samples remained. This data included six trials that were completed with the PVC and Gel filters along with the biosampler. Of those n=66 samples analyzed by bTEFAP, n=36 were filters, n=18 Gel and n=18 PVC. Of those n=36,n=17 were unable to be amplified for any organism and were marked as zero and the remaining n=19 had detectable counts of organisms, but only n=12 had counts for *B. atrophaeus.* From the original n=12 biosampler samples, seven samples were unable to be amplified for any

organism and were marked as zero. Five samples had detectable counts of organisms, but only three had counts for B. atrophaeus. The remaining n=18 samples from the original n=66 were chamber blanks and all came back as a zero except one Gel blank had a count of four of B. atrophaeus . All non-detects, meaning that there was no quantification of any organism, and zeros, which meant there was a zero count for *B. atrophaeus*, but had counts for other organisms, were both treated as zeros and were removed from analysis. Unfortunately, trial six was a complete non-detect and trial nine was partially non-detect and partially zero count. This caused number of trials t with countable data to decrease from six to four. This resulted in a smaller sample size than what was designed. Figure 6.1.1 illustrates the breakdown of samples.

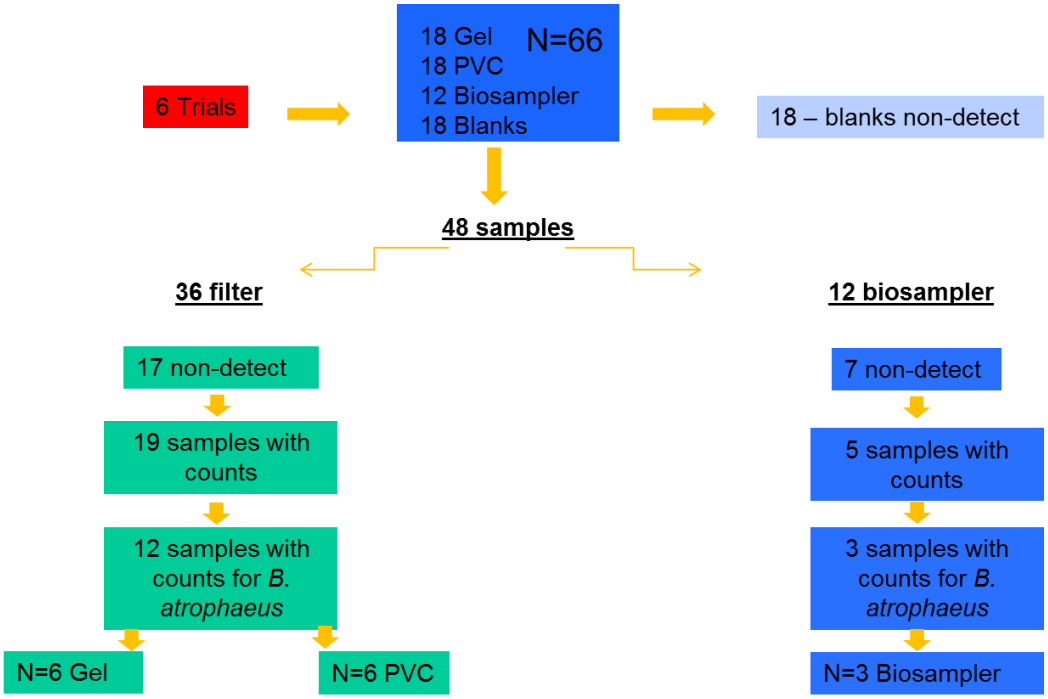

Figure 6.1.1: Sample analysis overview for filters, biosampler and blanks.

Due to the design of the chamber and sampling stand, there was concern that the location in which the samplers were placed would affect sampling performance. It was found

that location did not have an effect on sampling performance and locations did not interact with one another. With location removed from the analysis, trial and media were investigated. The culturable results come from a serial dilution completed with the post-aerosolization media from the biosampler. 100 µl was pipetted into the 10-fold series dilution and agar was plated in duplicates. Table 5.5.1 illustrates those results with back calculated CFUs/m^3 based on dilutions. The average culturable CFU/m^3 for all biosamplers was 40,900.±29,200. The results are only for those biosamplers used in the analysis and were based on the findings from culturable growth. The usable pyrosequencing results of the biosamplers, one each from trial 7, 8 and 11 indicated an average count/m^3 of 9±3.. These results do not correlate or agree with other studies that have suggested that the bTEFAP method is capable of detecting higher numbers of bacteria when compared to culturable methods. Pyrosequencing should enumerate or better detect airborne microorganisms as has been shown by Nonnenmann 2010 and others. For a reason that has not been illuminated yet, only 25% of of the biosamplers actually detected the challenge organism and detected it only in very small quantities compared to what was cultured. For example, the biosampler from trial eight indicated only 5. count/m^3 post pyrosequencing whereas when the same media was cultured it was illustrated that the same biosampler should have had around 10, 200 count/m^3. Filters were not extracted for culturable analysis so this comparison cannot me made. A stock sample (positive control) was sent for analysis with one µl of $2.2x10^9$*B. atrophaeus* and it returned a count of 17,000. This sample was not cultured therefore a comparison between stock pyrosequencing results and culturable results could not be made.

Due to culturable media having a significant (p=0.003) effect when compared to the other media types, it was removed in order to investigate the effects of trials and or any interaction amongst media and trials. After trial was found to not have a significant effect on the data, the data was log transformed and it was found that media from the biosampler was significantly different from both the Gel (p=0.0006) and the PVC (p=0.0002) filters. Figure 5.5.2 illustrates the log-transformed data and the boxplot comparing biosampler, PVC and Gel. The data was log-transformed at this point because assumptions of normality and equal variance were not met through non-log-transformed data. No statistical difference was found between the PVC and Gel filter, however, when compared to the culturable results, they both had significantly lower average count/m^3. The PVC and Gel performed about equal, however, the biosampler severely underestimated chamber aerosol. Even though the Grimm data indicates that the aerosolization process was consistent according to the MMD and therefore reproducible, the results from the media were not. The statistical model used did suggest that the biosampler was significantly different from both of the filter types. The original sample size calculations that resulted in a 90% power were eight samples per media type. The results used only evaluated six gel filters, six PVC filters and only three biosampler media samples. Although the model does suggest a significant difference when comparing the means of count/m^3 per power used, actual practical significance suggests less strength in this reasoning.

The presence of *Flavobacterium* and *Pseudomonas* bacteria is a concern as these two genera were the second and third most common genera to show up in the results behind *Bacillus*. These genera are not similar to those genera found in the quality control study *(Synechococcus* and *Pelagibacter)*. However, these results have yet to be resolved from an on-

going method analysis. It should also be noted that all media types within this study had similar contaminating genus types including a similar contamination amongst different media. . *Ralstonia* once again appeared, but it has been noted that this once particular genus has been an in-house contamination issue for the Research & Testing Laboratory. It is surprising and concerning that background contamination occurred in the pyrosequencing results. The fact that the lab has indicated there is a known contamination problem (*Ralstonia)*, raises questions about the aseptic methods that are utilized. *Flavobacterium* and *Pseudomonas* are types of bacteria that are prolific in marine and fresh water and could suggest an additional contamination, especially from the numbers in which these two organisms were found. Some of the other species identified such as *Clostridium, Ruminococcus and Synechococus* are predominantly found through fecal transmission, so it is plausible that the lab has other areas of contamination from cattle samples are being analyzed.

Temperature and humidity values stayed consistent throughout trials, as would be expected from a controlled environment. The average temperature over all four trials was 25.25±0.03 ^{0}C and the average humidity over all four trials was 24.92±5.20%. Temperature is similar to summer time conditions found in milking parlors, however, humidity in this study was much lower even when compared to winter parlor conditions (Funk 2011), however, this is to be expected.

No significant correlations were found between filters, trials, locations or media during analysis.

Limitations

The primary limitation of this study is that the samples sizes were drastically cut down due to the inability to detect the challenge organism through pyrosequencing. From an original 36 filter samples, only had six remaining, with three that were PVC and three that were Gel. In addition, of the original 12 biosampler samples only three detected the challenge organism. Due to the fact that the sample sizes were so low and varied drastically even amongst individual groups, it cannot definitively or exactly suggest a sampling media that is more accurate, precise or reproducible. The reasons for low sample numbers are unclear. However, it has been suggested that other bacteria in the samples could have acted antagonistically during the identification of the challenge organism during pyrosequencing, although, no antagonistic effect has been illustrated in the literature. In addition, it is worth noting that because this method did not enumerate counts from all of the media types, it doesn't mean that there are no organisms present. Another method, such as QPCR might have the ability to quantify counts and could be used in conjunction to pyrosequencing. In most cases, QPCR would be appropriate enough when attempting to amplify a single known specie, such as was done in this project. However, a diversification analysis was made (pyrosequencing) because a goal for this study to characterize media types of any impeding microorganism prior to use in a field environment.

Another limitation to this study is even though the culturing technique enumerated a significant amount of bacteria, it was not possible to reproduce those results with a molecular method that should be more sensitive. It has been shown that non-culturable organisms could represent more than 95% of the total bioaerosol load (Schenker 1998), so it is possible that we were only culturing 5% of what we were aerosolizing. The remaining 95%, viable or non-viable,

should be detected through molecular methods; the data does not agree with this argument. The data for the biosamplers is significantly lower than either the PVC or the Gel, yet, aliquots from the biosamplers produced a quantifiable culturable amount and this has presented serious concerns about methodology.

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

This study was designed to test the null hypothesis that there was no difference between the SKC Gelatin filter, SKC Polyvinylchloride filter and the SKC biosampler impinger media (both cultured and molecularly analyzed). No other studies have compared these media types with the use of bTEFAP in a controlled aerosol chamber evaluation. This study showed that culturable microorganism levels collected from biosampler media in a controlled chamber aerosol study were significantly different than similar media that was analyzed by bTEFAP. In addition, it was illustrated that pyrosequencing is a useful non-culture analytical method capable of detecting *B. atrophaeus*. It was also illustrated that location and trial number had no significant effect on the overall results of this study. In addition, amongst the three media types the SKC Gelatin filter and the SKC Polyvinylchloride filter were significantly better at capturing the challenge organism compared to the SKC biosampler impinger.

Recommendations

A thorough analysis of the bTEFAP methods, specifically applied to bioaerosol air sampling, needs to be addressed before further assessments or research takes place. It should be alarming that a culturable process produces significant growth of viable organisms when a sensitive molecular method fails to even detect a challenge organism. It is possible that bTEFAP methods interfere with *B. atrophaeus* amplification and sequencing, so it is therefore recommended that further evaluations occur that challenges both gram-negative and gram-positive species in addition to spore formers. It is also suggested that varying degrees of aerosol

be produced so that the bTEFAP method can be evaluated at variable concentrations. It is also recommended that multiple pyrosequencing facilities be compared in a round robin manner similar to those that investigated endotoxin analysis. Until a standardized method of bTEFAP materializes for bioaerosol sampling, questions will remain about the accuracy of this process with use of bioaerosol sampling methods.

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APPENDIX 1:

Endotoxin Assay Operating Procedures

Filters were removed from sealed packages for endotoxin analysis. The endotoxin analysis was performed using the Pyrogene Recombinant Factor C (rFC) Endotoxin Assay (Lonza Inc, Walkersville, MD). All samples were extracted in 10 ml certified pyrogen-free (PF) water (Lonza, Inc.) with 0.05% Tween 20. Samples were vortexed and shaken for I hour at 20^0C at 100 rpm. Serial dilutions of endotoxin standards (Lonza, Inc.) and sample extracts were prepared using sterile, PF water with Tween 20. Samples were added to a 96-well plate followed by 100 µl of an enzyme mixture, buffer, and fluorogenic substrate. An endpoint method was performed with plates incubated at 37^oC for 1 hour and read in a fluorescence microplate reader (Biotek Instruments, Winooski, VT; FLX800TBIE) at excitation/emission 380/440 nanometers (nm). Results were given in endotoxin units (EU/mL).

APPENDIX 2:

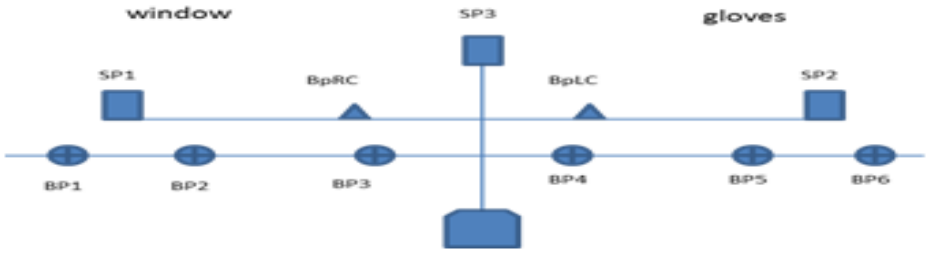
Sampling Data Sheet:

Date:	
Name:	
Trial Marked:	

Air	
Dilution	50
Neb	20

Pump Calibration	Pre-	Post-
SKC 1		
SKC 2		

AirCheck		
1		
2		
3		
4		
5		
6		



SP1:	SP2:	SP3:	BpRC:	BpLC:
Bp1:	Bp2:	Bp3:	Bp4:	Bp5:

Filter:
Filter

Time:	Temp:	Humidity: %

Data Saved as:	
Dilution air on:	
Spec on:	Spec off:
Neb on:	Neb off:
Pumps on:	Pumps off:
Pump down start:	Pump down stop:

Notes: