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DEVELOPMENT AND APPLICATION OF QUANTITATIVE BIOAEROSOL
ANALYSIS METHOD USING PCR

by
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ABSTRACT OF THE DISSERTATION

Development and application of quantitative bioaerosol analysis method using PCR

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The presence of harmful airborne particulate matter of biological origin has been associated with variety of negative health effects. In addition, there is a real threat of malicious release of hazardous bioaerosol to public sectors. To protect the population at risk from bioaerosol exposure, an effective bioaerosol detection system is urgently needed that enables a rapid and accurate bioaerosol sampling, identification, and quantification in air samples. As an effective bioaerosol monitoring system requires both an effective air sampling device and rapid sample analysis technique with high sensitivity, the performance of RCS High Flow was investigated with culture-based quantification technique in Chapter 1. The Results showed that the test sampler would collect more than 80 % of common fungal spores and more than 50 % of airborne bacteria larger than 1.1 μm . However the biological performance of the sampler determined using a culturable bacterial counting method was significantly affected by environmental conditions, characteristics of sampler type, and consequently caused an underestimation in quantification. Therefore, in Chapter 2 and Chapter 3, Quantitative Real-Time Polymerase Chain Reaction (QPCR)

was applied to count the total bioaerol number in air samples. The results showed that successful bioaerosol quantification using QPCR requires not only to understand the characteristics of bioaerosol to be investigated and its sampling methodology, but also to develop study-specific standard curves. To increase the reliability of the method, the study-specific standrad curves associated with factors such as bacterial species, cell suspension preparation methods, QPCR methods should be developed and used for quantifications. To this end, the developed QPCR assay was applied to test the performance of a novel bioaerosol sampler (EPSS). The test results indicated a successful application of QPCR method to test performance of bioaerosol samplers. By coupling with an effective bioaerosol sampling device, this QPCR assay could increase the reliability of bioaerosol sampling systems and allow timely and effective quantification of aerosol samples.

Overall, the findings in this dissertation provide the general guidelines to develop an effective bioaerosol monitoring system by setting-up the study-specific protocol of QPCR assay capable of determining total cell numbers in air samples. The improved bioaerosol sampling system enabling rapid quantification of bioaerosols with high sensitivity may be applied as a basis for developing bioaerosol detection systems capable of detecting even small bioaerosol concentrations thus providing useful information needed to understand the bioaerosol exposure dose and response relationship.

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Dedication

To my loving husband and daughter, Inkyu and Hannah

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Chapter 1

Background, Motivation, and Dissertation Overview

1.1 Background and Motivation

Airborne particulate matter of biological origin, bioaerosols, consisting of bacterial cells, cellular fragments, by-products of microbial metabolisms, viruses, protozoa, and fungal spore / fragments of fungal mycelium are a potential source of a wide variety of public and industrial health hazards. Interest in bioaerosol exposure has increased over the last few decades when it was realized that there are many adverse health effects such as infections, hypersensitivity pneumonitis and toxic reactions (Burge et al., 1989; Karol, 1991) associated with bioaerosol exposure occurring in domestic and industrial sector. For example, it has been found that occupational bioaerosol exposure to wood trimmers is associated with restrictive pulmonary dysfunction in response to heavy mold exposure and there was a significant relationship between the reduction in lung function and mold concentrations (Dahlqvist et al., 1992). Among workers employed in waste industry (collecting and composting waste), various adverse health effects due to bioaerosol exposure have been documented, including respiratory symptoms, febrile episodes, allergic disease such as hypersensitivity pneumonitis and asthma (Poulsen et al., 1995). In poultry houses, large-scale production leading to increased bird density within confined space can be a source of human health problems related to bioaerosol exposure generated by faeces, litter, feed, feather formation or when the fattened birds are collected for transportation to a slaughterhouse. Since the activity of catching and boxing birds generates supplementary bioaerosols (Oppliger et al., 2008).

Allergenic, toxic, and inflammatory responses are also caused by bioaerosol exposure not only to the viable but also to non-viable microorganisms present in the air (Robbins et al., 2000; Gorny et al., 2002). Endotoxin (lipopolysaccharide protein complexes which are integral parts of Gram-positive bacteria) is the main biohazard in indoor and outdoor environments that has been suggested to cause respiratory symptoms (Vogelzang et al., 1998; Thorn et al., 1998). Clinical symptoms are non-specific (fever, shortness of breath, cough, mild dyspnea, malaise, chest tightness, headache and nausea) and this self-limited flu-like syndrome beginning within hours after exposure to high concentration of endotoxins is known as organic dust toxic syndrome (ODTS) in the field of occupational health (Seifert et al., 2003). (1→3)- β -D-glucan, a fungal cell wall component, has also been considered as a potential biohazard found in organic dust which reacts synergistically with endotoxins and is an important agent associated with development of allergic alveolitis (Fogelmark and Rylander, 1993) causing symptoms like eye and throat irritation, dry cough, and itchy skin (Rylander, 1996). As seen in above cases, it is clear that bioaerosol exposure plays a causal role in the development of symptoms described above in numerous occupational and residential populations. However, despite the importance of bioaerosol exposure for human health, an exact relationship between the bioaerosol concentration and development of adverse health effects remains uncertain. One of the reasons is the uncertainty related to bioaerosol exposure assessment. For an improved bioaerosol exposure assessment, development and validation of advanced bioaerosol monitoring systems is urgently needed.

The recent spread and health problems posed by SARS and avian influenza viruses and a threat of malicious release of hazardous biological agents against

civil/governmental/military establishments further reinforced such a need. The bioterrorism acts such as the anthrax (*Bacillus anthracis*) attacks in 2001 and the ricin incident of 2003 in Washington D.C. have shown that such threats are real and we need to improve our capabilities to detect the release of dangerous bioaerosol particles. The above mentioned factors, together with the society's growing concern regarding human exposure to bioaerosol, makes the development of bioaerosol monitoring systems capable of early and reliable detection, quantification, and identification of pathogenic airborne biological particles in public and occupational environments exceptionally important and urgent. Thus, the primary goal of this study is to develop and validate the analysis methods that could contribute to more rapid and accurate bioaerosol detection and quantification, which will help to improve the efficiency of bioaerosol monitoring systems.

An effective bioaerosol monitoring system requires two components: a bioaerosol collector to efficiently collect airborne biological particles and an analyzer to provide timely and reliable bioaerosol detection and quantification. Depending on the design method, airborne biological particles are collected onto solid, liquid, or agar media by different bioaerosol samplers. Over the past years a number of bioaerosol samplers have been developed and evaluated (Kenny et al., 1998; Kenny et al., 1999; Mehta et al., 2000; Peter and Schillinger, 2001; Henningson and Ahlberg, 1994). One of the emerging trends in bioaerosol sampling is the reliance on portable samplers (Mark et al., 1995). In contrast to the samplers requiring external vacuum pumps, portable samplers, such as the Burkard air sampler (Burkard Manufacturing Co., Ltd., Hertfordshire, United Kingdom), the Surface Air Sampler (SAS) Super-90 (PBI International, Milan, Italy), the Reuters Centrifugal Sampler (RCS) (Biotest Diagnostics Corp., Denville, NJ) and others, do not require external pumps and could be used where electricity is not available. One such

recently-introduced sampler, the RCS High Flow (Biotest Diagnostics Corp., Denville, NJ), is battery-operated and collects airborne microorganisms on agar strips using centrifugal forces. However, the performance of the latest model of this centrifugal sampler, RCS High Flow, when collecting airborne culturable particles has been evaluated only to a limited extent. Therefore, the aim of Chapter 2 was to investigate the physical and biological efficiencies of the RCS High Flow sampler when collecting airborne culturable microorganisms in the laboratory and field environments and to compare its performance to a reference sampler.

The culture-based bioaerosol monitoring system which provides a number of Colony Forming Units (CFUs) in an air sample has certain limitations. Although several such bioaerosol monitoring systems have been developed and used in a number of residential and occupational bioaerosol exposure studies, no standard method has been agreed upon to date, mainly due to the inherited limitation of culture-based quantification assay. Because a majority ($\geq 90-99\%$) of naturally occurring microorganisms cannot be cultivated and readily identified using standard culture methods (Amann et al., 1995; DeLong and Pace, 2001), the use of culture-based quantification underestimates bioaerosol concentrations. Moreover, in epidemiological studies of allergic illnesses, non-viable airborne microorganisms, which cannot be detected using culture methods, still have allergic properties and are critically relevant to total bioaerosol exposure. Therefore, it is important to improve the reliability of bioaerosol monitoring systems that are capable of accurate total cell quantification irrespective of the culturability or even the viability of the collected cells.

Methods that are independent of culturing, such as epifluorescence and direct light microscopy, are often used to determine total microorganism concentrations in air samples,

but these microscopy-based methods are time and labor-consuming (Kildeso and Nielsen 1997; Williams et al. 2001a; Zeng et al. 2004) and are not species-specific. An alternative method for analyzing total bacterial load in bioaerosol samples is the Polymerase Chain Reaction (PCR) assay, a molecular technique, which amplifies target nucleic acids collected from air and can provide a qualitative or semi-quantitative sample assessment when used with gel electrophoresis to visualize the resulting PCR amplicon (Saiki et al. 1985). However, PCR analysis has certain limitations, particularly in its accuracy, reliability and reproducibility (Birch et al. 2001).

Quantitative Real-Time polymerase chain reaction (QPCR), has been widely applied in medical research and has gained popularity in environmental research as a rapid /reproducible /reliable method for quantifying total cell quantity and permitting species-specific identification. When bioaerosol sampling is conducted in either highly contaminated environments where the microbial composition may be highly dynamic or in ambient air where the hazardous microorganisms may be present in low concentration, the amenability, accuracy, and reliability of the QPCR could be advantageous over other methods for cell quantification. In bioaerosol research, however, the application of QPCR and its advantages and disadvantages for the determination of total microbial load has not been widely investigated. There is a need to establish protocols and develop standard methods for bioaerosol quantification.

Generally, QPCR based on standard curves has been used to determine total bacterial load in air samples. Construction of a standard curve is based on the relationship between the PCR output values and the threshold cycle number (C_T values) - on the y-axis and a corresponding cell number on the x-axis. To produce the standard curves, genomic DNA is purified from pure cultured cells and then used in PCR reaction in serial dilutions,

and the corresponding cell number is calculated based on the species-specific genomic DNA copy number. This is a theoretical number, however, and is based on known copy numbers confining the application of QPCR to a small bacterial pool. Adopting an actual corresponding cell number on the x-axis of the standard curve would be advantageous for using QPCR in large bacterial pools and for quantifying the bacteria in unknown samples. Moreover, such an approach, genomic DNA-based PCR, has a drawback: the DNA extraction efficiency may vary depending on the initial bacterial concentration in experimental samples. This extraction efficiency decreases either when the bacterial concentration is too high or too low. In air samples, relatively low bacterial concentrations lead to lower extraction efficiencies and can cause variations in the PCR quantification outcome (under / over estimation). Additionally, the lack of studies on bioaerosol quantification using a standard curve based on the QPCR assay decreases the reliability of the method. Most of the current research focuses on developing methods that are very specific to a particular QPCR instrument and associated amplification conditions, but the variability of standard curve-based QPCR has not been addressed. Moreover, a critical aspect that has not been studied in great detail is the validation of factors contributing to the variation in the output estimate of the QPCR assay in bioaerosol quantification.

To address these issues, in Chapters 3 and 4, our primary goal was to investigate the controlling factors in standard curve generation methods and to improve the method's accuracy and applicability for bioaerosol quantification. For the QPCR to be valid it is important to examine whether standard calibration curves are affected by various preparation factors. Since visual comparison of standard curves is potentially very subjective, there is a need for an objective statistical test to determine the difference (or similarity) of standard curves prepared by different methods and also to test the effects of

different preparation methods on QPCR output without subjective bias. As part of this testing we also examined the feasibility of whole-cell PCR, where cells in question are analyzed by QPCR directly without extracting the genomic DNA first. Such a method is much simpler, less time-consuming, and could be especially useful for analysis of low bacterial concentrations in environmental samples.

Once the QPCR method for the bioaerosol quantification has been developed and tested, it can be used as a part of a bioaerosol monitoring system, enabling the rapid response to bioaerosol release. As a part of this effort, a whole-cell QPCR method was developed as a part of this study, and used to analyze performance of a novel bioaerosol sampler and to compare its performance characteristics obtained using QPCR and epifluorescence microscopy. This research is described in Chapter 5. The bioaerosol sampler in question, Electrostatic Precipitator with Superhydrophobic Surface (EPSS), has been developed as a part of another study and its physical collection efficiency has been examined with non-biological particles (Han and Mainelis, 2008). The EPSS is an electrostatics bioaerosol sampler that removes particles in aspirated air into small droplets of liquid to facilitate subsequent analysis (Han and Mainelis, 2008). The main advantage of this new electrostatic sampler is the low power requirement compared to conventional techniques (inertial collection mechanism) and ability to achieve a high sample concentration rate, which is especially beneficial for detecting low bioaerosol concentrations. To increase the reliability of the monitoring system based on the EPSS, the sampler's performance when collecting bioaerosol particles was investigated using two different total cell counting methods: epifluorescence microscopy and the whole-cell QPCR, developed as a part of dissertation study. As a labor-effective and economic choice, whole-cell QPCR was applied and the study-specific standard curves were generated for

total cell quantification in air samples. The collection efficiency of the EPSS based on the generated standard curve was compared to the efficiency obtained with epifluorescence microscopy, a conventional total cell counting method.

1.2 Dissertation Overview

Chapter 1 described the motivation and primary goals of the dissertation. In each chapter, specific background information and research goals are presented in more detail. Chapter 1 also presents an overview of each chapter.

In **Chapter 2** the following null hypothesis was tested: the test sampler's (RCS High Flow) underperformance compared to the BioSampler (reference sampler) was caused by damage to sensitive microorganisms during the collection process, the test sampler's sensitivity to wind direction and speed, as well as break-up of particle aggregates during the impingement process in the BioSampler. This resulted in more CFUs counted by the reference sampler than by the test sampler. To test this hypothesis, the physical and biological efficiencies of the RCS High Flow sampler were examined when collecting airborne culturable microorganisms in the laboratory and field environments and its performance was compared to a reference sampler. To determine the physical collection efficiency of the RCS High Flow sampler, non-biological polydisperse oleic acid, PSL particles, and potassium chloride (KCl) particles were used. The biological efficiency of the test sampler was determined using *Bacillus subtilis* var. *niger* (BG) spores and vegetative cells, which are common environmental bacteria and known to be very resistant to various environmental stresses (Friis et al., 2000; Sneath, 1986). The sampler's cut-off size (d_{50}), the particle size at which the sampler has 50% collection efficiency, was determined. Details are found in Chapter 2.

In **Chapter 3** a bioaerosol monitoring system was developed that includes a BioSampler (bioaerosol collection device) and the use of quantitative Real-Time PCR (QPCR) (bioaerosol analysis technique) for rapid and accurate detection and quantification. In bioaerosol exposure studies, the ability to count total cells is needed because both viable and non-viable microorganisms can be associated with adverse health outcomes. However, the application of QPCR for the quantification of airborne microorganisms has not been widely investigated, and, therefore, there is a lack of established protocols for bioaerosol detection and quantification using this method. To test the effect of standard curve type on bioaerosol quantification using a QPCR assay, standard curves were constructed using different methods: PCR methods, sample suspension preparation methods, and cell counting methods. After completing the air sampling, the corresponding cell number was determined using both culture-based and non-culture based counting methods for the standard curve. Two different dilution methods were applied in sample preparation for the PCR assay and two different PCR methods were used to generate standard curves. Based on the constructed standard curves, the total cell number in unknown air samples were estimated and compared to the results determined by traditional counting methods and estimated cell number monitored by an optical particle counter. Details can be found in Chapter 3.

In **Chapter 4** statistical techniques were used to investigate the effect of three factors on the QPCR standard curves, and consequently, the influence of those factors on the bioaerosol quantification output. The following three factors were investigated: different bacterial species (gram negative vs. gram positive bacteria), different sample preparation methods (cells from culture suspension vs. cells from air samples), and different QPCR methods (use of extracted genomic DNA vs. whole-cell PCR). Once the

standard curves were constructed based on the variables above, they were compared statistically as a function of curve preparation factors. Details can be found in Chapter 4.

In **Chapter 5** the QPCR assay developed and evaluated in Chapter 3 and Chapter 4 was used to analyze the performance of a novel bioaerosol sampler: the Electrostatic Precipitator with Superhydrophobic Surface (EPSS) when collecting biological particles. For a successful application of the developed sampling system, the QPCR method was used to determine the sampler's collection efficiency and concentration rate. Two different, commonly used microorganisms were tested: *Pseudomonas fluorescens*, a gram negative organism and vegetative *Bacillus subtilis*, a gram positive organism. The standard curves were generated using whole-cell QPCR method following the guidelines determined in Chapter 4 (the study-specific standard curves). Total bioaerosol quantification in air samples was determined by standard curve-based whole-cell QPCR and the results were compared with the reference numbers determined by Acridine Orange microscopy and counting by a direct-reading aerosol instrument. Details can be found in Chapter 5.

Chapter 6 summarizes findings and implications of the performed research to bioaerosol exposure assessment. The Curriculum Vitae is also included.

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Chapter 2

Evaluation of a High Volume Portable Bioaerosol Sampler in Laboratory and Field Environments^{*}

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2.1 Abstract

In Chapter 2, the physical and biological performance of a portable centrifugal sampler for culturable bioaerosols, RCS High Flow was investigated. The performance of the test sampler in the laboratory and field environments was compared to that of a reference sampler, BioSampler. The laboratory experiments with non-biological particles of KCl, Oleic acid, and Polystyrene Latex showed that the test sampler's collection efficiency is about 22% for 0.5 μm particles, 48% for 1.0 μm particles, and close to 100% for particles of 2.5 μm and larger. These tests indicated that the sampler's cut-off size (d_{50}) was 1.1 μm . The test sampler's physical performance when collecting spores and vegetative cells of *B. subtilis var. niger* (BG) bacterium was similar to that when collecting non-biological particles of the same size. In the laboratory tests, the RCS High Flow sampler was found to enumerate approximately 40% of BG spores and cells relative to the reference sampler, BioSampler. A similar ratio was observed during testing in an indoor environment. This ratio decreased to below 10% when testing was performed in an outdoor environment. We hypothesize that the test sampler's underperformance compared to the BioSampler is caused by the damage to sensitive microorganisms during the collection process, test sampler's sensitivity to wind direction and speed as well as break-up of particle aggregates during the impingement process in BioSampler, which resulted in more CFUs counted by the reference sampler than by the test sampler. Overall, when the RCS High Plus is used to sample culturable airborne microorganisms, obtained results may have to be adjusted to avoid potential underestimation of microorganism concentration in the air.

2.2 Introduction

Bioaerosols are diverse and complex airborne particles of biological origin, including pollen, fungal spores, fragments of fungal mycelium, bacterial cells and endotoxins, viruses, protozoa, and fungal mycotoxins (Nevalainen et al., 1993). Exposure to airborne microorganisms in indoor and outdoor environments can result in many respiratory and other adverse health effects, such as infections, hypersensitivity pneumonitis and toxic reactions (Burge et al., 1989; Karol, 1991). The allergenic, toxic, and inflammatory responses are caused by exposure not only to the viable but also to non-viable microorganisms present in the air (Robbins et al., 2000; Gorny et al., 2002). The exposure to airborne infectious agents (e.g., *Legionella* spp. and *Mycobacterium tuberculosis*), indoor allergens (e.g., *Penicillium* spp., *Alternaria* spp., *Bacillus subtilis*, *Bacillus cereus*, and *Actinomyces* spp.), invasive infectious fungal agents (e.g., *Aspergillus fumigatus*, *Aspergillus flavus*) and other bioaerosol particles is usually determined by collecting bioaerosol particles onto solid, liquid, or agar media followed by microscopic, microbiologic, biochemical, immunochemical, or molecular biological analysis of the sample (Burge et al., 1987; Burge et al., 1989; Hyvärinen et al., 1993; Jensen et al., 1992; Madelin and Jonhson, 1992; Juozaitis et al., 1994; Pegues et al., 2001; Ramaswamy et al., 2004). Therefore, successful monitoring of bioaerosol particles in various environments requires development and validation of efficient bioaerosol samplers.

Methods for collecting biological airborne particles are classified as passive or active. Passive air samplers employ natural aerosol convection or diffusion to direct particles into the sampling device. Use of settling plates, where particles deposit by the influence of gravity is another example of passive sampling. Active sampling methods use

air movers, such as personal or stationary pumps, to extract sample from the environment and deliver it to the collection substrate. Over the past years a number of bioaerosol samplers have been developed and evaluated (Kenny et al., 1998; Kenny et al., 1999; Mehta et al., 2000; Peter and Schillinger, 2001; Agranovski et al., 2002, Henningson and Ahlberg, 1994). One of the emerging trends in bioaerosol sampling is the reliance on portable samplers (Mark et al., 1995). In contrast to bioaerosol samplers requiring external vacuum pumps, portable samplers, such as the Burkard air sampler (Burkard Manufacturing Co., Ltd., Hertfordshire, United Kingdom), the Surface Air Sampler (SAS) Super-90 (PBI International, Milan, Italy), the Reuters Centrifugal Sampler (RCS) (Biotest Diagnostics Corp., Denville, NJ) and others, do not require external pumps and can be used where electricity is not available or is hazardous. One such recently-introduced sampler, RCS High Flow (Biotest Diagnostics Corp., Denville, NJ), is battery-operated and collects airborne microorganisms on agar strips using centrifugal forces. This sampler operates at a flow rate of 100 L/min, which is 2-2.5 times higher than the flow rates of 40 and 50 L/min featured by the previous models of centrifugal sampler, RCS and RCS Plus, respectively. For the RCS and RCS Plus samplers, the flow rates of 40 and 50 L/min, respectively, are the effective flow rates, which are lower than total flow rates. The RCS High Flow features a different design of the sampling head and differently from its prototypes the effective flow rate is same as the total flow rate. The air stream enters the rotor from the front of the RCS High Flow and after impacting onto the collection surface the air is exhausted through two outlets situated in the back of the sampling head and positioned parallel with the instrument. Due to this design, there is no mixing of the incoming and exhaust air streams. Due to this design, there is no mixing of the incoming and exhaust air streams.

The ability of RCS and RCS Plus to collect and recover airborne bacteria and fungi has been evaluated in previous studies. These studies found that the sampling efficiency of the RCS sampler is comparable to that of the Andersen N-6 sampler and the slit sampler when enumerating airborne fungi (Smid et al., 1989). Mehta et al. (1996, 2000) showed that the efficiency of RCS Plus sampler is comparable to that of the SAS Super 90, but significantly lower than the efficiencies of Andersen 2-stage impactor and Burkard air sampler when determining airborne fungi. The number of bacteria determined by the RCS Plus was significantly lower than the number of bacteria enumerated by the SAS Super 90, the Andersen 2-stage impactor and Burkard air sampler. The performance of the latest model of this centrifugal sampler, RCS High Flow, when collecting airborne culturable particles has not yet been evaluated. Performance of any bioaerosol sampler depends on its ability to extract and collect a representative aerosol sample (its physical efficiency) and its effect on the biological activity of the captured microorganisms (its biological efficiency). These parameters are usually determined in the laboratory and field environments through the use of test particles and reference samplers. Therefore, the overall goal of this chapter was to investigate the physical and biological efficiencies of the RCS High Flow sampler when collecting airborne culturable microorganisms in the laboratory and field environments and to compare its performance to a reference sampler.

2.3 Materials and Methods

2.3.1 Test and reference samplers

In this research, I examined the physical and biological efficiencies of the battery-operated RCS High Flow bioaerosol collector (Biotest Diagnostics Corp., Denville, NJ) and evaluated its performance against a reference sampler, BioSampler

(SKC Inc., Eighty Four, PA). The RCS High Flow is designed to monitor microbial aerosols that are viable. The device collects microorganisms on agar strips using centrifugal forces at a sampling flow rate $Q_{RCS} = 100$ L/min. The sampler's limit of detection is 1 CFU per volume of air sampled. The upper limit of detection depends on the maximum number of CFUs on the agar strip that can be reliably distinguished and counted. The agar strip consists of 34 individual squares and, according to our estimate, one can distinguish about 15 - 20 bacterial CFUs in one square. Thus the upper limit of detection is about 500 -600 CFUs. As a reference sampler we chose Biosampler, because it has well-established performance characteristics (Willeke et al., 1998; Lin et al., 2000) and has been shown to induce minimum damage to sensitive airborne microorganisms (Lin et al., 2000). The BioSampler is designed to sample airborne microorganisms and biologically inert airborne particles at a standard sampling flow rate, $Q_{BIO} = 12.5$ L/min. In this sampler, the airborne microorganisms are drawn into 3 nozzles through which they are projected at an angle toward a curved surface, where the combined forces of impaction and centrifugation collect them into 5 mL of collection liquid. The collection liquid is then available for sample analysis.

2.3.2 Test particles

The physical collection efficiency of the RCS High Flow sampler was determined using the non-biological polydisperse oleic acid and potassium chloride (KCl) particles. Potassium chloride particles were produced by aerosolizing 1% w/w KCl solution, prepared by dissolving 5 g of reagent quality KCl (Mallinckrodt Baker, Inc. Phillipsburg, NJ) into 500 mL of deionized purified water. The collection efficiency curve obtained with polydisperse particles was validated by sampling monodisperse Polystyrene Latex (PSL)

(Bangs Laboratories, Fishers, IN) particles. In this chapter, PSL particles of 0.48, 0.93, 1.95, 2.13, and 2.81 μm in diameter were used to examine the physical performance of the RCS High Flow sampler.

The biological efficiency of the test sampler in the laboratory experiments was determined using *Bacillus subtilis* var. *niger* (BG) spores and vegetative cells, which are common environmental bacteria and known to be very resistant to various environmental stresses (Friis et al., 2000; Sneath, 1986). Another criterion for selecting BG spores and cells as test biological particles was their frequent use to simulate anthrax-causing *Bacillus anthracis* (Franz et al., 1997; Hill et al., 1999) because of their physical and biochemical similarity. In addition, in instrument testing performed with live *B. anthracis* and other biowarfare agents, agents are often aerosolized using Collison nebulizer (McBride et al., 2003), a microorganism dispersion method which will be used in laboratory phase of this research. Because of the use of *B. anthracis* simulants, the obtained results on the sampler's collection efficiency can be extrapolated to actual *B. anthracis* agents.

Dry BG spores were obtained from the US Army Edgewood Laboratories (Edgewood Research, Development and Engineering Center, Aberdeen Proving Ground, MD). Before their aerosolization, these spores were suspended in sterile deionized water, and then activated at 60°C for 25 min. Vegetative cells of *B. subtilis* were cultured from BG spores grown in trypticase soy broth (Becton Dickinson Microbiological System, Sparks, MD). Prior to experiments, freshly prepared test organisms were washed 3 times with sterile, deionized water by centrifugation at 6000g \times for 5 min, at 4°C (BR4; Jouan, Winchester, VA). The obtained suspension was diluted in some experiments to obtain

airborne bacterial concentrations resulting in 30-300 CFUs per agar strip of the RCS High Flow and agar plates inoculated with BioSampler's collection fluid.

2.3.3 Experimental setup and procedures

2.3.3.1 Laboratory testing

The experimental setup used in the laboratory evaluation is shown in Figure 2.1. Biological and non-biological particles were aerosolized from a suspension using an aerosol generator, Collison nebulizer (BGI Inc., Waltham, MA) operated at a flow rate $Q_{NEB} = 3.0$ L/min. The test aerosol was diluted with HEPA filtered compressed air, $Q_{DRY} = 150$ L/min, and after passing through a 10-mCi ^{85}Kr electrostatic charge equilibrators (model 3012, TSI Inc., St. Paul, MN) entered the test chamber housing the RCS High Flow sampler, shown as Test Sampler in Figure 2.1. The device was operated at its standard flow rate of $Q_{RCS} = 100$ L/min. The reference sampler was positioned in parallel to the test sampler. The concentration and size distribution of particles upstream, C_{UP} , and downstream, C_{DOWN} , of the samplers were monitored by either an optical particle counter (model 1.108, Grimm Technologies Inc., Douglasville, GA) or an aerodynamic particle size spectrometer (Aerosizer Mach II, TSI - Amherst Process Instruments, Hadley, MA). The Aerosizer operating at $Q_{AER} = 5.1$ L/min was used when determining the physical collection efficiency of the test sampler. The Grimm optical particle counter operating at $Q_{OPC} = 1.2$ L/min was used to monitor the concentration of airborne biological particles when determining the biological collection efficiency of the test sampler. The test sampler's physical collection efficiency, $E_{COLL, RCS}$, was determined using the following:

$$E_{COLL, RCS} = 1 - (C_{DOWN}/C_{UP}). \quad (2.1)$$

When the physical collection efficiency of the RCS High Flow is compared to that of the reference sampler (BioSampler in our tests), the relative physical collection efficiency can be determined as:

$$\text{Relative Physical Efficiency (RCS)} = \frac{E_{COLL, RCS}}{E_{COLL, BIO}}, \quad (2.2)$$

where $E_{COLL, BIO}$ is Biosampler's physical collection efficiency.

When determining the biological efficiency of the RCS high flow sampler, the device was operated at its standard sampling flow rate $Q_{RCS} = 100$ L/min for $t_{RCS} = 1$ min. The short sampling time was chosen not to overload the collection media (agar strips) with colonies. The reference sampler, BioSampler was operated at its standard flow rate of $Q_{BIO} = 12.5$ L/min for $t_{BIO} = 3$ min. During the experiment, both the test sampler and the reference sampler collected test microorganisms aerosolized from the same batch. The concentration and size distribution of particles upstream of the samplers, C_{UP} , was monitored by the Grimm optical particles counter. After completing the sampling, the collection media (agar strips) from the RCS High Flow were removed from the sampler and incubated. A 0.1 mL aliquot of BioSampler collection liquid was plated in triplicate on Trypticase Soy Agar (TSA) (Becton Dickinson Microbiological System, Sparks, MD) and incubated. Both the agar strips and cultivated plates containing *B. subtilis* were incubated for 18hr at 30°C. After the incubation, colony forming units (CFUs) formed on the agar strips, $N_{CFU, RCS}$ and on the agar plates, $N_{CFU, BIO}$, were counted. The CFUs obtained from each sampler were converted to the numbers of recovered airborne biological particles. The relative biological efficiency of the RCS High Flow was determined in terms of the relative CFU count and was determined as follows:

Relative Biological Efficiency = Relative CFU Count =

$$\frac{E_{REL, RCS}}{E_{REL, BIO}} = \frac{\frac{N_{CFU, RCS}}{C_{UP, RCS} Q_{RCS} t_{RCS}}}{\frac{N_{CFU, BIO}}{C_{UP, BIO} Q_{BIO} t_{BIO}}}, \quad (2.3)$$

where $C_{UP, RCS}$ and $C_{UP, BIO}$ are airborne particle concentrations observed during sampling by the RCS High flow and the BioSampler, respectively.

The entire test system was placed in a Class II biological safety cabinet so that any aerosol particles not collected by the sampler are properly eliminated. All experiments were performed no fewer than three times, so that proper descriptive statistics could be obtained.

2.3.3.2 Field testing

During field testing, the test and reference samplers were positioned about 30 cm apart. The measurements were performed by sampling the same air volume by the test and the reference samplers. Thus, the RCS High Flow sampling time was set to $t_{RCS} = 7.5$ min (750 Liters of air at $Q_{RCS} = 100$ L/min) and Biosampler was operated for $t_{BIO} = 60$ min (750 liters of air at $Q_{BIO} = 12.5$ L/min). To avoid potential agar strip overloading of the RCS High Flow, which could potentially lead to the underestimation of the collection efficiency, additional samples were collected for shorter time: $t_{RCS} = 2.5$ min (250 Liters of air at $Q_{RCS} = 100$ L/min).

One indoor and one outdoor location were selected to evaluate the performance of the RCS High Flow sampler in the field. A residential living room (200 ft²) was used for indoor testing, while an area outside of a midsize building was used for outdoor testing. These particular sites were selected to represent two very different sampling environments. One

reference and one test sample were collected during a period of 1 hour and three such sets of samples were collected at each testing site. To avoid artificial effects due to air turbulence, minimal activity occurred during sample collection indoors. The RCS High Flow sampler was supplied with Tryptic Soy Agar (TSA) and Rose Bengal agar strips (Biotest Diagnostics Corp., Danville, NJ) for collecting viable bacteria and fungi, respectively. The BioSampler was supplied with 0.9% saline solution. After completing the sampling, 0.1 mL of BioSampler's collection fluid was plated on TSA agar plates and on Rose Bengal agar plates with Antimicrobial Supplement C (Becton Dickinson and Company, Sparks, MD) in triplicate to recover culturable bacteria and fungi, respectively. The plates with TSA agar were incubated for 1-2 days at 30° C and those with Rose Bengal agar were incubated at 26° C for 5 days. After the incubation, all bacterial and fungal colonies formed on the agar strips, $N_{CFU, RCS}$ and on the agar plates, $N_{CFU, BIO}$, were counted. Use of overall bacterial and fungal colony counts without determining the collected species when comparing culturable bioaerosol samplers has been described by Lin et al. (1999). The number of CFUs obtained from each sampler was used to calculate the airborne concentrations of culturable organisms, C (CFU/m³):

$$C_{RCS \text{ HIGH FLOW}} = \frac{N_{CFU, RCS}}{Q_{RCS} t_{RCS}} ; C_{BIOSAMPLER} = \frac{N_{CFU, BIO}}{Q_{BIO} t_{BIO}} \frac{V_{BIO}}{V} , \quad (2.4)$$

where V_{BIO} is the final volume (in mL) of Biosampler's collection liquid and V is an aliquot used for plating (0.1 mL).

Statistical analysis of the obtained data was performed with SAS 8.2 (SAS Inc., Cary, NC) software.

2.4 Results

2.4.1 Size distribution of test airborne biological particles

When evaluating the physical and biological efficiencies of the test sampler in laboratory experiments, *B. subtilis* var. *niger* spores and vegetative cells were aerosolized and their size distributions were measured using both an aerodynamic particle spectrometer (Aerosizer Mach II), and the Grimm optical particle counter. In Figure 2.2, which presents the aerodynamic (Chart A) and optical (Chart B) size distributions of aerosolized biological particles, the ordinate presents the number of microorganisms registered in a specific size range. To avoid artifacts due to the variations in the width of the size ranges, the registered number of particles (ΔN) is divided by the difference in logarithm values of the upper (d_u) and lower (d_l) particle diameters of a particular size range. To facilitate the comparison of the size distributions of BG cells and spores, the peak of each size distribution was normalized to a unity. Figure 2.2.A shows that majority (about 90%) of *B. subtilis* spores had aerodynamic sizes ranging from 0.6 μm to 1.2 μm with their mean size being approximately 0.78 μm . The aerodynamic size of vegetative *B. subtilis* cells ranged from 0.6 μm to 1.6 μm , with the mean size being approximately 0.89 μm . The measured mean sizes of BG spores and cells were selected as representative sizes of these biological particles when measuring the physical collection efficiency of the RCS High Flow sampler.

As seen in Figure 2.2.B, determined optical sizes of both BG spores and cells ranged from approximately 0.5 μm to 2.0 μm . The mean optical size of measured BG spores and cells was approximately 0.73 μm and 0.8 μm , respectively, when measured with Grimm optical particle counter. The bacterial size distributions and their mean sizes measured with

the Grimm optical counter are somewhat different from those observed with the Aerosizer. We believe the difference is due to the different detection techniques employed by both samplers. The Aerosizer registers particles based on their aerodynamic properties, while the Grimm counter registers the particles based on optical light scattering. The aerodynamic particle size is a function of a square root of particle's density. From the obtained results it appears that density of BG spores and cells was higher than 1 g/cm^3 .

When determining the biological efficiency of the RCS High Flow sampler and measuring the particle concentration upstream of the sampler, only those particles that were larger than $0.5 \text{ }\mu\text{m}$ were counted as bacterial spores or cells. The particles measured between $0.3 \text{ }\mu\text{m}$ and $0.5 \text{ }\mu\text{m}$ are considered to be mostly droplet residues and bacterial fragments but not bacteria (Terzieva et al. 1996).

2.4.2 The physical and biological collection efficiencies of the test sampler

Figure 2.3 shows the physical collection efficiency of the RCS High Flow sampler when collecting non-biological particles of oleic acid, and Polystyrene Latex (PSL), and spores and vegetative cells of *B. subtilis* bacteria as determined using the Aerodynamic Particle Spectrometer. As seen from this Figure, the sampler's collection efficiency when sampling non-biological particles does not vary significantly with the type of test particle. The physical collection efficiency of the device was about 22% for $0.5\mu\text{m}$ particles, 48% for $1.0 \text{ }\mu\text{m}$ particles, and close to 100% for particles of $2.5 \text{ }\mu\text{m}$ larger. The collection efficiency obtained with polydisperse KCl particles (not shown in Figure 2.3) was almost identical to the curve obtained with oleic acid particles. Figure 2.3 shows that 27% of airborne *B. subtilis* spores were collected. The efficiency increased to 38%, when vegetative *B. subtilis* cells were collected with the test sampler. The data presented in

Figure 2.3 show that the biological test particles are collected with the same efficiency as non-biological test particles of the same size.

One of the important parameters describing the physical performance of bioaerosol samplers is their cut-off size, d_{50} . The d_{50} refers to an aerodynamic size at which 50% of the airborne particles are collected. Particles larger than d_{50} are collected with efficiencies higher than 50%, while particles smaller than d_{50} are collected with efficiencies lower than 50% (Hinds, 1982). As seen from Figure 2.3, the test sampler's cut-off size d_{50} is 1.1 μm , i.e., particles larger than 1.1 μm are collected with efficiency higher than 50%, while particles smaller than 1.1 μm are collected with efficiency lower than 50%. The RCS Plus, a predecessor of the current test sampler, was reported to have a d_{50} of 6 μm (Mehta et al. 1996). Thus, the d_{50} of this centrifugal sampler represents a significant improvement over its previous version. In comparison, the Burkard portable air sampler for agar plates and the Surface Air Sampler (SAS) Super-90 were reported have a d_{50} of 2.56, and 2 - 4 μm , respectively (Mehta et al., 1996). The size of most airborne bacteria ranges from 0.5 to 3 μm while size of most of the airborne fungal spores ranges from 2 to 10 μm (Nevalainen et al. 1993; Mehta et. al., 1996). Therefore, from the physical collection efficiency curve of the RCS High Flow (Figure 2.3) we can infer that the test sampler will collect more than 80% of common fungal spores and more than 50% of airborne bacteria larger than 1.1 μm . The experiments have shown that BG spores, which are often used as a simulant of *B. anthracis* spores, were collected with efficiency of 27%. However, the aerodynamic size of tested BG spores was measured to be approximately 0.8 μm , while the anthrax-causing *Bacillus anthracis* spores are rods with size of 1-1.5 μm x 3-10 μm (Friedlander, 1997) and their aerodynamic size of *B. anthracis* spores is considered to be 1-5 μm . Therefore, it

could be expected that the RCS High Flow will collect *B. anthracis* spores with efficiency of approximately 50% or higher.

2.4.3 The relative physical and biological efficiencies of the RCS High Flow

Figure 2.4 presents the relative physical and biological efficiencies of the RCS High Flow sampler as compared to the reference sampler, BioSampler. When determining relative physical collection efficiency of the RCS High Flow sampler, its efficiency data presented in Figure 2.3 were used. The BioSampler's physical collection efficiency data for the same size and type of test particles were determined from the literature: 90% for BG spores and 95% for BG cells (Lin et al., 2000). The relative biological efficiency of the RCS High Flow sampler was determined as described in Materials and Methods section, Equation 3. The coefficient of variation (CV) of CFUs obtained with the RCS sampler in laboratory tests, when collection was performed for 1 min, was approximately 10%. Figure 2.4 shows that the relative physical and biological efficiencies of the RCS High Flow when collecting *B. subtilis* var. *niger* spores were $30\pm 3\%$ and $37\pm 14\%$, respectively. For the vegetative cells of *B. subtilis* var. *niger*, the relative physical and biological collection efficiencies were $40\pm 1\%$ and $39\pm 11\%$, respectively, as compared to the BioSampler. The difference between the relative biological efficiencies when collecting BG spores and vegetative cells was not statistically significant ($p>0.05$).

2.4.4 Performance of the test sampler in indoor and outdoor environments

The concentrations of airborne culturable bacteria and fungi determined with the test and reference sampler in indoor and outdoor locations are presented in Figure 2.5. At

the indoor sampling site, the mean concentration of airborne culturable bacteria and fungi as determined with the Biosampler was about 300 CFU/m³. The average bioaerosol concentrations determined with the RCS High Flow sampler operating for 2.5 and 7.5 minutes were lower and ranged from 150 to 250 CFU /m³.

At the outdoor sampling site the highest number of CFU concentration, 3000/m³, was observed with the Biosampler when determining bacteria. The concentration of airborne culturable bacteria determined with the test sampler in outdoor location ranged from 80 to 100 CFU/m³. The difference in bacterial CFU concentrations outdoors determined with the test and the reference samplers was statistically significant ($p < 0.01$). Also, at the outdoor sampling site, the concentration of airborne fungi determined with the test sampler was about 5 CFU/m³, while the concentration of culturable fungi determined with the reference sampler was about 200 CFU/m³. Again, the observed difference in airborne fungi concentration determined with the test and reference samplers was statically significant ($p < 0.0004$). The RCS sampler's coefficient of variation (CV) in indoor tests when sampling bacteria and fungi varied from 25% to 70%. In outdoor tests when sampling bacteria the CV varied from 20% to 50%; the CV was larger when sampling fungi. For both indoor and outdoor sampling locations, the Tukey's statistical test, which analyzed the RCS High Flow data obtained at different sampling volume, determined that there was no statistically significant difference in CFU concentrations obtained from 2.5 min and 7.5 min sampling intervals.

Figure 2.6 shows the ratios of the CFU concentrations determined with the test sampler relative to the CFU concentrations determined with the reference sampler. The ratios were determined using Equation 4 as described in Materials and Methods section for

each sampling set and averaged over three sampling sets. Figure 2.6.A shows that for the indoor sampling location, the test sampler operated for 2.5 min (250 Liters of air collected) on average enumerated 72% and 41% of bacteria and fungi, respectively, relative to the Biosampler. For the test sampler operated for 7.5 min (750 Liters of air collected), the average observed ratio was 55% for fungi and 38% for bacteria. The latter number, 38%, is very similar to relative biological efficiency observed in laboratory experiments (Figure 2.4). The statistical analysis of the data presented in Figure 2.6.A indicates that there is no statistically significant difference between CFU ratios for enumerated bacteria and fungi. The difference between CFU ratios obtained with the test sampler operated for 2.5 and 7.5 min is also not statistically significant.

The results obtained from the outdoor experiments indicate that the test sampler enumerated between 1.5 and 5% of airborne culturable microorganisms relative to the reference sampler, as shown in Figure 2.6.B. The fungal spores are larger than bacterial particles and, thus, they should be collected with higher efficiency than bacteria and should result in higher colony counts. However, there was no statistical difference between the ratios of enumerated bacteria and fungi. This result may indicate that the sampling flow rate of 100 L/min subjects fungal spores to strong shear forces which render the spores non-viable. The results from outdoor testing indicate that, relative to the reference sampler the test sampler, enumerated many fewer culturable organisms outdoors than in the indoor testing site. We believe there are three factors contributing to this result. First, visual observation of fungal and bacterial colonies indicated that morphology (shape, size, color) of the colonies enumerated at indoor and outdoor sampling sites were somewhat different. The test sampler, RCS High Plus, collects airborne microorganisms at a flow rate of 100 L/min using impaction technique, which is known to damage certain organisms (Stewart et

al., 1995). The Biosampler, on the other hand, was shown to induce less damage. Thus, it is possible that a significant fraction of microorganisms outdoors was more prone to damage than microorganisms collected in the indoor environment. However, since no testing was done to determine species of the collected organisms, this hypothesis remains speculative in nature.

Second, collection at the indoor sampling site was performed in calm air conditions and the result observed here was similar to a laboratory evaluation, where sampling was performed at isokinetic sampling conditions. The outdoor environment, on the other hand, was somewhat windy on the day of testing. Thus, it is possible that the test sampler is sensitive to wind direction and speed, causing an under-sampling of the airborne microorganisms. This hypothesis is reinforced by Grinshpun et al. (1994) who postulate that a major reason for differences in samplers' performance may be samplers' dependence on wind speed and direction, which may vary from test to test. In case of the RCS High Flow, the effect of wind speed and direction on the instrument's performance would have to be investigated in further research.

Third, when particles are collected by impingement, air flow velocity through the collector's nozzles often approaches sonic velocity, which is known to break-up clumps of culturable propagules into individual cells. This way, a particle initially containing several propagules will be broken up into several separate cells to be counted as multiple propagules. On the other hand, in an impactor, like the RCS High Flow, a particle impacting on the agar surface will be counted as one CFU even if it contains several culturable propagules. Thus, a higher number of CFUs consistently obtained by the BioSampler could also be caused by the particle deagglomeration during the impingement process.

Overall, at the laboratory and field testing locations, the test sampler, RCS High Flow, enumerated fewer airborne microorganisms than the reference sampler, BioSampler. On the other hand, the test sampler is portable, battery-operated and collects biological aerosols directly on agar at a flow rate of 100L/min which may improve detection of hardy organisms. Thus, the RCS High Flow sampler can find applications in certain environments where low concentrations of such microorganisms need to be detected. However, as the results from this chapter have shown, the culturable microorganism concentration obtained in such testing may have to be adjusted to avoid underestimation of microorganism concentration in the air.

2.5 Conclusions

A portable bioaerosol sampler, such as RCS High Flow, offers certain advantages over other samplers for viable bioaerosols: it is lightweight, battery operated, and collects viable microorganisms directly on agar media. The results obtained in this chapter indicate that the RCS High Flow has a $d_{50} = 1.1 \mu\text{m}$, i.e., microorganisms larger than $1.1 \mu\text{m}$ will be collected with efficiency of 50% or higher. The aerodynamic size of anthrax-causing *B. anthracis* spores is considered to be 1-5 μm . Therefore, it could be expected that the RCS High Flow will collect *B. anthracis* spores with efficiency of approximately 50% or higher. Field testing indicated that test sampler's performance may significantly depend on the testing environment. During the outdoor testing, the RCS High Flow on average recovered less than 5% of organisms enumerated with the test sampler, BioSampler. We hypothesize that the low microorganism recovery was due to the presence of sensitive microorganisms that were damaged during the collection process. Other possible causes of the low microorganism recovery include the RCS High Plus sampler's sensitivity to wind direction and speed as well as break-up of particle aggregates during the impingement process in the reference sampler (BioSampler), which resulted in more CFUs counted by the reference sampler than by the test sampler. These hypotheses require further investigation. Overall, this chapter has shown that concentration of airborne culturable microorganisms determined with the RCS High Flow sampler in a certain environment may have to be adjusted to avoid underestimation of the microorganism concentration in the air.

2.6 References

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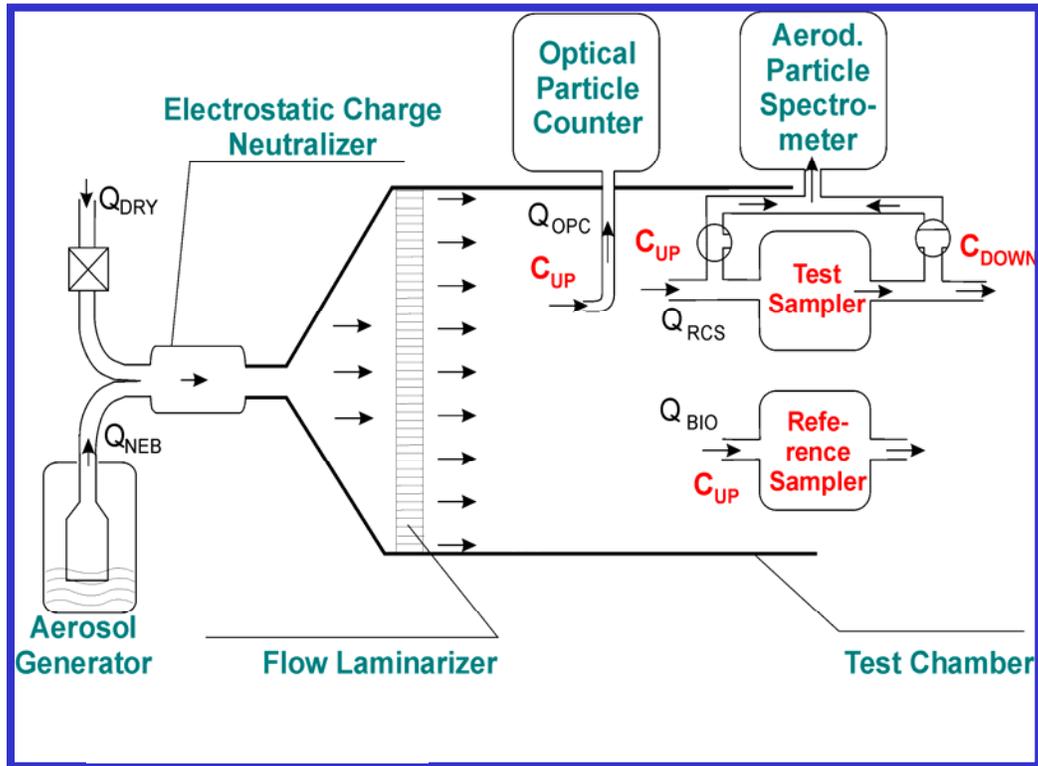


Figure 2.1. Experimental Setup

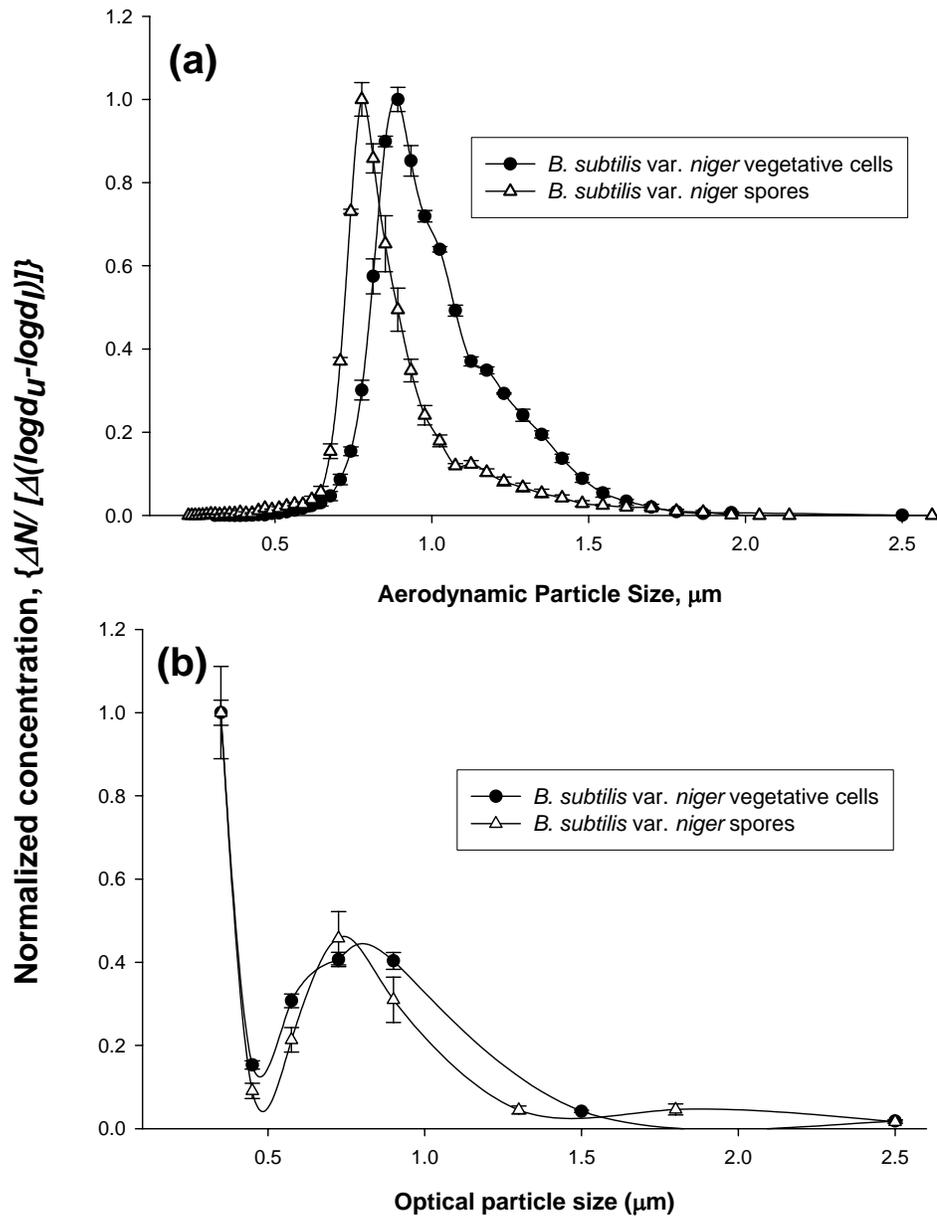


Figure 2.2. Size distribution of *B. subtilis* var. *niger* spores and vegetative cells as determined by an aerodynamic particle spectrometer (A) and by an optical particle counter (B).

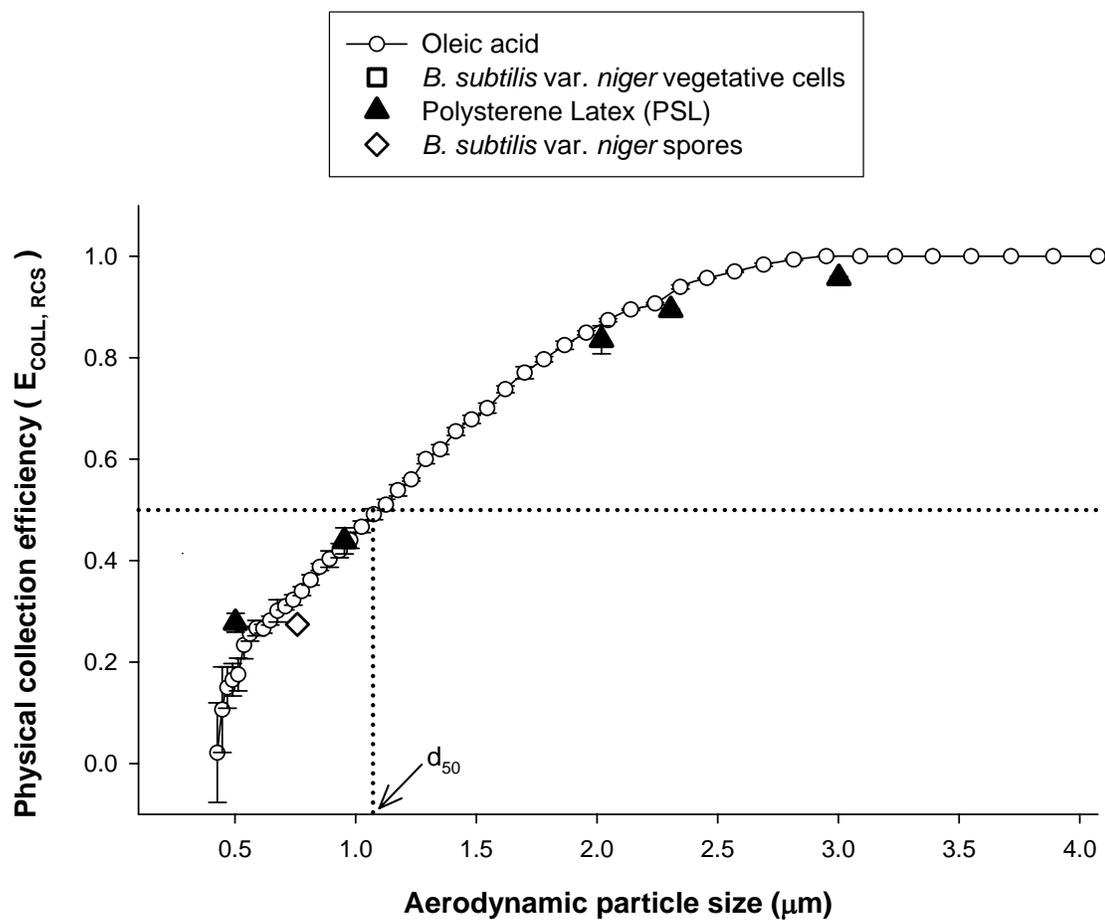


Figure 2.3. The physical collection efficiency of the RCS High Flow sampler when collecting biological and non-biological particles as determined with an aerodynamic particle spectrometer.

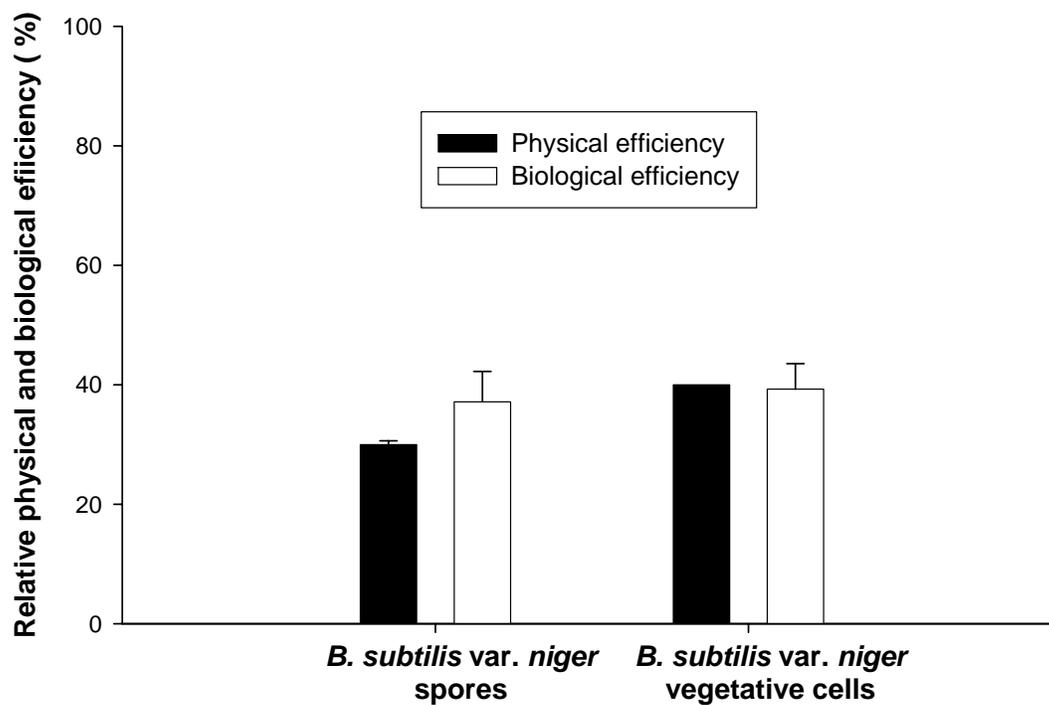


Figure 2.4. The relative physical and biological efficiencies of the RCS High Flow sampler as compared to the BioSampler. The tests were performed with *B. subtilis* var. *niger* spores and vegetative cells.

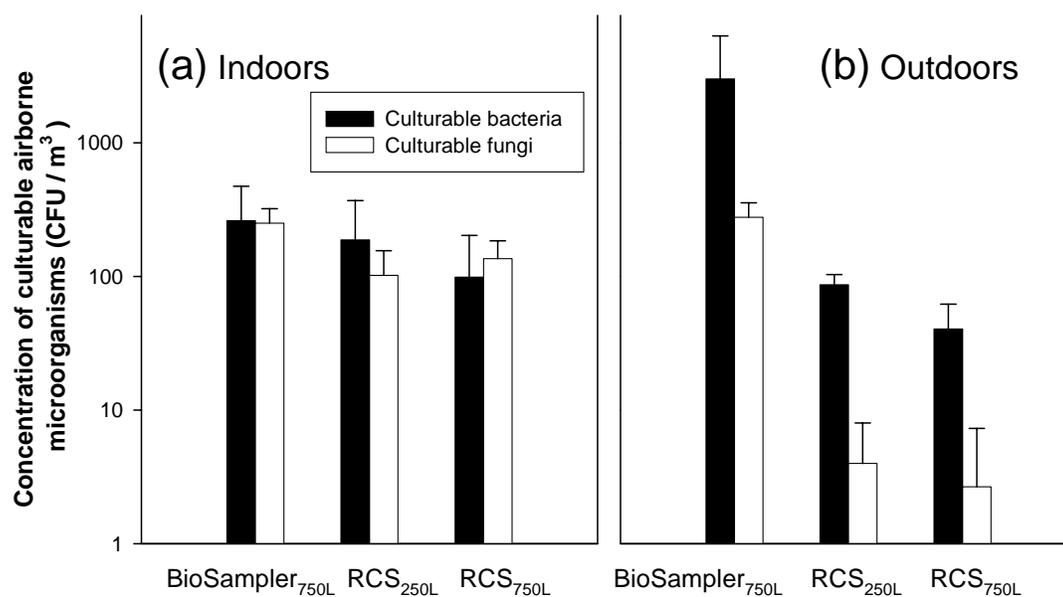


Figure 2.5. The concentration of colony forming units measured by the test sampler (RCS High Flow) and the reference sampler (BioSampler) in two different field environments. The subscripts indicate the air volume sampled.

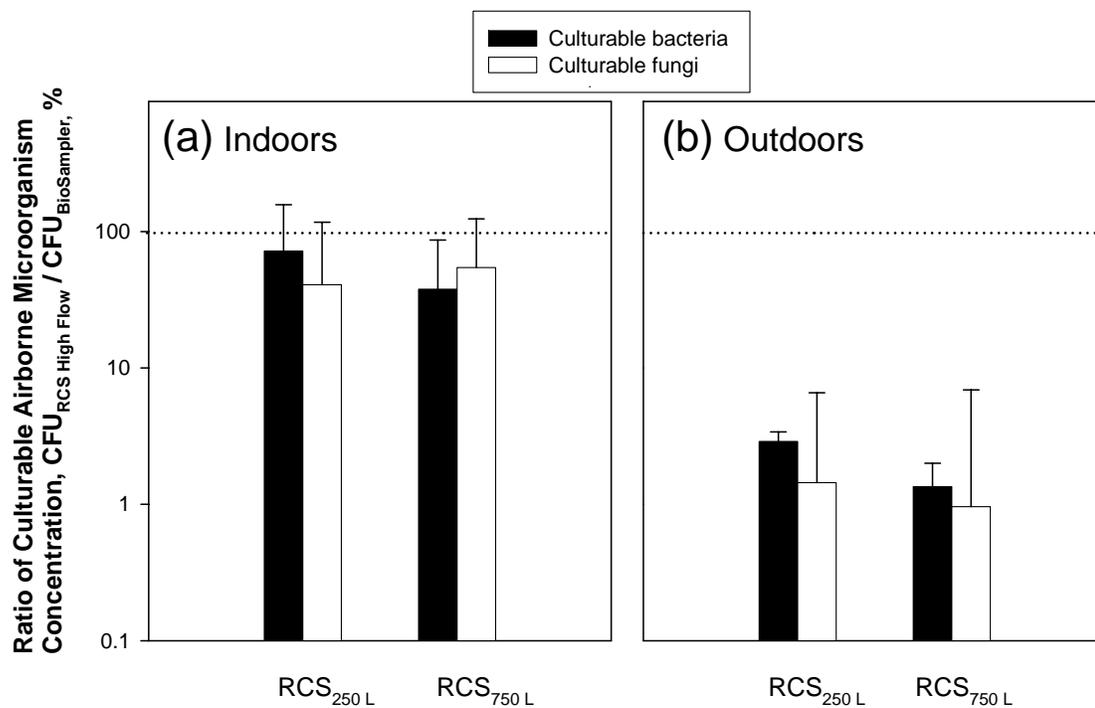


Figure 2.6. Ratio of the culturable microorganism concentrations determined by the test sampler (RCS High Flow) compared to the culturable microorganism concentrations determined by the reference sampler (BioSampler). The measurements were performed in two different field environments.

Chapter 3

Development and Calibration of Real-Time PCR for Quantification of Airborne Microorganisms in Air Samples[†]

[†] This chapter is modified from the manuscript by **An, H.R.**, Mainelis, G., and White L. (2006) Development and Calibration of Real-Time PCR for Quantification of Airborne Microorganism in Air Sample, *Atmospheric Environment*. 40:7924-7939.

3.1 Abstract

This manuscript describes the coupling of bioaerosol collection and the use of Real-Time PCR (QPCR) to quantify the airborne microorganisms. The quantity of collected microorganisms determined by QPCR is compared with conventional quantification techniques, such as culturing, microscopy and airborne microorganism counting using Optical Particle Counter (OPC). Our data show that an experimental approach used to develop standard curves for use with QPCR is critical for accurate sample quantification. Using universal primers we generated 12 standard curves by various methods and these curves were used to quantify model organism *Escherichia coli* (*Migula*) *Catellani* from air samples. Standard curves prepared using a traditional approach, where genomic DNA is extracted from pure cultured bacteria, diluted in series, and then amplified by PCR yielded significant underestimation of sample quantities compared to airborne microorganism concentration as measured by an OPC. The underestimation was especially pronounced when standard curves were built using colony forming units (CFUs). In contrast, the estimate of cell concentrations in an air sample by QPCR was more accurate (~60% compared to the airborne microorganism concentration) when the standard curve was built using aerosolized *E. coli*. The accuracy improved even further (~100%) when air samples used to built the standard curves were diluted first, then the DNA extracted from each dilution was amplified by the QPCR – to mimic the handling of air samples with unknown and possibly low concentration. Therefore, our data show that QPCR can be a rapid and accurate method to quantify airborne microbes. However, the standard curve used for quantification needs to be prepared using the same environmental matrix and procedures as handling of the environmental sample in question. Reliance on

the standard curves generated with cultured bacterial suspension (a traditional approach) may lead to substantial underestimation of microorganism quantities in environmental samples.

3.2 Introduction

The presence of harmful airborne microorganisms in various indoor and outdoor environments has been associated with a variety of illnesses, including allergic reactions, toxicoses, and infections (Agranovski et al. 2002; Li et al. 1999; Burge et al. 1989; Karol 1991a; Nevalainen et al. 1993). Moreover, there is a threat of malicious release of hazardous microorganisms against civil/governmental/military establishments. To protect the populations at risk, efficient bioaerosol monitoring tools are required. Exposure to bioaerosol particles, including airborne infectious agents, indoor allergens, fungal agents and others, is usually determined by collecting such particles into solid, liquid, or agar media followed by qualitative and/or quantitative sample analysis using microscopic, microbiologic, biochemical, immunochemical, or molecular techniques which offer different degrees of sensitivity and specificity (Burge et al. 1989; Burge and Solomon 1987; Hybarinen et al. 1993; Juozaitis et al. 1994; Madelin and Johnson 1992).

Conventional quantitative bioaerosol detection methods often rely on the culture-based analysis which provides a number of Colony Forming Units (CFUs) in an air sample (Williams et al. 2001a; Zeng et al. 2004). When the health outcome is an allergenic response, the human immune system responds not only to culturable, but also to non-culturable microorganisms (Gorny et al. 2002a; Robbins et al. 2000a). Since not all microorganisms in an air sample are culturable, the determination of only the culturable airborne microorganism concentration may lead to an underestimation of the total microorganism concentration in the sample (Cox 1989a) and, therefore, is not the most accurate method for many exposure assessment purposes. Methods that are independent of

culturing, such as epifluorescence and direct light microscopy, are often used to determine total microorganism concentrations in air samples, but these microscopy-based methods are time and labor-consuming (Kildeso and Nielsen 1997; Williams et al. 2001a; Zeng et al. 2004) and are not species-specific. An alternative method for analyzing total bacterial load in bioaerosol samples is the Polymerase Chain Reaction (PCR) assay which can provide qualitative or semi-quantitative sample assessment when used with gel electrophoresis to visualize the resulting PCR amplicon (Saiki et al. 1985). The PCR assay has been applied to analyze air samples for the presence of endemic microorganisms (Alvarez et al. 1994), biowarfare agents (Higgins et al. 2003a), and fungi commonly associated with adverse health effects (Williams et al. 2001a; Cruz-Perez et al. 2001). PCR has an advantage over many conventional techniques because by using specific primer sets, this technique can also be used to identify a particular microbe. However, PCR analysis has certain limitations, particularly in its accuracy, reliability and reproducibility (Birch et al. 2001).

Currently, the Real-Time PCR (QPCR) is evolving into a promising tool capable of reproducible and accurate measurements of total microorganism concentrations in environmental samples. This detection system combines a thermalcycler coupled to an optical module. At the end of the extension phase of each PCR cycle, the optical module measures the fluorescence intensity of each reaction which is generated either by hybridization probes (TaqMan, molecular beacon, or fluorescence resonance energy transfer (FRET)), or by double stranded DNA dyes such as Sybgreen green (SYBR) (Stetzenbach et al. 2004a; O'Mahony and Hill 2002a). The data analysis software provided in a QPCR system calculates a threshold based on the background fluorescence, and determines the threshold cycle number, C_T , at which the fluorescence in the sample crosses this threshold. The C_T is inversely correlated with the DNA concentration in the sample.

By using known DNA concentrations as template, standard curves can be produced to quantify the total DNA. Such a calibration curve can then be used to analyze samples with unknown microorganism quantities.

QPCR is a sensitive and accurate method that also can be used for high throughput screening. Furthermore, QPCR does not require the post-PCR analysis such as gel-electrophoresis that is need for traditional PCR. This method has been successfully used for the quantification of several microorganisms, such as *Mycobacterium avium* subsp. *paratuberculosis* (O'Mahony and Hill 2002a), and *Salmonella* in pure culture (Kimura et al. 1999a), *Escherichia coli* O157:H7 in soil, and dairy waste washwater (Ibekwe and Grieve 2003), *Vibrio vulnificus* in water (Panicker et al. 2004a) as well as for the detection of airborne fungal spores in environmental samples (Williams et al. 2001a; Stetzenbach et al. 2004a; Schweigkofler et al. 2004a). However, the application of QPCR for the quantification of airborne microorganisms has not been widely investigated, and, therefore, there is the lack of established protocols for bioaerosol detection and quantification.

In many studies, standard curves used to quantify microorganisms are generated using purified DNA isolated from cultured cells and then applied to quantify environmental samples collected from different matrices, i.e. air, water or soil (Li et al., 1999; Robbins et al., 2000). Such a protocol may not be applicable for airborne microorganisms because their culturability can be affected by the air sampling process (Stetzenbach et al. 2004a) in which case the standard curves generated with purified DNA would not provide accurate quantification of microorganisms in air samples (Zeng et al. 2004). One of the possible solutions to this problem is to generate standard curves using actual air samples of known concentrations. Standard curve preparation in previous reports

also relied on the serial dilutions of DNA isolated from the concentrated bacterial solution for the template. Such an approach does not take into account that the yield of DNA extraction kits may differ depending on the bacterial concentration. Potential differences may be exacerbated when using the DNA extraction kits for low concentration air samples. This issue may play a role in applying Real-Time PCR for quantification of air samples and should also be addressed.

The main goal of this paper was to contribute towards addressing the issues above by comparing several standard curve preparation methods so that a protocol leading to a more accurate quantification of the microorganism load in air samples using Real-Time PCR could be developed. For this purpose, we used a model organism and constructed a family of standard curves using purified genomic DNA not only from cultured bacteria but also from bacteria collected by air sampling. Potential differences in DNA yield from high concentration and low concentration samples were investigated using two sample dilution approaches as described in the Material and Methods. When developing the standard curves, the concentration of bacteria corresponding to a certain C_T value was determined by three different methods: culture-based counting (CFUs), epifluorescence and direct light microscopy. Overall, we developed twelve standard curves by different methods which were then used to quantify the amount of total bacteria in air samples of different concentrations. The number of bacteria in air samples determined by Real-Time PCR and the developed different standard curves were compared with traditional sample analysis methods: culture-based counting (CFUs), epifluorescence and direct light microscopy. We believe that the developed method for QPCR assay calibration will facilitate quantification of total bacterial load in air samples and will contribute towards improved methods for bioaerosol exposure assessment.

3.3 Materials and Methods

3.3.1 Bacterial strain and its preparation

As a test organism for this work, we used *Escherichia coli* (Migula) *Catellani* strain ATCC11775 (American Type Culture Collection, Manassas, VA). This organism is easy to culture and the suitable universal primers for Real-Time PCR assay are already available (Nadkarni et al. 2002). The *E. coli* bacteria have been suggested as one of the standards when testing bioaerosol sampling technologies (Macher 1997) and have been used when testing bioaerosol samplers (Li et al. 1999). The potential of respiratory disease when exposed *E. coli*-containing droplets (Berge 1995) and a recent study implicating airborne spread of *E. coli* O157 during outbreak investigation (Varma et al. 2003) further justifies the use of *E. coli* (non-pathogenic strain) bacteria as a test microorganism. The *E. coli* strain used in our experiments was cultured in Trypticase Soy Broth (TSB) (Becton Dickinson Microbiological System, Sparks, MD) at 37 °C for 16 hrs. Prior to experiments, the bacteria were washed 3 times with sterile, deionized water by centrifugation at 6000g \times for 5 min, at 4 °C (BR4; Jouan, Winchester, VA).

3.3.2 Development of Real-Time PCR standard curves

For each experimental condition, a standard curve was obtained by plotting each threshold cycle (C_T) value against the log of corresponding *E. coli* cell quantity. The cell quantity was determined by using three different methods: culture-based method (CFUs mL⁻¹, culturable number of cells), direct light microscopy (cells mL⁻¹, total number of cells) and epifluorescence microscopy (cells mL⁻¹, total number of cells). These methods

for estimating cell quantity were applied for both cultured *E. coli* cells and for *E. coli* cells collected in air samples. The overall diagram of experimental procedures used to generate a family of standard curves by Real-Time PCR is shown in Figure 3.1. The standard curves were constructed with genomic DNA extracted from either cultured *E. coli* or air sampled *E. coli*. Each template DNA was prepared using two different methods: (1) the genomic DNA was first extracted from 1 mL of undiluted (10^0 dilution) cell suspension and the resulting genomic DNA was then diluted in a series of 10-fold dilutions (later in the text this method is called “Isolated-Diluted”); (2) the cells in the undiluted suspension (10^0 dilution) were first diluted in a series of 10-fold dilutions and genomic DNA was then extracted from 1 mL of each dilution (later in the text this method is called “Diluted-Isolated”). The Diluted-Isolated set is designed to mimic application of Real-Time PCR to an air sample of unknown and, possibly low, concentration. This dual method of template DNA preparation was applied for both cultured *E. coli* and air-sampled *E. coli*.

Genomic DNA was extracted from both cultured *E. coli* and air sampled *E. coli* using the DNease tissue protocol (Dneasy Tissue Kit, Qiagen, Velencia, CA), according to the manufacturer’s protocol. An aliquot of 1 mL of cultured cells and 1 mL of air sampled cells was used per extraction. For cultured cells, the dilution of cell suspension or of extracted genomic DNA ranged from 10^{-1} to 10^{-6} , while for air sampled cells the dilution range was from 10^0 to 10^{-5} . 5 μ l of prepared template DNA was used to generate standard curves in a triplicate PCR reaction. Sterile purified water was used for all dilutions.

When preparing the standard curves the sensitivity (limit of quantification) of *E. coli* detection by the Real-Time PCR was determined from the highest titration of genomic DNA isolated from either cultured cells or air sampled cells at which a threshold value (C_T)

could be reliably measured. The Real-Time PCR was determined to operate below the quantification limit if the relationship between C_T values and the high-titration template DNA concentration became nonlinear, or if the C_T value for a particular dilution was approximately the same as the C_T values for negative controls (no template present).

Using the methods described above we prepared a family of twelve standard curves that correlate C_T values from two types of samples (cultured bacteria and air-sampled bacteria) and two different methods of genomic DNA extraction (“Isolated-Diluted” and “Diluted-Isolated”) with three methods of estimating the cell quantity in a sample (CFUs, direct light microscopy and epifluorescence microscopy). The details of the cell counting methods are described below.

3.3.3 Determination of culturable bacterial number by CFU count

The freshly prepared *E. coli* was harvested and serially diluted in 10-fold. 0.1 mL aliquots from the 10^{-4} to 10^{-8} water-based serial dilutions were plated in triplicate onto Trypticase Soy Agar (TSA) (Becton Dickinson Microbiology Systems). The plates were incubated at 37 °C for 16 hrs and the number of Colony Forming Units (CFUs) was counted. The same procedure, except for the used dilution factor, was used to enumerate CFUs from air samples. The cell numbers in higher/lower dilutions were determined by applying dilution factor.

3.3.4 Determination of total bacterial number by microscopy

Total numbers of bacteria in liquid suspensions were determined both by direct light microscopy and epifluorescence microscopy using the Axioskop 20 (Carl Zeiss Inc., Thornwood, NY). For direct light microscopy, we used Petroff-Hausser counting chamber (Hausser Scientific, Horsham, PA) and for each aliquot of a serially-diluted sample we counted bacteria in all 25 chamber's fields in duplicate. For epifluorescence microscopy, two replicate slides were prepared by filtering 0.1 mL aliquots of a selected dilution through a black polycarbonate filter (Fisher Scientific, Suwanee, GA) and then staining it with the Acridine Orange (Becton Dickinson Microbiology Systems, Sparks, MD). At least 20 microscopic fields were counted using an oil-immersion objective. The cell numbers for dilutions other than those counted were determined by applying dilution factor.

3.3.5 Real-Time PCR amplification

Amplification and detection of DNA by real-time PCR was performed on iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) using the iQ SYBR Green supermix PCR Kit (Bio-Rad Laboratories, Hercules, CA) consisting of 2× SYBR Green supermix (with the hot-start enzyme, iTaq™ DNA polymerase, SYBR PCR buffer, dNTP mix, SYBR I, 20 nM fluorescein for dynamic well factor collection, and 6 mM MgCl₂). The purified *E. coli* genomic DNA both from cultured *E. coli* and air sampled *E. coli* were used as the templates in PCR reaction. Reaction mixes were prepared by combining 12.5 µl of 2× SYBR Green supermix, primers and H₂O to a total volume of 25 µl for each reaction. A 25 µl reaction volume including template (genomic DNA) was transferred to a thin 96-well PCR plate (Bio-Rad Laboratories, Hercules, CA). The plate

set-up contained two negative controls (one without primers and the other without template DNA) in triplicate. The plate was covered with optical-quality sealing tape (Bio-Rad Laboratories, Hercules, CA) and centrifuged briefly to bring all reagents to the bottom of the wells. The universal primers originally described by Nadkarni et al. (2002) including the forward primer, 5'-TCCTACGGGAGGCAGCAGT-3' and the reverse primer, 5'-GGACTACCAGGGTATCTAATCCTGTT-3' were used at final concentration of 0.25 μ M for each primer and produced 466bp amplicon (between residues 331 and 797 on the *E. coli* 16S rRNA gene). Amplification reaction was performed using the following program with iCycler iQTM thermal cycler (Bio-Rad Laboratories, Hercules, CA): 10 min at 95 °C; 40 cycles of (15 s at 95 °C 1 min at 60 °C). Data analysis was performed using iCycler iQTM Real-Time detection system software.

After completing the PCR amplification cycles, a melt curve was generated for the resulting amplicon by measuring loss of fluorescence over a temperature range of 55-92 °C. In these curves, a negative first derivative plot is presented as the rate of change in fluorescence over temperature range. This graph represents the desired amplicon as distinct melting peaks with specific melting temperature (T_m). If there is a contamination during the reaction, a peak, other than desired amplicon peak, would appear in melting curve thus indicating the contamination of DNA, non-specific binding, or occurrence of primer dimers.

3.3.6 Aerosolization and sampling of *E. coli* bacteria when developing standard curves

The experimental setup used to aerosolize and collect *E. coli* is shown in Figure 3.2. Freshly harvested *E. coli* suspension was aerosolized using a Collison nebulizer (BGI Inc.,

Waltham, MA) operated with filtered air at 10 psi and a flow rate $Q_{NEB} = 3.0$ L/min. The test aerosol was diluted with HEPA filtered air $Q_{DRY} = 100$ L/min, passed through a flow-laminarizing honeycomb and entered the test chamber housing a liquid microbial sampler, BioSampler equipped with 5 mL sampling cup and operated at a flow rate $Q_{BIO} = 10$ L/min (Fig. 2). The BioSampler (SKC Inc., Eighty Four, PA) has established performance characteristics and features collection efficiency of approximately 100% for bacterial cells (X Lin et al. 2000; Willeke et al. 1998). During each test, the BioSampler collected airborne *E. coli* into 5 mL of purified and sterilized water for 5 minutes. The concentration of airborne *E. coli* inside the test chamber, C_{OPC} , was monitored by an Optical Particle Counter (OPC) (model 1.108, Grimm Technologies Inc., Douglasville, GA). The Grimm optical particle counter operates at a flow rate $Q_{OPC} = 1.2$ L/min and measures particles in sixteen size channels ranging from 0.3 to 20 μm . Analysis of the size distribution of aerosolized particles indicated that vast majority (~95%) of airborne bacteria were single particles. Control aerosolization of water without bacteria indicated low particle concentration background in the sampling chamber, approximately 10 particles / L. The entire test system was placed in a Class II biological safety cabinet so that any aerosol particles not collected by the sampler are properly eliminated.

When the airborne *E. coli* bacteria were collected for the generation of standard curves, the concentration of bacteria in the test chamber was approximately $8 \times 10^5/\text{L}$. Given the sampling time of $t = 5$ min, sampling flow rate of $Q_{BIO} = 10$ L/min, BioSampler's sample volume $V = 5$ mL, and BioSampler's collection efficiency $E \approx 100\%$, this concentration of airborne bacteria was used to calculate the expected cell concentration N_L (cells/ 5mL) in the collection liquid:

$$N_L = C_{OPC} \cdot t \cdot Q_{BIO} \cdot E / V. \quad (3.1)$$

In this experiment the N_L was expected to be approximately 8×10^6 cells/mL. After each air sample was collected, it was processed as follows: (1) 1 mL of sample suspension was transferred to sterilized microcentrifuge tubes and 0.1 mL aliquot of the sample was used to obtain 10^0 to 10^{-8} serial water-based dilutions. The dilutions ranging from 10^{-4} to 10^{-8} were used to obtain CFU counts as described above; (2) the second 1 mL of sample suspension was transferred to sterilized microcentrifuge tubes and used to prepare 10-fold serial water-based dilutions. The aliquots of dilutions were used to determine the concentration of collected bacteria using direct light and epifluorescence microscopy; (3) the third 1 mL of sample suspension was transferred to sterilized microcentrifuge tubes and diluted in 10-fold. The extracted genomic DNA from each dilution was used in Real-Time PCR reaction (Diluted-Isolated set); (4) the fourth 1 mL of sample suspension was used for DNA extraction, then the isolated genomic DNA was diluted serially in 10-fold and used in Real-Time PCR reaction (Isolated-Diluted set).

3.3.7 Application of developed standard curves for the quantification of air samples

Twelve standard curves were developed using methods described above and used to quantify concentration of *E. coli* bacteria in air samples. The experimental setup shown in Figure 3.2 and described above was also used here. To test the sensitivity of the method we produced three distinctly different concentrations of airborne cells: $C_{OPC} = 2.74 \times 10^6$, 2.47×10^5 and 2.25×10^4 cells / L of air as determined by the optical particle counter. Based on the Equation 3.1, we expected the concentrations of airborne bacteria to result in the following concentrations of bacteria in liquid samples: 2.74×10^7 , 2.47×10^6 , and 2.25×10^5 cells/mL. Further in the text these concentrations are designated as High, Medium,

and Low concentrations of airborne bacteria. After 5-minute sampling, 3 mL of each sample was used to determine the concentration of collected *E. coli* by using CFU counting, direct light, and epifluorescence microscopy. Another 2 mL was used for template DNA preparation: Diluted-Isolated and Isolated-Diluted sets. These sets were then used in Real-Time PCR with universal primers according to conditions described in Real-Time PCR amplification section above. Once the C_T value was determined, the family of twelve standard curves was used to quantify the cell concentration in the samples. The number of collected *E. coli* cells quantified by Real-Time PCR was compared to that quantified by traditional counting methods: CFUs, direct light, and epifluorescence microscopy as well as the expected cell concentration N_L .

3.4 Results

3.4.1 Results of Real-Time PCR

The universal primers originally described by Nadkarni et al. (Nadkarni et al. 2002) were used in QPCR reactions containing SYBR Green as a fluorescence marker. Results from gel electrophoresis confirmed that the molecular weight of the amplicon was the correct size (466 bp), which indicates that the primers are specific (data not shown). To confirm that no products of non-specific priming, including primer dimers, were contributing to the signal, all QPCR reactions were followed by melting curve analysis, which indicated a single melting point of the *E. coli* amplicon (89 °C). As shown in Figure 3.3.A, no melting peaks were observed for nonspecific amplicons, except for a weak signal

appearing at low concentrations of template DNA and identified as primer dimers ($T_m = 75$ °C, if present).

An example of the graphical output of QPCR for the concentrations of Isolated-Diluted DNA obtained from cultured *E. coli* cells is shown in Figure 3.3.B. As could be seen from this graph, the C_T values for 10-fold DNA dilutions ranging from 10^{-2} to 10^{-6} are evenly spaced, which indicates linearity of the reaction within investigated concentration range. For higher dilutions the relationship between the DNA concentration and C_T values became non-linear indicating that we were below the limit of quantification. These DNA concentrations were excluded from standard curve preparation. Thus, for the investigated conditions and the concentration range, dilution 10^{-6} presented the limit of quantification. When very high DNA concentrations (10^0 and 10^{-1} dilutions) were amplified, the relationship between the C_T value and DNA concentration became non-linear indicating that the DNA amplification reached its saturation. Such high DNA concentrations were also excluded from standard curve preparation.

The C_T values obtained for different DNA concentrations and corresponding cell concentrations determined by various methods are shown in Tables 3.1 and 3.2. For both Isolated-Diluted and Diluted-Isolated sets of cultured *E. coli* (Table 3.1) the highest reliably detectable microorganism concentration was 2.69×10^7 CFU/mL (culture method), 3.24×10^7 cells/mL (epifluorescence microscopy), and 1.97×10^7 cells/mL (direct light microscopy). These cell concentrations yielded C_T values of 10.60 ± 0.26 and 10.40 ± 0.10 for Isolated-Diluted and Diluted-Isolated sets, respectively. The limit of quantification for cultured *E. coli* was 2.67×10^3 CFU/mL (culture method), 3.24×10^3 cells/mL (epifluorescence microscopy), and 1.97×10^3 cells/mL (direct light microscopy) associated

with $C_T = 24.57 \pm 0.15$, and $C_T = 25.17 \pm 0.21$ for Isolated-Diluted and Diluted-Isolated DNA preparation sets, respectively. As the microorganism concentrations are expressed as cells/mL and only 5 μ L of DNA suspension was used for amplification, the actual calculated microbes will be lower by a factor of 200: 20-30 cells according to the counting by microscopy. The C_T values did not show a significant difference between two template DNA preparation methods ($p > 0.05$). The C_T values for negative controls were $C_T = 30.2 \pm 0.36$.

The results of Real-Time PCR with air sampled *E. coli* are shown in Table 3.2. The highest investigated microorganism concentration (4.38×10^4 CFU/mL; 7.29×10^6 cells/mL, epifluorescence microscopy; and 8×10^6 cells/mL, direct light microscopy) yielded similar C_T values for both Isolated-Diluted and Diluted-Isolated DNA sets. The C_T values did not show a significant difference between two template DNA preparation methods ($p > 0.05$). The limit of quantification, however, for the Isolated-Diluted set was by an order of magnitude lower than that for Diluted-Isolated set and corresponded to 4.38×10^0 CFU/mL; 7.29×10^2 cells/mL, epifluorescence microscopy and; 8×10^2 cells/mL, direct light microscopy. This cell concentration yielded C_T value of 29.40 ± 0.17 . Negative controls had a C_T value of 30.2 ± 0.36 . For both air-sampled *E. coli* sets, the melting temperature was approximately 89 °C.

The results presented in Tables 3.1 and 3.2 indicate that C_T values for both DNA preparation methods (Isolated-Diluted and Diluted-Isolated) are similar, except in the lower microorganism concentrations (Table 3.2). In this case the Diluted-Isolated DNA set, which was designed to mimic environmental microbial samples, showed lower sensitivity by an order magnitude compared to Isolated-Diluted set. This result indicates that

efficiency of the DNA extraction from low microbial concentrations may play a significant role in the overall sensitivity of detection by the QPCR technique. Therefore, insufficient cell concentration in environmental samples along with the inability to consistently extract sufficient amount of DNA from these samples may result in inconsistent and unreliable PCR output. One of the possible solutions to this issue would be the use of air samplers capable of concentrating high volumes of air into small amounts of liquid thus increasing bacterial concentration (Mainelis et al. 2005).

3.4.2 Comparison of standard curves

Using the linear-range data presented in Tables 3.1 and 3.2 we constructed a twelve standard curves that correlate C_T values from two types of samples (cultured *E. coli* and air sampled *E. coli*), and two different methods of template DNA preparation (“Isolated-Diluted” and “Diluted-Isolated”) with three methods of estimating corresponding cell quantity in a sample (CFUs, direct light microscopy and epifluorescence microscopy). These standard curves are shown in Figure 3.4. As could be seen from this Figure, the standard curves do not substantially depend on the DNA preparation method. This is true for both cultured *E. coli* and air sampled *E. coli*. However, differences are observed in curves prepared with cultured *E. coli* and air sampled *E. coli*. The highest difference was recorded with standard curves based on CFU counting. For the same C_T value, standard curve based on cultured cells would yield almost 100 times higher cell concentration compared to a standard curve based on air sampled cells. The trend was reversed for curves built using direct light and epifluorescence microscopy as cell counting methods. Here, for the same C_T value, standard curves based on cultured cells would yield

approximately 5-times lower microorganism concentration compared to a standard curve based on air sampled cells. These data indicate that the standard curve preparation method can substantially affect quantification of environmental microbial samples using QPCR assay.

The linear amplification ranges for each curve as well as equations for the fitting curves are presented in Table 3.3. The C_T values are expressed as

$$C_T = k \cdot \log(x) + b, \quad (3.2),$$

where k is the coefficient of the slope of the standard curve, x is the microorganism concentration, and b is a numerical constant. Using the value of k we can estimate the amplification efficiency, E , as suggested by (Ibekwe and Grieve 2003):

$$E = (10^{1/k}) - 1. \quad (3.3)$$

A PCR reaction with 100 % efficiency would generate a slope of -3.32 (Ibekwe and Grieve 2003). The amplification efficiency for all curves was greater than 85% and the correlation coefficient r^2 for all standard curves was approximately 0.99 (Table 3.3).

3.4.3 Quantification of airborne *E. coli*

The suitability of the standard curves for quantification of bacterial concentrations in air samples was tested by aerosolizing, sampling and analyzing *E. coli* bacteria. In order to evaluate the sensitivity of the detection methods we aerosolized three different concentrations of bacteria (High, Medium and Low) differing by approximately a factor of 10. The concentration of airborne bacteria was monitored by an Optical Particle Counter (OPC) and its data were used to compute expected bacterial concentration N_L in liquid samples (5mL) (Equation 1). After 5 min. sampling, the culturable and total cell number in

each sample were determined by the following methods: CFUs, direct-light microscopy, epifluorescence microscopy, and QPCR. For the latter, the amount of collected bacteria was determined using the obtained C_T value and standard curves generated earlier (Table 3.3). Bacterial numbers determined with all methods were converted to cells/5mL and compared to the concentration of cells N_L expected to be collected by the microbial sampler. The results of this experiment are presented in Table 3.4. As could be seen from this table, the number of cells in a high-concentration sample as determined by microscopy is in very good agreement with the number based on OPC measurements N_L . The microscopy method, however, became unreliable at lower cell concentrations and could not provide accurate microorganism concentration estimates at Medium and Low concentrations. The traditional CFU counting method is very sensitive and, theoretically, can be used to detect a single bacterium. However, the cell concentrations provided by CFU counting was approximately 3 orders of magnitude lower compared to those based on OPC measurements.

When the cell concentration in air samples was estimated using QPCR, the accuracy of the estimate (as compared to OPC measurements) was dependant on the standard curve used. The curves based on CFU counts underestimated sample concentrations from 10 times (DNA from cultured bacteria) to about 200 times (DNA from air sampled bacteria). This is not unexpected given the comparison between the CFU estimation and the OPC measurement. Compared to CFU-based curves, standard curves based on microscopy counts provided a better estimate of *E. coli* concentration in air samples. Among these, standard curves built with air-sampled *E. coli* provided a more accurate sample concentration measurement compared to standard curves prepared with cultured bacteria.

The effect of standard curve type on the quantification of microorganisms in air samples becomes even more obvious when sample concentrations, as determined by traditional counting methods and QPCR are expressed as fractions of the expected microorganism concentrations based on OPC measurements and Equation 1. The averages of such fractions for three different airborne microorganism concentrations are presented in Figure 3.5. The y-axis of the graphs is the ratio of the mean bacterial number determined by various counting methods over mean number of cells N_L expected to be collected by the microbial sampler. For traditional detection methods, this ratio was 0.12 %, 95.3 %, and 97.2 % for CFUs, epifluorescence and direct light microscopy, respectively. For QPCR assay, the highest ratio was achieved when applying the standard curves based on air sampled *E. coli* and DNA prepared as Diluted-Isolated with total cell number determined by microscopic counting. In this case, the average ratio of bacterial number determined by QPCR over N_L was approximately 90 % or higher (depending whether epifluorescence or direct-light microscopy was used). The difference between the two microscopic methods was not statistically significant ($p > 0.05$). When standard curves based on air sampled *E. coli*, microscopy counting and Isolated-Diluted DNA were used, the ratio decreased to approximately 60%. The difference between the Isolated-Diluted and Diluted-Isolated sets for air-sampled bacteria was statistically significant ($p < 0.05$). When standard curves prepared with cultured *E. coli* and microscopy counting were applied for sample quantification, the ratio of determined cell concentration vs. N_L was approximately 10-20% for both methods of DNA preparation (Isolated-Diluted or Diluted-Isolated). Compared to corresponding air-sampled sets, the difference was statistically significant ($p < 0.05$).

The results also indicate that use of only culturable bacteria, or CFUs, when preparing standard curves, may not be suitable for quantification of airborne bacterial concentrations and may yield significant underestimations of sample concentration. The standard curve prepared with air sampled *E. coli* and Diluted-Isolated DNA was designed to closely mimic the processing of actual air samples and this curve yielded the most accurate results compared to N_L . It seems that the method of standard curve preparation can play a significant role in sample quantification. Thus, our results suggest that standard curves should be prepared in a way that simulates the collection and processing of actual environmental samples. For airborne microorganisms this could be accomplished by aerosolizing and collecting the known quantities of target microorganisms in the laboratory prior to sampling in the field. Another possibility would be comparison of Real-Time PCR output with other quantitative methods.

3.5 Discussion

Quantification of microbial concentrations in air samples is an active research area and development of rapid and reliable assays for microbial detection is needed for exposure assessment and control purposes. In recent years, RT - PCR has been gaining popularity as a tool for detection and quantification of microorganisms in environmental samples. However, reliable quantification of bacteria from air samples using this method is still not fully developed. This chapter used a model organism to demonstrate the calibration of the QPCR protocol for quantification of airborne bacteria. For this purpose, we developed a group of standard curves and used those curves to quantify air samples. The obtained results were compared with those received using traditional air sample

analysis methods to determine the standard curve yielding the most accurate sample concentration. Our results indicate that the methods used to develop standard curves may play a significant role in accuracy of sample quantification by the Real-Time PCR.

Standard curves are a fundamental and important element of any QPCR analysis; however, methods to select ideal standard curves that would improve the accuracy of QPCR assay for quantification of air samples have received little attention. Since the standard curve is usually generated based on the relationship between C_T values and corresponding cell number, the accurate determination of cell number is essential for an accurate curve. In most studies, the standard curves were generated based on the cell number determined with either 16S rDNA copy number (Nadkarni et al. 2002) or CFUs (Ibekwe and Grieve 2003). Since the number of 16S rDNA genes per genome in bacteria may vary (Anderson et al. 1995; Wilkinson and Young 1995), not knowing the exact number of 16S rDNA operons in any given species at the time of sampling is a substantial limitation. The QPCR assay based on standard curves built with CFUs also has inherent limitations: 1) the enumeration is based only on culturable cells and total number of cells could be higher, which is especially important when allergenic health outcomes are considered; and 2) if bacterial agglomerates are collected, one colony could represent more than one cell. Another alternative is the QPCR assay based on total cell number determined by microscopy. As shown in Tables 3.1 and 3.2, the ranges of bacterial number used for standard curves varied depending on the cell counting method which resulted in substantially different standard curves. In some cases, when air sampled *E. coli* was used, the number of cells determined by microscopy was 200-fold higher compared to CFU counting. Based on the existing research, it could be expected that culturable counting method (CFUs), which relies on the enumeration and identification of only those cells that

are culturable, may underestimate total bacterial number. Aerosolization and sampling stress may reduce the viability and culturability of vegetative cells in air sample even further (Cox 1989a; Buttner and Stetzenbach 1991a; Martinez et al. 1988a). Therefore, a reliable and accurate primer detection limit for QPCR should be determined by a total cell counting method, such as microscopy or flow cytometry, which permits enumeration of cells regardless of their metabolic state.

Our data indicate that, in order to improve the accuracy of the QPCR assay, a standard curve should be prepared in a way that mimics the handling of environmental sample in question. Use of standard curves that rely only on laboratory-cultured bacterial suspension may lead to underestimation of microorganism quantities in environmental samples. Although the described calibration with a family of standard curves was performed with one model organism, we believe that the demonstrated principles of Real-Time PCR calibration for airborne samples will serve as a prototype for other single and mixed microbial populations. Future studies will address the application of this calibration principle for field sampling where a variety of mixed microbial populations can be encountered.

3.6 References

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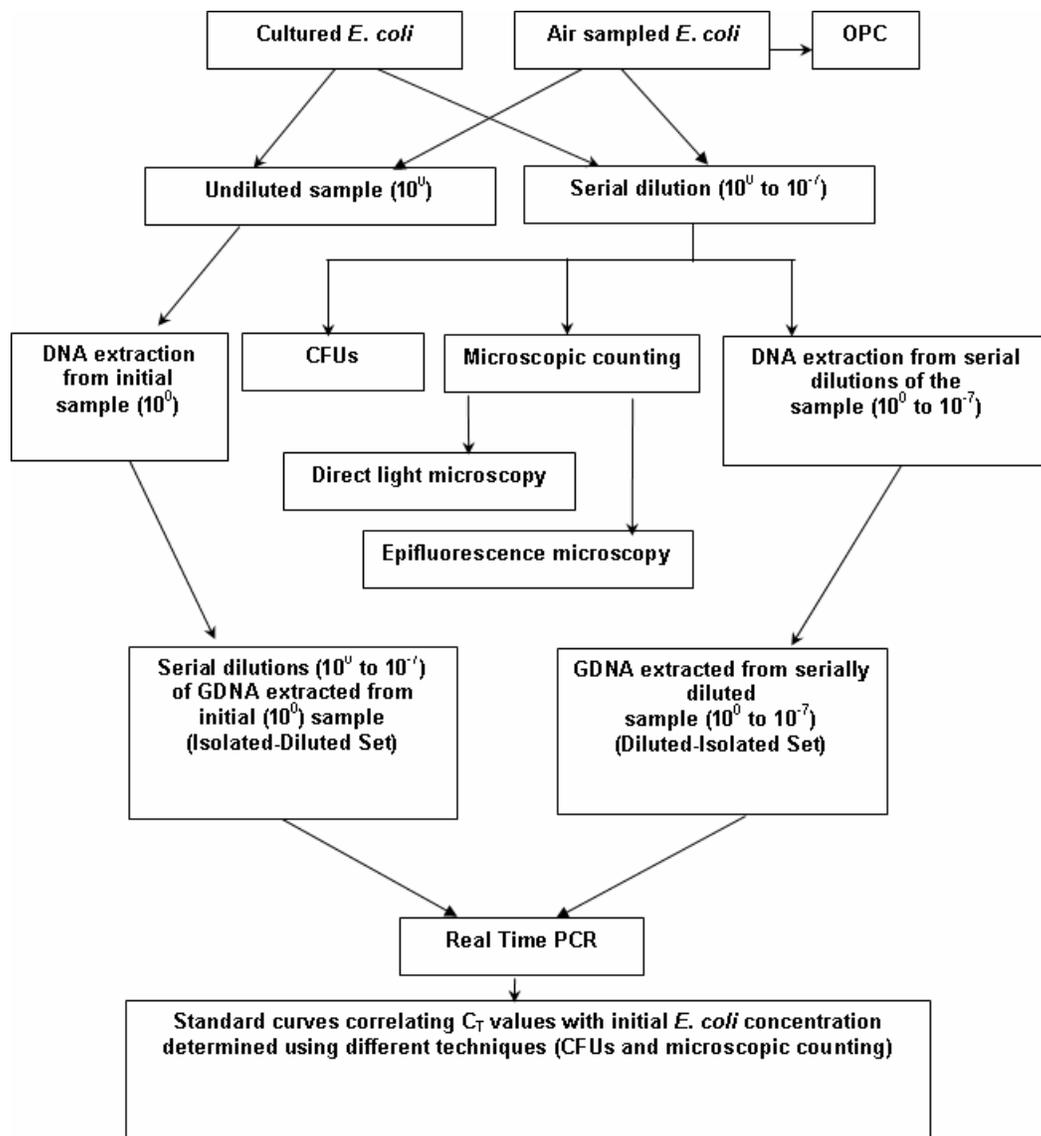


Figure 3.1. Outline of the experiments used to generate standard curves by Real-Time PCR. Abbreviations: OPC – optical particle counter, C_T – threshold cyclevalue, GNDA – genomic DNA.

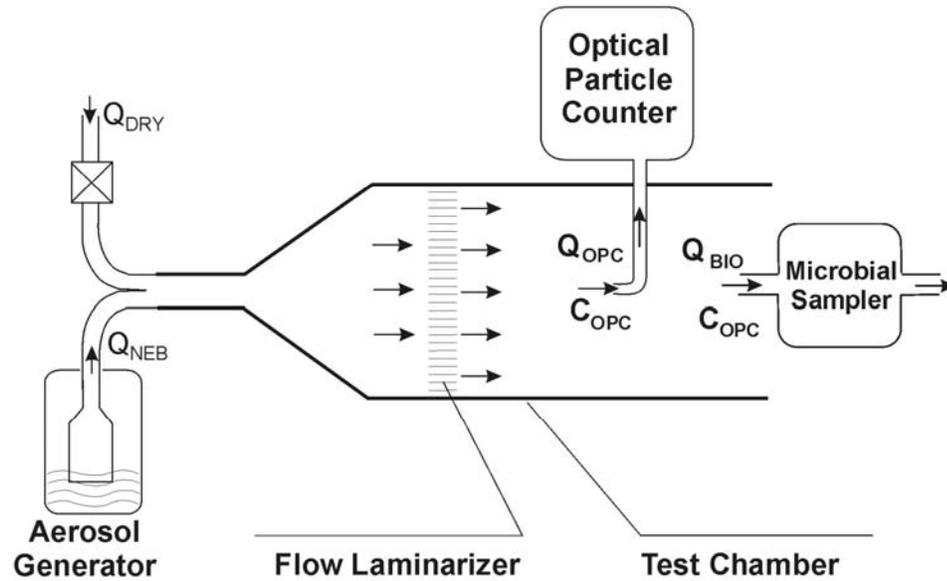


Figure 3.2. Experimental setup. Abbreviations: Q_{NEB} – aerosolization air flow, Q_{DRY} – dilution air flow, Q_{OPC} – sampling flow rate of the optical particle counter, C_{OPC} – bacterial concentration measured by the optical particle counter, Q_{BIO} – microbial sampler’s flow rate.

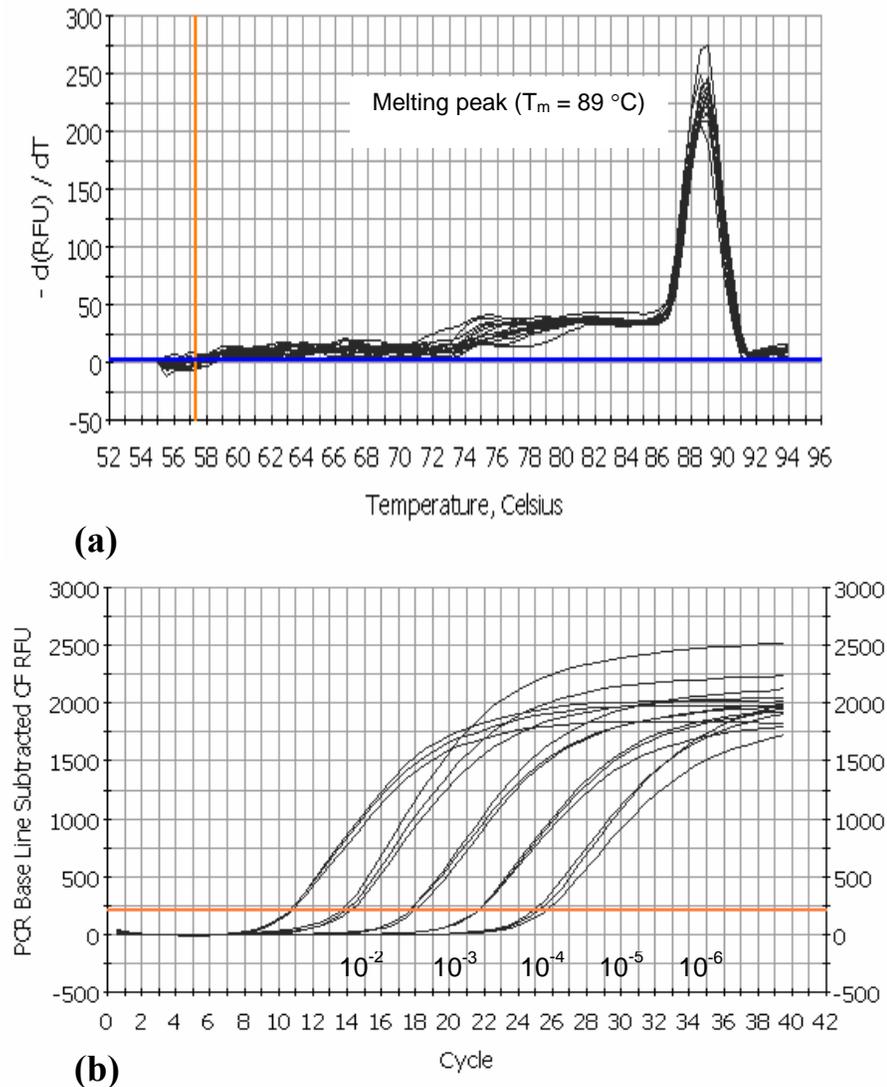


Figure 3.3. Typical Real-Time PCR output in our experiments: (A) The melting curves analysis presenting the amplicon as a distinct melting peak with specified melting temperature ($T_m=89^\circ\text{C}$); (B) Sensitivity of SYBR Green based real-time PCR using purified genomic DNA with universal primers when detecting *Escherichia coli* in pure culture at different dilution factors. Purified DNA was extracted from serially diluted *E. coli* with concentrations from 10^3 to 10^7 , based on microscopic counts.

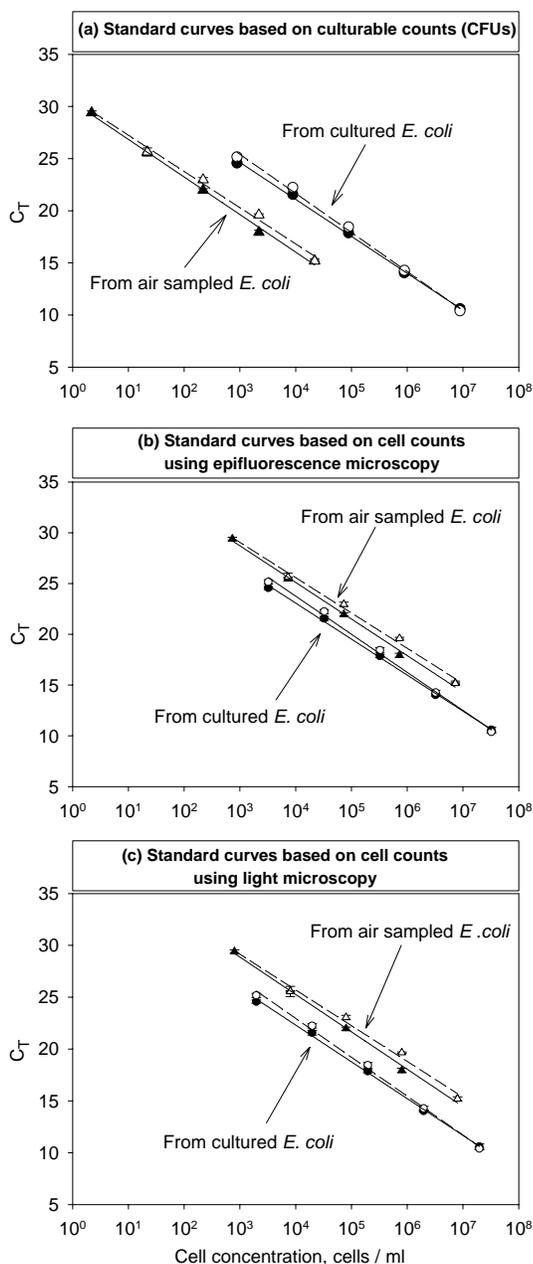


Figure 3.4. Standard curves based on the relationship between C_T values and the bacterial concentration as determined by three different counting methods: (a) using culturable counts, (b) using epifluorescence microscopy, and (c) using light microscopy. The circles represent C_T values of the genomic DNA isolated from cultured *E. coli*: open circle (○) for Isolated-Diluted, and closed circle (●) for Diluted-Isolated. The triangles represent C_T values of the genomic DNA isolated from air sampled *E. coli*: open triangle (△) for Isolated-Diluted, and closed triangle (▲) for Diluted-Isolated.

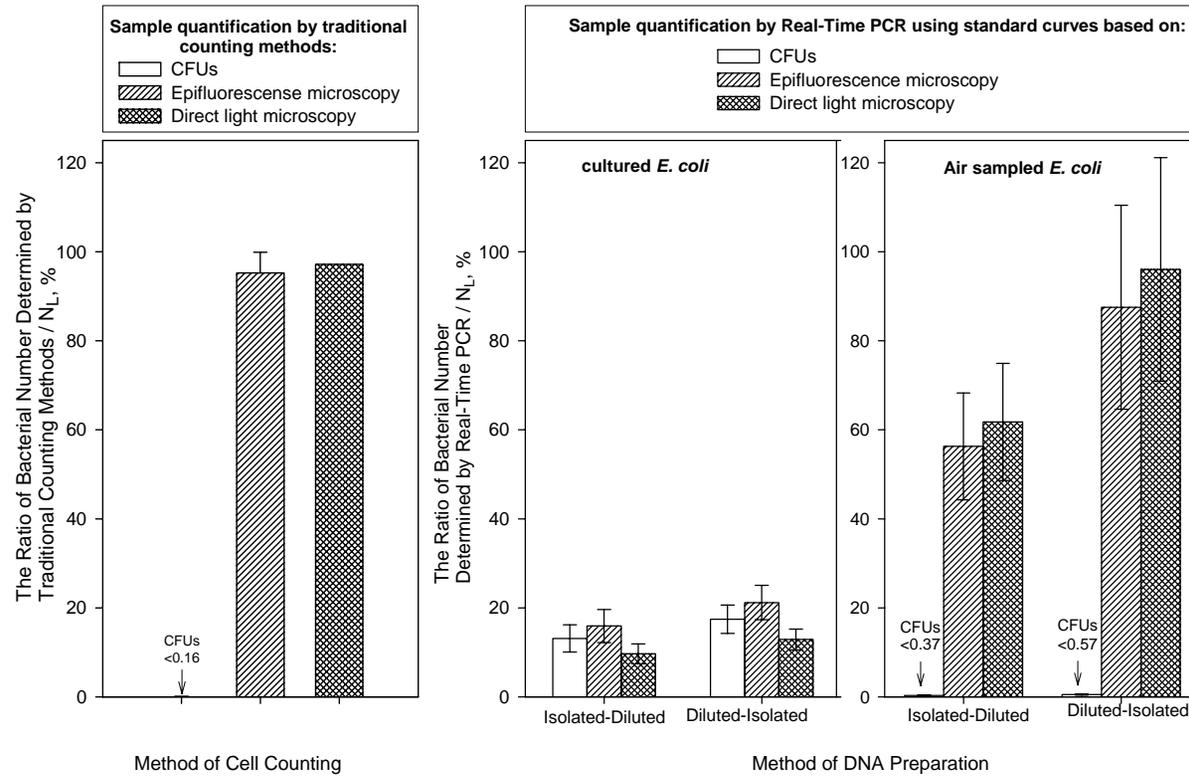


Figure 3.5. The quantification of *E. coli* in air sample using colony counting, epifluorescence microscopy, direct light microscopy, and real time PCR based detection methods. The data are average of six trials and the errors represent standard deviation

Table 3.1. Results of Real-Time PCR assay with cultured *E. coli* cells within linear reaction range.

Dilution factor	Bacterial concentration determined using different counting methods, cells / mL			C _T values	
	CFUs	Epifluorescence microscopy	Direct light microscopy	Isolated-Diluted	Diluted-Isolated
10 ⁻²	2.67 x 10 ⁷	3.24 x 10 ⁷	1.97 x 10 ⁷	10.60 ± 0.26	10.40 ± 0.10
10 ⁻³	2.67 x 10 ⁶	3.24 x 10 ⁶	1.97 x 10 ⁶	14.03 ± 0.12	14.27 ± 0.21
10 ⁻⁴	2.67 x 10 ⁵	3.24 x 10 ⁵	1.97 x 10 ⁵	17.87 ± 0.15	18.43 ± 0.25
10 ⁻⁵	2.67 x 10 ⁴	3.24 x 10 ⁴	1.97 x 10 ⁴	21.57 ± 0.21	22.23 ± 0.21
10 ⁻⁶	2.67 x 10 ³	3.24 x 10 ³	1.97 x 10 ³	24.57 ± 0.15	25.17 ± 0.21
Negative control	—	—	—	30.2 ± 0.36	30.2 ± 0.36

* C_T values in triplicate for each processed genomic DNA extracted from cultured *E. coli* cells. The results are presented as mean values ± 1 standard deviation, where applicable. The Standard Error for CFU counting was < 1%, for microscopy < 15%.

Table 3.2. Results of Real-Time PCR assay with air sampled *E. coli* cells.

Dilution factor	Bacterial concentration determined using different counting methods, cells / mL			C _T values	
	CFUs	Epifluorescence microscopy	Direct light microscopy	Isolated-Diluted	Diluted-Isolated
10 ⁰	4.38 x 10 ⁴	7.29 x 10 ⁶	8.00 x 10 ⁶	15.20 ± 0.20	15.17 ± 0.06
10 ⁻¹	4.38 x 10 ³	7.29 x 10 ⁵	8.00 x 10 ⁵	17.93 ± 0.21	19.60 ± 0.10
10 ⁻²	4.38x 10 ²	7.29 x 10 ⁴	8.00 x 10 ⁴	21.97 ± 0.15	22.97 ± 0.21
10 ⁻³	4.38 x 10 ¹	7.29 x 10 ³	8.00 x 10 ³	25.57 ± 0.06	25.63 ± 0.38
10 ⁻⁴	4.38 x10 ⁰	7.29 x 10 ²	8.00 x 10 ²	29.40 ± 0.17	<i>Below LOQ</i>
Negative Control	—	—	—	30.2 ± 0.36	30.2 ± 0.36

* C_T values in triplicate for each processed genomic DNA extracted from air sampled *E. coli* cells. The results are presented as mean values ± 1 standard deviation, where applicable. The Standard Error for CFU counting was < 1%, for microscopy < 10%. *Below LOQ* indicates below limit of quantification.

Table 3.3. The linear dilution range of bacterial concentration and amplification efficiency for a family of standard curves.

	Cell counting method	Cell suspension	Template DNA	Standard curve	Amplification efficiency, E	Correlation coefficient, R ²	Linear range (bacterial concentration, cells/mL)
1	CFUs	Cultured <i>E. coli</i>	Isolated-Diluted	$C_T = -3.55 \log(x) + 36.97$	0.914	0.999	$2.67 \times 10^3 - 2.67 \times 10^7$
2			Diluted-Isolated	$C_T = -3.75 \log(x) + 38.45$	0.848	0.997	$2.67 \times 10^3 - 2.67 \times 10^7$
3		Air sampled <i>E. coli</i>	Isolated-Diluted	$C_T = -3.60 \log(x) + 31.53$	0.895	0.997	$4.38 \times 10^0 - 4.38 \times 10^4$
4			Diluted-Isolated	$C_T = -3.48 \log(x) + 31.76$	0.939	0.987	$4.38 \times 10^1 - 4.38 \times 10^4$
5	Epifluorescence microscopy	Cultured <i>E. coli</i>	Isolated-Diluted	$C_T = -3.55 \log(x) + 37.27$	0.914	0.999	$3.24 \times 10^3 - 3.24 \times 10^7$
6			Diluted-Isolated	$C_T = -3.75 \log(x) + 38.77$	0.848	0.997	$3.24 \times 10^3 - 3.24 \times 10^7$
7		Air sampled <i>E. coli</i>	Isolated-Diluted	$C_T = -3.60 \log(x) + 39.54$	0.895	0.997	$7.29 \times 10^2 - 7.29 \times 10^6$
8			Diluted-Isolated	$C_T = -3.48 \log(x) + 39.49$	0.939	0.987	$7.29 \times 10^3 - 7.29 \times 10^6$
9	Direct light microscopy	Cultured <i>E. coli</i>	Isolated-Diluted	$C_T = -3.55 \log(x) + 36.50$	0.914	0.999	$1.97 \times 10^3 - 1.97 \times 10^7$
10			Diluted-Isolated	$C_T = -3.75 \log(x) + 37.95$	0.848	0.993	$1.97 \times 10^3 - 1.97 \times 10^7$
11		Air sampled <i>E. coli</i>	Isolated-Diluted	$C_T = -3.60 \log(x) + 39.68$	0.895	0.997	$8.00 \times 10^2 - 8.00 \times 10^6$
12			Diluted-Isolated	$C_T = -3.48 \log(x) + 39.63$	0.939	0.987	$8.00 \times 10^3 - 8.00 \times 10^6$

Table 3.4. Quantification of airborne *E. coli* bacteria in air samples collected by BioSampler. Cell quantity determined by CFUs, epifluorescence microscopy, direct light microscopy, and Real-Time PCR assay. The results are presented as mean values \pm 1 standard deviation.

Concentrations	Expected cell concentrations N_L based on optical particle counting, cells / 5 mL	Cell quantity determined by Real-Time PCR using standard curves developed using Isolated-Diluted <i>E. coli</i> genomic DNA, cells / 5mL								
		Traditional counting methods, cells / 5mL			Standard curves developed using cultured <i>E. coli</i> and different cell counting methods			Standard curves developed using air sampled <i>E. coli</i> and different cell counting methods		
		CFUs	Epifluorescence microscopy	Light microscopy	CFUs	Epifluorescence microscopy	Light microscopy	CFUs	Epifluorescence microscopy	Light microscopy
High	$2.74 \times 10^7 \pm 1 \times 10^6$	$2.93 \times 10^4 \pm 1.15 \times 10^3$	$2.71 \times 10^7 \pm 1.15 \times 10^5$	$2.66 \times 10^7 \pm 7.35 \times 10^6$	3.73×10^6	4.53×10^6	2.75×10^6	9.29×10^4	1.55×10^7	1.70×10^7
Medium	$2.47 \times 10^6 \pm 5 \times 10^4$	$3.33 \times 10^3 \pm 1.4 \times 10^2$	$2.28 \times 10^6 \pm 2.86 \times 10^5$	<i>Below LOQ</i>	3.93×10^5	4.77×10^5	2.90×10^5	1.01×10^4	1.69×10^6	1.85×10^6
Low	$2.25 \times 10^5 \pm 8 \times 10^3$	$2.93 \times 10^2 \pm 6.11 \times 10^0$	<i>Below LOQ</i>	<i>Below LOQ</i>	2.21×10^3	2.68×10^3	1.63×10^3	5.96×10^2	9.92×10^4	1.09×10^5

Concentrations	Expected cell concentrations N_L based on optical particle counting, cells / 5mL	Cell quantity determined by Real-Time PCR using standard curves developed using Diluted-Isolated <i>E. coli</i> genomic DNA, cells / 5mL								
		Traditional counting methods, cells / 5mL			Standard curves developed using cultured <i>E. coli</i> and different cell counting methods			Standard curves developed using air sampled <i>E. coli</i> and different cell counting methods		
		CFUs	Epifluorescence microscopy	Light Microscopy	CFUs	Epifluorescence microscopy	Light microscopy	CFUs	Epifluorescence microscopy	Light microscopy
High	$2.74 \times 10^7 \pm 1 \times 10^6$	$2.93 \times 10^4 \pm 1.15 \times 10^3$	$2.71 \times 10^7 \pm 1.15 \times 10^5$	$2.66 \times 10^7 \pm 7.35 \times 10^6$	4.38×10^6	5.32×10^6	3.23×10^6	1.56×10^5	2.60×10^7	2.85×10^7
Medium	$2.47 \times 10^6 \pm 5 \times 10^4$	$3.33 \times 10^3 \pm 1.4 \times 10^2$	$2.28 \times 10^6 \pm 2.86 \times 10^5$	<i>Below LOQ</i>	5.22×10^5	6.33×10^5	3.85×10^5	1.57×10^4	2.62×10^6	2.87×10^6
Low	$2.25 \times 10^5 \pm 8 \times 10^3$	$2.93 \times 10^2 \pm 6.11 \times 10^0$	<i>Below LOQ</i>	<i>Below LOQ</i>	3.43×10^4	4.16×10^4	2.53×10^4	8.35×10^2	1.39×10^5	1.52×10^5

Below LOQ indicates below limit of quantification.

Chapter 4

Quantitative Real-Time PCR for Bioaerosol Detection: Analysis of Factors Affecting Standard Curves[‡]

[‡] This chapter is modified from the manuscript by **An, H.R.**, Mainelis, G., and White L. (2009) Quantitative Real-Time PCR for Bioaerosol Detection: Analysis of Factors affecting Standard Curves, to be submitted, *Environmental Science and Technology*.

4.1 Abstract

Availability of rapid and reliable methods to quantify total bioaerosols is one of the important components of any bioaerosol monitoring system enabling detection of both endemic and intentionally released hazardous biological particles. Although the Quantitative Real-Time polymerase chain reaction (QPCR) has recently gained popularity and has been applied in many research areas, relatively little information is available about the factors affecting its accuracy in bioaerosol quantification. Thus, in this chapter, I investigated the effect of the three following factors on the QPCR standard curves and, consequently, the effect of those variables on the QPCR output: different bacterial species (gram negative and gram positive bacteria), different sample preparation methods (cells from culture suspensions and cells from air samples), and different QPCR methods (use of extracted genomic DNA and whole-cell PCR). Once the standard curves were constructed based on the variables above, they were statistically analyzed for differences in slopes and intercepts as a function of curve preparation factors. As part of this testing we also examined the feasibility of the whole-cell PCR, where cells in question are analyzed by QPCR directly without extracting the genomic DNA first. The whole-cell PCR is much simpler, less time-consuming and could be especially useful to analyze low bacterial concentrations in environmental samples. First, the results showed a statistically significant difference between the species-specific standard curves, most likely due to the chemical / physical differences between the two species that affect the DNA extraction efficiency and the extent of damage cells sustain during the aerosolization and air-sampling process. Second, the standard curves with air-sampled cells showed higher uncertainty compared to standard curves prepared from culture suspensions regardless of

the bacterial species used. Third, our results showed that the easy-to-use whole-cell QPCR method could be a reliable and cost / labor effective alternative to the isolated-genomic DNA QPCR method in bioaerosol quantification. This work improves our understanding of factors affecting the accuracy of standard curves that ultimately will increase the accuracy of the QPCR assay for bioaerosol quantification. The improved QPCR accuracy would help to develop a better understanding of bioaerosol exposure and related adverse health outcomes.

4.2 Introduction

Bioaerosols are diverse and complex airborne particles of biological origin, including pollen, fungal spores, fragments of fungal mycelium, fungal mycotoxins, bacterial cells, bacterial endotoxins, viruses, and protozoa (Nevalainen et al. 1993). The interest in bioaerosol exposure has increased over the last few decades when it was realized that exposures to airborne biological particles in indoor and outdoor environments are associated with adverse health effects, such as respiratory diseases, contagious infectious diseases, acute toxic reactions, and allergies (Burge et al. 1989; Agranovski 2002; Karol 1991b; Douwes et al. 2000; Lange et al. 1997; Van Tongeren et al. 1997). In addition to endemic microorganisms, certain biological agents (e.g., anthrax-causing *Bacillus anthracis* spores) may be used as biological weapon (Franz and Zajtchuk 2002; Higgins et al. 2003b).

Despite the importance of human exposure to bioaerosols, an exact relationship between the bioaerosol concentration and development of adverse health effects remains uncertain. One of the reasons is the uncertainty related to bioaerosol measurement in exposure assessment. To improve bioaerosol exposure assessment, development and validation of advanced bioaerosol monitoring systems are urgently needed. A successful bioaerosol monitoring system needs two components: efficient air sampling techniques and bioaerosol sample quantification methods that are rapid, sensitive and accurate. Numerous studies have successfully developed and validated various air sampling devices. However, most applications rely on culturable particles for the detection and quantification of bioaerosols, and culturable particles are only a fraction of all (total) bioaerosol particles collected in an air sample. Although counting culturable bioaerosol is widely used due to

ease-of-use, potential sensitivity and accessibility, it has serious drawbacks related to the accuracy of bioaerosol measurement in exposure assessment, including poor reproducibility, preferential detection of certain species and possible underestimation of exposure due to undetected viable cells that are not cultured, cell debris, and toxic microbial components. The non-viable bioaerosols present in the air are also known to cause adverse health effects: the allergenic, toxic, and inflammatory responses in respiratory disease (Gorny et al. 2002b; Robbins et al. 2000b). In addition, bioaerosol sampling process itself can affect culturability of the microorganisms, leading to a substantial underestimation of microbial quantity (Buttner and Stetzenbach 1991b; Cox 1989b; Martinez et al. 1988b; Lin 2000; Stewart 1995).

In contrast to culture based methods, non-culture based methods enumerate total cell count and include both live and dead cells. Since the 1980s several assays have been developed to quantify bioaerosols using total cell counts: light microscopy (LM), epifluorescence microscopy (EFM), electron microscopy (EM), scanning EM (B Lin et al. 2000; Prigione et al. 2004), and flow cytometry (FCM). However, these methods are laborious, underestimate the cell counts in small size ranges, and the limit of taxonomic identification due to a general lack of morphological distinction between species (Lange et al. 1997; Prigione et al. 2004).

Therefore, among the molecular methods, the quantitative Real-Time PCR assay (QPCR) has gained popularity and been applied as a tool to determine the presence of microorganisms and to estimate their total concentration in pure cultures and environmental samples (Kimura et al. 1999b; O'Mahony and Hill 2002b; Panicker et al. 2004b; Schweigkofler et al. 2004b; Stetzenbach et al. 2004b; Williams et al. 2001b). Generally, when applying the QPCR to quantify environmental samples, one uses standard

(or calibration) curves generated by measuring output values of PCR reactions (the threshold cycle number or C_T) from known or estimated quantities of target microorganisms. The cell quantity in an environmental sample can then be estimated using QPCR output value and the standard curve generated in a laboratory.

In bioaerosol research the QPCR has also been applied to detect the presence of microorganisms (Oppliger et al. 2008), but the ability of the method to quantify the total loads of airborne bacteria has not been widely investigated. Wider application of QPCR for bioaerosol quantification will require the analysis of factors affecting the accuracy of the PCR output.

Chapter 3 demonstrated that the standard curves generated using different protocols resulted in variations in the QPCR output and showed that the use of a properly constructed standard curve can substantially and significantly increase the accuracy of bioaerosol quantification (Burns et al. 2004). When generating standard curves, there are a number of factors to consider. For example, standard curves generated from hardy and sensitive bacteria may differ even if the concentrations of both bacterial species are identical. However, to simplify the application of QPCR, it would be much more convenient to use one universal standard curve to quantify various bioaerosol species in different environments, instead of developing standards for multiple species. For this approach to be valid it is important to examine whether standard calibration curves are affected by various preparation factors. Since visual comparison of standard curves is potentially very subjective, there is a need for an objective statistical test to determine the difference (or similarity) of standard curves prepared by different methods and also to test the effects of different preparation methods on QPCR output without subjective bias. As part of this testing we also examined the feasibility of whole-cell PCR, where the cells in question are

analyzed by QPCR directly without extracting the genomic DNA first. This method is simpler, less time-consuming and could be especially useful for analysis of low bacterial concentrations in environmental samples.

Thus, the primary objective of this chapter was to investigate the effect of three factors on the QPCR standard curves and, consequently, the effect of those variables on the QPCR output. The following three factors were investigated: different bacterial species (hardy and sensitive bacteria), different sample preparation method (cells from culture suspensions and cells from air samples), and different QPCR methods (use of extracted genomic DNA and whole-cell PCR). Once the standard curves were constructed based on the variables above, they were statistically analyzed to determine whether slopes and intercepts differ as a function of curve preparation factors. This comparative approach and statistical analysis will improve our understanding of the variables affecting the standard curves and will increase the accuracy of the QPCR assay in bioaerosol quantification.

4.3 Materials and Methods

4.3.1 Bacterial strains and their preparation

Two types of microorganisms were used to examine the effect of bacterial species on QPCR standard curves. We used *Pseudomonas fluorescens* strain ATCC17559 (American Type Culture Collection, Manassas, VA), a gram negative microorganism and *Bacillus subtilis* var. *niger* (BG) vegetative cells, often used as a simulant of anthrax-causing *Bacillus anthracis* (Franz and Zajtchuk 2002), a gram positive microorganism. *P. fluorescens* were cultured on nutrient agar (BD 213000) and in nutrient broth (BD 234000) (Becton Dickinson Microbiological Systems, Sparks, MD) at 26 °C for

18 hrs. Vegetative cells of *B. subtilis* were prepared by using dry-from *BG* spores which were suspended in sterile deionized water, activated at 60°C for 25 min and then grown on nutrient agar medium (BD 213000, Becton Dickinson Microbiological Systems) at 30 °C for 4 days. Prior to experiments, all freshly grown test organisms were washed 3 times with sterile, deionized water followed by centrifugation at 6000g for 5 min, at 4°C (BR4; Jouan, Winchester, VA). When used for aerosolization, the obtained bacterial suspensions were diluted with sterile deionized water to obtain a desired bacterial concentration.

4.3.2 Determination of cell number to generate standard curves

Culturable cell number. The freshly cultured test organisms, both *P. fluorescens* and *B. subtilis*, were harvested and serially diluted in 10-fold. 0.1 mL aliquots from the 10^{-4} to 10^{-8} water-based serial dilutions were plated in triplicate onto nutrient agar (BD 213000, Becton Dickinson Microbiological Systems). The plates containing cells were incubated at growth conditions described above. After incubation, the number of Colony Forming Units (CFUs) was counted to determine the corresponding cell number used to construct the standard curves. When test bacteria were first aerosolized and then collected by a microbial sampler, the same CFU determination procedure was used, but the dilution factor ranged from 10^0 to 10^{-5} .

Total cell number. Total number of each test organism (sum of viable and non-viable cells) for each test condition was determined by epifluorescence microscopy using the Axioskop 20 (Carl Zeiss Inc., Thornwood, NY) following procedures described in chapter 3 (An et al. 2006). For each test organism, both cultured cells and aerosollized and air-sampled cells were prepared in 10-fold serial dilutions in ionized sterilized water.

The dilutions of 10^{-4} and 10^{-2} were used for counting cultured cells and air-sampled cells, respectively.

4.3.3 Aerosolization and sampling of test organisms to generate standard curves

Figure 4.1 shows the experimental setup used to aerosolize and collect test organisms (both *P. fluorescens* and *B. subtilis*). The microorganisms were freshly prepared and aerosolized using a Collison nebulizer (BGI Inc., Waltham, MA) operated at a flow rate, $Q_{NEB} = 3.0$ L/min and pressure of 15 psi. The aerosolized test organisms were diluted with HEPA filtered air at a flow rate $Q_{DRY} = 100$ L/min and passed through a flow-laminarizing honeycomb. The generated bioaerosol was isokinetically collected by a liquid impingement sampler, BioSampler (SKC Inc., Eighty Four, PA) operated at a flow rate $Q_{BIO} = 10$ L/min (Figure 4.1). During each test, the BioSampler collected airborne test organisms into 5 mL of ionized and sterilized water for 5 minutes. The concentration of airborne test organisms inside the test chamber, C_{OPC} , was monitored by an Optical Particle Counter (OPC) (model 1.108, Grimm Technologies Inc., Douglasville, GA) operated at a flow rate $Q_{OPC} = 1.2$ L/min. The OPC measures particles in sixteen size channels ranging from 0.3 to 20 μm . Prior to every experiment we determined the background particle concentration by aerosolizing purified water without the bacteria. The usual background concentration was less than 50 particles/L in the sampling chamber. After collecting the air samples, the chamber was disinfected by aerosolizing 70% ethyl alcohol for at least 2 hrs followed by the aerosolization of sterilized water while monitoring the background particle concentration by the OPC. The entire test system was placed in a Class II biological safety

cabinet (NUAIRE Inc., Plymouth, MN) to capture any bioaerosol particles not collected by the sampler.

Based on the OPC measurements, we determined the cell concentration that could be expected to be collected in the BioSampler's liquid, N_L :

$$N_L = C_{OPC} \cdot t \cdot Q_{BIO} \cdot E / V, \quad (4.1)$$

where C_{OPC} is the airborne particles concentration, t is sampling time, E is BioSampler's collection efficiency and V is liquid volume. In our experiments $t = 5$ min, and $V = 5$ mL. BioSampler's collection efficiency $E \approx 100\%$ for investigated bacterial particles (B Lin et al. 2000).

4.3.4 Preparation of sample suspensions and generation of standard curves

The overall diagram of experimental procedures used to prepare samples and generate a family of standard curves by QPCR is shown in Figure 4.2. For standard curve generation, culture suspensions and air-sampled cell suspensions were processed as follows: (1) determination of culturable cell number (CFUs): 1 mL of sample suspension was transferred to a sterilized microcentrifuge tube and 10-fold serial dilutions of 1 mL final volume were prepared with filtered sterilized water. The dilutions ranging from 10^0 to 10^{-8} and from 10^0 to 10^{-5} for cultured cells and air-sampled cells, respectively, were used to obtain CFU counts as described above; (2) determination of total cell number: the second 1 mL of sample suspension was prepared in 10-fold serial water-based dilutions ranging from 10^0 to 10^{-3} and then used for epifluorescence microscopy as described above; (3) PCR using genomic DNA: the third 1 mL of sample suspension was transferred to

sterilized microcentrifuge tubes and serially diluted in 10-fold ranging from 10^0 to 10^{-7} . The genomic DNA was extracted from each dilution and then used in QPCR reaction. Later in the text this method is called Isolated-genomic DNA-PCR; (4) PCR using whole cell: the fourth 1 mL of sample suspension was prepared in 10-fold serial dilutions and then aliquots of each dilution were directly (without extracting genomic DNA first) used in PCR reaction. Later in the text this method is called whole-cell PCR.

The output from a QPCR reaction is a threshold cycle (C_T) value which is defined as the PCR cycle number at which the fluorescence of the amplicon exceeds a calculated threshold value. The C_T value is inversely proportional to the initial DNA (or cell) concentration with which the reaction was started. Thus, for each experimental condition, a standard curve was obtained by plotting C_T values against the log of corresponding cell quantity in a sample. The corresponding cell quantity was determined by culture-based method (culturable cells, CFUs mL^{-1}) and epifluorescence microscopy (total cells, cells mL^{-1}). These methods were applied for both cultured cells and for cells that were aerosolized and then collected by air sampling. Two different DNA preparation methods were used to generate standard curves with QPCR:

(i) Isolated-genomic DNA-PCR in which genomic DNA was extracted from each bacterial suspension of different dilutions and aliquots were then used in PCR reactions as a template. This method is described our earlier research (An et al. 2006), where it was referred to as “Diluted-Isolated”. This DNA preparation method was applied for both cultured and air-sampled bacterial cells.

(ii) whole-Cell PCR, where aliquots from diluted cell suspensions were used in PCR reaction directly without extracting DNA from the cells first. The cell dilutions ranged from 10^0 to 10^{-7} for cultured cells and from 10^0 to 10^{-5} for air-sampled cells.

QPCR amplification was performed using iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) using the iQ SYBR Green supermix PCR Kit (Bio-Rad Laboratories, Hercules, CA). The information about the primer set and PCR amplification conditions are described in detail in chapter 3 .

4.3.5 Statistical analysis

Statistical analysis of the obtained data was performed using SAS 9.1.3 (SAS Institute Inc., Cary, NC). The standard (or calibration) curve was generated based on the relationship between the threshold cycle (C_T) value and the logarithm of the corresponding cell quantity (Cell). The logarithm of the corresponding cell quantity was treated as an independent variable and the C_T value was considered as a dependent variable. The standard curves are usually linear; however we hypothesized that their slope and intercept values depend on their preparation method, which, in turn, affects the quantification of samples when the curves are applied. Thus, the slope and intercept of QPCR standard curves were compared separately for bacterial species (hardy *B. subtilis* cells and sensitive *P. fluorescens* cells), sample preparation method (cultured cells and air-sampled cells), and QPCR method (Isolated-genomic DNA-PCR and whole-cell PCR).

We conducted two multiple linear regression analyses to examine the effect of the factors on standard curves. In Model 1, the data were pooled from all experiments, and the threshold cycle values (C_T) were treated as the response (dependent variable), and the log-transformed concentrations of cells (hardy *B. subtilis* and sensitive *P. fluorescens*) as predicting variables. The form of the model is as follows:

$$C_i = \beta_0 + \beta_1 X_{cell} + \beta_2 X_{bac} + \beta_3 X_{cell} X_{bac} + \varepsilon, \quad (4.2)$$

where C_t is the value of the threshold cycle values (C_T); X_{cell} represents the log-transformed cell number (continuous variable); X_{bac} represents the bacterial species (categorical variable; 0 for *B. subtilis* and 1 for *P. fluorescens*); $X_{cell}X_{bac}$ represents the interaction effect between the log-transformed cell number and the bacterial species; and ε represents the random error, which is assumed to be normally distributed. β_0 is the intercept value, β_1 represents the effect of cell concentrations without considering bacterial species, while β_2 represents the effect of bacterial species without considering cell concentration ranges tested in the experiments, and β_3 represents the interaction effects between log-transformed cell number and bacterial species, indicating that the slopes do differ between two bacterial species across different cell number concentrations. Intercept β_0 difference between bacterial species also reveals whether there are significant differences in the C_T values caused by the difference in bacterial species.

The effect of sample preparation method, and QPCR method were examined in Model 2 with data stratified by bacterial species. The form of the model is as follows:

$$C_t = \beta_0 + \beta_1 X_{cell} + \beta_2 X_{sam} + \beta_3 X_{pcr} + \beta_4 X_{cell} X_{sam} + \beta_5 X_{cell} X_{pcr} + \beta_6 X_{sam} X_{pcr} + \beta_7 X_{cell} X_{sam} X_{pcr} + \varepsilon \quad (4.3)$$

Where, C_t is the value of the threshold cycle values (C_T); X_{cell} represents the log-transformed cell concentration (continuous variable); X_{sam} represents the sample preparation method (categorical variable; 0 for Air Sample and 1 for Pure Culture); X_{pcr} represents the QPCR method (categorical variable; 0 for Isolated-genomic DNA-PCR and

1 for whole-cell PCR); $X_{cell}X_{sam}$ represents the interaction effect between the log-transformed cell concentration and the sample preparation method; $X_{cell}X_{pcr}$ represents the interaction effect between the log-transformed cell concentration and the QPCR method; $X_{sam}X_{pcr}$ represents the interaction effect between the sample preparation method and the QPCR method; $X_{cell}X_{sam}X_{pcr}$ represents the interaction effect among the log-transformed cell concentration, the sample preparation method, and the QPCR method; and ε_{ij} represents the random error, which is assumed to be normally distributed. β_0 is the intercept value, β_1 represents the effect of cell concentrations without considering sample preparation method and QPCR method, β_2 represents whether there is difference between sample preparation method without considering tested cell concentration ranges and QPCR method, and β_3 only represents the effect of PCR method on C_t value without considering sample preparation method and range of cell concentrations tested in the experiments, β_4 indicates whether the slopes are different in sample preparation method across the tested range of cell concentrations regardless of PCR method, β_5 also indicates whether the slopes between Isolated-genomic DNA-PCR and whole-cell PCR are different across the tested range of cell concentrations without considering sample preparation method, β_6 represents the interaction effect between sample preparation method and PCR method on threshold cycle values without reflecting the range of cell concentrations. To test the hypothesis, β_7 explains the interaction effect among the log-transformed cell concentrations, sample preparation method, and the RCR method on whether the slopes do differ among sample preparation method and PCR method across different cell number concentrations. The model considers the effect of cell concentration, sample preparation method, QPCR method and their interactions. In Model 2, we were primarily interested in determining whether the QPCR method (Isolated-genomic DNA-PCR vs. whole-cell PCR) and sample

preparation method (air sampled vs. pure cultured cells) produce statistically significant differences in standard curves, which then results in differences when quantifying samples. Therefore, a test of the regression coefficient β_4 reveals whether there are significant differences in standard curves caused by sample preparation methods and regression coefficient β_7 reveals whether there are significant differences in the threshold cycle values depending on QPCR method adjusted for sample preparation method. Intercept differences (β_0) between sample preparation method and QPCR method were also tested.

We report the determined differences in intercepts and regression coefficients as well as standard errors and 95% confidence intervals (CIs) of the differences resulting from the effect of bacterial species, sample preparation methods, and QPCR extraction methods.

4.4 Results and discussion

4.4.1 Determination of corresponding cell number for standard curve generation

Culturable and total cell concentrations of test organisms in suspensions were determined by culturable cell count via plating (CFUs/mL) and total cell count via epifluorescence microscopy (cells/mL) and the results are presented in Figure 4.3. Figure 4.3a shows results for cell suspensions prepared from cultured bacteria, and Figure 4.3b for cells that were aerosolized and then collected by air sampling. For air-sampled cells, the expected cell concentration N_L , based on OPC measurements and Equation 1 was added as a reference and ranged from approximately 10^6 to 10^7 cells / mL (Figure 4.3b). This concentration was mostly in good agreement with total concentration determined by epifluorescence microscopy for both test organisms.

As could be seen in Figure 4.3a, for *P. fluorescens*, the concentration of culturable cells (CFUs mL⁻¹) was as low as 17% of the total cell quantity (determined by epifluorescence microscopy) in cultured *P. fluorescens* suspension which was not exposed to additional stress through the aerosolization and air sampling process. The culturability of *P. fluorescens* which were aerosolized and then collected by an air sampler was further decreased to as low as 7 % of the total cell concentration as shown in Figure 4.3b. Heidelberg et al. (1997) observed a dramatic loss of culturability of sensitive bacteria following bioaerosol generation while most of the bacteria remained viable (Heidelberg et al. 1997). Even so, the bioaerosol that becomes non-culturable may still be capable of causing negative health effects (Gorny et al. 2002b; Robbins et al. 2000b)

This result indicates that the culturability of sensitive bacteria can easily decrease due to many factors including temperature, humidity, aerosolization and air sampling (Heidelberg et al. 1997; Marthi et al. 1990). Thus, in case of stress-sensitive bioaerosols, the use of a culture-based method to determine corresponding cell number for QPCR standard curves and use of those curves may lead to a dramatic underestimation of the bioaerosol quantity in air samples. Similar results were observed in Chapter 3 using *E. coli*, also a sensitive bacterial species (Burns et al. 2004) and in other studies (Buttner and Stetzenbach 1991b; Cox 1989b; Martinez et al. 1988b; Heidelberg et al. 1997). In contrast, *B. subtilis*, the culturable cell concentration was only 2 – 3 % lower compared to the total cell concentration (Figure 4.3a). Even after the bioaerosol generation and air sampling process, there was only a 7 % loss of the culturable cell count compared to the total cell quantity (Figure 4.3b). Thus, for *B. subtilis*, the chosen cell counting method would not make a substantial difference. However, the culture-based counting method requires more time compared to the non-culture based method such as microscopy. Thus, to improve the

accuracy of QPCR assay, it is necessary to use a cell counting method that reflects the total cell number in a sample, including culturable, non-culturable but viable, and even dead cells. Use of total cell number when generating standard curves would allow more accurate determination of cell concentrations in unknown samples.

4.4.2 Standard curves

Figure 4.4 presents an example of QPCR progress curves and a generated standard curve where genomic DNA purified from cultured *P. fluorescens* was used in PCR reaction; cell concentrations ranged from 7×10^2 to 7×10^6 total cells/mL. These data show an inverse relationship between the C_T value and the corresponding cell quantity was linear over 5 orders of magnitude with $r^2 = 0.99$. Figure 4.5 presents the standard curves for *P. fluorescens* obtained using two different QPCR methods (Isolated-genomic DNA-PCR and whole-cell PCR) and two cell preparation methods (culture suspensions and air-sampled cell suspensions). The corresponding cell concentration (cells/ mL) was determined by epifluorescence microscopy. Each experiment was performed in duplicate and then suspension from each experiment was analyzed by PCR in two independent trials and each trial contained triplicate samples. Thus, 12 C_T values were generated for each condition. When analyzing the data, we defined the primer detection limit (cells / PCR reaction) as the lowest bacterial concentration in PCR reaction to be able to produce the PCR output (C_T) value in the linear response range. As seen in Figure 4.5, with Isolated-genomic DNA-PCR methods, the primer detection limit for cultured *P. fluorescens* was estimated as $> 1.42 \times 10^3 \pm 2.00 \times 10^1$ cells / mL and for air-sampled *P. fluorescens* $> 4.58 \times 10^3 \pm 1.55 \times 10^3$ cells/mL. For the whole-cell PCR method, the primer

detection limit was $3.97 \times 10^3 \pm 1.72 \times 10^2$ cells/mL for cultured *P. fluorescens* and $4.58 \times 10^3 \pm 1.55 \times 10^3$ cells / mL for air-sampled bacteria. The cell concentrations corresponding to the primer detection limit were determined by epifluorescence microscopy and the uncertainty (standard deviation) was based on duplicate counting of each sample. Since only 5 μ L of template was used in the PCR reaction, then accounting for dilution, the estimated primer detection limit per PCR reaction was lowered by a factor of 200. Therefore, the primer detection limit for cultured *P. fluorescens* was 7.10 ± 0.1 cells $5\mu\text{L}^{-1}$ for the Isolated-genomic DNA-PCR method, and 19.85 ± 0.86 cells $5\mu\text{L}^{-1}$ for whole-cell PCR method. In case of air-sampled *P. fluorescens*, both methods had same primer detection limit (22.9 ± 7.75 cells $5\mu\text{L}^{-1}$). The combination of Isolated-genomic DNA-PCR method and culture suspension resulted in the lowest detectable cell number (highest primer detection limit) (7.10 ± 0.1 cells $5\mu\text{L}^{-1}$), while other combinations yielded primer detection limits of ~ 20 cells $5\mu\text{L}^{-1}$. Thus, when air-sampled *P. fluorescens* are used to generate standard curves, the whole-cell PCR produces the same output compared to Isolated-genomic DNA-PCR, while being less labor intensive and more cost effective). However, more uncertainty (higher standard deviation) in PCR output was observed when air-sampled *P. fluorescens* was used as a template in PCR reaction compared to pure cultured cells. A more detailed statistical analysis of standard curves is presented in the following paragraphs.

For the gram positive test organism *B. subtilis* more cells were needed for the PCR output signal to cross the threshold compared to *P. fluorescens* (Figure 4.6). For cultured *B. subtilis*, the primer detection limit with genomic DNA method was 46.9 ± 1.86 cells $5\mu\text{L}^{-1}$ (higher by a factor of ≈ 7 compared to *P. fluorescens*), and for the whole-cell PCR method it

was 469 ± 18.6 cells $5\mu\text{L}^{-1}$ (higher by a factor of ≈ 20 compared to *P. fluorescens*). In the case of air-sampled *B. subtilis*, the primer detection limit was 36.75 ± 2.15 cells $5\mu\text{L}^{-1}$ for both Isolated-genomic DNA-PCR and whole-cell PCR method. The concentrations of cells and their standard deviations were determined based on epifluorescence microscopy following the procedure mentioned above. The use of cultured *B. subtilis* and whole-cell PCR required the highest number of cells for detection in linear PCR range, ≈ 10 times higher than the other method combinations.

When cultured *B. subtilis* coupled with the whole-cell PCR method was used, the primer detection limit was 10 times lower compared to that observed with the combination of cultured *B. subtilis* and Isolated-genomic DNA-PCR. Application of the whole-cell PCR yielded similar primer detection limit compared to Isolated-genomic DNA-PCR for air-sampled *B. subtilis*. Since the whole-cell PCR method uses an untreated cell suspension as a template in the PCR reaction instead of extracted genomic DNA, the physical characteristics of cells can affect the PCR amplification efficiency. Through the process of aerosolization and air sampling, the *B. subtilis* cells likely sustained some damage that may facilitate the release of genomic DNA during the PCR reaction while with the cultured cells; most of the cells remain intact in the reaction tube and release the genomic DNA less readily, thus yielding lower concentrations of template DNA (a hardy bacterium). However, unlike a gram positive bioaerosol of *B. subtilis*, in case of a gram negative *P. fluorescens*, there was no apparent difference between cultured and air-sampled cells in PCR output when using the whole-cell PCR method. Compared to *B. subtilis*, *P. fluorescens* has a thinner membrane and most of the cells in culture suspension are easily further lysed during the PCR reaction and any damage from the aerosolization and air

sampling process does not improve the release of genomic DNA any further (a sensitive bacterium).

For all experiments described above, non-specific PCR products from bacterial samples, such as primer-dimers or non-specific priming amplicons were not observed by melting curve analysis. If a negative control (no template added in PCR reaction) had C_T values of ≥ 30 , such C_T values from test samples in the same PCR reaction were discarded and not used for standard curve construction. The apparent increase in fluorescence from a negative control in a SYBR Green-based PCR assay could have resulted from 16S rDNA contamination of assay reagent, Tag polymerase or water used for the reagents or PCR reactions can contain contaminated DNA that results in non-specific priming amplicons.

4.4.3 Effects of bacterial species on standard curves

All standard curves showed strong linear relationships between corresponding cell quantity and C_T values for both *P. fluorescens* and *B. subtilis*. The differences in standard curves between the bacterial species were examined using Model 1 (Eq. 4.2) and the data are presented in Table 4.1. The slope for *P. fluorescens* was 12% lower than that for *B. subtilis* and the difference was statistically significant ($p=0.045$). The intercept for *P. fluorescens* was 3% lower than that for *B. subtilis*, but the difference was not statistically significant. Since there is a significant difference between the species-specific standard curves, application of the QPCR assay for bioaerosol quantification using a non-species specific standard curve would lead to an over- or under-estimation of bioaerosol quantity. For instance, QPCR analysis of a *P. fluorescens* sample performed by using the standard curve obtained with *B. subtilis* would yield concentrations higher by a factor 3 compared to

the application of a standard curve obtained with *P. fluorescens*. Conversely, analysis of a *B. subtilis* sample using a standard curve obtained with *P. fluorescens* would underestimate the sample concentration by a factor of 3.

The result indicates that bioaerosols with different characteristics will introduce differences in QPCR standard curves. The chemical/physical differences of the two species (gram-negative *P. fluorescens* and gram-positive *B. subtilis*) used in this research was likely responsible for the differences in their respective QPCR standard curves: the DNA extraction from gram-positive bacteria is usually harder than from gram-negative bacteria due to thicker cell wall in different molecular composition. This may lead to a difference in PCR amplification efficiency. Additionally, variation in the molarity of the guanine-plus-cytosine (G+C) content of template DNA, and the influence of template folding have been reported as plausible factors influencing PCR amplification (Suzuki and Giovannoni 1996; Dutton et al. 1993) among different species and could affect the QPCR amplification process (e.g., amplification efficiency, melting temperature, etc.) leading to more variation in the standard curves) In fact, the melting temperature of the two studied species was different.

The air sampling process can also magnify the differences between bacterial species. The air sampling process places the test organisms under stress resulting in potential alteration of bacterial culturability and viability, especially in the case of sensitive organisms. In addition, depending on the amount of damage for a particular species, including fragmentation of the cell and cell wall during sampling, the efficiency of whole-cell PCR amplification could be affected by the release of genomic DNA as discussed in the previous section. Therefore, a difference in the physiology of different bacterial species is one factor affecting the accuracy of bioaerosol quantification by QPCR.

For more accurate sample quantification, species-specific standard curves should be developed.

4.4.4 Effects of sample preparation method on standard curves

The effects of sample preparation method (cultured cell suspension vs. air-sampled cell suspension) on QPCR standard curves are presented in Table 4.2. Here, the data for each organism were analyzed separately, while the data from Isolated-genomic DNA-PCR and whole-cell PCR were pooled. For the *P. fluorescens* standard curves, neither the difference in slope nor the difference in intercept was statistically significant. Thus, the sample preparation method was not a significant contributor to the variations in standard curves prepared with *P. fluorescens*. Although there were no statistically significant differences in the standard curves of *P. fluorescens* due to sample preparation methods, the results indicated that there was more uncertainty in slopes / intercepts among standard curves prepared with air-sampled cells than with cultured cells. For instance, for air-sampled cells the slopes were -3.47 with a standard deviation of 0.35, but the slopes for cultured cells were -3.19 with a standard deviation of 0.17. Most likely, a lower initial cell quantity in air-sampled *P. fluorescens* suspensions (10^5 to 10^6 cells / mL) compared to their culture suspensions (10^8 to 10^{10} cells / mL) lead to the greater variation in the PCR amplification efficiency potentially introducing more uncertainty in the standard curves.

In contrast, for *B. subtilis*, as shown in Table 4.2, the differences in both slopes and intercepts of standard curves constructed by using cultured and air-sampled cells were statistically significant ($p < 0.001$). The slopes were -3.27 ± 0.12 for cultured *B. subtilis*, and -1.96 ± 0.26 for air-sampled *B. subtilis*. The intercepts were 42.6 ± 0.8 and 35.1 ± 1.4 for

cultured and air-sampled *B. subtilis*, respectively. Thus, in the case of gram-positive bacteria such as *B. subtilis*, it is very important that the standard curve preparation method mimic the handling of actual samples. Otherwise, the application of QPCR may lead to substantial inaccuracies when estimating cell quantity in environmental samples when gram positive bacteria are presented in air samples.

In general, the uncertainty (standard deviations) of slopes and intercepts of standard curves prepared with air-sampled cells was consistently higher than the uncertainty in curves prepared with cultured cells. This was true for both *B. subtilis* and *P. fluorescens*. The larger variability from the air-sampled cells is likely due to the lower initial cell concentrations in air samples and greater biochemical alterations / damage resulting from the air sampling process. The degree of damage may affect the amount of genomic DNA released during PCR amplification which, in turn, would lead to a higher / lower initial template concentration in the PCR tube. Additionally, for outdoor sampling any possible contamination introduced into air samples can affect the efficiency of both the DNA extraction and PCR amplification and may lead to increase of non-specific binding products.

For the QPCR bioaerosol quantification using standard curves, the slope and intercept of a standard curve are the key elements used to estimate the cell number in unknown bioaerosol samples. Even when the standard curves are constructed using the same species as a sample in question (the species-specific standard curve), the differences in cell preparation methods when generating standard curves can influence the accuracy of the PCR results. Especially for bioaerosol quantification, it is important to consider that the characteristics of bioaerosol particles collected from the air will differ from those of laboratory-cultured cells. According to the data in Table 4.2, the concentration of *B.*

subtilis in an unknown air sample estimated using standard curves prepared with air-sampled *B. subtilis* cells would be higher by a factor of 3 compared to that estimated using standard curves prepared with cultured cells. Use of a particular air sampling device also might affect the characteristics of bioaerosol particles. Therefore, to increase the accuracy of QPCR assay in bioaerosol quantification, it is necessary to take the bioaerosol generation /collection process into a consideration when preparing cell suspensions to generate standard curves. In the best case scenario, one would prepare study-specific standard curves. In chapter 3 with *Escherichia coli* (An et al. 2004), the accuracy in the QPCR assay was increased when the cells for the generation of standard curves were handled the same way as the *E. coli* cells from air samples of unknown concentration.

4.4.5 Effects of Real-Time PCR method on standard curves

Table 4.3 shows statistical analysis results for standard curve slope and intercept differences when the curves were prepared using two different PCR methods: Isolated-genomic DNA-PCR and whole-cell PCR. Here, the statistical tests were performed separately for each organism and each cell preparation method.

When using cultured *P. fluorescens*, we observed a statistically significant difference in the slope between the Isolated-genomic DNA-PCR (-3.37 ± 0.09) and the whole-cell PCR (-3.18 ± 0.07), $p=0.029$. The intercept difference associated with PCR methods was also statistically significant (37.1 ± 0.5 for Isolated-genomic DNA-PCR vs. 41.6 ± 0.4 for whole-cell PCR, $p < 0.001$). In case of air-sampled *P. fluorescens*, there was no statistical slope difference between PCR methods ($p=0.828$), but the intercept difference

was statistically significant (37.0 ± 0.9 for genomic DNA vs. 41.5 ± 0.7 for whole-cell PCR, $p < 0.001$).

As could be seen in Table 4.3, for the hardy test organism, the cultured *B. subtilis* standard curves showed no significant slope difference between the two PCR methods (-3.36 ± 0.12 for genomic DNA vs. -3.35 ± 0.10 for whole-cell PCR, $p = 0.924$), while there was a statistically significant difference in intercepts (41.6 ± 0.8 for Isolated-genomic DNA vs. 44.8 ± 0.7 for whole-cell PCR, $p < 0.001$). The same statistical results were observed for standard curves generated with air-sampled *B. subtilis*: statistically significant difference was found only for intercepts (34.3 ± 1.7 for Isolated-genomic DNA PCR vs. -40.0 ± 1.4 for whole-cell PCR, $p < 0.001$), but not for slopes.

Since both the slope and intercept of a standard curve affect the test outcome, it could be concluded that a particular PCR method used for sample analysis introduces another uncertainty factor. The advantage of QPCR with genomic DNA is the ability to measure the extracted DNA concentration and thus to know the approximate initial DNA concentration in the template which allows for more control when preparing standard curves. On the other hand, in the case of the whole-cell PCR, the DNA concentration necessary for standard curve generation may be hard to predict prior to experiments, however this method reduces the time of experiments and is labor and cost effective. Additionally, for the bioaerosol quantification, the relatively low bacterial concentration in samples collected from ambient air may make it difficult to extract sufficient amounts of genomic DNA. In such situations, the whole-cell PCR would be advantageous.

For a successful bioaerosol monitoring system, a rapid and accurate quantification method is urgently required. As part of the method development, QPCR assay has been applied for bioaerosol quantification and is gaining popularity. We examined the factors

that contribute to variations in the standard curve that can potentially lead to significant uncertainty in quantification outcome of the QPCR assay. Our data indicate that when culture-based methods are used to determine the quantity of cells when generating standard QPCR curves, the accuracy of the cell count could be substantially affected by the bioaerosol generation and sampling process. Use of non-culture based methods, such as microscopy, takes into account culturable, non-culturable but viable (NCBV) and even dead cells, and yields a more accurate estimate of total cell quantity for QPCR standard curve generation. Thus, a non-culture based counting method is recommended to increase the reliability and accuracy of assay.

In the second part of the study, we examined three factors affecting the variability and accuracy of the standard curve associated with QPCR measurement: bacterial species, a method to prepare bacterial suspension, and the PCR method. The results indicated that all three investigated factors may affect the slopes and intercepts of generated standard curves, thus affect the output of the PCR assay when a bioaerosol sample is analyzed. Thus, control of experimental factors to generate a study-specific standard curve would increase the reliability of the QPCR assay to quantify bioaerosol samples. Although only three factors were examined here, and the approach has limitations inherent in the application of any statistical method, the results emphasize the need for more studies to examine various factors that may affect the accuracy of QPCR assay for bioaerosol quantification.

In conclusion, successful bioaerosol quantification using QPCR method requires not only an understand of the characteristics of bioaerosol to be investigated and its sampling methodology, but also the need to develop study-specific standard curves. Poor understanding of factors affecting the standard curves may lead to uncertainty in QPCR output. The approach and procedures described in this paper potentially provide guidelines

to construct study-specific standard curves leading to more accurate bioaerosol quantification using QPCR.

4.5 References

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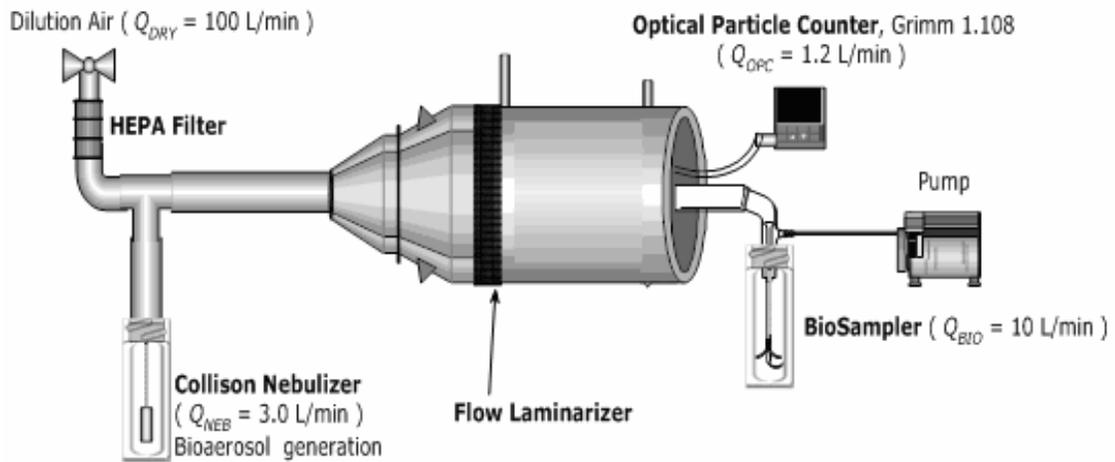


Figure 4.1. The schematic diagram of experimental setup. Abbreviations: Q_{NEB} – aerosolization flow rate, Q_{DRY} – dilution air flow rate, Q_{OPC} – the optical particle counter’s sampling flow rate, and Q_{BIO} – BioSampler’s sampling flow rate

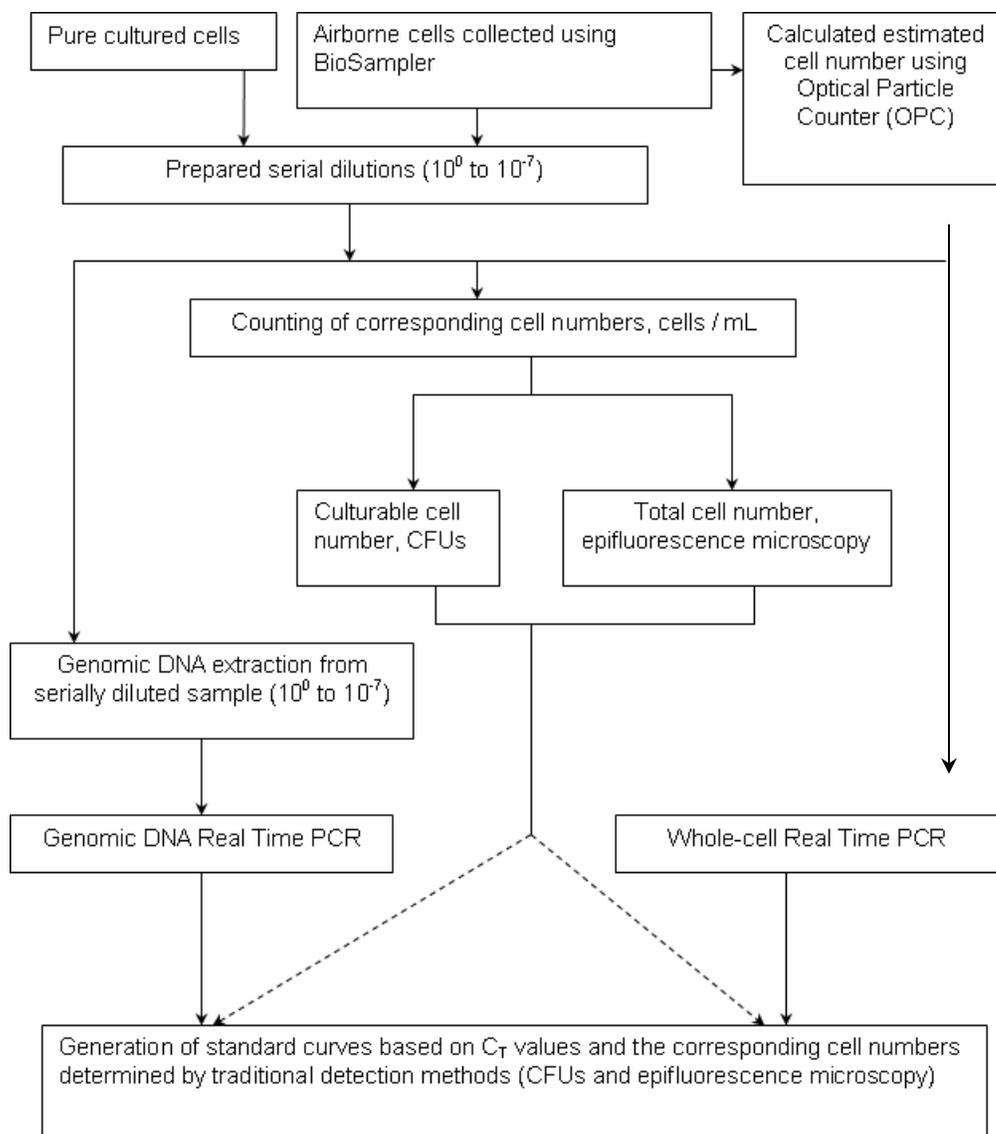


Figure 4.2. The overall diagram of experimental procedures used to generate standard curves using QPCR.

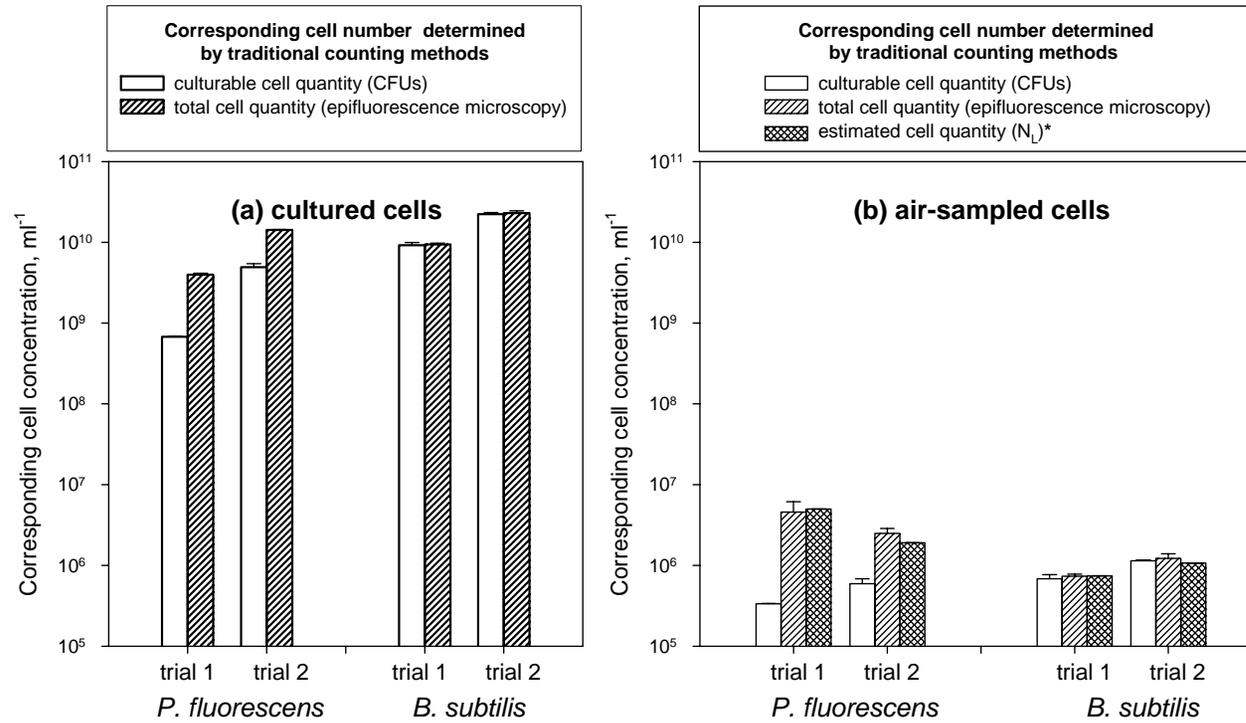


Figure 4.3. The corresponding cell quantity determined by traditional counting methods. Based on this method, the culturable and total cell quantity were determined for standard curve generation. CFUs were counted in triplicate and epifluorescence microscopy was conducted in duplicate in each trial. (a) the corresponding cell numbers of cultured cell suspension, (b) the corresponding cell concentration in air samples. * N_L was calculated based on the cell number monitored by optical particle counter.

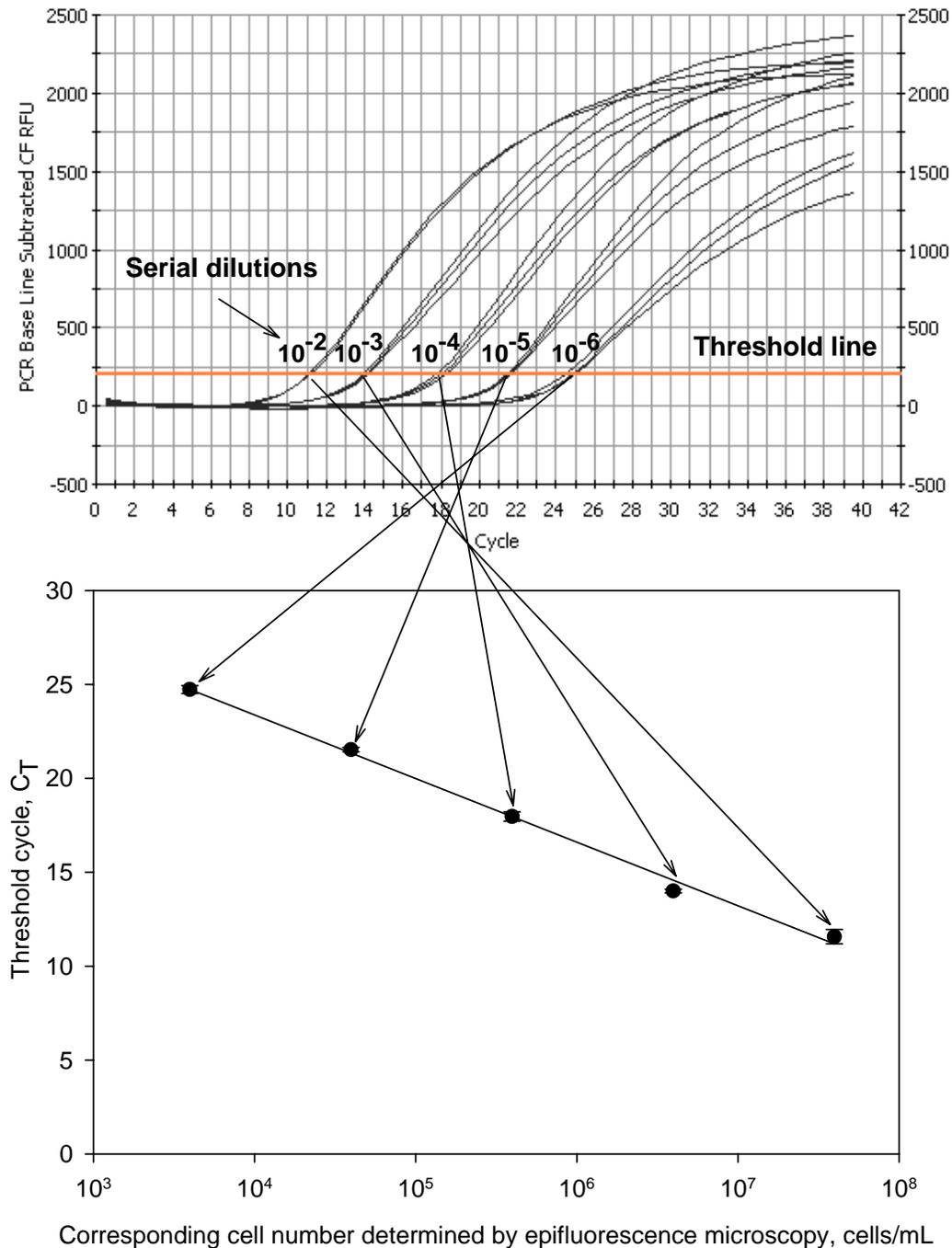


Figure 4.4. Example of Real-Time PCR output and standard curve generation: SYBR Green based Real-Time PCR using purified genomic DNA of cultured *Pseudomonas fluorescens* prepared in 10-fold serial dilutions using universal primers for bacterial 16 s rRNA gene amplification. The circles represent the C_T values of cultured *P. fluorescens* corresponding to the cell number determined by epifluorescence microscopy.

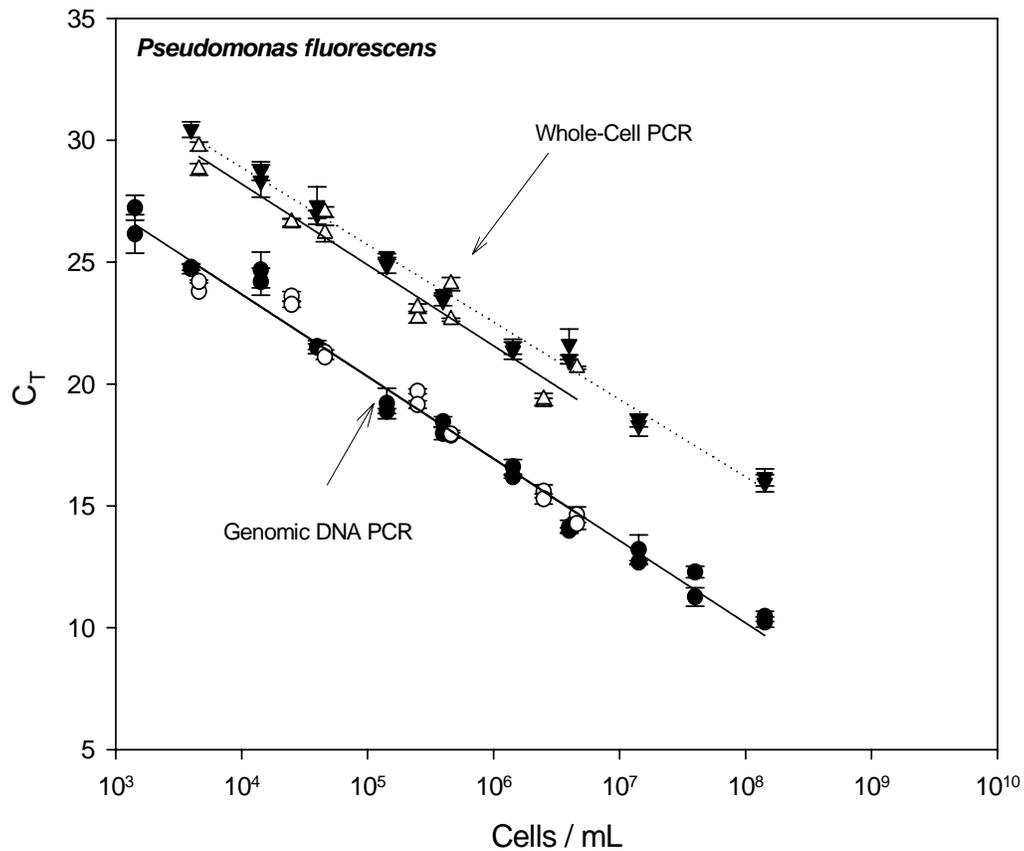


Figure 4.5. Standard curves of *P. fluorescens* based on the relationship between C_T values and the bacterial concentration as determined by epifluorescence microscopy. The circles represent C_T values for genomic DNA isolated from *P. fluorescens*: open circle (○) for air-sampled cells, and closed circle (●) for cultured cells. The triangles represent C_T values for whole-cell PCR using *P. fluorescens*: open triangle (△) for air-sampled, and closed triangle (▲) for cultured cells.

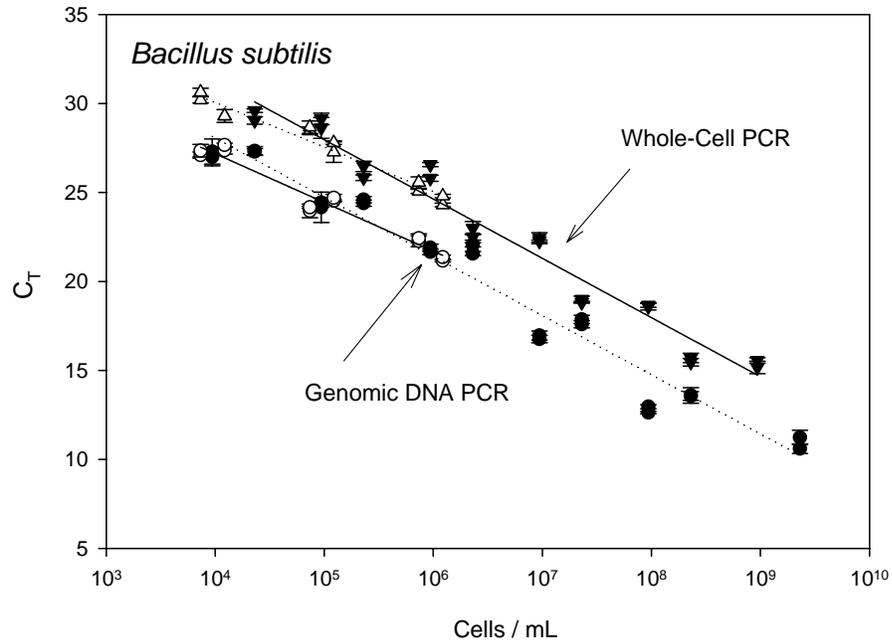


Figure 4.6. Standard curves of *B. subtilis* based on the relationship between C_T values and the bacterial concentration as determined by epifluorescence microscopy. The circles represent C_T values for genomic DNA isolated from *B. subtilis*: open circle (○) for air-sampled cells, and closed circle (●) for cultured cells. The triangles represent C_T values for the whole-cell PCR using *B. subtilis*: open triangle (△) for air-sampled, and closed triangle (▲) for cultured cells.

Table 4.1. Comparison of standard curve slopes and intercepts by bacterial species

Species	Standard curves	Intercept difference		Slope difference	
		Difference (95% CIs)	<i>p</i>	Difference (95% CIs)	<i>p</i>
<i>P. fluorescens</i>	$C_T = -3.24 \log(x) + 38.8$				
		1.1 (-0.8, 3.1)	0.259	0.34 (0.01, 0.69)	0.045
<i>B. subtilis</i>	$C_T = -2.90 \log(x) + 39.9$				

Table 4.2. Comparison of standard curve slopes and intercepts by sample preparation methods

Species	Standard curves	Intercept difference		Slope difference	
		Difference (95% CIs)	<i>p</i>	Difference (95% CIs)	<i>p</i>
<i>P. fluorescens</i>					
Air-sampled	$C_T = -3.47 \log(x) + 39.8$	1.3 (-2.5, 5.1)	0.477	-0.28 (-0.97, 0.40)	0.419
Cultured	$C_T = -3.19 \log(x) + 38.5$				
<i>B. subtilis</i>					
Air-sampled	$C_T = -1.96 \log(x) + 35.1$	-7.5 (-10.4, -4.5)	<0.001	1.31 (0.76, 1.87)	< 0.001
Cultured	$C_T = -3.27 \log(x) + 42.6$				

Table 4.3. Comparison of slopes and intercepts by PCR detection method

Species	Standard curves	Intercept difference		Slope difference	
		Difference (95% CIs)	<i>p</i>	Difference (95% CIs)	<i>p</i>
<i>P. fluorescens</i>					
Cultured cells:					
genomic DNA	$C_T = -3.37 \log(x) + 37.1$	-4.5 (-5.5, -3.5)	< 0.001	-0.19 (-0.36, -0.02)	0.029
whole-cell	$C_T = -3.18 \log(x) + 41.6$				
Air-sampled cells:					
genomic DNA	$C_T = -3.36 \log(x) + 37.0$	-4.5 (-6.4, -2.7)	< 0.001	-0.04 (-0.37, 0.30)	0.828
whole-cell	$C_T = -3.32 \log(x) + 41.5$				
<i>B. subtilis</i>					
Cultured cells:					
genomic DNA	$C_T = -3.36 \log(x) + 41.6$	-3.2 (-4.9, -1.5)	<0.001	0.01 (-0.24, 0.23)	0.924
whole-cell	$C_T = -3.35 \log(x) + 44.8$				
Air-sampled cells:					
genomic DNA	$C_T = -2.20 \log(x) + 34.3$	-5.7 (-8.6, -2.8)	<0.001	0.29 (-0.20, 0.86)	0.331
whole-cell	$C_T = -2.49 \log(x) + 40.0$				

Chapter 5

Application of a Whole-Cell QPCR Method to Evaluate the Performance Characteristics of a Novel Bioaerosol Sampler When Collecting Airborne Bacteria[§]

[§] This chapter is written based on the manuscript by Han, T., **An, H.R.**, and Mainelis, G. (2009) Performance of an Electrostatic Precipitator with Superhydrophobic Surface (EPSS) when Collecting Airborne Bacteria, to be submitted, *Aerosol Science and Technology*.

5.1 Abstract

Modern bioaerosol detection systems enabling rapid detection of low bioagent concentrations in various environments are needed to help us understand the causal relationship between adverse health effects and bioaerosol exposure and also to enable the timely biohazard detection in case of intentional release. In this chapter of the thesis, the study-specific whole-cell QPCR assay was applied to evaluate the collection efficiency of novel bioaerosol sampler, the Electrostatic Precipitator with Superhydrophobic Surface (EPSS), when collecting biological particles. The EPSS, a combination of electrostatic collection mechanism with superhydrophobic collection surface, allows for efficient particle collection, removal and concentration in water droplets as small as 5 μL . Additionally, this sampling concept was found to have very high sample concentration rates (1 million and higher) when testing with non-biological polystyrene latex particles and could potentially be applied to detect low concentrations of bioaerosols in various environments. The tests were performed with two common bacteria, *Pseudomonas fluorescens* and *Bacillus subtilis*. The study-specific standard curves were generated using whole-cell QPCR and epifluorescence microscopy and were used to determine collection efficiency of the sampler. The results showed good agreement with those obtained using the traditional method of microscopic counting. The collection efficiencies for both bacteria ranged from 50 to 72% and were substantially higher compared to the collection efficiencies for PSL particles of similar size as determined by Han and Mainelis (2008). Therefore, as a cost and labor-effective alternative to microscopic counting, whole-cell

QPCR, enabling rapid and accurate bioaerosol quantification, could be a useful tool when developing an effective bioaerosol monitoring system.

5.2 Introduction

The interest in estimating bioaerosol exposure has increased over the past few decades when it was realized that many adverse health effects such as infections, hypersensitivity pneumonitis and toxic reactions are associated with bioaerosol exposure occurring in the domestic and industrial sector (Burge et al., 1989; Karol, 1991). A significant relationship was found between the reduction in lung function and airborne mold concentrations (Dahlqvist et al., 1992). Respiratory symptoms, febrile episodes, allergic diseases such as hypersensitivity pneumonitis and asthma due to bioaerosol exposure have been documented among workers employed in the waste industry (Chiba et al., 2009; Poulsen et al., 1995). It was also found that not only viable, but also non-viable bioaerosol particles are capable of causing adverse health effects (Adhikari et al., 2009; Gorny et al., 2002; Robbins et al., 2000).

The presence of bioaerosols in various environments is determined by sampling airborne particles and analyzing the sample by various techniques. Our desire to better understand the relationship between bioaerosol exposures and health effects as well as the need to detect harmful biological agents in case of their malicious release reinforced the need to develop and test bioaerosol monitoring systems capable of early and reliable detection of biological agents in near real-time. To satisfy such requirements, the monitoring system should feature a particle collection unit capable of detecting low bioaerosol concentrations and an analysis unit capable of fast and reliable identification and quantification of total (viable and non-viable) bioaerosol particles as shown in Figure 5.1. Ideally, the system should also have a small footprint and low power requirements for

its wide distribution. A system satisfying these requirements would enable early detection of bioaerosol release and would help us develop a dose-response relationship for bioaerosol exposures.

The liquid-based collectors usually feature satisfactory collection efficiency, allow sample analysis by multiple techniques (Eduard and Heederik, 1998), and thus are candidates for the system suggested in Figure 5.1. A key parameter defining their ability to detect low concentrations of bioaerosol particles is their concentration rate (a ratio of particle concentration in the collection liquid versus the airborne particle concentration per time unit). Several new samplers capable of providing a concentrated sample in a small volume of liquid have recently been suggested. For example, a wetted-wall bio-aerosol cyclone (Seo, 2007) has concentration rates of $\sim 1 \times 10^5$ for single bacteria-sized particle; among the electrostatic precipitators, a briefcase-sized sampler (Carlson et al., 2004) has concentration rates of about 15,000. A previous study by Han and Mainelis (2008) presented the Electrostatic Precipitator with Superhydrophobic Surface (EPSS), which combines an electrostatic collection technique with superhydrophobic collection surface and allows efficient particle collection, removal and concentration in water droplets of 5 and 40 μL . When collecting polystyrene latex particles 3 μm in diameter, this sampler achieved a concentration rate of 1×10^6 . Given the high concentration rate and low power requirements of the sampler, it makes a good candidate for the detection system shown in Figure 5.1. However, the efficiency of this sampler has not yet been tested with biological particles.

Another key component in any bioaerosol detection system is sample analysis. The used culture-based sample analysis methods (CFUs, Colony Forming Units) is potentially

very sensitive (one organism), but time-consuming, and organisms may become damaged and non-culturable during sample collection (Buttner et al., 1991; Martinez et al., 1988; Lin et al., 2000). Moreover, the majority of naturally occurring microorganisms cannot be cultivated using standard culture methods ($\geq 90-99\%$) or readily identified in culture (Amann et al., 1995; DeLong and Pace, 2001). Therefore, a fast analysis method providing a total cell concentration would be a better candidate for the monitoring system suggested in Figure 5.1. Quantitative real-time PCR (QPCR), allows not only the identification of collected species, but also their quantification, once proper standard (calibration) curves have been prepared. Our earlier study (Chapters 3 and 4) showed that to improve the accuracy of the bioaerosol quantification, the calibration curves should be study-specific, i.e., they have to take into account the sampling device, target bioaerosol, its counting method, and the DNA preparation method for the PCR reaction (An et al., 2006). We also showed that the QPCR analysis could be simplified and accelerated by not extracting the genomic DNA, but by using the whole cells in the reaction (whole-cell PCR) (An et al., 2009). As such, the whole-cell QPCR analysis method could be a candidate for an advanced bioaerosol monitoring system shown in Figure 5.1.

Thus, the main goal of this chapter was to apply whole-cell QPCR to analyze the performance of the Electrostatic Precipitator with Superhydrophobic Surface (EPSS) when collecting biological aerosols. The data obtained using the whole-cell QPCR were compared with those obtained with a more traditional total cell counting method using acridine orange epifluorescence microscopy (AOEM). The settings of EPSS collection voltage and charging conditions were the same as in a previous study by Han and Mainelis (2008) and this study analyzed its biological performance. The experiments were

performed with vegetative cells of two different, test microorganisms commonly used in bioaerosol research: Gram-negative *Pseudomonas fluorescens* as a sensitive organism and Gram-positive *Bacillus subtilis* var. *niger* as a hardy organism. The obtained data were used to determine the feasibility of the suggested sampling and analysis methods for incorporating into the bioaerosol monitoring system suggested in Figure 5.1.

5.3 Materials and Methods

5.3.1 Biological Test Particles

Test microorganisms used in this chapter, *P. fluorescens* and *B. subtilis*, are representatives of sensitive or hardy organisms and commonly found in indoor and outdoor environments (Hill et al., 1999; Johnson et al., 1994; Neidhardt et al., 1990). *P. fluorescens* (ATCC 13525) was obtained from American Type Culture Collection (Rockville, MD). *P. fluorescens* cells were grown in a nutrient broth (Becton Dickinson Microbiology Systems, Cockeysville, MD) at 26°C for 18 hours in a shaking incubator. After growth, the cells were harvested by centrifugation at 7000 rpm for 5 min, at 4 °C (BR4, Jouan, Winchester, VA) and then washed 3 times with sterile deionized water under the same conditions. Prior to experiments, the resulting cell pellet was resuspended in sterile deionized water to obtain suspension with the target cell concentration of $\sim 10^9$ cells per mL, as determined by epifluorescence microscopy.

Dry *B. subtilis* spores were obtained from the US Army Edgewood Laboratories (Edgewood Research, Development and Engineering Center, Aberdeen Proving Ground, MD). The *B. subtilis* spores were suspended in sterile deionized water, and then activated at 60 °C for 25 min. The activated *B. subtilis* was cultured in nutrient broth for 18 hrs at 30

°C to obtain vegetative *B. subtilis* cells. Prior to experiments, freshly grown vegetative *B. subtilis* cells were prepared using the same method as described above.

5.3.2 Test Sampler and Sample Collection

The test sampler and the experimental setup are shown in Figure 5.2. The EPSS (bottom right figure) has the shape of a closed half-pipe, where the top surface serves as a ground electrode while the narrow collection electrode (2.1 mm width and 254 mm length) covered by a superhydrophobic substance (HIREC-1450, NTT Corporation Inc., Japan) is positioned on the opposite plate slightly below (0.3 to 0.5 mm) the surface of the plate for improved guidance of the collecting droplet (Han and Mainelis, 2008). As the positively charged particles are drawn into the sampler, they are deposited on the collection electrode by the action of the electrostatic field. After collection, a tiny liquid droplet (5 μ L) is introduced at the top of the collection electrode, rolls down under gravity and removes the deposited particles.

For each test, about 30 mL of fresh cell suspension was prepared for nebulization as described above. The collecting electrode was prepared by coating it with a superhydrophobic spray followed by drying at 60 °C for at least 1 hour. The test bacteria were aerosolized using a Collison nebulizer (BGI Inc., Waltham, MA) operated at a flow rate, Q_A (4 L/min) and diluted with HEPA-filtered air flow, Q_D (36 L/min). The 40 L/min aerosol stream was passed through a 2-mCi Po-210 charge neutralizer. The electrically neutralized bioaerosols then passed through a 0.035 m duct housing a vertically oriented ionizer (Wein Products Inc., Los Angeles, CA) which imparted a positive charge on the particles under controlled operating conditions (12V/50mA). The positively charged

bioaerosols passed through a flow straightener and entered the test chamber where they were collected by the EPSS operated at a sampling flow rates, Q_s (10 L/min). After sampling time t (10 min), the bacterial particles deposited on the superhydrophobic surface were removed by a rolling water droplet (5 μ L) which was collected in a vial. Then, sterile and purified water was added to increase the liquid volume to 1 mL and it was used for further analysis: total cell quantification using acridine orange microscopy counting and whole-cell quantitative Real-Time PCR as described below.

One stable DC power supply (BK Precision, Yorba Linda, CA) provided power to the ionizer, while another stable DC high voltage power supply (Bertan Associates, Inc, Valhalla, NY) provided power to the EPSS (-7kV). The operating values for these two voltages were established in the previous study by Han as yielding the most efficient deposition of particles inside the EPSS (Han and Mainelis, 2008). The entire experimental setup was housed inside a Class II Biosafety cabinet (NUAIRE Inc., Plymouth, MN).

5.3.3 Determination of Total Bacterial Number

The total number of cells collected by the EPSS was determined by both acridine orange epifluorescence microscopy (AOEM) using the Axioskop 20 (Carl Zeiss MicroImaging Inc., Thornwood, NY) and the whole-cell Quantitative Real-Time PCR (whole-cell QPCR) assay. When counting cells using epifluorescence microscopy, the droplet (5 μ L) containing particles collected by the EPSS was diluted by adding sterile and purified water to increase the liquid volume to 1 mL. The resulting 1 mL sample was further serially diluted in 10-fold dilutions with sterilized water to achieve a concentration that could be comfortably counted using epifluorescence microscopy (generally less than

100 cells per microscopic field). Each slide was prepared by filtering 1 mL aliquot of a selected dilution through a 25 mm black polycarbonate filter (Fisher Scientific, Suwanee, GA) and then staining it with 1 mL of 0.1 µg/mL Acridine Orange solution (Becton Dickinson Microbiology Systems, Sparks, MD) for 10 min. After washing with 3 mL of sterilized water and air-drying, at least 40 microscope fields were counted using the 100x oil-immersion objective. The total cell number in each sample, C_{sample} , was calculated as follows:

$$C_{sample} = N \times X \times D \quad (5.1)$$

Here, N is the average cell count in each microscope view field, X is the number ($X=6125$) of fields in the entire filter area, and D is the dilution factor.

When a biological sample is analyzed by the QPCR, the output from the reaction is the threshold cycle (C_T) value which is defined as the PCR cycle number at which the fluorescence of the amplicon exceeds a calculated threshold value. The C_T value is inversely proportional to the initial DNA (or cell) concentration, corresponding cell number with which the reaction was started. Thus, a standard (calibration) curve could be prepared based on the relationship between the corresponding cell number (determined by epifluorescence microscopy) and the C_T values.

Our earlier research indicated that the standard curves have to be process-specific, i.e., they have to be prepared using the sampling protocol as will be used to collect actual samples (An et al., 2006, 2009). In addition, we have shown that the whole-cell QPCR method could be a viable alternative to genomic DNA QPCR (An et al., 2009) for bioaerosol quantification. This is a labor and time efficient method, where aliquots from untreated cell suspensions are directly used in a PCR reaction as template unlike in the

Isolated-genomic DNA-PCR where DNA has to be extracted from cells prior to PCR reaction.

When preparing the standard curves for the whole-cell QPCR, the volume of samples collected by the EPSS (5 μ L) was increased to 1 mL and this 1 mL suspension was further diluted in 10-fold serial water-based dilutions ranging from 10^0 to 10^{-5} . The corresponding cell number in each dilution was determined by epifluorescence microscopy as described above and was related to the PCR output (C_T value) of each dilution. During the PCR reaction, 5 μ L aliquots of each dilution were directly (without extracting genomic DNA first) used as template. Later in the text this method is called whole-cell QPCR. PCR amplification was performed using iCycler iQTM Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) using the iQ SYBR Green supermix PCR Kit (Bio-Rad Laboratories, Hercules, CA). The information about the primer set and PCR amplification conditions are described in detail in our previous study (Chapter 3 based on An et al., 2006). The PCR reactions were performed in triplicate and two sets of air samples were collected and used in the PCR reaction separately for each test organism. The standard curves obtained for *P. fluorescens* and *B. subtilis* bacteria used in this chapter are shown in Figure 5.4. Each point represents an average and standard deviation of three C_T values.

When analyzing performance of the EPSS using the PCR method, the collected particles were removed by the 5 μ L droplet and the droplet volume was increased to 1 mL, which was divided into two parts: one analyzed by AOEM another by PCR. For the PCR, 5 μ L were taken from the 1mL aliquot and then used directly in the PCR reaction as a

template. The equations resulting from the generated calibration curves shown in Figure 5.4 were used to determine the number of collected cells in air samples.

5.3.4 Determination of the EPSS Collection Efficiency

Before and after sampling with the EPSS, the reference concentration of bacteria was determined by an Aerodynamic Particle Sizer (APS, Model 3321, TSI Inc., St. Paul, MN) and an isokinetic probe as shown in bottom left of Figure 5.2. The accuracy of the reference concentration measurements was verified by comparing the bacterial concentrations determined by the APS against those measured using a reference filter. Here, the aerosolized bacteria were isokinetically collected on a 47 mm membrane filter (Pall Inc., East Hills, NY) and then were extracted into liquid using the procedure reported by Wang et al. (2001): the filter was soaked in 30 mL of sterile deionized water for 10 minutes, followed by vortexing for 2 minutes and sonicating for 15 minutes. The number of bacteria in the resulting suspension was determined using epifluorescence microscopy. It was found that the two number concentrations (APS reading versus reference filter) agreed within 8%. Given the inherent uncertainty when microorganism number is determined by microscopy (standard deviation is usually ~20%), this was acceptable agreement. Thus, the collection efficiency of the EPSS was for each sampling condition was defined as:

$$\eta = \frac{C_{sample}}{C_{reference}} = \frac{C_{sample}}{R_{APS} \cdot 1000 \cdot Q \cdot t} \quad (5.2)$$

Here, C_{sample} is the number of cells in a water droplet determined either by microscopy or PCR, $C_{reference}$ is the reference cell number measured by the APS, R_{APS} is the average cell

concentration ($\#/cm^3$) measured by the APS every 6 seconds for 150 seconds before and after each sample, Q_s (L/min) is the sampling flow rate, and t (min) is the sampling time period.

In addition to the collection efficiency, another metric that describes the performance of a liquid-based aerosol collector is the concentration factor, R_C , which represents the ratio of particle concentration in liquid versus the concentration of particles in air per time period. The concentration rate R_C with units of min^{-1} could be expressed as follows:

$$R_C = \frac{Q_s}{V_{WD}} \times \eta \quad (5.3)$$

where Q_s (L/min) is the sampling flow rate (L/min) and V_{WD} is the volume of the water droplet.

Since the earlier investigation by Han and Mainelis (2008) observed that the majority of particles deposited on the collection electrode is removed by the 1st water droplet, the collection efficiency and the concentration rate were calculated based on the water droplet.

5.4 Results and Discussion

The primary goal of this study was to apply the whole-cell QPCR in the investigation of the performance of the Electrostatic Precipitator with Superhydrophobic Surface (EPSS) when sampling bacterial aerosols. Furthermore, we wanted to investigate the compatibility of the sampler with whole-cell QPCR analysis method for their potential use in the bioaerosol monitoring system shown in Figure 5.1. The number concentrations

and particle size distributions of airborne *P. fluorescens* and *B. subtilis* bacterial cells measured by the APS are shown in Figure 5.3. As could be seen, the mean aerodynamic diameters of the bacteria were $0.82 \pm 0.02 \mu\text{m}$ and $0.89 \pm 0.03 \mu\text{m}$, respectively, and the particle agglomerates were absent. Figure 5.4 shows the study-specific standard curves generated for each test organism based on the relationship between the C_T values and the corresponding cell number determined earlier by using microscopy. Two sets of air samples were analyzed by whole-cell QPCR and each PCR reaction was performed in triplicate. As could be seen, for *P. fluorescens* the inverse relationship between C_T value and the corresponding cell quantity was linear over 4 orders of magnitude with $R^2 = 0.99$. For *B. subtilis* the inverse relationship between C_T value and the corresponding cell quantity was linear over 3 orders of magnitude with $R^2 = 0.99$. The primer detection limit (cells / PCR reaction) defined as the lowest bacterial concentration in PCR reaction to be able to produce the PCR output (C_T) value in the linear response range (An et. al 2009) was estimated as $> 8.79 \times 10^3 \pm 2.00 \times 10^2$ cells / 5 μL . The cell concentrations corresponding to the primer detection limit were determined by the epifluorescence microscopy and uncertainty (standard deviation) was based on duplicate counting of each sample as described in previous Chapter 3.

For the hardy test organism *B. subtilis*, the standard curve was generated in a smaller range, and the primer detection limit was $> 1.75 \times 10^5 \pm 4.01 \times 10^4$ cells / 5 μL (higher by a factor of ≈ 20 compared to *P. fluorescens*) (Figure 5.4). Also as concluded in previous chapters, the whole-cell PCR needs more *B. subtilis* cells in PCR reaction compared to *P. fluorescens*. Since the whole-cell PCR method uses an untreated cell suspension as a template in the PCR reaction instead of extracted genomic DNA, the

physical characteristics of cells can affect the PCR amplification efficiency. Through the process of aerosolization and air sampling, the *P. fluorescens* cells have likely sustained some damage facilitating the release of genomic DNA during PCR reaction with hot temperature start while with *B. subtilis*, most of the cells remain intact in the reaction tube and release the genomic DNA less readily, thus yielding lower concentration of genomic DNA in the reaction tube.

The collection efficiency of the EPSS was determined by counting the collected cells using the equation generated from the standard curves and presented in Figure 5.4. The number of collected cells (average \pm standard deviation) for each test microorganism determined by the QPCR, by the epifluorescence microscopy, the reference cell number (average \pm standard deviation) as measured by the APS and the resulting collection efficiencies are presented in Table 5.1. The presented data also show the uncertainty of each measurement for each measurement technique. When calculating the collection efficiency uncertainty, the error was propagated. The average collection efficiency values for each microorganism determined by the two different methods are shown in Figure 5.5. For *P. fluorescens*, the average collection efficiency of the EPSS determined by the microscopy was 51% and by the QPCR 66%. The difference was not statistically significant. For *B. subtilis*, the average collection efficiency was 64% and 54% determined by microscopy and QPCR, respectively. The concentration rate averaged for both microorganisms and both methods was $\sim 1.2 \times 10^6$. When the difference of the average collection efficiency between AOEM and QPCR was tested using the Wilcoxon-Mann-Whitney test for *P. fluorescens*, as a sensitive bacteria, the average collection efficiency of QPCR was not significantly different with that of AOEM ($p=0.194$,

n=4). Similarly, for the *B. subtilis*, no statistically significant difference was observed between the two methods ($p=0.065$, $n=6$). Thus, the two sample analysis methods yielded collection efficiency values for the EPSS that were not statistically different. In addition, the whole-cell PCR method offers certain advantages. The PCR method is more sensitive compared to microscopy (An et al., 2006) and is suitable to measure particle concentrations that are too low to determine using microscopy, e.g., air samples containing harmful bioaerosol in low concentration. Additionally, the whole-cell PCR does not require DNA extraction prior to PCR reaction, is more labor and time efficient comparing to microscopy, and allows processing more samples over the same amount of time. We believe that the whole-cell PCR provides a promising alternative to the microscopic counting method in bioaerosol quantification. One potential drawback is the need for a sampler and microorganism-specific standard curve.

Tests with two common test microorganisms (*Pseudomonas fluorescens* and *Bacillus subtilis*) have shown that the novel bioaerosol sampler with superhydrophobic collection surface can efficiently collect and concentrate airborne biological particles in small amounts of liquid (5 μL). The collection efficiencies for both bacteria ranged from 50 to 72% and were substantially higher compared to the collection efficiencies for PSL particles of similar size as determined by Han and Mainelis (2008). The sampler can achieve a very high concentration rate of 1.2×10^6 and thus is suitable to detect low microorganism concentrations. It was also shown that whole-cell QPCR can be a good alternative to acridine orange microscopic counting to investigate performance of a bioaerosol sampler, in this case EPSS. Both techniques (EPSS sampler and whole-cell QPCR method) could be incorporated into an advanced bioaerosol monitoring system

suggested in Figure 5.1 and thus would allow measurement of low bioaerosol concentrations in various environments.

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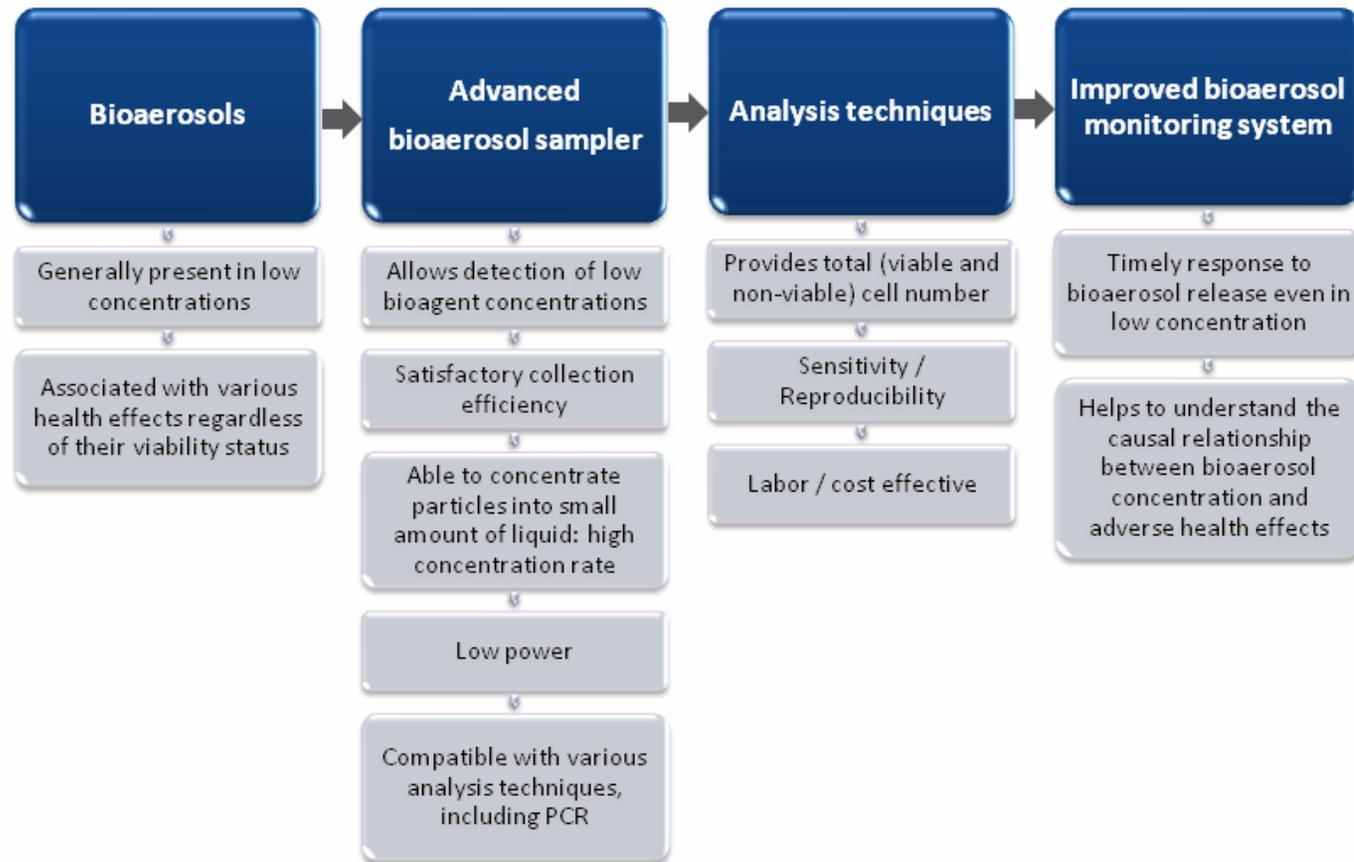


Figure 5.1. Structure of an advanced bioaerosol monitoring system

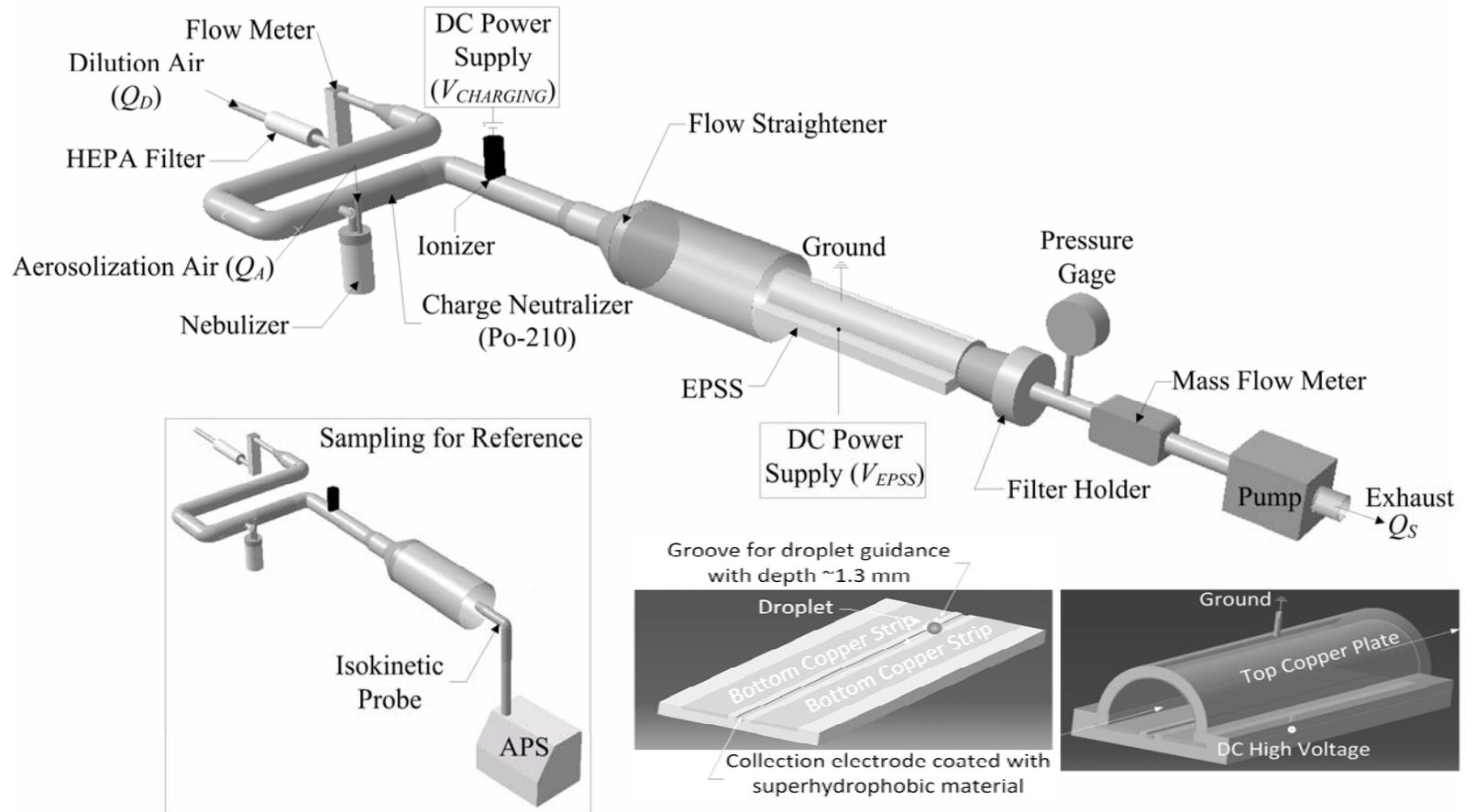


Figure 5.2. The schematic diagram of experimental set-up. The figure in left bottom corner shows an alternative set-up used to determine the bioaerosol reference concentration. The bottom right figure shows the schematic of the novel bioaerosol sampler.

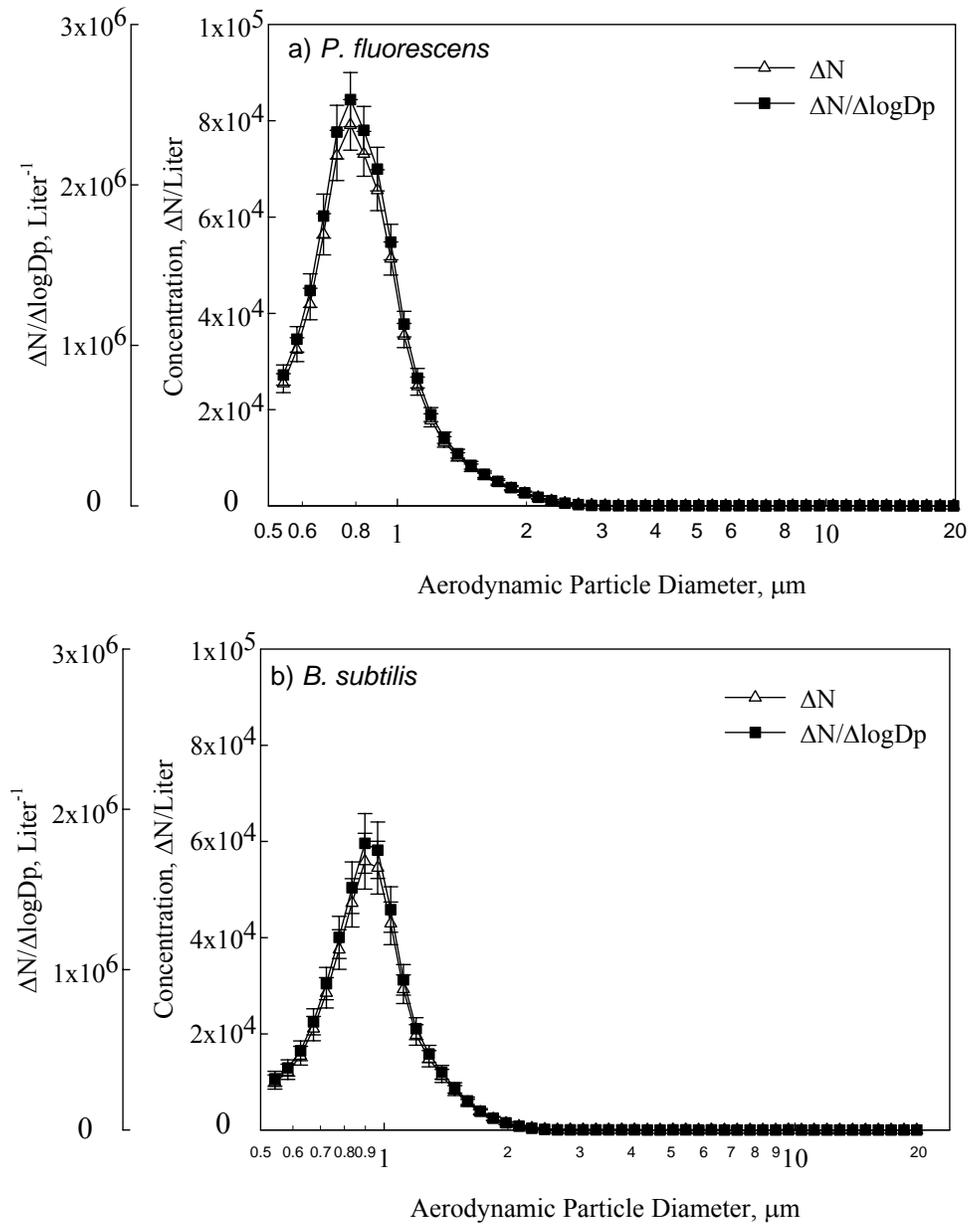


Figure 5.3. Particle size distributions according to number concentration (ΔN) and normalized concentration ($\Delta N / \Delta \log D_p$) for two biological particles, (a) *P. fluorescens* and (b) *B. subtilis*.

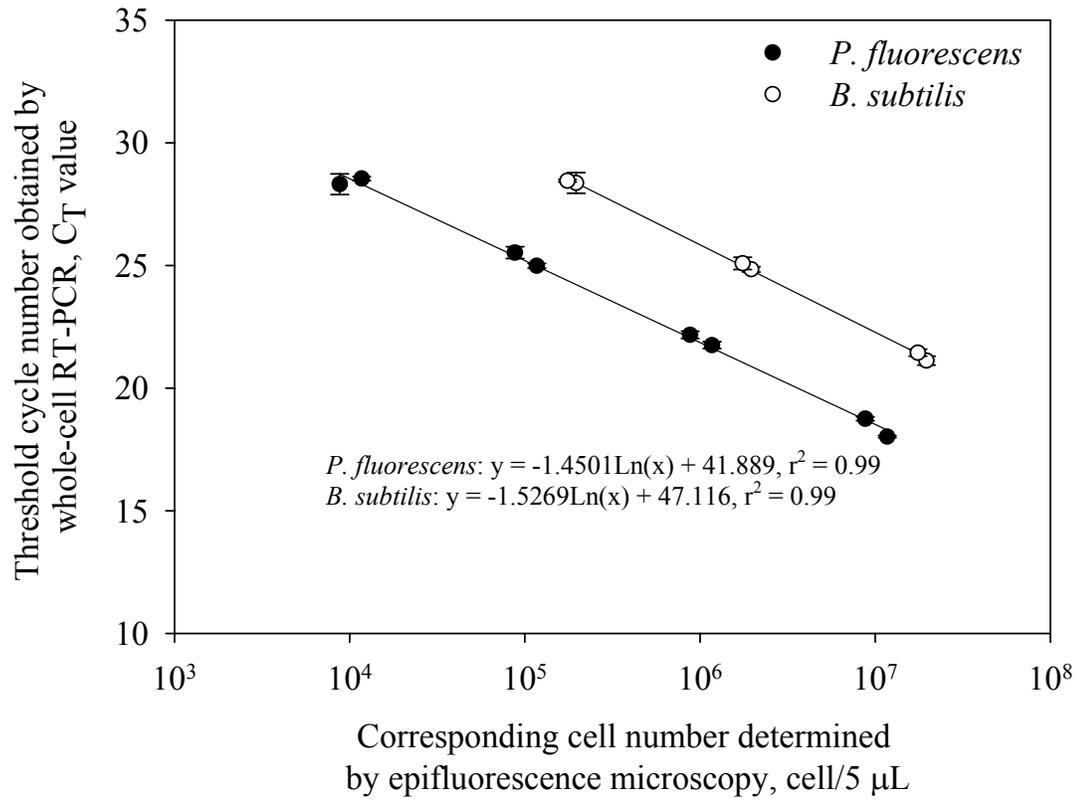


Figure 5.4. Standard curves of *P. fluorescens* and *B. subtilis* based on the relationship between C_T values and the bacterial concentration as determined by epifluorescence microscopy.

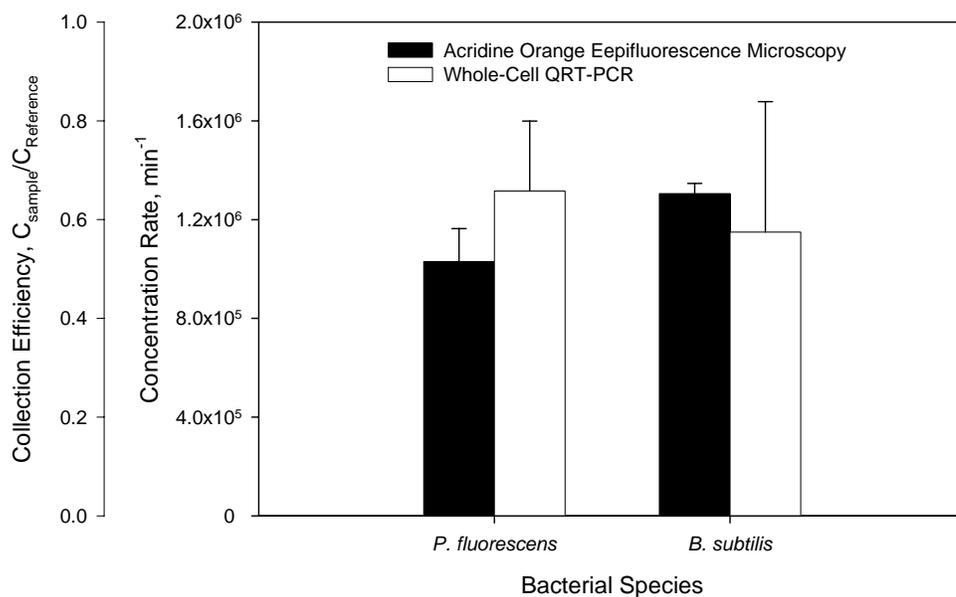


Figure 5.5. Comparison of collection efficiency of the EPSS based on the bacterial concentration determined by different quantification methods; black bars present data based on the acridine orange epifluorescence microscopy (AOEM) and white bars present data based on whole-cell QPCR method.

Table 5.1. Collection efficiency of the EPSS detecting *P. fluorescens* and *B. subtilis* based on total bioaerosol quantification.

Sample	Reference cell number by APS	Total cell number determined by AOEM	Total cell number determined by whole cell QPCR	Collection efficiency (%) AOEM/APS	Collection efficiency (%) whole cell QPCR/APS
<i>P. fluorescens</i>					
Sample 1	$(3.78 \pm 0.17) \times 10^6$	$(1.72 \pm 0.47) \times 10^6$	$(3.06 \pm 0.42) \times 10^6$	46 ± 13	81 ± 12
Sample 2	$(3.88 \pm 0.09) \times 10^6$	$(1.96 \pm 0.40) \times 10^6$	$(1.84 \pm 0.20) \times 10^6$	51 ± 10	47 ± 5
Sample 3	$(3.78 \pm 0.18) \times 10^6$	$(1.83 \pm 0.52) \times 10^6$	$(2.47 \pm 0.31) \times 10^6$	48 ± 14	65 ± 9
Sample 4	$(3.93 \pm 0.13) \times 10^6$	$(2.38 \pm 0.76) \times 10^6$	$(2.72 \pm 0.14) \times 10^6$	61 ± 19	70 ± 4
	Average Collection Efficiency of EPSS (%)			51 ± 7	66 ± 4
<i>B. subtilis</i>					
Sample 1	$(2.14 \pm 0.14) \times 10^6$	$(1.43 \pm 0.26) \times 10^6$	$(1.28 \pm 0.08) \times 10^6$	67 ± 13	60 ± 5
Sample 2	$(2.37 \pm 0.08) \times 10^6$	$(1.50 \pm 0.28) \times 10^6$	$(1.22 \pm 0.06) \times 10^6$	63 ± 12	51 ± 3
Sample 3	$(2.21 \pm 0.11) \times 10^6$	$(1.47 \pm 0.34) \times 10^6$	$(1.22 \pm 0.20) \times 10^6$	67 ± 16	55 ± 9
Sample 4	$(2.61 \pm 0.07) \times 10^6$	$(1.66 \pm 0.34) \times 10^6$	$(1.24 \pm 0.15) \times 10^6$	64 ± 13	48 ± 6
Sample 5	$(2.48 \pm 0.12) \times 10^6$	$(1.52 \pm 0.15) \times 10^6$	$(0.51 \pm 0.02) \times 10^6$	61 ± 7	20 ± 1
Sample 6	$(2.39 \pm 0.10) \times 10^6$	$(1.53 \pm 0.05) \times 10^6$	$(2.11 \pm 0.22) \times 10^6$	64 ± 3	88 ± 10
	Average Collection Efficiency of EPSS (%)			64 ± 7	54 ± 4

Chapter 6

Research Outlook

6.1 Applications and Implications

Growing concerns regarding human exposure to bioaerosols along with the possibility of endemic or malicious release of hazardous bioaerosol agents against civil/governmental/military establishments have reinforced the urgent need to investigate and develop advanced, reliable and efficient bioaerosol monitoring systems. The primary goal of this dissertation was to develop and evaluate techniques that would contribute towards more accurate detection and quantification of bioaerosols thus improving the efficiency of bioaerosol monitoring systems. To achieve the goals of this dissertation, the key components of any bioaerosol detection system, including a bioaerosol sampling device and a bioaerosol analysis technique, were investigated. While both components play important roles in detection, quantification, and identification of bioaerosols, is research primarily focused on the development of protocols for the QPCR technique to be applied for bioaerosol analysis.

The emerging trend in the use of bioaerosol sampling devices is the use of portable samplers. Unlike to the commonly used bioaerosol samplers such as the BioSampler collector, the Andersen-type impactor and AGI-30 impinger, the battery operated portable sampler does not require an external pump and is easy to use in remote sampling sites. A RCS High Flow is a recently developed portable bioaerosol sampler that is lightweight, battery operated, and collects viable microorganisms directly on agar media. However, relatively very little information is available about the performance characteristics of RCS High Flow which could affect the interpretation of the data obtained by this sampler.

Performance of any bioaerosol sampler depends on its ability to collect a representative aerosol sample (physical efficiency) and its effect on culturability /viability of the captured microorganisms (biological efficiency). Therefore, in this study, the physical and biological performance of this sampler was investigated in an indoor and outdoor environment. One of the important parameters describing the physical performance of bioaerosol samplers is their cut-off size, d_{50} . The d_{50} refers to an aerodynamic size at which 50% of the airborne particles are collected. Particles larger than d_{50} are collected with efficiencies higher than 50%, while particles smaller than d_{50} are collected with efficiencies lower than 50% (Hinds, 1982). As could be seen from Figure 2.3, our test results showed that the RCS High Flow would collect more than 80% of common fungal spores and more than 50% of airborne bacteria larger than 1.1 μm . Moreover, the biological collection efficiency of this sampler when collecting *Bacillus anthracis* spores could be approximately 50 % or higher. In terms of biological recovery, a relatively low enumeration rate was found with the RCS High Sampler comparing to the BioSampler as shown in Figure 2.6. The results showed that the performance of the test sampler was significantly affected by environmental conditions such as wind direction and speed. For the successful outdoor application of this sampler, further investigation regarding the parameters affecting sampler's performance is required. However, it is hoped that the information presented here would be useful to field professionals performing bioaerosol monitoring.

The conventional bioaerosol detection methods relying on culture-based counting result in a bioaerosol monitoring system with relatively low accuracy and sensitivity, because the majority of naturally occurring microorganisms can not be detected at

laboratory culture conditions (Amann et al., 1995; DeLong and Pace, 2001) and even non-viable microorganisms are still capable of causing various adverse health effects (Robbins et al., 2000; Gorny et al., 2002). Thus, the development and validation of bioaerosol analysis assays enabling a rapid, reproducible, reliable quantification of total (viable and non-viable) bacteria and permitting species-specific identification are urgently needed in the field of bioaerosol exposure assessment. The Quantitative Real-Time polymerase chain reaction (QPCR) has gained popularity and has been applied in many research areas. However, the application of this technique for the bioaerosol quantification is only beginning and there are few studies of bioaerosol quantification using standard curve-based QPCR assay. Most of the current research focuses on development of the methods that are very specific for the QPCR equipment and its amplification condition and not on the sensitivity or variability of this method. Therefore, a critical aspect to be investigated in this area is the investigation of factors contributing toward the variation QPCR assay output during bioaerosol quantification. To address those problems, a group of standard curves was generated under different experimental conditions, those curves were applied for sensitive and hardy bacteria and the results were statistically analyzed.

In chapter 3, we used universal primers and generated 12 standard curves by various methods and these curves were used to quantify model organism *Escherichia coli* (*Migula*) *Catellani* from air samples. Standard curves are a fundamental and important element of any QPCR analysis; however, methods to select optimal standard curves that would improve the accuracy of QPCR assay for quantification of air samples have received little attention in bioaerosol research. Since the standard curve is usually generated based on the relationship between C_T values and corresponding cell number, the accurate

determination of corresponding cell number is essential for an accurate standard curve. As shown in Tables 3.1 and 3.2, the ranges of bacterial number used to generate the standard curves varied substantially depending on the cell counting method which resulted in substantial differences in the generated standard curves. In some cases, when air sampled *E. coli* was quantified using those standard curves, the number of cells determined by microscopy was 200-fold higher compared to CFU counting. Based on the existing research, it could be expected that culturable counting method (CFUs), which relies on the enumeration and identification of only those cells that are culturable, may underestimate total bacterial number. Aerosolization and sampling stress may reduce the viability and culturability of microorganisms in air sample even further (Cox 1989a; Buttner and Stetzenbach 1991a; Martinez et al. 1988a). Therefore, for the reliable and accurate bioaerosol quantification by using QPCR, one should use total cell counting methods to determine the corresponding cell number to generate standard curves, such as microscopy or flow cytometry, which permits enumeration of cells regardless of their metabolic state. Our data also indicated that in order to improve the accuracy of the QPCR assay, a standard curve should be prepared in a way that mimics the handling of an environmental sample in question. Use of standard curves that rely only on bacterial suspensions cultured in a laboratory may lead to underestimation of microorganism quantities in environmental samples. Although the described calibration with a family of standard curves was performed with one model organism, we believe that the demonstrated principles of Real-Time PCR calibration for airborne samples will serve as a prototype for other single and mixed microbial populations.

Based on the guidelines developed in Chapter 3, the three key factors affecting the standard curves and, consequently affecting the reliability of the QRP-PCR assay, were investigated and statistically analyzed in detail in Chapter 4. The following factors were investigated: different bacterial species (hardy and sensitive bacteria), different sample preparation method (cells from culture suspension and cells from air samples), and QPCR method (use of Isolated-genomic DNA and a whole-cell PCR).

First of all, as presented in Table 4.1 there is a significant difference between the species-specific standard curves. The chemical/physical differences of the two species (gram-negative *P. fluorescens* and gram-positive *B. subtilis*) used in this research was likely responsible for the differences in their respective QPCR standard curves. For instance, the variation in the molarity of the guanine-plus-cytosine (G+C) content of template DNA and of template folding could affect QPCR amplification process such as amplification efficiency and melting temperature (Suzuki and Giovannoni 1996; Dutton et al. 1993). As a result, it will affect the PCR outcomes and increase the differences in the generated standard curve using different bacterial species. Additionally, when whole-cell PCR is applied to generate the standard curves, the template DNA concentration in PCR reaction tube can also be affected by the different species. DNA extraction from gram-positive bacteria is usually harder than from gram-negative bacteria due to thicker cell wall in different molecular composition of gram-positive bacteria which will affect the DNA concentration in PCR reaction in the begin with. Moreover, the degree of damage among different bacterial species due to the air sampling process affecting the template DNA concentration in PCR reaction could magnify the differences among the standard curves. Therefore, application of the QPCR assay without developing species-specific

standard curve would lead to inaccurate quantification of air samples. This finding should be taken under consideration when QPCR is applied for bioaerosol quantification.

Statistical analysis of different sample preparation methods and their effect on standard curves was performed. The slope and intercept of a standard curve are the key elements needed to estimate the cell number in unknown bioaerosol samples. In our study, the higher uncertainty in slopes and intercepts of standard curves was found with air-sampled cells compared to cultured cells regardless of bacterial species. Even when species-specific standard curve was applied, the different sample preparation methods caused differences among the generated standard curves thus affecting the reliability of PCR results. Especially for the bioaerosol quantification, it is important to consider that the characteristics of bioaerosol collected from the air will differ from those of laboratory-cultured cells. For example, according to the data in Table 4.2, the concentration of *B. subtilis* in an unknown air sample estimated by using *B. subtilis*-specific standard curves prepared by air-sampling, would be higher by a factor of 3 compared to the results obtained by using standard curves prepared with cultured cells. Besides, selection of bioaerosol generation and collection method also may affect the characteristics of bioaerosol particles. Therefore, to increase the accuracy of QPCR assay in bioaerosol quantification, it is necessary to take the bioaerosol generation and collection process into a consideration when preparing cell suspension to generate the standard curves. In the best case scenario, one would prepare study-specific standard curves. In our previous study with *Escherichia coli* (Chapter 3), the accuracy in QPCR assay was increased when the cells for the generation of standard curves were handled the same way as the *E. coli* cells from air samples of unknown concentration.

Our results also indicated that a particular PCR method used for sample analysis introduces another uncertainty factor in a standard curve generation and suggested that the whole-cell PCR can be a reasonable alternative to Isolated-genomic DNA-PCR which commonly used in bioaerosol research, irrespective of the bacterial species. The whole-cell PCR is less labor intensive and more cost effective compared to Isolated-genomic DNA-PCR. Especially, with air sample where the relatively low cell concentration can hamper the DNA extraction process, the use of whole-cell PCR can be advantageous in accurate quantification.

Thus, successful bioaerosol quantification using QPCR method requires not only to understand the characteristics of bioaerosol to be investigated and its sampling methodology, but also to develop study-specific standard curves. Poor understanding of factors affecting the standard curves may lead to uncertainty in QPCR output and errors in sample quantification. The approach and procedures described in Chapter 4 provide guidelines for constructing study-specific standard curves leading to more accurate bioaerosol quantification using QPCR.

After completing the investigation of factors affecting the quantification of PCR output, the performance of a prototype novel bioaerosol sampler was analyzed using the methods developed in Chapter 3 and 4. The investigation of the Electrostatic Precipitator with Superhydrophobic Surface (EPSS) performance is described in Chapter 5. Following the guidelines suggested above, the study-specific standard curves were generated using whole-cell PCR method and the quantification results were compared with a traditional total cell counting method, Acridine Orange Epifluorescence Microscopy (AOEM). As could be seen from the results (Figure 5.5), the quantification results using whole-cell PCR

method and in good agreement with one determined by AOEM. Moreover it has an advantage over the microscopy method which becomes unreliable at lower cell concentrations and could not always provide accurate microorganism concentration estimates. The developed study-specific whole-cell PCR seems to be a promising alternative to the microscopic method in bioaerosol quantification due to its sensitivity and time and labor efficiency. The newly developed bioaerosol sampler coupled with QPCR analysis allows for both effective bioaerosol collection with low energy consumption and timely as well as accurate total bioaerosol quantification.

The main reason obstructing a rapid response to the release of hazardous bioaerosols in public sector is the lack of effective bioaerosol monitoring systems. In this dissertation, we investigated several important issues to address the problem, and developed and validated study-specific bioaerosol quantification methods. Unlike other environmental samples, the biochemical characteristics of biological particles in air samples could be affected by the bioaerosol sampling process itself. Additionally, due to the relatively low bacterial concentration in an average environment, it is even harder to accurately determine the total cell number in air samples. The results demonstrated in this dissertation suggest the QPCR is an effective total bioaerosol counting method featuring high sensitivity. The results also provide guidance how to set up protocols to generate the study-specific standard curves to improve the reliability of QPCR results and where the factors like sampling device, media and characteristics of targeted bioaerosol should be considered. To this end, the developed QPCR assay was applied to test the performance of a novel bioaerosol sampler (EPSS). The test results indicated a successful application of QPCR method to test performance of bioaerosol samplers. By coupling with an effective

bioaerosol sampling device, this QPCR assay could increase the reliability of bioaerosol sampling systems and allow timely and effective quantification of aerosol samples.

However, to effectively use QPCR assay for bioaerosol quantification, generation of the standard curve is one of the most important steps. Thus, I would like to suggest the following procedure:

- ✓ Study the characteristics of bioaerosol sampler to determine how much damage this sampler would cause to collected organisms and whether the sampling method can be incorporating with PCR assay
- ✓ Determine the target bioaerosol (general bioaerosol population or specific bioaerosol)
- ✓ Determine the sampling environment (indoor / outdoor or residential / occupational etc.)
- ✓ Determine or design a primer set based on the information of target gene (universal primer or target-specific primer)
- ✓ Determine an optimal PCR amplification condition for target bioaerosol and primer sets
- ✓ Select a method to generate the corresponding cell number in the x-axis of a standard curve (e.g., a theoretical cell number, culturable cell number, or total cell number)
- ✓ Select a PCR method (Isolated-genomic DNA PCR or whole-cell PCR)
- ✓ Generate the standard curve(s)

Overall, the findings in this dissertation provide the general guidelines to develop an effective bioaerosol monitoring system by setting-up the study-specific protocol of QPCR assay capable of determining total cell numbers in air samples. The improved

bioaerosol sampling system enabling rapid quantification of bioaerosols with high sensitivity may be applied as a basis for developing bioaerosol detection systems capable of detecting even small bioaerosol concentrations thus providing useful information needed to understand the bioaerosol exposure dose and response relationship.

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