INVESTIGATION OF BIOAEROSOL CHARACTERIZATION BIAS DUE TO AEROSOLIZATION AND SAMPLING

by

HUAJUN ZHEN

A dissertation submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Environmental Sciences

Written under the direction of

Gediminas Mainelis

And approved by

_____

_____

_____

_____

New Brunswick, New Jersey

May, 2015

ABSTRACT OF THE DISSERTATION

Investigation of Bioaerosol Characterization Bias due to Aerosolization and Sampling

by HUAJUN ZHEN

Dissertation Director:
Gediminas Mainelis

Bioaerosols have been investigated for their adverse effects on human health and also their roles in cloud formation, precipitation and atmospheric chemical reactions. In these studies, it is often necessary to collect biological cells from the airborne state or to disperse the cells into the air using a variety of sampling and aerosolization devices. These devices inevitably exert stress on the cells and result in changes in their biological characteristics, e.g. loss of culturability, impairment of cell membrane, and change in cellular activities. It is highly likely that after experiencing such stress, collected microorganisms are not representative of cells prior to sampling and aerosolization, and thus could introduce biases in their characterization. In this dissertation, the potential bioaerosol characterization biases induced by several commonly used sampling and aerosolization devices were investigated.

The stress experienced by *E. coli* during aerosolization was found to depend on a particular aerosolization device. Particularly, a newly developed pneumatic nebulizer, the Single-Pass Aerosolizer, was shown to better preserve cell culturability

and membrane integrity compared to the commonly used Collison nebulizer at similar biological particles output concentrations.

Several bioaerosol samplers that employ filtration, impingement, impaction and electrostatic precipitation for sample collection were tested with respect to their effects on the cell membrane integrity and cellular 16S rRNA content of *Escherichia coli* cells. Sampling stress resulted in severe membrane impairment to *E. coli* aerosols, leading to the release of genomic DNA as extracellular molecules. Extracellular DNA should be taken into account when analyzing bioaerosol samples to more accurately quantify bacterial presence. Cell membrane damage to bioaerosols depended on which sampler was used and could be reduced by modifying specific operational parameters. *E. coli* cells exhibited variation in 16S ribosomal RNA (rRNA) level when exposed to long-term air sampling in laboratory experiment, suggesting a change of biological activity in response to sampling stress. The importance of this effect for those taxa in airborne bacterial community from a variety of environments should be examined. The abundance of bacterial 16S rRNA in bioaerosols collected from an outdoor environment was about two orders of magnitude higher than that of 16S rRNA gene. However, the sampler-dependent bias effect on analysis of 16S rRNA sequences for bacterial community composition was minimal in investigated outdoor bioaerosols.

Acknowledgements

First of all, I would like to express my sincere gratitude to my advisor Dr. Gediminas Mainelis for his continuous support of my Ph.D. research. He gave me great freedom to pursue my own interests, and at the same time provided invaluable guidance on my research. I am very grateful for his patience, motivation, enthusiasm and immense knowledge. I would like to thank Dr. Donna E. Fennell for being my mentor in the first two years of my Ph.D. study and serving on my dissertation committee. I would also like to thank Dr. John Reinfelder and Dr. Stephan Schwander for their insightful discussion and valuable comments on my dissertation.

I acknowledge the grants that supported me during my Ph.D. study at Rutgers: Excellence Fellowship and Teaching Assistantship from the Department of Environmental Sciences, and grants from CDC/NIOSH (R01-OH009783) and New Jersey Agricultural Experiment Station (NJAES) at Rutgers University (Project 07160).

My sincere thanks also go to all my lab colleagues, Dr. Taewon Han, Dr. Zuocheng Wang, Dr. Yevgen Nazarenko, Leonardo Calderon, Jennifer Therkorn and Dr. Jessica Sagona, for their support and friendship in the past few years. I would like to express my thankfulness to Dr. Valdis Krumins who taught me many experimental techniques and helped me a lot in my research. I would also like to express my gratitude to all my friends in the department who made my journey towards a Ph.D. full of wonderful memories.

Last but not the least, I would like to thank my parents for their endless support and unconditional love for me.

Table of Contents

List of Tables

List of Figures

Chapter 1: Background, Motivation and Dissertation Overview

1.1 Background and Motivation

Bioaerosols are defined as airborne particles of biological origin, which may affect living things through infectivity, allergenicity, toxicity, pharmacological or other processes (1). They may consist of viruses, bacteria, fungi, pollen, plant debris and their by-products such as endotoxins, β(1→3) -glucans, peptidoglycans, allergens and mycotoxins, *etc.* (2-4)

Bioaerosols have drawn attention of the public and scientific community largely due to the adverse health effects they might cause to individuals and large populations (2, 5, 6). Exposure to high concentration of bioaerosols, mainly through the respiratory route, may cause respiratory distress, microbial infection, allergenic reaction, respiratory sensitization and toxicological reaction (2, 7) and other adverse health effects. Indoor environments such as residential houses and confined office spaces have become major sources of human exposure to bioaerosols (5, 6, 8). It was estimated that people spent on average 85% of their time indoors and many chronic respiratory illnesses including asthma have been linked to indoor bioaerosols exposure, especially in poor ventilate environments (9-12). A nonspecific building-related illness, also known as sick building syndromes (SBS), which manifests through membrane irritation, headaches, fatigue, cognitive complaints, nausea, skin rash and dizziness, has been frequently associated with bioaerosols (8). In addition, bioaerosols pose health concerns in certain industries, e.g. workers from industries such as waste composting, biosolid land application and poultry houses, were reported with high incidence of adverse health symptoms such as febrile episodes, hypersensitivity pneumonitis and asthma (13, 14). Bioaerosols could also have broad health impact if they are intentionally released as biowarfare agents or used in bioterrorism events such as the anthrax attacks in 2001 (3).

In addition to human health reasons, bioaerosols have also been widely studied for their ecological significance and their impact on biogeochemical (15-17) and hydrological cycles (18-20). It was estimated that bioaerosols constitute ~25% of aerosol particles in the size range 0.2–50 μm both by mass and number globally (21), while in some particular environment such as the Amazonian region bioaerosols contribute up to 85% of coarse particle mass (19, 22). Bioaerosols are excellent cloud condensation nuclei (CCN) (18, 20) and ice nuclei (IN) (18, 23), and thus they have a wide-scale impact on clouds and precipitation and can greatly influence the regional and global climate (21). Besides, bioaerosols could potentially have a significant role in atmospheric chemistry by actively metabolizing the organic compounds present in the air (15-17).

The first step in bioaerosol research in many areas is usually to accurately and comprehensively characterize the airborne microorganisms. For instance, for bioaerosols exposure studies, it is important to know not only the microorganism presence and its concentration, but also its physiological status in the air (2). Certain kinds of microorganisms can cause infectious diseases to the human host and their infectivity relies on the microorganism viability when in contact with the host (2). For example, tuberculosis, an infectious disease known to affect human lungs, is caused by various strains of *mycobacteria*, usually *Mycobacterium tuberculosis* (2, 24); Legionnaires disease and Pontiac fever are high profile infections that are caused by exposures to *Legionellae* (particularly *Legionella pneumophila*) (2). In addition, the physiological state of bacteria and fungi also determine their abilities to successfully colonize, amplify and disseminate large quantities of allergenic particles into the environment (25), causing respiratory diseases such as asthma, rhinitis, chronic bronchitis, *etc.* (2) For other

bioaerosol studies, such as investigation on its role in atmospheric chemistry and cloud formation, the activities of bacteria, especially from the perspective of cell metabolism and protein synthesis , is also of particular interest to microbiologists and atmospheric scientists (16, 17, 26).

The ambient air has been commonly viewed as a hostile environment for microorganisms to survive (26). This is largely due to the various stressors that the microorganisms may experience in the airborne state. These stressors include, but are not limited to, lack of nutrients, unfavorable temperature and humidity, ultraviolet radiation and chemical pollutants (26-32). The physiological status of airborne microorganisms is subjected to change due to the joint effects of all types of stressors they may experience in the air. Therefore, airborne microorganisms can be classified as culturable, viable but non-culturable (VBNC), non-viable but able to maintain the cell membrane integrity, and cell fragments (32-34). The culturable fraction of airborne microorganisms refers to those cells which can form colonies on specific nutrient media (liquid or semi-solid) after collection. It was estimated that less than 1% of microorganisms in natural water and soil samples are culturable (35). The VBNC microorganisms represent those cells which could not form colonies on media but retain metabolic activity (36). When cells reach the end of their life cycle, they lose their viability completely and become non-viable cells. However, depending on whether the cell membrane is completely impaired and lysed, they may still exist as whole cells but without any cellular functions, or become cell fragments although some organelles and molecules such as DNA may persist (37-39). In previous studies, the first three cell categories (culturable, VBNC and nonviable) have

been well documented as bioaerosols (33, 34, 40), however, few studies have investigated the fourth bioaerosol category: cell fragments (32).

Currently, a wide variety of bioaerosol collection devices have been developed, and the major types of mechanisms used by these devices include impaction, impingement and filtration (3). Ideally, a bioaerosol collection device should be able to collect a representative sample from an environment (7), which means that the bioaerosol sample retrieved by the device is identical to the airborne microorganisms prior to sampling in both their physical and biological characteristics. However, this becomes a great challenge for almost all samplers due to multiple reasons. First, the ambient air is not homogenous; as a result, it often requires multiple grab samples to achieve a representative sample from the studied environment. Second, the samplers vary in their collection efficiencies for particles with different sizes, and therefore no single sampler or sampling protocol is likely to be adequate for collection of bioaerosols from all size ranges (7). Another factor is that the biological and physical characteristics of microorganism are subjected to change with changes in time and environmental conditions (7), for example, these characteristics may vary constantly for airborne microorganisms due to their reaction to environmental stressors. It is also reasonable to hypothesize that the physiological status of biological cells could be altered due to effects of sampling stressors such as impaction, impingement, desiccation and osmotic presses, *etc.* This becomes especially important when considering the fact that a variety of mechanical forces are applied continuously for long periods of time (hours to days) in most bioaerosols sampling protocols. For example, the cell injury and loss of culturability have been observed due to dehydration when microorganisms were collected by filters

(41) and portable microbial impactors (40) for times as short as a few minutes. As a result, it is very likely that the results from samples collected with a particular technique or method could be biased compared to the actual information about the bioaerosols prior to their collection. Thus, there is a great need to investigate the potential bias caused by bioaerosol sampling technique to analysis results.

To date, a number of techniques have been developed and utilized to analyze bioaerosols. Depending on the particular principle of the analysis technique, each technique discovers different fraction of microorganism from mixed environmental samples (7). The most traditional method is the agar-based cultivation technique which can detect and enumerate the culturable microorganism but fail to detect the non-culturable fraction of an airborne microbial community. Methods independent of culturing are able to present more accurate information about bioaerosols especially considering that less than 1% of airborne bacteria are culturable (3, 35). Among these methods, epifluorescence and direct light microscopy are often used to determine the total numbers of microorganism in air samples, but they are both labor and time-consuming, and fail to reveal any species information (42, 43). In addition, the microscopy technique is inefficient in discovering the cell fragments in an environmental sample.

In the last few decades, the polymerase chain reaction (PCR)-based technologies are increasingly being applied in analysis of bioaerosol samples (44, 45). In general, specific DNA fragments could be amplified by PCR to increase the initial DNA quantity by several orders of magnitudes; thus this technique greatly lowers the detection limit for bioaerosols compared with other quantification methods (3, 45). The amplified DNA

fragments can be quantified to determine the DNA copy numbers in initial samples, in which case the method is called quantitative-PCR (qPCR) (44, 45). The amplified DNA fragments can also be passed to downstream analysis to investigate microbial communities using a sequencing-based approach. More importantly, the primer sets for PCR reaction can be deliberately designed to target a group of bacteria at different taxonomic levels (species, phylum, etc.) or even the entire bacteria community. For example, the most commonly used primers target the 16S rRNA gene, which is ubiquitous in genomes of all bacterial species (46).

However, the qPCR technique alone cannot reveal any information regarding the fraction of microorganisms in each physiological state. After modifying sample extraction protocols prior to a PCR reaction, it is feasible to selectively detect viable cells within a complex microbial sample. For example, a number of previous studies have reported that sample treatment with ethidium monoazide or propidium monoazide and subsequent light radiation can destroy the nucleic acids in non-viable cells (47). As a result, the DNA from viable cells could be selectively saved and analyzed. In addition, the culturable fraction in a bioaerosol sample could also be simply analyzed with traditional agar plating method (40). However, no study has reported the selective analysis of cell fragments or debris which is different from the other three aforementioned cells categories (culturable, VBNC and nonviable). In this dissertation, we made such investigation by targeting the free DNA molecules outside of biological cell, i.e. extracellular DNA (eDNA), which is also known as a persistent component after cell lysis (37-39) from a bioaerosol sample. Previously, the eDNA has been intensively studied in microbial samples collected from natural water (48), sediment (49, 50), biofilm

(51) and sludge (52), however, reporting on eDNA for bioaerosols is still lacking. The research on this topic is covered in Chapters 2, 3 and 5 throughout this dissertation.

Other than DNA, the RNA has previously been proposed as an alternative target molecule to analyze environmental bacteria, particularly the active members within a microbial community (53). The utilization of RNA instead of DNA has an advantage because the DNA-based analysis of environmental samples may detect dead cells. This is because the DNA is generally stable in an environment (37-39) even if the cells are dead or lysed, but the RNA is directly linked to cell physiology (54, 55) and is unstable outside of cell (56). Of the two commonly investigated RNA types, the study of messenger RNA transcripts have been limited to tracking particular bacterial functional groups, while the ribosomal RNA (rRNA) transcripts could be used to study all active members of the microbial community (53). Previously, investigations of rRNA especially the 16S rRNA have been widely conducted with samples from a variety of environments by two typical approaches (57): the quantitative approach, which enumerates the rRNA transcripts level, e.g. 16S rRNA: rRNA gene ratio, can be used to determine the relative change in protein synthesis potential for specific bacterial taxa (54, 55, 58); the qualitative approach, which utilizes the 16S rRNA sequences as well as abundance of each sequence type, can be used to identify the active microbial populations in a mixed community (59, 60). To the best of our knowledge, only one study has used the quantitative approach to investigate the RNA abundance from a bioaerosol sample (26), and no study on investigation of airborne microbial community using the qualitative approach has been published. Moreover, a large knowledge gap still exists regarding the question whether the retrieved bioaerosol sample after a long term of sampling period could still represent the actual

rRNA levels of airborne microbes before sampling. In this dissertation, one objective was to investigate the changes in cellular rRNA level due to sampling stress and the results are presented in Chapters 4 and 5.

The physiological status change for bioaerosols is not limited to the process of sample collection where the microorganisms are removed from an airborne phase and concentrated into/onto liquid or solid material. In many other scenarios, researchers need to perform an opposite process: to intentionally disperse the microorganisms in a liquid culture or powders into the air, i.e. aerosolization. Similar to sample collection, this aerosolization process can be achieved by utilizing many types of bioaerosol generators (aerosolizer) which inevitably employ a variety of mechanical forces for effective dispersal of microorganisms into the air. Bioaerosol generator is an essential component in nearly all laboratory-based bioaerosol research projects, and its applications include, but are not limited to, testing performance of bioaerosol collectors, investigating exposure and health effects of airborne microorganisms, studying transport and deposition of biological particles, evaluating effectiveness of bioaerosol control techniques (61). In most of these applications, a stable and reliable aerosol generator is often required to produce bioaerosols with high particle concentrations but with minimal disturbance to biological and physical characteristics of cells. Currently, the most frequently used generator, the Collison nebulizer was originally designed for research with inorganic aerosols (62). However, the Collison nebulizer has been shown to cause severe damage to bacterial cells in a liquid suspension after only a few minutes operation (63). In recent years, many aerosol generators have been developed and some were designed to lessen the stress to microorganisms due to various mechanical forces during

aerosolization (61, 62, 64-66). However, there is no systematic comparison of those commonly used generators regarding the effects on physiological state of microorganisms due to aerosolization. A part of this dissertation focused on such an investigation and the relevant content is presented in Chapter 3.

1.2 Goals and Hypotheses

The **overall goal** of this dissertation was to systematically investigate the change of bioaerosol characteristics due to the stress during sampling and aerosolization processes. This overall goal was achieved by fulfilling **three objectives**. The **first** objective was to investigate the damage to cell integrity during bioaerosol sample collection. The **second** objective was to study the change of bioaerosol 16S ribosomal RNA transcripts level during sampling collection. The **third** objective was to investigate the damage to microorganism integrity and culturability loss due to aerosolization by different techniques. It is hoped that the results from this dissertation will provide guidance for selecting appropriate sampling and aerosolization protocols so that the physiological status of bioaerosols is minimally affected, leading to more accurate sample analysis and more reliable aerosol generation in bioaerosol studies.

Three major **hypotheses** were tested in this dissertation:

1. The stressors caused by a variety of mechanical forces when performing bioaerosol sampling and aerosolization can cause severe damage to bacterial cells and produce extracellular DNA; this effect of aerosolization and sampling is device-dependent.

2. The ribosomal RNA level of a bioaerosol sample is subjected to change due to sampling stress during long-term air sampling, this effect is sampling device-dependent.

3. The impact of aerosolization stress on bacterial cells varies among different devices and techniques.

1.3 Dissertation Overview

Chapter 1 introduces the motivation, primary goals and hypotheses of the dissertation. A brief description for each chapter of this dissertation is also presented.

In **Chapter 2**, we investigated the release of DNA as extracellular molecules by membrane-damaged microorganisms due to the stress imposed on them during air sampling. Samplers using different collection mechanisms of filtration, impaction, impingement and electrostatic precipitation were tested. There were four objectives in this chapter: 1) to verify the occurrence of eDNA in lab-generated *E. coli* bioaerosol samples collected by multiple devices; 2) to study whether the adjustment of operational parameters for a particular sampler can alleviate the damage to cell membrane; 3) to test whether there is a difference between gram-positive and gram-negative species in their capability to withstand the sampling stress; 4) to investigate whether the eDNA could be detected from environmental bioaerosols collected by multiple devices.

Chapter 3 presents our research on a systematic comparison of the performance of four common bioaerosol generators with respect to the physical properties of generated bioaerosols such as concentration and size distribution, and the biological characteristics of generated bioaerosols including the culturability loss and impairment of structural integrity. The investigated aerosol generators include a three-jet Collison nebulizer and a newly developed Single-Pass Aerosolizer, a Liquid Sparging Aerosolizer and a C-Flow nebulizer**.**

Chapter 4 is a chapter focusing on the development of a dual-internal-reference technique for accurate determination of bacterial 16S rRNA: rRNA gene ratio by reverse

transcription qPCR (RT-qPCR) for application to bacterial samples including bioaerosols. The work in this chapter prepares methodology for a project described in the following Chapter 5. In Chapter 4, we explain in detail how the dual internal references are prepared and how the operational protocol for this technique is developed. At the end of this Chapter, we describe the application of this technique to determine the temporal variation of 16S rRNA: rRNA gene ratio for *E. coli* aerosols when exposed to active sampling stress after being collected on filter.

In **Chapter 5**, we investigate three different sampling devices for their applicability to study 16 rRNA of bioaerosol samples. The work from this chapter is fundamental for all future studies dealing with bioaerosol ribosomal RNA and investigates the potential bias of experimental results due to sampling stress. Three objectives were set: 1) to investigate the change in 16S rRNA: rRNA gene ratio for *E. coli* in response to long-term (two hours) bioaerosol sampling stress by using the dual-internal-reference technique developed in Chapter 4; 2) to assess whether the change of 16S rRNA: rRNA gene ratio for *E. coli* bioaerosols is sampler-dependent; 3) to study the effect of sampling stress on analysis of 16S rRNA by pyrosequencing from bioaerosol samples collected in an outdoor environment.

In **Chapter 6**, the main results and conclusions from Chapters 2-5 are summarized. In addition, we discuss the implications of the work performed in this dissertation on future bioaerosol research.

References

1. **Hirst, J. M.** 1995. Bioaerosols: introduction, retrospect and prospect, p. 5. *In* C. S. Cox and C. M. Wathes (ed.), Bioaerosols handbook. CRC Press, New York.
2. **Douwes, J., P. Thorne, N. Pearce, and D. Heederik.** 2003. Bioaerosol health effects and exposure assessment: progress and prospects. Ann. Occup. Hyg. **47:**187-200.
3. **Xu, Z. Q., Y. Wu, F. X. Shen, Q. Chen, M. M. Tan, and M. S. Yao.** 2011. Bioaerosol science, technology, and engineering: past, present, and future. Aerosol Sci. Technol. **45:**1337-1349.
4. **Macher, J.** 1999. Bioaerosols: assessment and control. American Conference of Governmental Industrial Hygienists, Inc., Cincinnati, OH.
5. **Fung, F., and W. G. Hughson.** 2003. Health effects of indoor fungal bioaerosol exposure. Appl. Occup. Environ. Hyg. **18:**535-544.
6. **Robbins, C. A., L. J. Swenson, M. L. Nealley, R. E. Gots, and B. J. Kelman.** 2000. Health effects of mycotoxins in indoor air: a critical review. Appl. Occup. Environ. Hyg. **15:**773-784.
7. **Cox, C. S., and C. M. Wathes.** 1995. Editors' introduction, p. 3-4. *In* C. S. Cox and C. M. Wathes (ed.), Bioaerosols handbook. CRC Press, New York.
8. **Laumbach, R. J., and H. M. Kipen.** 2005. Bioaerosols and sick building syndrome: particles, inflammation, and allergy. Current opinion in allergy and clinical immunology **5:**135-139.
9. **Klepeis, N. E., W. C. Nelson, W. R. Ott, J. P. Robinson, A. M. Tsang, P. Switzer, J. V. Behar, S. C. Hern, and W. H. Engelmann.** 2001. The National Human Activity Pattern Survey (NHAPS): a resource for assessing exposure to environmental pollutants. J. Expo. Anal. Environ. Epidemiol. **11:**231-252.
10. **Brasche, S., and W. Bischof.** 2005. Daily time spent indoors in German homes--baseline data for the assessment of indoor exposure of German occupants. Int. J. Hyg. Environ. Health. **208:**247-253.
11. **Yang, W., K. Lee, C. Yoon, S. Yu, K. Park, and W. Choi.** 2011. Determinants of residential indoor and transportation activity times in Korea. J. Expo. Sci. Environ. Epidemiol. **21:**310-316.
12. **Hospodsky, D., J. Qian, W. W. Nazaroff, N. Yamamoto, K. Bibby, H. Rismani-Yazdi, and J. Peccia.** 2012. Human occupancy as a source of indoor airborne bacteria. Plos One **7:**e34867.
13. **Poulsen, O. M., N. O. Breum, N. Ebbehøj, A. M. Hansen, U. I. Ivens, D. van Lelieveld, P. Malmros, L. Matthiasen, B. H. Nielsen, and E. M. Nielsen, *et al.*** 1995. Collection of domestic waste. Review of occupational health problems and their possible causes. Sci. Total Environ. **170:**1-19.
14. **Oppliger, A., N. Charrière, P. O. Droz, and T. Rinsoz.** 2008. Exposure to bioaerosols in poultry houses at different stages of fattening; use of real-time PCR for airborne bacterial quantification. Ann. Occup. Hyg. **52:**405-412.
15. **Herlihy, L. J., J. N. Galloway, and A. L. Mills.** 1987. Bacterial utilization of formic and acetic acid in rainwater. Atmos. Environ. **21:**2397-2402.

16. **Vaitilingom, M., L. Deguillaume, and V. Vinatier, *et al.*** 2013. Potential impact of microbial activity on the oxidant capacity and organic carbon budget in clouds. Proc. Natl. Acad. Sci. U.S.A. **110:**559-564.
17. **Konstantinidis, K. T.** 2014. Do airborne microbes matter for atmospheric chemistry and cloud formation? Environ. Microbiol. **16:**1482-1484.
18. **Christner, B. C.** 2012. Cloudy with a chance of microbes. Microbe **7:**70-74.
19. **Pöschl, U., S. T. Martin, B. Sinha, Q. Chen, S. S. Gunthe, and J. A. Huffman, *et al.*** 2010. Rainforest Aerosols as Biogenic Nuclei of Clouds and Precipitation in the Amazon. Science **329:**1513-1516.
20. **Bauer, H., H. Giebl, and R. Hitzenberger, et al.** 2003. Airborne bacteria as cloud condensation nuclei. J. Geophys. Res. **108:**4658.
21. **Jaenicke, R.** 2005. Abundance of cellular material and proteins in the atmosphere. Science **308:**73.
22. **Jaenicke, R., S. Matthias-Maser, and S. Gruber.** 2007. Omnipresence of biological material in the atmosphere. Environ. Chem. **4:**217-220.
23. **Vali, G., M. Christensen, and R. W. Fresh, *et al.*** 1976. Biogenic ice nulei. Part II: Bacterial sources. J. Atmos. Sci. **33:**1565-1570.
24. **Kassim, I., and C. G. Ray.** 2004. Sherris Medical Microbiology (4th ed.) McGraw Hill.
25. **Ness, S. A.** 1996. NIOSH case studies in Bioaerosols Government Institutes, Inc., Rockville, Maryland.
26. **Krumins, V., G. Mainelis, L. J. Kerkhof, and D. E. Fennell.** 2014. Substrate-dependent rRNA production in an airborne bacterium. Environ. Sci. Technol. Letters **1:**376-381.
27. **Handley, B. A., and A. J. F. Webster.** 1995. Some factors affecting the airborne survival of bacteria outdoors. J. Appl. Bacteriol. **79:**368-378.
28. **Hatch, M. T., and R. L. Dimmick.** 1966. Physiological responses of airborne bacteria to shifts in relative humidity. Bacteriol. Rev. **30:**597-602.
29. **Tang, J. W.** 2009. The effect of environmental parameters on the survival of airborne infectious agents. J. R. Sco. Interface **6:**S697-S702.
30. **Tong, T., and B. Lighthart.** 1997. Solar radiation has a lethal effect on natural populations of culturable outdoor atmospheric bacteria. Atmos. Environ. **31:**897-900.
31. **Zentner, R. J.** 1966. Physical and chemical stresses of aerosolisation. Bacteriol. Rev. **30:**551-557.
32. **Zhen, H. J., T. Han, D. E. Fennell, and G. Mainelis.** 2013. Release of free DNA by membrane-impaired bacterial aerosols due to aerosolization and air sampling. Appl. Environ. Microbiol. **79:**7780-7789.
33. **Oliver, J. D.** 2005. The viable but nonculturable state in bacteria. J. Microbiol. **43:**93-100.
34. **Caron, G. N., P. Stephens, and R. A. Badley.** 1998. Assessment of bacterial viability status by flow cytometry and single cell sorting. J. Appl. Microbiol. **84:**988-998.
35. **Barcina, I., and I. Arana.** 2009. The viable but nonculturable phenotype: a crossroads in the life-cycle of non-differentiating bacteria? Rev. Environ. Sci. Biotechnol. **8:**245-255.

36. **Xu, H. S., N. Roberts, F. L. Singleton, R. W. Attwell, D. J. Grimes, and R. R. Colwell.** 1982. Survival and viability of nonculturable Escherichia coli and Vibrio cholerae in the estuarine and marine environment. Microbial Ecology **8:**313-323.
37. **Josephson, K. L., C. P. Gerba, and I. L. Pepper.** 1993. Polymerase chain reaction detection of nonviable bacterial pathogens. Appl. Environ. Microbiol. **59:**3513-3515.
38. **Keer, J. T., and L. Birch.** 2003. Molecular methods for the assessment of bacterial viability. J. Microbiol. Methods **53:**175-183.
39. **England, L. S., S. B. Holmes, and J. T. Trevors.** 1998. Persistence of viruses and DNA in soil. Would Journal of Microbiology & Biotechnology **14:**163-169.
40. **Mainelis, G., and M. Tabayoyong.** 2010. The effect of sampling time and the overall performance of portable microbial impactors. Aerosol Sci. Technol. **44:**75-82.
41. **Wang, Z., T. Reponen, S. A. Grinshpun, R. I. Gorny, and K. Willeke.** 2001. Effect of sampling time and air humidity on the bioefficiency of filter samplers for bioaerosol collection. J. Aerosol Sci. **32:**661-674.
42. **Kildeso, J., and B. H. Nielsen.** 1997. Exposure assessment of airborne microorganisms by fluorescence microscopy and image processing. The Annals of Occupational Hygiene **41:**201-216.
43. **Williams, R. H., E. Ward, and H. A. McCartney.** 2001. Methods for integrated air sampling and DNA analysis for detection of airborne fungal spores. Appl. Environ. Microbiol. **67:**2453-2459.
44. **Peccia, J., and M. Hernandez.** 2006. Incorporating polymerase chain reactionbased identification, population characterization, and quantification of microorganisms into aerosol science: a review. Atmos. Environ. **40:**3941-3961.
45. **An, H. R., and G. Mainelis.** 2006. Development and calibration of real-time PCR for quantification of airborne microorganisms in air samples. Atmos. Environ. **40:**7924-7939.
46. **Lane, D. J., B. Pace, G. J. Olsen, D. A. Stahl, M. L. Sogin, and N. R. Pace.** 1985. Rapid-determination of 16s ribosomal-RNA sequences for phylogenetic analyses. Proc. Natl. Acad. Sci. U.S.A. **82:**6955-6959.
47. **Pan, Y., and F. Breidt.** 2007. Enumeration of viable *Listeria Monocytogenes* cells by real-time pCR with propidium monoazide and ethidium monoazide in the presence of dead cells. Appl. Environ. Microbiol. **73:**8028-8031.
48. **Liang, Z. B., and A. Keeley.** 2013. Filtration recovery of extracellular DNA from environmental water samples Environ. Sci. Technol. **47:**9324-9331.
49. **Corinaldesi, C., R. Danovaro, and A. Dell'Anno.** 2005. Simultaneous recovery of extracellular and intracellular DNA suitable for molecular studies from marine sediments. Appl. Environ. Microbiol. **71:**46-50.
50. **Mao, D. Q., Y. Luo, J. Mathieu, and Q. Wang, *et al.*** 2014. Persistence of extracellular DNA in river sediment facilitates antibiotic resistance gene propagation. Environ. Sci. Technol. **48:**71-78.
51. **Cheng, M., A. E. Cook, T. Fukushima, and P. L. Bond.** 2011. Evidence of compositional differences between the extracellular and intracellular DNA of a granular sludge biofilm. Letters in Applied Microbiology **53:**1-7.

52. **Zhang, Y. P., D. D. Snow, D. Parker, Z. Zhou, and L. Xu.** 2013. Intracellular and extracellular antimicrobial resistance genes in the sludge of livestock waste management structures. Environ. Sci. Technol. **47:**10203-10213.
53. **Pitkänen, T., H. Ryu, and M. Elk, *et al.*** 2013. Detection of fecal bacteria and source tracking identifiers in environmental waters using rRNA-based RT-qPCR and rDNA-based qPCR assays. Environ. Sci. Technol. **47:**13611-13620.
54. **Kemp, P. F., S. Lee, and J. LaRoche.** 1993. Estimating the growth rate of slowly growing marine bacteria from RNA content. Appl. Environ. Microbiol. **59:**2594-2601.
55. **Poulsen, L. K., G. Ballard, and D. A. Stahl.** 1993. Use of rRNA fluorescence in situ hybridization for measuring the activity of single cells in young and established biofilms. Appl. Environ. Microbiol. **59:**1354-1360.
56. **Deutscher, M. P.** 2003. Degradation of stable RNA in bacteria. J. Biol. Chem. **278:**45041-45044.
57. **Blazewicz, S. J., R. L. Barnard, R. A. Daly, and M. K. Firestone.** 2013. Evaluating rRNA as an indicator of microbial activity in environmental communities: limitations and uses. ISME J. **7:**2061-2068.
58. **Kerkhof, L., and B. Ward.** 1993. Comparison of nucleic acid hybridization and fluorometry for measurement of the relationship between RNA/DNA ratio and growth rate in a marine bacterium. Appl. Environ. Microbiol. **59:**1303-1309.
59. **Campbell, B. J., L. Yu, J. F. Heidelberg, and D. L. Kirchman.** 2011. Activity of abundant and rare bacteria in a coastal ocean. Proc. Natl. Acad. Sci. U.S.A. **108:**12776-12781.
60. **Kamke, J., M. W. Taylor, and S. Schmitt.** 2010. Activity profiles for marine sponge-associated bacteria obtained by 16S rRNA vs 16S rRNA gene comparisons. ISME J. **4:**498-508.
61. **Simon, X., P. Duquenne, V. Koehler, and C. Piernot, *et al.*** 2011. Aerosolisation of *Escherichia coli* and associatedendotoxinusinganimproved bubbling bioaerosol generator. J. Aerosol Sci. **42:**517-531.
62. **May, K. R.** 1973. The Collison nebulizer: description, performance and application. J. Aerosol Sci. **4:**235-243.
63. **Reponen, T., K. Willeke, V. Ulevicius, S. A. Grinshpun, and J. Donnelly.** 1997. Techniques for dispersion of microorganisms into air. Aerosol Sci. Technol. **27:**405-421.
64. **Mainelis, G., D. Berry, H. R. An, M. S. Yao, K. DeVoe, D. E. Fennell, and R. Jaeger.** 2005. Design and performance of a single-pass bubbling bioaerosol generator. Atmos. Environ. **39:**3521-3533.
65. **Thomas, R. J., D. Webber, W. Sellors, and A. Collinge, *et al.*** 2009. Generation of large droplet aerosols within microbiological containment using a novel flow-focussing technique. Aerobiologia **25:**75-84.
66. **Ganan-Calvo, A. M., and A. Barrero.** 1999. A novel pneumatic technique to genrate steady capillary microjets. J. Aerosol Sci. **30:**117-125.

Chapter 2: Release of DNA as Extracellular Molecules by Membrane-Impaired Bacterial Aerosols Due to Aerosolization and Air Sampling[1]

[1] This chapter is modified from the paper publication by Huajun Zhen, Taewon Han, Donna E. Fennell, and Gediminas Mainelis 2013. Release of free DNA by membrane-impaired bacterial aerosols due to aerosolization and air sampling. *Applied and Environmental Microbiology.* **79:**7780-7789.

2.1 Abstract

We report here that stress experienced by bacteria due to aerosolization and air sampling can result in severe membrane impairment, leading to the release of DNA as extracellular molecules. *Escherichia coli* and *Bacillus atrophaeus* bacteria were aerosolized and then either collected directly into liquid or using other collection media and then transferred into liquid. The amount of DNA released was quantified as the cell membrane damage index (I_D), the number of 16S rRNA gene copies in the supernatant liquid relative to the total number in the bioaerosol sample. During aerosolization by a Collison nebulizer, the I_D of *E. coli* and *B. atrophaeus* in the nebulizer suspension gradually increased during 60 min of continuous aerosolization. We found that the I_D of bacteria during aerosolization was statistically significantly affected by the material of Collison jar (glass > polycarbonate, $p<0.001$) and bacteria species (*E. coli* > *B. atrophaeus*, $p<0.001$). When *E. coli* was collected for 5 minutes by filtration, impaction and impingement, its I_D values were within the following ranges: 0.051–0.085, 0.16–0.37, and 0.068–0.23, respectively; when collected by electrostatic precipitation, the I_D values (0.011–0.034) were significantly lower ($p<0.05$) compared to other sampling methods. Air samples collected inside an equine facility for two hours by filtration and impingement exhibited I_D in the range of 0.30-0.54. The data indicate that the amount of cell damage during bioaerosol sampling and the resulting release of DNA can be substantial and that this should be taken into account when analyzing bioaerosol samples.

2.2 Introduction

Investigation of the presence of airborne microorganisms (bioaerosols) in the ambient air is of interest due to their environmental and human health effects (1, 2). Numerous studies have shown that increased exposure to bioaerosols is positively correlated with the incidence of negative respiratory health effects, including lung irritation, asthma, rhinitis, allergy, and cough (1-4). It is generally accepted that health effects caused by exposure to bioaerosols depend not only on the organism and its concentration, but also on its physiological status in the air, because viable and nonviable microorganisms have different potentials for causing adverse respiratory health effects (2, 5, 6).

Microorganisms in the airborne state may experience a variety of stressors, including unfavorable temperature and humidity, lack of nutrients, UV radiation, chemical pollutants, and other variables that affect their physiological status (7-11). Depending on that status, airborne cells could be classified as culturable, viable but not culturable, nonviable but maintaining membrane integrity, and cell fragments (12, 13). When bioaerosols are collected for environmental or health investigations or other purposes, it is desirable that the sampling method maintains their physiological status to minimize bias when quantifying and identifying microorganisms in the sample.

Numerous sampling devices have been developed and used to collect bioaerosols using filtration, impaction, impingement, electrostatic precipitation, and other methods. However, during each sampling process, the microorganisms are inevitably exposed to additional stress, which affects their viability and culturability. It has been observed that dehydration during sampling by filters (14, 15) and portable microbial impactors (16)

may cause cell injury and loss of culturability, especially in sensitive species. While one of the liquid-based bioaerosol samplers, the BioSampler (SKC Inc., Eighty Four, PA), is considered to be a relatively low-stress sampling device for collecting bioaerosols (17), one study has demonstrated that certain collection fluids, including glycerol and surfactant, greatly decreased the viability of *Legionella pneumophila*, presumably due to the elevated osmotic pressure (18). Stewart *et al.* observed that 49% of *Pseudomonas fluorescens* bacteria lost their culturability after impacting an agar surface at a speed of 40 m/s, most likely due to mechanical stress (19). Another study showed that the intactness of the genomic DNA was impaired due to the stress of impaction onto collection surface (20). Recently, Zhao *et al.* (2011) found that sampling stress from a variety of bioaerosol samplers decreased the bacterial culturability (21, 22). In addition to the sampling process, microorganisms may also experience substantial stress during aerosolization. It was found in our earlier study that the viability of *P. fluorescens* bacteria aerosolized by a Collison nebulizer (BGI Inc., Waltham, MA) decreased by over 50% after 90 minutes of continuous aerosolization (23). Similarly, Thomas *et al.* indicated that 99.9% of an *Escherichia coli* population suffered sublethal injury after a 10-min-long aerosolization by a Collison nebulizer (24). They also concluded that the cell membrane was the major site of damage due to impaction and shear force stress that disturbed membrane homeostasis (24). In light of this study, we hypothesized that the cell membrane could also be a major site of damage during bioaerosol sampling when cells experience substantial mechanical stress, such as during impaction and impingement. In addition, the elevated osmotic stress resulting from non-mechanical sampling factors,

such as desiccation, would make cell membranes more vulnerable to mechanical stress, possibly even leading to cell rupture.

In the past few years, quantitative polymerase chain reaction (qPCR) has gained popularity in bioaerosol research due to its capacity to rapidly quantify and identify microorganisms in air samples (25, 26). The collected microorganisms must first be lysed, but the method depends on how air samples are collected. Very often bioaerosols are collected directly into liquid by using impingers or first collected onto filters and then transferred into liquid. To concentrate such samples, liquid is centrifuged and only the pelleted cells are used for DNA extraction, while the rest of the liquid is discarded (18, 27-30). However, if we consider that a large fraction of cells experience severe stress during aerosolization and air sampling leading to the loss of their structural integrity, it becomes highly likely that genomic DNA from the ruptured cells is released into the liquid. If this DNA-rich liquid is discarded and not included as part of sample analysis, qPCR performed only on DNA extracted from the pelleted cells would lead to an underestimation of the collected bioaerosol quantity, resulting in an underestimation of their airborne concentration as well.

Thus, the goal of this study was to investigate the release of DNA as extracellular molecules by membrane-damaged microorganisms due to the stress imposed on them during aerosolization and air sampling. To the best of our knowledge, this is the first study to investigate such an effect. The tests were performed with four bioaerosol sampling devices: the Button Aerosol Sampler (SKC Inc., Eighty Four, PA), an Anderson-type impactor (BioStage, SKC Inc.), the BioSampler (SKC Inc.), and a newly developed Electrostatic Precipitator with Superhydrophobic Surface (EPSS) (31-33). We

also assessed the effect of various aerosolization and air sampling parameters on cell integrity and DNA release, including the material of the Collison nebulizer jar (either glass or polycarbonate), the sampling time when collecting on a filter using the Button Aerosol Sampler, the jet-to-plate distance and jet velocity of an Anderson-type impactor, and the type of collection fluid used in the BioSampler. The tests were performed with both gram-negative *E. coli* and gram-positive *Bacillus atrophaeus* bacteria to investigate how different cell wall structures withstand the aerosolization and air sampling stress. It is hoped that the results of this study will provide guidance for selecting appropriate sampling and aerosolization protocols so that the physiological status of bioaerosols is minimally affected, leading to a more accurate sample analysis in bioaerosol studies.

2.3 Materials and Methods

2.3.1 Test Microorganisms

The sensitive gram-negative bacterium *E. coli* (ATCC 15597) and the hardy gram-positive bacterium *B. atrophaeus* (ATCC 49337) were selected as test microorganisms. These two organisms have been widely used in bioaerosol research to represent bacteria with different cell wall types and levels of hardiness (15, 18, 25, 34-36). Both organisms were cultivated on nutrient agar (Becton, Dickinson and Company, Sparks, MD) and stored at 4°C for less than three months prior to transfer. Prior to experiments, *E. coli* and *B. atrophaeus* were pre-cultured in nutrient broth (Becton, Dickinson and Company, Sparks, MD) for 18 hours at 37°C and 30°C, respectively. After growing for 18 hours, both bacterial cultures were in stationary phase, and over 99% of *B. atrophaeus* were present as vegetative cells as verified by Schaeffer-Fulton method for staining endospores (37). All freshly prepared test organisms were washed 3 times with sterile, deionized (DI) water (Millipore, Billerica, MA) by centrifugation at 7000×g for 5 min at 4°C (BR4; Jouan, Winchester, VA). The concentrated bacterial cells were then diluted with sterile DI water to prepare a final bacterial suspension with a concentration ranging from 1–3×10^8 cells/ml, as determined by microscopy.

2.3.2 Experimental Setup

A schematic of the experimental setup is presented in Figure 2.1. Bacterial suspensions were aerosolized using a three-jet Collison nebulizer (BGI Inc., Waltham, MA) with either a glass or polycarbonate jar by passing HEPA-filtered air at a flow rate Q_{AER} = 4 L/min (pressure of 12 psi). The relatively low aerosolization pressure and flow rate were

chosen to minimize potential damage to the bacterial cells due to mechanical stress during aerosolization (24). The aerosolized test organisms were diluted with HEPA-filtered air at a flow rate Q_{DIL} = 80 L/min and passed through a flow-laminarizing honeycomb inside the test chamber. For tests with the EPSS, a 2-mCi Po-210 charge neutralizer was placed in the air stream before it entered the test chamber. During each test, the concentration of airborne microorganisms inside the chamber was monitored continuously by an Aerodynamic Particle Sizer (APS 3321, TSI Inc., Shoreview, Minnesota, USA). The initial volume of freshly prepared culture within the Collison nebulizer was 20 ml during each experiment. The bioaerosol generated was collected by four air sampling devices, as described below. For each repeat with a particular sampling device, a fresh batch of the test bacteria was used. Samples were collected for 5 minutes immediately after starting the aerosolization to minimize cell damage. At least three repeats were conducted for each sampling device or sampling parameter. All experiments were performed inside a Class II Biosafety cabinet (NUAIRE Inc., Plymouth, MN). Humidity and temperature inside the cabinet were monitored by a traceable hygrometer (Fisher Scientific, Pittsburgh, PA) during each test. The relative humidity (RH) ranged between 40%-45% depending on a day, while the temperature stayed in 24-26 °C range.

2.3.3 Bioaerosol Collection System

The aerosolized microorganisms were collected using a BioStage Impactor (SKC Inc., Eighty Four, PA), a Button Aerosol Sampler (SKC Inc.), a BioSampler (SKC Inc.), or an EPSS as shown in Figure 2.1.

A BioStage impactor was used to investigate the effect of impaction on cell integrity (Figure 2.1A). We found the recovery of DNA from agar plates to be extremely low (a few percent, data not shown). Thus, to facilitate effective recovery of collected bacteria and extracellular DNA for analysis by qPCR we used a sheet of aluminum foil positioned on an adjustable support pad inside a Petri dish instead of agar as our collection surface. Since our previous study showed that jet velocity and jet-to-plate distance affect the collection efficiency of microbial impactors (38), we investigated whether these two factors affect the integrity of *E. coli* cell structure as well. Thus, the impactor was operated for 5 min at different combinations of flow rate Q_A (nominal flow rate of 28.3 L/min with jet velocity of 23.7 m/s or increased flow rate of 47 L/min with jet velocity of 39.3 m/s) and simulated agar volume (40 ml with jet-to-plate distance of 2.2 mm or 48 ml with jet-to-plate distance of 1.3 mm). To achieve the desired flow rates, two vacuum pumps were connected to the impactor using a Y-splitter (Figure 2.1A). A Millipore filter holder (Millipore, Billerica, MA) with 0.45-µm pore size polycarbonate filter (Millipore) was connected at the inlet of one of the vacuum pumps to collect those particles that were smaller than the impactor's cut-off size (d_{50} = 0.6 µm as per manufacturer) or escaped due to bouncing from the aluminum foil surface. The total number of such particles was calculated based on the flow rate proportion of the sampling pumps. We were not able to connect the filter directly at the impactor's outlet due to a substantial pressure drop across the impactor and filter. After sampling, particles collected on the aluminum foil and filters were eluted by vortexing the collection media for 2 min in 10 ml and 5 ml of elution solution, respectively.

To investigate the effect of filter collection on bioaerosol cell integrity, a Button Aerosol Sampler was selected as a filter holder and used with a 0.6-μm pore size polycarbonate filter (Millipore, Billerica, MA). The sampler was operated at a flow rate of Q_B = 7.5 L/min, the maximum flow rate achieved in our setup (Figure 2.1B). The Button Aerosol Sampler is designed to operate at 4 L/min so that its inlet aspiration efficiency follows the inhalable sampling convention (39), but has been used with flow rates as high as 10 L/min (40), and the use of 7.5 L/min flow rate instead of the nominal 4 L/min allowed us to collect more bacteria in a short time. The effect of sampling time on cell membrane integrity was investigated by using two different sampling protocols: 1) sampling of aerosolized *E. coli* for 5 minutes; 2) sampling of aerosolized *E. coli* for 5 minutes followed by the passing of particle-free air though the filter for 2 hours. Once the sampling was completed, the filter was removed from the sampler and placed into a 10-ml Tween mixture solution containing 0.1% peptone (Fisher, Fair Lawn, NJ), 0.01% Tween 80 (Fisher, Fair Lawn, NJ), and 0.005% Antifoam Y-30 (Sigma, St. Louis, MO) (18). Vortexing is generally accepted as an efficient way to elute bioaerosol particles from filters (41, 42), and our preliminary experiments showed that short-term vortexing (<2 min) did not result in significant release of DNA from freshly grown *E. coli* cells (data not shown). It was also shown that the use of ultrasonic agitation after vortexing improves the recovery of samples (15), but we were concerned that ultrasonic agitation might affect cell membrane integrity. To investigate this effect, some samples collected on filters were eluted by vortexing for 2 minutes while the rest were first vortexed for 2 minutes and then further treated with ultrasonic agitation for five minutes, and the membrane integrity between the two methods was compared.

An SKC BioSampler with a 5-ml sampling cup was operated at a flow rate of Q_C = 12.5 L/min during each 5-minute sampling period. A 0.45-µm pore size polycarbonate filter (Millipore) was connected to the BioSampler outlet to collect those particles that were either not collected or reaerosolized (Figure 2.1C). Two types of collection fluid, sterile DI water and Tween mixture solution (0.1% peptone, 0.01% Tween80 and 0.005% antifoam), which are commonly used in bioaerosol sampling (15, 18, 34, 43), were tested. During the BioSampler's operation, the bacteria were first impinged into the collection liquid and then subjected to centrifugal motion for the remainder of the sampling period. To test only the effect of centrifugal motion on the membrane integrity of bacteria, 5 ml of sampling fluid was spiked with a known number of *E. coli*, placed into a collection cup, and particle-free air at 12.5 L/min was aspirated by the sampler for 5 min. In the control group, 5 ml of sampling fluid with the same number of *E. coli* bacteria was placed into a collection cup and kept static for 5 min.

A novel electrostatic precipitator with a superhydrophobic surface (EPSS) has been designed in our laboratory and was used as the fourth sampling device (Figure 2.1D). The device is described in detail elsewhere (32, 33), but briefly, it has the shape of a closed half-cylinder positioned at an angle to the horizontal, where the round top part contains an ionizer, while the flat bottom plate holds a narrow collection electrode covered by a superhydrophobic substance positioned slightly below the surface. Particles that enter the sampler are electrically charged and then deposited onto the collection electrode by electrostatic forces. Once the sampling is completed, a 40-µl water droplet is introduced at the top of the collection electrode. Due to gravitational force, the droplet rolls down and gathers the deposited particles. The droplet containing the particles is

collected in a vial and then diluted by adding 960 µl of sterile deionized water for subsequent analysis. The EPSS was operated at a flow rate of Q_D = 10.0 L/min for 5 min. A Millipore filter holder (Millipore) with 0.45-µm pore size polycarbonate filter (Millipore) was placed downstream of the EPSS to collect those particles that were not captured.

The four sampling devices were also used to collect the gram-positive bacterium *B. atrophaeus* to test the effect of sampling method on their membrane integrity. The experimental conditions were chosen only to assess the stress due to one of the four tested collection methods but without considering other variables such as sampling time, filter elution with ultrasonic agitation, and the use of Tween mixture. Thus, the aerosolized *B. atrophaeus* were collected under the following experimental conditions: 1) the Button Aerosol Sampler with filter was operated for 5 minutes and the collected bacteria were eluted from the filter by vortexing for 2 minutes; 2) the BioStage Impactor was operated for 5 minutes with a jet velocity of 39.3 m/s and jet-to-plate distance of 1.3 mm; 3) the BioSampler with 5 ml of DI water was operated for 5 minutes at a sampling flow rate of 12.5 L/min; 4) the EPSS was operated for 5 minutes at a sampling flow rate of 10.0 L/min.

2.3.4 Cell Membrane Damage Index

To assess cell membrane damage during aerosolization and air sampling, 1 ml of solution taken from a liquid sample or sample eluted from a filter was centrifuged at 16,100×g for 5 minutes at 4°C. Then, 950 µl of supernatant liquid was carefully transferred into a new 1.5-ml centrifuge tube by gentle pipetting, while the remaining 50 µl liquid containing

pellet was mixed with 950 µl of sterile DI water. We assumed that in each sample, the DNA in the supernatant liquid originated from the bacterial cells that lost membrane integrity, while the DNA in a pellet represented cells that maintained membrane structure.

In order to validate our assumption, experiments were carried out in triplicates by spiking a known quantity of either freshly grown *E. coli* cells (7×10^7), or *E. coli* genomic DNA (6.5×10^6 copies of *E. coli* genome) or their mixture into 1 ml of sterile DI water. After vortexing for 30 seconds, the samples were processed using the same procedures as described above. The *E. coli* cells in the pellet and DNA in the supernatant liquid were quantified by microscopic counting and qPCR, respectively. When the *E. coli* cells and *E. coli* genomic DNA were spiked separately, their recovery was 101.0 ± 6.7% and 97.1 ± 10.9%, respectively. When they were spiked together into the same 1 mL sterile DI water sample, the recoveries for *E. coli* cells and *E. coli* genomic DNA were 106.7 ± 11.2% and 117.8 ± 18.9%, respectively. This indicates that extracellular DNA in the supernatant liquid could be efficiently separated from the DNA in the pellet cells by our method.

The extent of membrane damage for different sampling conditions was calculated as the cell membrane damage index (I_D): the ratio of 16S rRNA gene copies in the supernatant liquid to the entire number of 16S rRNA gene copies in the sample:

$$\mathrm{I_D} = \frac{\mathrm{N_s}}{\mathrm{N_s}+\mathrm{N_p}} \qquad (1)$$

where: N_S (#/ml) is the number concentration of target 16S rRNA gene copies in the supernatant phase of the liquid sample after centrifugation, as determined by qPCR; N_P (#/ml) is the number concentration of target 16S rRNA gene copies in the pellet sample determined using cell counts from epifluorescence microscopy and the number of 16S

rRNA genes for a specific bacterial genome. The determination of N_S and N_P are described below. Depending on the stress that the bacteria experience, the I_D value could range from 0–1, with higher values indicating more damage.

When sampling bacteria with the BioStage impactor, particles smaller than the impactor's cut-off size (d_{50} = 0.6 μm as per manufacturer) as well as particles that bounced off the collection surface (aluminum foil) escaped the impactor and were collected on the filter mounted at the inlet of one of the vacuum pumps (Figure 2.1A). Therefore, when determining N_S and N_P, we considered that the bacteria and their fragments collected not only on the aluminum foil in the impactor but also on the pump filter:

$$\mathrm{N_s} = \frac{\mathrm{C_{s\_filter}} \times \mathrm{V_{filter}}}{\eta} + \mathrm{C_{s\_foil}} \times \mathrm{V_{foil}} \qquad (2)$$

$$\mathrm{N_p} = \left(\frac{\mathrm{C_{p\_filter}} \times \mathrm{V_{filter}}}{\eta} + \mathrm{C_{p\_foil}} \times \mathrm{V_{foil}}\right) \times \mathrm{n} \qquad (3)$$

where C_{s_filter} (#/ml) and C_{s_foil} (#/ml) are the number concentrations of target 16S rRNA gene copies in the supernatant phase of liquid samples eluted from filter and aluminum foil, respectively. C_{p_filter} and C_{p_foil} are the number concentrations of intact cells in the resuspended pellet samples from filter and aluminum foil, respectively. V_{filter} and V_{foil} are the solution volumes into which the bacteria were eluted from the filter and aluminum foil (5 ml and 10 ml, respectively). n is the number of target gene copies per cell; η is the air flow fraction passing through the filter:

$$\eta = \frac{\mathrm{Q_{filter}}}{\mathrm{Q_A}} \qquad (4)$$

where Q_{filter} (L/min) is the flow rate though the filter; Q_A (L/min) is the BioStage impactor sampling flow rate as shown in Figure 2.1A. Both Q_{filter} and Q_A were measured by a mass flow meter (TSI Inc. Shoreview, MN).

2.3.5 Counting by Microscopy

The number concentration of bacteria in liquid or resuspended cell pellets was determined by epifluorescence microscopy using an Axioskop 20 microscope (Carl Zeiss Inc., Thornwood, NY), according to a previously published method (25). Depending on the initial concentration of bacteria in each sample, a dilution factor was chosen to yield 10– 40 stained bacteria per microscope view field. At least 40 random fields were counted for each sample and the number concentration of bacteria, $C_{Bacteria}$ (#/ml), was calculated as:

$$C_{Bacteria} = \frac{N \times X \times F}{V} \qquad (5)$$

where N is the average number of bacteria per microscope view field, X is the number of fields for the entire filter, F is the dilution factor, and V is the volume of liquid sample used to prepare the microscope slide (ml). N_p (#/ml) was then determined as:

$$N_{p=}C_{Bacteria} \times n \qquad (6)$$

where n is the number of target gene copies per cell (n = 7 for both *E. coli* and *B. atrophaeus;* GenBank accession numbers NC_010473.1 and NC_014639, respectively).

2.3.6 DNA Extraction and Quantitative PCR

Quantitative PCR was performed on an iCycler iQ5 RT-PCR Detection System (Bio-Rad Laboratories, Hercules, CA). As in previous studies, the universal primer pairs (forward: 5'-TCCTACGGGAGGCAGCAGT-3'; reverse: 5'-GGACTACCAGGGTATCTAATCCTGTT-3') for the bacterial 16S rRNA gene were selected with a target amplicon size of 466 bp and 467 bp for *E. coli* and *B. atrophaeus*, respectively (25, 44). Reaction mixtures were prepared by combining 10 µl of 2×SYBR Green supermix (Bio-Rad Laboratories, Hercules, CA), 2 µl of each 2.5-µM primer, 5 µl of template DNA, and 1 µl PCR-grade water to a total volume of 20 µl for each reaction. The amplification reaction was performed with an iCycler iQ thermal cycler (Bio-Rad Laboratories, Hercules, CA) using the following temperature program: 10 min of denaturation at 95°C; 40 cycles of 15 s of denaturation at 95°C and 1 min of annealing/extension at 60 °C. Data analysis was performed using iCycler iQ Real-Time detection system software. After completing PCR amplification in each reaction, a melting curve test was made to check the purity of the generated amplicons.

To prepare standard curves for qPCR reaction, a batch of freshly harvested cells was first quantified by epifluorescence microscopy and genomic DNA was extracted from a known number of cells using the DNeasy Blood & Tissue Kit protocol (Qiagen, Valencia, CA) for qPCR quantification. Standard curves were prepared by plotting each cycle threshold (C_T) value against the log of target gene copy number (Eq. 6). The number of 16S rRNA gene copies present in the supernatant liquid (*Ns*) was determined by purifying the DNA fragments present in the supernatant according to the DNeasy Blood & Tissue Kit protocol (Qiagen, Valencia, CA), performing qPCR, and applying the standard curves.

2.3.7 Collection of Environmental Air Samples

An air sample was collected inside an equine facility at the Rutgers Equine Science Center in New Jersey. Three samplers, one Button Aerosol Sampler and two BioSamplers, were operated concurrently for two hours inside a stall with no horse present. The samplers were placed 0.6 m above the stall bedding. The Button Aerosol Sampler was used with a 0.6-µm pore size polycarbonate filter (25 mm diameter, Millipore, Billerica, MA) to collect an air sample at a flow rate of 6.5 L/min. Two SKC BioSamplers with 5-ml sampling cups were operated at a flow rate of 12.5 L/min for 2 hours. One BioSampler used sterile DI water and another used the Tween mixture solution as collection liquid. Due to liquid evaporation during sampling, the collection liquid was refilled to 5 ml every 15 minutes for both BioSamplers. After sampling, filters and liquid samples were immediately placed in a cooler, transported within minutes to the laboratory and immediately processed as follows.

Particles collected on a filter were eluted by vortexing for 2 minutes in 5 ml sterile DI water. Liquid suspensions from each BioSampler were transferred into 50 ml sterile tubes. Then, 5 ml sterile DI water or Tween mixture was added to each BioSampler, and it was vigorously shaken for 15 s to remove any particles that remained on inner walls of each sampler. Liquid suspensions from the second wash were then combined with the initial samples. Then, 1 ml liquid was taken from the pooled sample from each sampler for subsequent analysis. Specifically, the samples were centrifuged at 16,100×g for 5 minutes at 4°C, and 950 µl supernatant liquid was transferred to a new 1.5-ml centrifuge tube by gentle pipetting. The DNA in the pellet sample and supernatant liquid were extracted and purified by using the DNeasy Blood & Tissue Kit (Qiagen,

Valencia, CA). The copy numbers of 16S rRNA gene in each sample were then determined by qPCR.

2.3.8 Statistical Analysis

Statistical analysis was performed using Statistica software version 10.0 (StatSoft Inc., Tulsa, OK). Factorial ANOVA was used to analyze the I_D as a function of the Collison nebulizer jar material, bacterial species, and aerosolization time. For samples collected by the Button Aerosol sampler and BioStage Impactor, factorial ANOVA was performed to analyze I_D as a function of the sample collection/filter elution method and jet-to-plate distance/jet velocity, respectively. For samples collected by the BioSampler, single-factor ANOVA was conducted to analyze the I_D as a function of the collection fluid type. For each sample collection device, student's *t*-test was applied to compare the results between *E. coli* and *B. atrophaeus*. For each bacterial species, comparisons between the EPSS and the other three collection devices were made with student's *t*-test. For all tests, a statistically significant difference was defined as $p<0.05$.

2.4 Results

2.4.1 Aerosolization by Collison Nebulizer

Figure 2.2 presents the cell membrane damage index (I_D) for *E. coli* bacteria as a function of aerosolization time. When pure *E. coli* culture was suspended in water for 0–60 min without aerosolization, the I_D stayed below 0.01, showing no significant effect of time (p=0.16). When the Collison nebulizer was in operation, the I_D of the *E. coli* culture in the nebulizer's reservoir exhibited a clear increase over time, and the increase depended on the Collison jar material (glass vs. polycarbonate). For the Collison nebulizer with a glass jar, the I_D values were 0.003 ± 0.002, 0.060 ± 0.022, 0.085 ± 0.042, and 0.142 ± 0.056 for 0, 10, 30, and 60 min of nebulization time, respectively. When a polycarbonate jar was used, the I_D values were 0.003 ± 0.002, 0.048 ± 0.022, 0.073 ± 0.038, and 0.085 ± 0.027 for 0, 10, 30, and 60 min of nebulization time, respectively. For each aerosolization time >0 min, the I_D values for a polycarbonate jar were significantly lower than with a glass jar (p<0.001). In order to minimize the mechanical stress imposed on *E. coli* bacteria during aerosolization in subsequent experiments, we chose to use a Collison nebulizer with the polycarbonate jar and an aerosolization time of 5 min.

2.4.2 Sampling by Filtration

The I_D values of samples collected by filtration using a Button Aerosol Sampler are shown in Figure 2.3. In Method A, the bacteria were collected on a filter for 5 minutes and then eluted from it by either vortexing for 2 minutes or vortexing for 2 minutes followed by 5 minutes of ultrasonic agitation. The I_D value was 0.051 ± 0.014 when only vortexing was used, but it increased to 0.063 ± 0.019 when ultrasonic agitation was

applied after vortexing. In Method B, the bacteria were sampled for 5 min and then particle-free air was pulled through the filter for 2 hours to test the effect of extended sampling time on cell membrane integrity. In this case, the I_D value for samples treated by vortexing was 0.058 ± 0.018, while the combination of vortexing and ultrasonic agitation increased the I_D to 0.085 ± 0.022. For both filter elution methods, I_D values were greater after the exposure of collected bacteria to particle-free air for 2 hours compared with I_D values without such exposure. Factorial ANOVA analysis showed that both sampling and filter elution methods had significant effects on the I_D of *E. coli* bacteria: p=0.007 for the sampling method and p=0.001 for the filter elution method. No significant interaction between these two factors was found (p=0.189).

2.4.3 Sampling by Impaction

Two factors, jet velocity and jet-to-plate distance, were investigated for their effects on cell membrane integrity of *E. coli* collected by a BioStage impactor. The standard air sampling flow rate of 28.3 L/min resulted in a jet velocity of 23.7 m/s, while a higher sampling flow rate of 47 L/min yielded a jet velocity of 39.3 m/s. Two simulated agar volumes of 48 ml and 40 ml yielded jet-to-plate distances of 1.3 and 2.2 mm, respectively. As shown in Figure 2.4, a jet velocity of 23.7 m/s with a jet-to-plate distance of 2.2 mm resulted in an I_D value of 0.159 ± 0.008. However, the I_D increased to 0.175 ± 0.017 when the jet velocity was increased to 39.3 m/s for the same jet-to-plate distance. When the jet velocity was maintained at 23.7 m/s but the jet-to-plate distance was decreased from 2.2 mm to 1.3 mm, the I_D increased from 0.159 ± 0.008 to 0.205 ± 0.010. The highest I_D was observed when the jet velocity was increased to 39.3 m/s at a lower jet-to-plate distance of 1.3 mm, where the value reached as high as 0.368 ± 0.009. According to

the factorial ANOVA analysis, both jet-to-plate distance and jet velocity had a significant effect on cell membrane damage (p<0.001), and there was also a significant interaction between these two factors (p<0.001).

2.4.4 Sampling by Impingement

In experiments with the BioSampler, a filter was placed at the sampler's outlet to capture particles or their fragments that were not collected or were reaerosolized and escaped the sampler. Neither extracellular DNA nor intact whole cells were detected on those filters, indicating that the particle escape rate was low and thus could be neglected in our study. This finding was consistent with other studies (34, 45). An effect of collection fluid type (sterile DI water or Tween mixture) on I_D values after a 5-min collection time is shown in Figure 2.5. When sterile DI water was used, the I_D was 0.068 ± 0.029. However, when Tween mixture was used, the value increased to 0.234 ± 0.088, and the increase was statistically significantly (p=0.009).

In order to further assess the effect of collection fluid on the cell integrity of *E. coli* bacteria, a known number of *E. coli* from a fresh culture was added into BioSampler cups filled with either 5 ml of DI water or 5 ml of Tween mixture. The solutions were kept static for 5 min and then aliquots of liquid samples were taken out for analysis. As shown in Figure 2.5, the I_D value was 0.008 ± 0.002 for *E. coli* suspended in DI water, but increased to 0.012 ± 0.002 for cells suspended in Tween mixture (p=0.01). In the next step, a known number of *E. coli* from a fresh culture was added into two 5-ml collection cups filled with DI water or Tween mixture and then two BioSamplers aspirated particle-free air at 12.5 L/min for 5 min. As a result, the I_D was found to be 0.014 ± 0.005 for the

sample that was suspended in DI water, but increased significantly to 0.066 ± 0.004 for the sample that was suspended in Tween mixture (p<0.001). Also, when particle-free air was aspirated into the BioSampler, both I_D values were significantly higher than those when the BioSampler sampling cups were kept static (p=0.034 for DI water and p<0.001 for Tween Mixture) but significantly lower than the I_D values when the aerosolized *E. coli* were actively collected by the BioSampler (p=0.008 for DI water and p=0.004 for Tween Mixture).

2.4.5 Sampling by Electrostatic Precipitation

The I_D value for *E. coli* bacteria collected by the EPSS was 0.016 ± 0.016. Similar to the results with the BioSampler, neither the extracellular DNA nor intact *E. coli* cells were detected on the filter downstream of the sampler. The result indicates that few particles escaped from the EPSS, which was consistent with a previously demonstrated high collection efficiency for this newly designed sampler (31, 32).

2.4.6 Aerosolization of the Gram-positive Bacterium *B. atrophaeus*

Figure 2.6 shows the effect of aerosolization time on the extent of cell membrane damage of a *B. atrophaeus* culture suspension in the Collison nebulizer. When using a glass jar, the I_D values were 0.004 ± 0.002, 0.043 ± 0.001, 0.040 ± 0.007, and 0.031 ± 0.007 for 0, 10, 30, and 60 min of aerosolization, respectively. However, when using a polycarbonate jar, the I_D values were significantly lower for the same aerosolization times: 0.001 ± 0.001, 0.005 ± 0.001, 0.008 ± 0.005, and 0.013 ± 0.009 for 0, 10, 30, and 60 min of aerosolization, respectively (p<0.001). These results were similar to the findings for *E. coli* bacteria, supporting our conclusion that the polycarbonate jar induces less damage to

cell membranes than the glass jar. For comparison, the I_D values for *B. atrophaeus* bacteria kept in a liquid reservoir without aerosolization were 0.000 ± 0.000, 0.000 ± 0.000, 0.001 ± 0.000, and 0.002 ± 0.000 for 0, 10, 30, and 60 min, respectively. When the two bacterial species were compared, the I_D values for *B. atrophaeus* bacteria were significantly lower than those of *E. coli* bacteria when using either glass jar (p<0.001) or polycarbonate jar (p<0.001).

2.4.7 Collection of *B. atrophaeus* by Four Different Collection Devices

The cell membrane damage index of *B. atrophaeus* bacteria was compared with that of *E. coli* when collected with the four tested devices. The sampling conditions and results are presented in Figure 2.7. The I_D values for *B. atrophaeus* ranged from 0.002 ± 0.003 when sampled with the EPSS to 0.052 ± 0.008 when sampled with the BioSampler. The I_D values for *E. coli* ranged from 0.016 ± 0.016 when sampled with the EPSS to 0.368 ± 0.009 when sampled with the BioStage impactor. According to the *t*-test, there was no significant difference between I_D values for *E. coli* and *B. atrophaeus* when using Button Aerosol Sampler (p=0.961), BioSampler (p=0.234), or EPSS (p=0.213). However, the I_D value for *B. atrophaeus* was significantly lower than that for *E. coli* when using the BioStage Impactor (p<0.001).

Among the four sampling devices, the EPSS showed the lowest average I_D value for both microorganisms. For *E. coli*, this result was statistically significant for all samplers: the BioSampler (p=0.041), the Button Aerosol Sampler (p=0.026), and the BioStage Impactor (p=0.014). For *B. atrophaeus*, the I_D value when sampling with the EPSS was significantly lower than when using the BioSampler (p=0.010) and Button

Aerosol Sampler (p=0.001), but not significantly different compared with the BioStage Impactor (p=0.109).

2.4.8 Detection of Extracellular DNA in Environmental Aerosol Samples

Quantities of DNA in environmental samples were determined by qPCR using *E. coli* 16S rRNA gene as standard curve. The PCR efficiencies of the *E. coli* 16S rRNA gene and the environmental samples were between 90% and 105%, and no inhibitor effect was observed. The qPCR results were converted to number of bacteria per m^3 assuming four 16S rRNA gene copies per bacterial genome (46). When only the pellet sample was considered, the airborne bacteria concentration inside the equine facility was found to be 2.8×10^6, 5.8×10^6 and 1.4×10^7 bacteria/m^3 for samples collected by BioSampler with Tween mixture, BioSampler with water and Button Aerosol Sampler, respectively. A comparable amount of DNA was detected in all three supernatant liquid samples, the 16S rRNA gene copy numbers of which were determined to be 1.2×10^6, 6.7×10^6 and 1.5×10^7 bacteria/m^3 for samples collected by BioSampler with Tween mixture, BioSampler with water and Button Aerosol Sampler, respectively. Accordingly, the I_D values were calculated to be 0.30, 0.54 and 0.52 for samples collected by the three devices, respectively.

2.5 Discussion

Numerous studies have reported that bacteria experience stress during aerosolization and collection due to mechanical forces (19, 24) and, possibly, exposure to dry air (15, 16). Bacteria that sustain sublethal injury could easily become viable but non-culturable or even lose their viability (24, 47, 48). Here we demonstrate for the first time that, under certain conditions, the stress of aerosolization and the air sampling process is strong enough to break cell membranes and release the genomic DNA as extracellular molecules. Furthermore, we introduce the concept of the cell membrane damage index (I_D) to reflect the magnitude of the membrane damage that was experienced by the bacteria. The I_D value could range from "0" indicating no damage to "1" indicating that all bacteria in a sample had lost their cell membrane integrity. This index could be used as an indicator of the physiological status to the collected bacteria, and it could also provide a useful way to evaluate sampling protocols and adjust the design parameters of bioaerosol samplers, with the goal of minimizing damage to bioaerosol samples.

The Collison nebulizer has been widely used to generate bioaerosols in laboratory experiments, even though studies suggested that the recirculation of culture suspension exerts a strong stress on the bacteria due to shear forces and impaction onto the inside wall of the container (24, 49). As a result, the loss of culturability and fragmentation of cells were frequently observed for the aerosolized bacteria (23, 49, 50). In a recent study, the cell membrane was suggested as a major site of damage during aerosolization by the Collison nebulizer (24). Bacterial cells under aerosolization stress were discovered to have a loss of respiratory enzymatic activities, membrane depolarization, or even loss of membrane integrity (24). Here, we confirm that the Collison nebulizer can cause severe

damage to the bacterial cell membrane and report that the release of genomic DNA was observed, presumably due to the mechanical stress of shear force and wall impaction. Interestingly, our findings show that the Collison nebulizer container made of polycarbonate material induces less stress on bacterial cultures than glass. We speculate that a greater amount of the kinetic energy of the impacting bacteria is transferred to the polycarbonate material than with glass, presumably due to the greater deformation of polycarbonate material (51). Accordingly, when impacting onto the polycarbonate surface, less of the remaining energy acts back onto the biological particle, thus resulting in less damage.

Jet-to-plate distance and jet velocity are two important factors that determine the collection efficiency of impaction-based aerosol samplers. Our experiments with the BioStage impactor demonstrated that an increase in jet velocity and decrease in jet-to-plate distance result in the increase of the cell membrane damage index. The experiments also showed that *E. coli* bacteria experience more stress than *B. atrophaeus*. Considering that viability of bacteria is highly correlated with the integrity of the cell membrane, our results confirm an earlier study that suggested that jet-to-plate distance and jet velocity affect the culturability of microorganisms collected by impaction (38). According to that study, an increase in jet-to-plate distance leads to the dissipation of air jets, which means lower jet velocity and impaction of bacteria onto the collection surface with lower kinetic energy. Since the recovery of DNA from agar plates was found to be extremely low, aluminum foil was used as collection surface to facilitate effective recovery of collected bacteria and extracellular DNA. As a result, a large fraction of impact energy was transferred back to the bacteria, causing damage to their membranes. When bacteria are

collected onto a semi-solid surface like agar instead of a hard surface, damage to the cell membrane is likely to be lower, since the agar will absorb some of the impact energy. On the other hand, even for collection on agar using impaction, the damage to bacteria is still considerable, as demonstrated by earlier studies (19).

The stress on bacteria due to collection by the BioSampler comes from a variety of sources, including, but not limited to, the impingement of bacteria into the collection fluid, particle bounce and reaerosolization due to high-speed centrifugal motion, and the possible detrimental effect of substances present in the collection fluid. First, we hypothesized that the Tween mixture might be toxic to *E. coli* and thus decrease cell membrane integrity. This was demonstrated by a higher I_D for *E. coli* bacteria that was added into the Tween mixture and kept static compared with I_D for bacteria that was added into sterile deionized water and also kept static. An antifoam agent present in the Tween mixture has been found to reduce the growth of *Helicobacter pylori* (52) and *Hyphomicrobium zavrzinii ZV 580* (53). Moreover, our finding was similar to that of a previous study showing that DI water preserved the viability of *L. pneumophila* better than the Tween mixture when sampling by BioSampler (18). Second, centrifugal motion during the sampling process could add to the damage to the cell structure. It has been shown that liquid loss during BioSampler operation increases the chance of particle bounce and reaerosolization, which adds extra stress to the bacterial cells and impairs their membrane structure (18, 34). In our study, after five minutes of sampling, the Tween mixture lost 1.3 ml of volume, which was greater than that of DI water (0.9 ml), based on the initial volume of 5 ml for both fluids. This greater volume loss of Tween mixture compared with DI water and the resulting increase in particle bounce could

partially explain the much higher I_D value observed for the Tween mixture compared with DI water. Nonetheless, the I_D value when the particle-free air was aspirated into the BioSampler cup containing *E. coli* culture was still much lower than the value observed when actively collecting airborne *E. coli* bacteria using the BioSampler, indicating additional stress from either the aerosolization process, impingement, or, most likely, a combination of both.

In addition to DNA release due to stress from mechanical processes, we also found that a non-mechanical stress like desiccation also facilitated the release of DNA by the impaired bacterial cells. Studies applying filtration for bioaerosol collection have used sampling times ranging from a few minutes to several hours (28, 54) or even as long as 24 hours (55, 56). It has been shown that prolonged sampling periods by impaction-based samplers increase the risk of microorganism viability loss (16, 57, 58). The desiccation of the already collected bioaerosols together with the desiccation of agar media contributed to decreased microorganism recovery (16). We found no reports on the impact of dehydration on cell membrane integrity; however the protein coating of airborne *Gumboro* virus was reported to be damaged at lower humidity level (59). The data presented in our study clearly show that cell membrane rupture is more substantial with prolonged sampling periods, which was demonstrated by higher I_D values for samples exposed to dry air for an extended sampling period in comparison with those without such exposure. Thus, the sampling time should be as short as reasonably possible in order to reduce the desiccation effects on the collected samples when operating both filtration- and impaction-based samplers.

We also found that the filter elution method can contribute to cell membrane damage. The increased recovery of bacteria from filter samples has been reported by adding the extra step of ultrasonic agitation (15), but our findings here show that this treatment can add more stress to the collected bacteria and result in an increased release of DNA. On the other hand, vortexing is generally accepted as an efficient way to elute bioaerosol particles from filters (41, 42), and we found that short-term vortexing (<2 min) did not result in significant release of DNA from freshly grown *E. coli* cells (data not shown). Thus, to minimize the stress on the bacteria collected on the filter, including for molecular analysis techniques, sampling protocols should also consider filter elution methods.

Our findings also showed that for the same aerosolization and air sampling conditions, the gram-positive bacterium *B. atrophaeus* was less susceptible to mechanical stress, such as from impaction and shear forces, compared with the gram-negative bacterium *E. coli*. The higher resistance of *B. atrophaeus* to stress is likely due to its thicker and more rigid peptidoglycan layer, which is responsible for its cell wall strength, compared with more sensitive Gram-negative bacteria (19). This result also suggests that, when sampling ambient microorganisms using inertia-based methods, e.g. impaction, we could selectively enrich gram-positive bacteria over gram-negative bacteria if we apply an enumeration method based on intact cells or do not take into account the released DNA. Consequently, it would bias our information regarding the relative abundance of various bacterial species within the complex airborne microbial community.

When comparing the four bioaerosol sampling devices used in our study, we found that samples collected by our newly developed EPSS had the lowest I_D, followed

by the BioSampler with DI water and the Button Aerosol Sampler when only vortexing was used to extract bacteria. By contrast, *E. coli* bacteria when collected by the BioStage impactor or the BioSampler with Tween mixture seemed to be exposed to greater stress, which was high enough to affect the cell membrane integrity and release as much as one third of the total amount of DNA material. Electrostatic collection results in a low velocity of bacterial deposition onto the collection surface, which is conducive to cell membrane preservation. This particular sampler prototype collects bioaerosols on a superhydrophobic surface and then concentrates them into small volumes of liquid (40 µl or less), thus allowing one to achieve very high concentration rates (31, 33). Our earlier study showed that exposure to strong electrostatic fields while airborne does not induce appreciable cell damage (60). These features of the new electrostatic precipitator—a high sample concentration rate and an ability to maintain cell integrity—should be valuable for bioaerosol detection, especially when high sensitivity and a low detection limit are desired.

It should be noted that *B. atrophaeus* used in our study were mostly vegetative cells, while in the natural environment some gram-positive bacteria, e.g. *Bacillus* and *Clostridium* spp., often exist in spore form (61). Those spores are known to have resistant structures which protect bacteria from unfavorable environmental conditions, e.g. desiccation (62) and mechanical stress (63). In addition, the aerosolized bacteria were mostly single cells as verified by the measured bioaerosol size distribution. However, airborne microorganisms in the natural environment often form aggregates of multiple cells or attach to particulate matter (64). Thus, it was of great interest to test how these factors affect susceptibility of natural bioaerosols to sampling stress. Our samples

collected by a filter sampler and two BioSamplers inside an equine facility for two hours accumulated a sufficient amount of bacteria for subsequent analysis by qPCR. The data showed that extracellular DNA was detected in air samples collected by all three devices. It was rather surprising to find that the amount of extracellular DNA was comparable to or even higher than that in intact cells, depending on the sampling method. While our sampling protocol did not separately determine what fraction of extracellular DNA was captured directly from air and what fraction was released due to sampling stress, our findings do indicate that a substantial amount (~50%) of the DNA in processed sample could be extracellular DNA. This demonstrates that commonly used protocols for bioaerosol sampling and sample processing could underestimate the presence of airborne microbial content in the natural environment by a large fraction (up to 50%).

2.6 Conclusions

Our results strongly suggest that bioaerosol quantification using molecular methods, such as qPCR, should include not only DNA in intact cells, but also DNA released by the cells damaged during aerosolization and air sampling, i.e., the extracellular DNA from the supernatant should not be discarded but should be included in the sample analysis. Otherwise, bioaerosol concentrations might be substantially underestimated. A negative bias of more than 20% was observed when a BioSampler containing Tween mixture sampling solution was used to sample bacteria for only 5 minutes. A negative bias as high as 50% was observed in environmental bioaerosol samples collected and processed by commonly used bioaerosol protocols. It is hoped that this study will provide guidance for selecting bioaerosol aerosolization and sampling methods and their analysis protocols that minimize bioaerosol quantification bias using molecular tools.

2.7 Acknowledgments

The publication was supported by Grant R01-OH009783 “Advanced Sampler for Measuring Exposure to Biological Aerosols” from CDC/NIOSH and Project 07160 funded by the New Jersey Agricultural Experiment Station (NJAES) at Rutgers, The State University of New Jersey. Its contents are solely the responsibility of the authors and do not necessarily represent the official view of the CDC/NIOSH or the NJAES.

References

1. **Burge, H.** 1990. Bioaerosols: Prevalence and health effects in the indoor environment. J. Allergy. Clin. Immunol. **86:**687-701.
2. **Douwes, J., P. Thorne, N. Pearce, and D. Heederik.** 2003. Bioaerosol health effects and exposure assessment: Progress and prospects. Ann. Occup. Hyg. **47:**187-200.
3. **Fung, F., and W. Hughson.** 2003. Health effects of indoor fungal bioaerosol exposure. Appl. Occup. Environ. Hyg. **18:**535-545.
4. **Herr, C. E., A. Zur Nieden, M. Jankofsky, N. I. Stilianakis, R. H. Boedeker, and T. F. Eikmann.** 2003. Effects of bioaerosol polluted outdoor air on airways of residents: a cross sectional study. Occup. Environ. Med. **60:**336-342.
5. **Lee, T., S. A. Grinshpun, D. Martuzevicius, A. Adhikari, C. M. Crawford, and T. Reponen.** 2006. Culturability and concentration of indoor and outdoor airborne fungi in six single-family homes. Atmos. Environ. **40:**2902-2910.
6. **Hirvonen, M. R., M. Ruotsalainen, K. Savolainen, and A. Nevalainen.** 1997. Effect of viability of *actinomycete* spores on their ability to stimulate production of nitric oxide and reactive oxygen species in RAW264.7 macrophages. Toxicology **124:**105-114.
7. **Tang, J. W.** 2009. The effect of environmental parameters on the survival of airborne infectious agents. J. R. Sco. Interface **6:**S697-S702.
8. **Handley, B. A., and A. J. F. Webster.** 1995. Some factors affecting the airborne survival of bacteria outdoors. J. Appl. Bacteriol. **79:**368-378.
9. **Hatch, M. T., and R. L. Dimmick.** 1966. Physiological responses of airborne bacteria to shifts in relative humidity. Bacteriol. Rev. **30:**597-602.
10. **Tong, T., and B. Lighthart.** 1997. Solar radiation has a lethal effect on natural populations of culturable outdoor atmospheric bacteria. Atmos. Environ. **31:**897-900.
11. **Zentner, R. J.** 1966. Physical and chemical stresses of aerosolisation. Bacteriol. Rev. **30:**551-557.
12. **Caron, G. N., P. Stephens, and R. A. Badley.** 1998. Assessment of bacterial viability status by flow cytometry and single cell sorting. J. Appl. Microbiol. **84:**988-998.
13. **Oliver, J. D.** 2005. The viable but nonculturable state in bacteria. J. Microbiol. **43:**93-100.
14. **Willeke, K., and Macher, J. M.** 1999. Air Sampling, p. 11-11--11-25. *In* J. Macher (ed.), Bioaerosols: Assessment and Control. American Conference of Governmental Industrial Hygienists, Cincinnati, OH.
15. **Wang, Z., Reponen, T., Grinshpun, S. A., Gorny, R. L., and Willeke, K.** 2001. Effect of sampling time and air humidity on the bioefficiency of filter samplers for bioaerosol collection. J. Aerosol Sci. **32:**661-674.
16. **Mainelis, G., and M. Tabayoyong.** 2010. The effect of sampling time and the overall performance of portable microbial impactors. Aerosol Sci. Technol. **44:**75-82.

17. **Nevalainen, A., K. Willeke, F. Liebhaber, J. Pastuszka, H. Burge, and E. Henningson.** 1993. Bioaerosol sampling: Aerosol measurement principles, techniques, and applications. Van Nostrand Reinhold, New York.
18. **Chang, C.-W., and F.-C. Chou.** 2011. Assessment of bioaerosol sampling techniques for viable *Legionella pneumophila* by ethidium monoazide quantitative PCR. Aerosol Sci. Technol. **45:**343 - 351.
19. **Stewart, S. L., S. A. Grinshpun, K. Willeke, S. Terzieva, V. Ulevicius, and J. Donnelly.** 1995. Effect of impact stress on microbial recovery on an agar surface. Appl. Environ. Microbiol. **61:**1232-1239.
20. **King, M. D., and A. R. McFarland.** 2011. Bioaerosol sampling with a wetted wall cyclone: Cell culturability and DNA integrity of *Escherichia coli* bacteria. Aerosol Sci. Technol. **46:**82-93.
21. **Zhao, Y., A. J. A. Aarnink, P. Doornenbal, T. T. T. Huynh, P. W. G. Groot Koerkamp, M. C. M. de Jong, and W. J. M. Landman.** 2011. Investigation of the efficiencies of bioaerosol samplers for collecting aerosolized bacteria using a fluorescent tracer. I: effects of non-sampling processes on bacterial culturability. Aerosol Sci. Technol. **45:**423-431.
22. **Zhao, Y., A. J. A. Aarnink, P. Doornenbal, T. T. T. Huynh, P. W. G. Groot Koerkamp, W. J. M. Landman, and M. C. M. de Jong.** 2011. Investigation of the efficiencies of bioaerosol samplers for collecting aerosolized bacteria using a fluorescent tracer. II: sampling efficiency and half-life time. Aerosol Sci. Technol. **45:**432-442.
23. **Mainelis, G., D. Berry, H. R. An, M. S. Yao, K. DeVoe, D. E. Fennell, and R. Jaeger.** 2005. Design and performance of a single-pass bubbling bioaerosol generator. Atmos. Environ. **39:**3521-3533.
24. **Thomas, R. J., D. Webber, R. Hopkins, A. Frost, T. Laws, P. N. Jayasekera, and T. Atkins.** 2011. The cell membrane as a major site of damage during aerosolization of *Escherichia coli*. Appl. Environ. Microbiol. **77:**920-925.
25. **An, H. R., G. Mainelis, and L. White.** 2006. Development and calibration of real-time PCR for quantification of airborne microorganisms in air samples. Atmos. Environ. **40:**7924-7939.
26. **Peccia, J., and M. Hernandez.** 2006. Incorporating polymerase chain reaction-based identification, population characterization, and quantification of microorganisms into aerosol science: a review. Atmos. Environ. **40:**3941-3961.
27. **Schafer, M. P., K. F. Martinez, and E. S. Mathews.** 2003. Rapid detection and determination of the aerodynamic size range of airborne *Mycobacteria* associated with whirlpools. Appl. Occup. Environ. Hyg. **18:**41050.
28. **Chen, P. S., and C. S. Li.** 2005. Quantification of airborne *Mycobacterium tuberculosis* in health care setting using real-time qPCR coupled to an air-sampling filter method. Aerosol Sci. Technol. **39:**371-376.
29. **Pascual, L., S. Perez-Luz, C. Moreno, D. Apraiz, and V. Catalan.** 2001. Detection of *Legionella pneumophila* in bioaerosols by polymerase chain reaction. Can. J. Microbiol. **47:**341-347.
30. **Wilson, K. H.** 2002. High-density microarray of small subunit ribosomal DNA probes. Appl. Environ. Microbiol. **68:**2535-2541.

31. **Han, T., Y. Nazarenko, P. J. Lioy, and G. Mainelis.** 2011. Collection efficiencies of an electrostatic sampler with superhydrophobic surface for fungal bioaerosols. Indoor Air **21:**110-120.
32. **Han, T., and G. Mainelis.** 2008. Design and development of an electrostatic sampler for bioaerosols with high concentration rate. J. Aerosol Sci. **39:**1066–1078.
33. **Han, T., H. R. An, and G. Mainelis.** 2010. Performance of an electrostatic precipitator with superhydrophobic surface when collecting airborne bacteria. Aerosol Sci. Technol. **44:**339-348.
34. **Han, T., and G. Mainelis.** 2012. Investigation of inherent and latent internal losses in liquid-based bioaerosol samplers. J. Aerosol Sci. **45:**58-68.
35. **Hospodsky, D., N. Yamamoto, and J. Peccia.** 2010. Accuracy, precision, and method detection limits of quantitative PCR for airborne bacteria and fungi. Appl. Environ. Microbiol. **76:**7004-7012.
36. **Lee, B. U., and S. S. Kim.** 2003. Sampling *E. coli* and *B. subtilis* bacteria bioaerosols by a new type of impactor with a cooled impaction plate. J. Aerosol Sci. **34:**1097-1100.
37. **Gerhardt, P., R. G. E. Murray, W. A. Wood, and N. R. Krieg.** 1994. Methods for general and molecular bacteriology. ASM Press, Washington, DC.
38. **Yao, M., and G. Mainelis.** 2006. Effect of physical and biological parameters on enumeration of bioaerosols by portable microbial impactors. J. Aerosol Sci. **37:**1467-1483.
39. **Aizenberg, V., S. A. Grinshpun, K. Willeke, J. P. Smith, and P. A. Baron.** 2000. Performance characteristics of the button personal inhalable aerosol sampler. Am. Ind. Hyg. Assoc. J. **61:**398-404.
40. **Hauck, B. C., S. A. Grinshpun, A. Reponen, T. Reponen, K. Willeke, and R. L. Bornschein.** 1997. Field testing of new aerosol sampling method with a porous curved surface as inlet. Am. Ind. Hyg. Assoc. J. **58:**713-719.
41. **Wang, Z., Reponen, T., Willeke, K., and Grinshpun, S. A.** 1999. Survival of bacteria on respirator filters. Aerosol Sci. Technol. **30:**300-308.
42. **Burton, N. C., A. Adhikari, S. Grinshpun, R. Hornung, and T. Reponen.** 2005. The effect of filter material on bioaerosol collection of *Bacillus subtilis* spores used as a *Bacillus anthracis* simulant. J. Environ. Monit. **7:**475-480.
43. **Willeke, K., Lin, X., and Grinshpun, S. A.** 1998. Improved aerosol collection by combined impaction and centrifugal motion. Aerosol Sci. Technol. **28:**439-456.
44. **Nadkarni, M. A., F. E. Martin, N. A. Jacques, and N. Hunter.** 2002. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology **148**.
45. **Lin, X., T. A. Reponen, K. Willeke, S. A. Grinshpun, K. K. Foarde, and D. S. Ensor.** 1999. Long-term sampling of airborne bacteria and fungi into a non-evaporating liquid. Atmos. Environ. **33:**4291-4298.
46. **Lee, Z.-P., B. C, and T. M. Schmidt.** 2009. rrnDB: documenting the number of rRNA and tRNA genes in bacteria and archaea. Nucleic Acids Research **37:**D489-D493.

47. **Heidelberg, J. F., M. Shahamat, M. Levin, I. Rahman, G. Stelma, C. Grim, and R. R. Colwell.** 1997. Effect of aerosolization on culturability and viability of gram-negative bacteria. Appl. Environ. Microbiol. **63:**3585-3588.
48. **Colwell, R. R., P. R. Brayton, D. J. Grimes, D. B. Roszak, S. A. Huq, and L. M. Palmer.** 1985. Viable but non-culturable *Vibrio cholerae* and related pathogens in the environment: implications for release of genetically engineered microorganisms. Biotechnology **3:**817-820.
49. **Reponen, T., K. Willeke, V. Ulevicius, S. A. Grinshpun, and J. Donnelly.** 1997. Techniques for dispersion of microorganisms into air. Aerosol Sci. Technol. **27:**405-421.
50. **Mainelis, G., K. Willeke, P. Baron, T. Reponen, S. A. Grinshpun, R. L. Gorny, and S. Trakumas.** 2001. Electrical charges on airborne microorganisms. J. Aerosol Sci. **32:**1087-1110.
51. **Wall, S., W. John, H. C. Wang, and S. L. Goren.** 1990. Measurements of kinetic energy loss for particles impacting surfaces. Aerosol Sci. Technol. **12:**926-946.
52. **Kane, A. V., and A. G. Plaut.** 1996. Unique susceptibility of *Helicobacter Pylori* to simethicone emulsifiers in alimentary therapeutic agents. Antimicrob. Agents Chemother. **40:**500-502.
53. **Jerome, V., M. Hermann, F. Hilbrig, and R. Freitag.** 2007. Development of a fed-batch process for the production of a dye-linked formaldehyde dehydrogenase in *Hyphomicrobium Zavarzinii ZV 580*. Appl. Microbiol. Biotechnol. **77:**779-788.
54. **Wu, Z., G. Blomquist, S. Westermark, and X. R. Wang.** 2002. Application of PCR and probe hybridization techniques in detection of airborne fungal spores in environmental samples. J. Environ. Monit. **4:**673-678.
55. **Maher, N., H. K. Dillon, S. H. Vermund, and T. R. Unnasch.** 2001. Magnetic bead capture eliminates PCR inhibitors in samples collected from the airborne environment, permitting detection of *Pneumocystis carinii* DNA. Appl. Environ. Microbiol. **67:**449-452.
56. **Brodie, E. L., T. Z. DeSantis, J. P. M. Parker, I. X. Zubietta, Y. M. Piceno, and G. L. Andersen.** 2007. Urban aerosols harbor diverse and dynamic bacterial populations. Proc. Nat. Acad. Sci. USA **104:**299-304.
57. **Juozaitis, A., K. Willeke, S. A. Grinshpun, and J. Donnelly.** 1994. Impaction onto a glass slide or agar versus impingement into a liquid for the collection and recovery of airborne microorganisms. Appl. Environ. Microbiol. **60:**861-870.
58. **Hensel, A., and K. Petzoldt.** 1995. Biological and biochemical analysis of bacteria and viruses. *In* C. S. Cox and C. M. Wathes (ed.), Bioaerosols Handbook. Lewis Publishers, New York.
59. **Zhao, Y., A. J. A. Aarnink, R. Dijkman, T. Fabri, M. C. M. de Jong, and P. W. G. Groot Koerkamp.** 2011. Effects of temperature, relative Humidity, absolute humidity, and evaporation potential on survival of airborne Gumboro vaccine virus. Appl. Environ. Microbiol. **78:**1048-1054.
60. **Yao, M. S., G. Mainelis, and H. R. An.** 2005. Inactivation of microorganisms using electrostatic fields. Environ. Sci. Technol. **39:**3338-3344.

61. **Leggett, M. J., G. McDonnell, S. P. Denyer, P. Setlow, and J. Y. Maillard.** 2012. Bacterial spore structures and their protective role in biocide resistance. J Appl Microbiol **113:**485-498.
62. **Cano, R. J., and M. K. Borucki.** 1995. Revival and identification of bacterial spores in 25- to 40-million-year-old Dominican amber. Science **268:**1060-1064.
63. **Jones, C. A., N. L. Padula, and P. Setlow.** 2005. Effect of mechanical abrasion on the viability, disruption and germination of spores of *Bacillus subtilis.* J Appl Microbiol **99:**1484-1494.
64. **Adhikari, A., A. Reponen, S. A. Grinshpun, D. Martuzevicius, and G. LeMasters.** 2006. Correlation of ambient inhalable bioaerosols with particulate matter and ozone: a two-year study. Environ. Pollut. **140:**16-28.

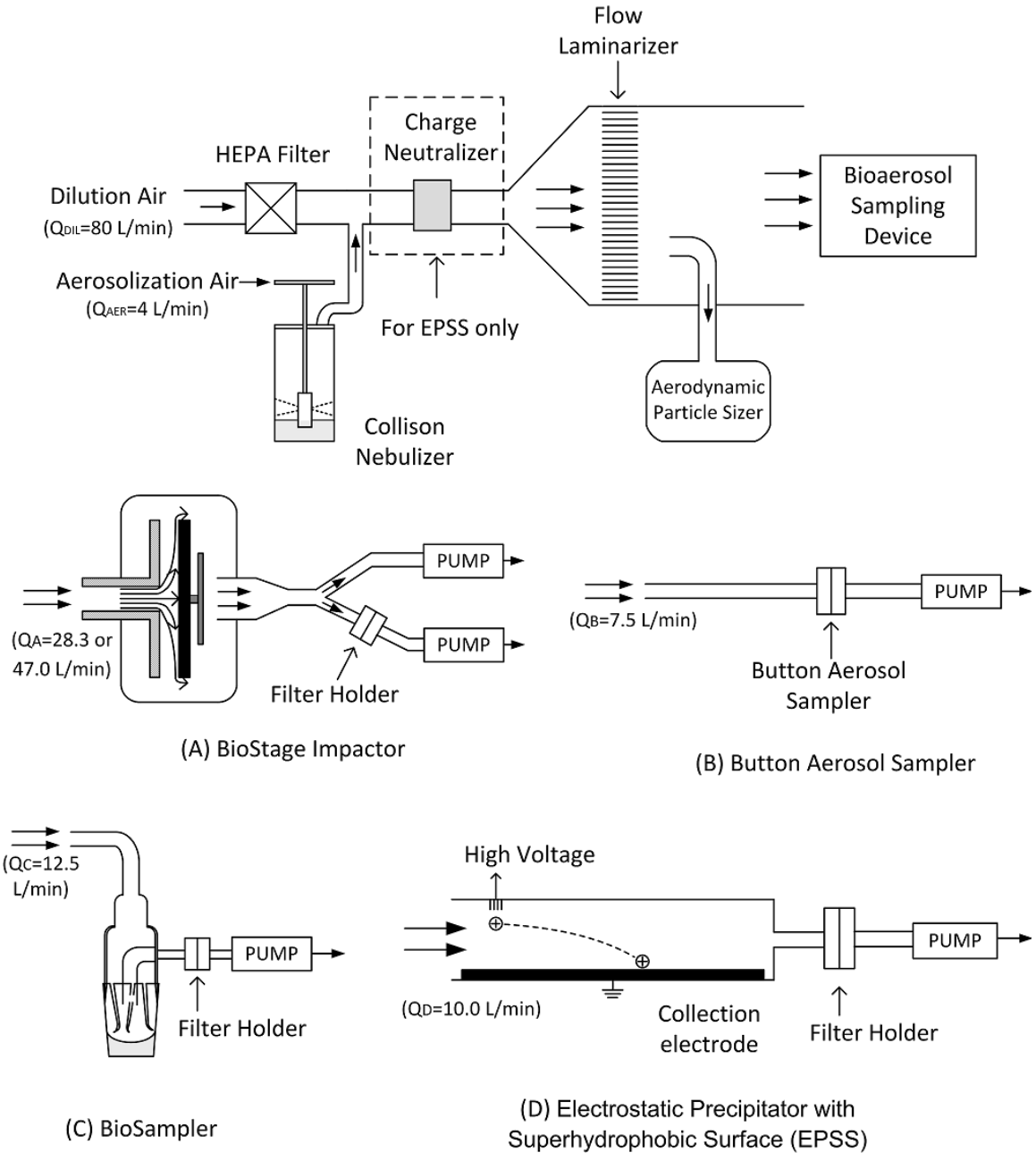

Figure 2.1 Experimental setup used to aerosolize and collect bioaerosols with the BioStage Impactor (A), Button Aerosol Sampler (B), BioSampler (C), and Electrostatic Precipitator with Superhydrophobic Surface (EPSS) (D).

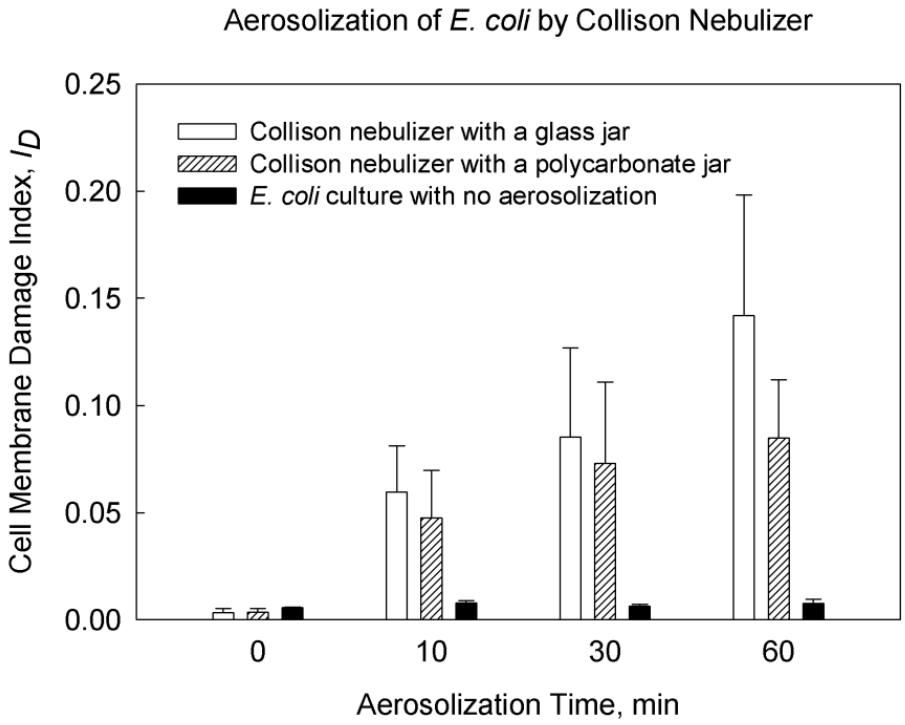

Figure 2.2 The effect of aerosolization time on *E. coli* cell integrity using the Collison nebulizer. Each bar is the average of triplicate samples and error bars are one standard deviation.

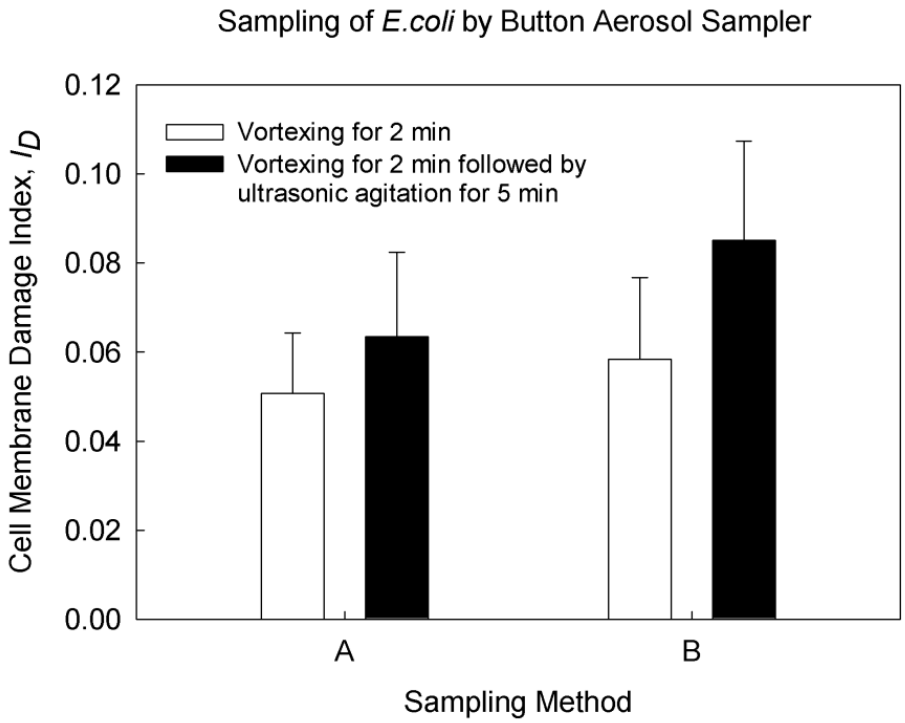

Figure 2.3 The effect of sampling and filter elution methods on cell integrity of *E. coli* cells when collected on filters. Method A: sampling for 5 minutes; Method B: sampling for 5 minutes followed by the passing of particle-free air for 2 hours. Each bar is the average of triplicate samples and error bars are one standard deviation.

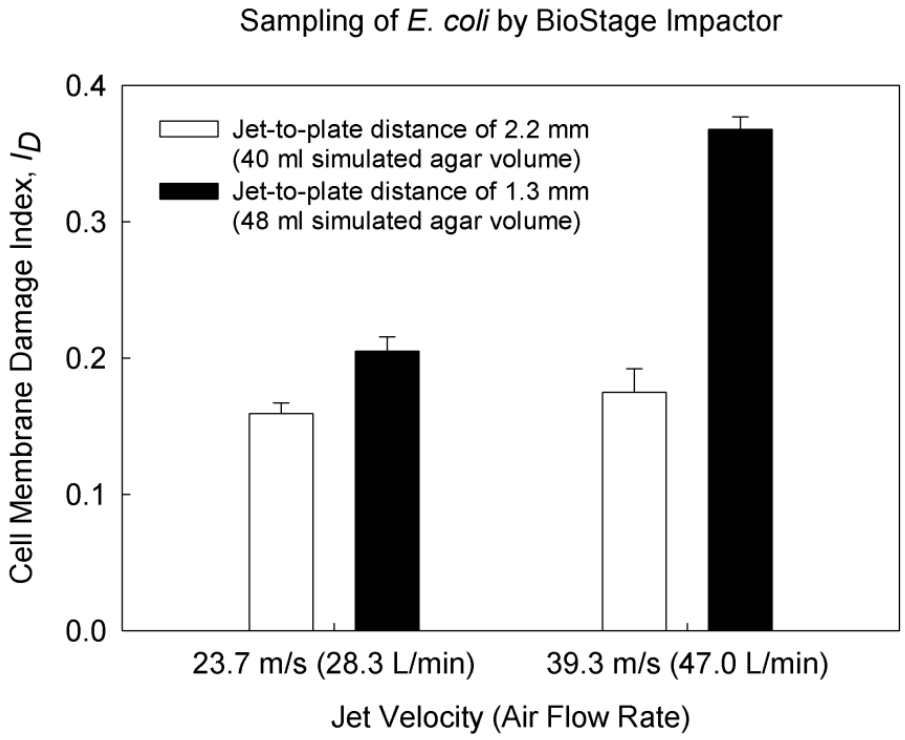

Figure 2.4 The effect of different jet-to-plate distances and jet velocities on *E. coli* cell integrity when sampling with the BioStage impactor. Each bar is the average of triplicate samples and error bars are one standard deviation.

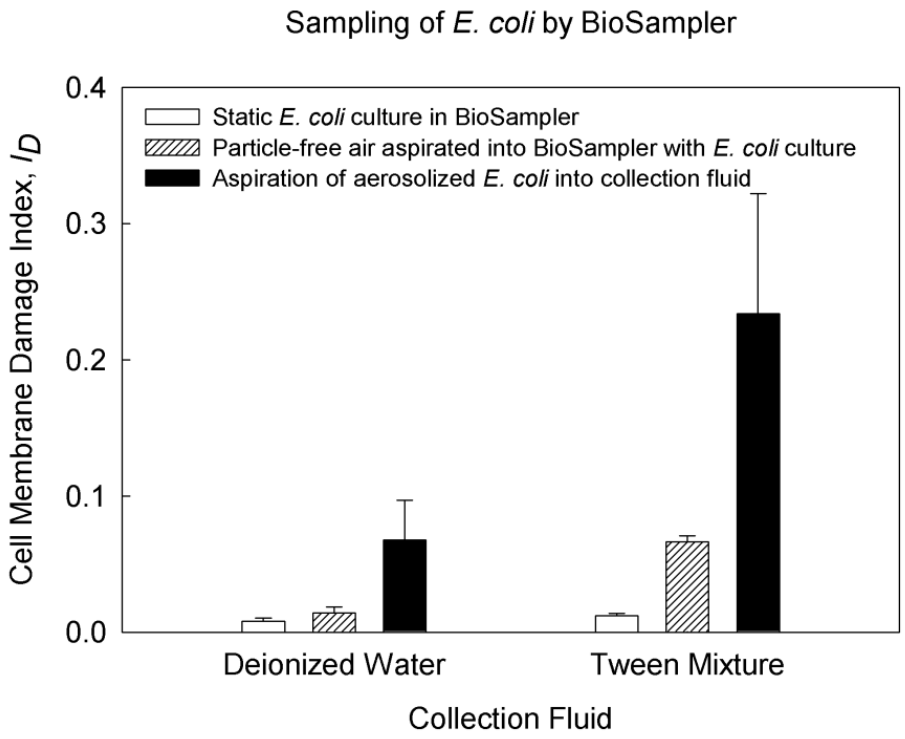

Figure 2.5 The effect on *E. coli* cell integrity of using the BioSampler under three conditions: static *E. coli* culture in the BioSampler, particle-free air aspirated into the BioSampler with the *E. coli* culture, and aspiration of aerosolized *E. coli* into the collection fluid (sterile DI water or Tween mixture). Each bar is the average of triplicate samples and error bars are one standard deviation.

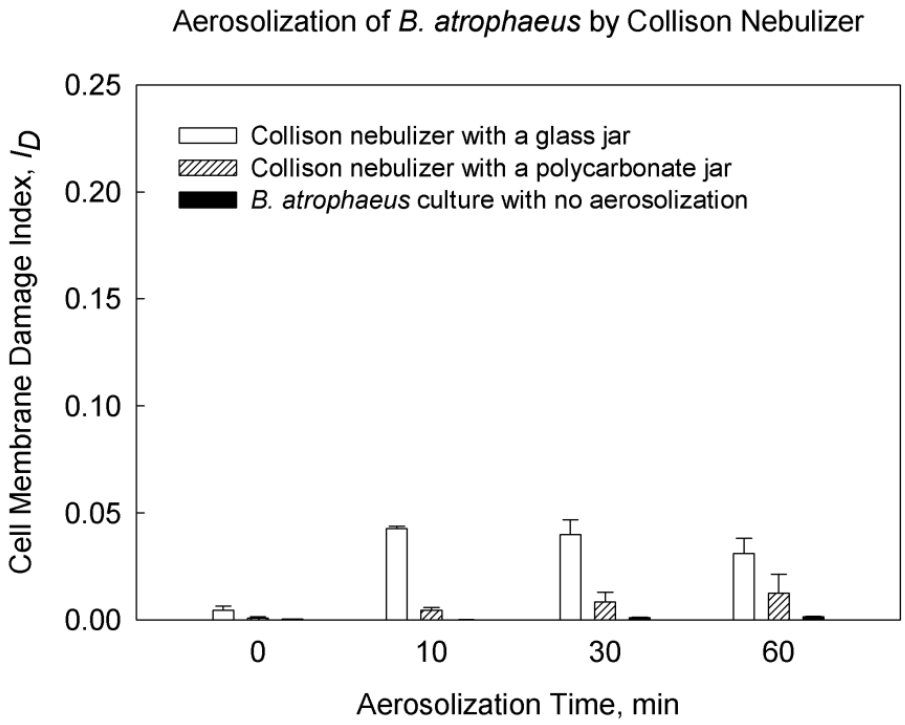

Figure 2.6 The effect of aerosolization time on *B. atrophaeus* cell integrity when using the Collision nebulizer. Each bar is the average of triplicate samples and error bars are one standard deviation.

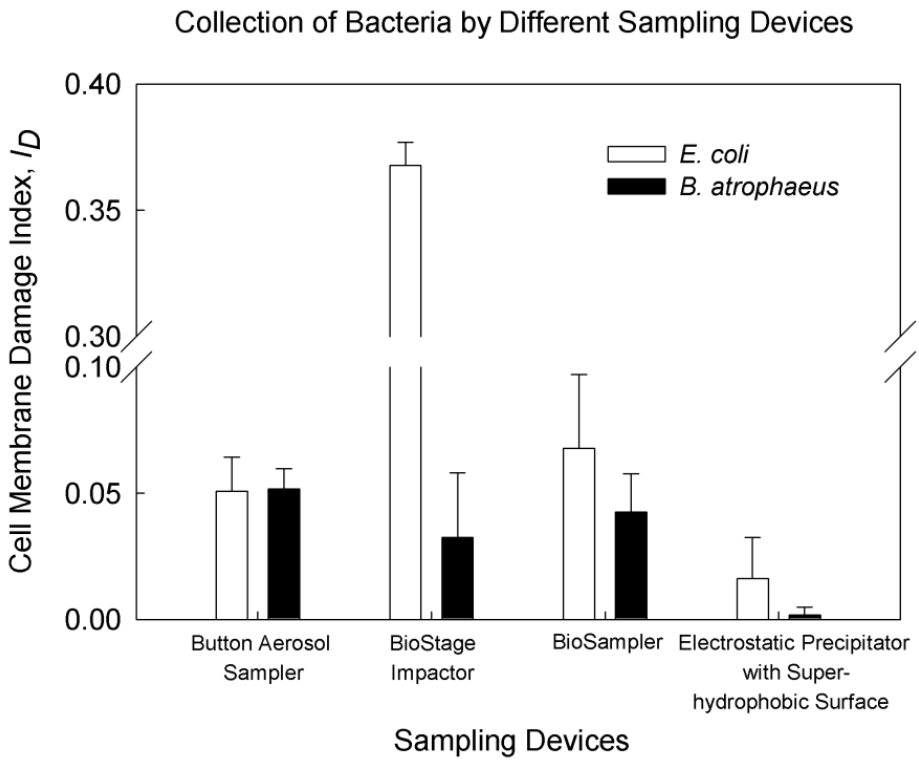

Figure 2.7 Comparison of the cell membrane damage index (I_D) for *B. atrophaeus* and *E. coli* bacteria using four different samplers for 5 min. Button Aerosol Sampler: bacteria collected on a filter eluted by vortexing for only 2 minutes; BioStage impactor: jet velocity of 39.3 m/s and a jet-to-plate distance of 1.28 mm; BioSampler: 5 ml of sterile DI water as collection fluid; EPSS: sampling flow rate of 10.0 L/min. Each bar is the average of triplicate samples and error bars are one standard deviation.

Chapter 3: A Systematic Comparison of Four Bioaerosol Generators: Affect on Culturability and Cell Membrane Integrity when Aerosolizing *Escherichia coli* Bacteria[2]

[2] This chapter is modified from the paper publication by Huajun Zhen, Taewon Han, Donna E. Fennell, and Gediminas Mainelis 2014. A systematic comparison of four bioaerosol generators: affect on culturability and cell membrane integrity when aerosolizing *Escherichia coli* bacteria. *Journal of Aerosol Science.***70:**67-79.

3.1 Abstract

Bioaerosol research requires stable and reliable aerosol generators that can produce high particle concentrations with minimal damage to microorganisms. This study compared Collison nebulizer, Liquid Sparging Aerosolizer (LSA), C-Flow nebulizer, and a newly designed Single-Pass Aerosolizer with respect to their physical performance, and ability to preserve the culturability and structural integrity of bacteria. *Escherichia coli* bacteria were aerosolized at different air pressures, collected by a BioSampler and their Cell Membrane Damage Index (I_D), expressed as the fraction of 16S rRNA gene copies in the supernatant liquid versus the amount of 16S rRNA gene copies in the total sample (cell pellet plus supernatant), was determined. The I_D of *E. coli* aerosolized by the Collison and C-Flow nebulizers at 40 psi compared to aerosolization at 5 and 15 psi was significantly higher (p<0.05). However, the I_D of *E. coli* aerosolized with the LSA and Single-Pass Aerosolizer did not seem to significantly depend on aerosolization pressure. The I_D of *E. coli* collected with a BioSampler was found to positively and significantly correlate (p=0.043) with the presence of airborne bacterial fragments (aerodynamic size range 0.37-0.523 μm) as measured by the Aerodynamic Particle Sizer. Increased loss of culturability was observed for bacteria aerosolized by the Collison nebulizer and Single-Pass Aerosolizer with increasing aerosolization pressure (p<0.05), while no significant change in culturability was found for the other two generators as a function of aerosolization pressure. At particle output concentration of ~100 particles/cm^3, the Single-Pass Aerosolizer preserved the culturability of bacteria significantly better than the other three generators (p<0.05). It also exhibited a significantly lower I_D (p<0.001) and less culturability reduction (p=0.03) compared to the Collison nebulizer at particle

output concentrations of ~1,000 particles/cm^3. It is hoped that this study will help bioaerosol researchers select a bioaerosol generator and method best suiting their studies.

3.2 Introduction

Stable and reliable bioaerosol generation is one of the important elements of bioaerosol research in a laboratory setting. Bioaerosol generators are used to test performance of bioaerosol collectors, to investigate exposure and health effects of airborne microorganisms, to study transport and deposition of biological particles, to evaluate effectiveness of bioaerosol control techniques and other projects (1). A number of bioaerosol generators employing various principles to produce biological particles have been used and described (1-6) .

Currently, pneumatic nebulization is probably the most commonly used method to aerosolize microorganisms (7-9). The Collison nebulizer (4), one of the most frequently used nebulizers in bioaerosol research, is able to produce high concentrations of aerosol, but also has been shown to injure and fragment microorganisms due to strong impaction and shear forces (10). Frequent recirculation of the cell suspension, e.g., 20 ml is recirculated about every six seconds (4), increases fragmentation of bacteria during prolonged nebulization (11).

In recent years, several new generators have been designed for bioaerosol research with a particular goal of minimizing damage to microorganisms. Mainelis *et al.* presented a single-pass bubbling generator, called Liquid Sparging Aerosolizer (LSA), which employed a concept of bursting bubbles to aerosolize particles while avoiding recirculation of suspension (3). They showed that after 90 minutes of continuous aerosolization by the LSA culturability of *Pseudomonas fluorescens* did not change, while culturability of the same bacteria decreased by 50% when a Collison nebulizer was

used for the same duration. A modified LSA-type bubbling generator was recently developed and showed good stability and reproducibility when generating bacteria and endotoxin (1). Ganan-Calvo *et al.* described a pneumatic technique to generate monodisperse aerosols (2). Here, the aerosol particles are produced by the breakup of generated capillary microjets which are driven by the aerodynamic suction of a highly accelerated co-flowing gas stream (2). It appears that two different generators have been developed based on this mechanism: a flow-focusing aerosol generator (FFAG) (5, 12) and a commercially available C-Flow concentric nebulizer (by Savillex Inc., Eden Prairie, MN). A comparative study showed that *E. coli* aerosolized by a flow-focusing aerosol generator experienced significantly lower losses of membrane homeostasis and respiratory enzyme activity compared to aerosolization by a Collison nebulizer (12). Thomas *et al.* also suggested that the bacterial membrane was a major site of damage during aerosolization (12). Another study confirmed this observation and demonstrated that stress exerted onto *E. coli* by a Collison nebulizer could lead to cell membrane rupture and release of genomic DNA as extracellular DNA molecules (13). Previously, Mainelis *et al.* (2001) introduced the idea of improved pneumatic nebulization without liquid recirculation, aiming to reduce the damage to bacterial culturability and structural integrity. A prototype device has been utilized when investigating electrical charges on airborne microorganisms (11, 14). Recently, another such device, namely the Single-Pass Aerosolizer, was designed by CH Technologies (Westwood, NJ). It utilizes pneumatic nebulization to generate particles in a way similar to Collison nebulizer, but without the recirculation of the liquid cell suspension. Also, in contrast with the Collison nebulizer,

the droplets produced by the Single-Pass Aerosolizer do not impact onto a surface, which should minimize the stress to bacterial cells due to impaction.

While the studies described above present a few options for selecting a device for bioaerosol generation, there is still lack of information on which device can produce high bioaerosol concentrations with minimal effects on the bacterial culturability and structural integrity and is also easy to operate. Thus, the main goal of this study was to systematically analyze and compare the performance of common bioaerosol generators with respect to produced particle concentration and size distribution as well as bacterial cell damage as a function of aerosolization pressure when aerosolizing microbial suspension of the same cell concentration. Damage to the bacterial cells was evaluated in terms of loss of culturability and cell membrane integrity. Four aerosol generators: a three-jet Collison nebulizer, a Liquid Sparging Aerosolizer, a C-Flow nebulizer and the newly developed Single-Pass Aerosolizer were tested and compared. We believe that these generators represent the main types of technologies used for bioaerosol generation today.

3.3 Materials and Methods

3.3.1 Test Microorganisms

Gram-negative bacteria *Escherichia coli* (ATCC 15597, Manassas, VA) was selected as our test bacterial species in this study. *E. coli* cells are rod-shaped with a nominal size of 1.67-3.08 μm in length and 0.68-0.84 μm in width depending on the growth phase and nutrient conditions (15). This organism is often used in bioaerosol research and has been suggested as a standard test bacterium (16-19). *E. coli* was cultivated on Tryptic Soy Agar (Becton, Dickinson and Company, Sparks, MD) and stored at 4 °C for less than three months prior to transfer. Prior to experiments, *E. coli* were pre-cultured overnight in 50 ml Tryptic Soy broth (Becton, Dickinson and Company, Sparks, MD) at 37 °C. Then, the bacteria were washed 3 times with sterile deionized water by centrifugation at 7000×*g* for 5 min at 4 °C (Jouan Inc., Winchester, VA). Afterwards, *E. coli* were resuspended in sterile deionized water to prepare a bacterial solution of approximately 1×10^9 cells/ml as verified by microscopic counting.

3.3.2 Test System

A schematic diagram showing the experimental setup is presented in Figure 3.1. All experiments were performed inside a Class II Biosafety cabinet (NUAIRE Inc., Plymouth, MN). Bacterial suspensions were aerosolized using one of the four aerosol generators tested in this study: a three-jet Collison nebulizer (BGI Inc., Waltham, MA), a Single-Pass Aerosolizer (CH Technologies Inc., Westwood, NJ), a C-Flow nebulizer (Savillex Inc., Eden Prairie, MN) and a Liquid Sparging Aerosolizer (LSA) (3) operated at different pressures and aerosolization flow rates as described below. For tests with the C-

Flow nebulizer, a liquid trap was positioned after the generator's outlet to collect large droplets that settle out from the airstream before reaching the dilution air stream. The smaller droplets were diluted and desiccated with HEPA-filtered compressed air to achieve a final air flow rate of 80 L/min and then passed through a flow-laminarizing honeycomb inside a test chamber (approximately 0.36 m in length and 0.10 m in diameter). The only exception was the Single-Pass Aerosolizer, where the dilution air was supplied and mixed with the generated droplets inside the generator chamber and the settled droplets were drained downstream of the generation chamber. In this case, the dilution air from the main air stream was not utilized. Temperature and relative humidity (RH) inside the test chamber were monitored by a traceable hygrometer (Fisher Scientific, Pittsburgh, PA) during each test. The temperature stayed in the range of 24-26°C. The RH varied depending on the aerosolization condition (device and working pressure), mixing ratio of aerosolization air with dilution air and is provided below. The size distribution and concentration of *E. coli* bioaerosol inside the chamber were monitored by an Aerodynamic Particle Sizer (APS, Model 3321, TSI Inc., St. Paul, MN) during each test. The number concentration of *E. coli* bioaerosol was based on the total number concentration of particles with an aerodynamic diameter larger than 0.523 µm. The aerosolized bacteria were collected with a BioSampler (SKC Inc., Eighty Four, PA) with a 5 ml cup using sterile deionized water as collection fluid. Each sample was collected for 5 minutes at a BioSampler nominal flow rate of 12.5 L/min. According to our previous study, this sampling protocol largely preserves the structural integrity of cell membrane (13). After sampling, the BioSampler cup was removed, closed and then vigorously

vortexed for 30 seconds to homogenize the collected sample; then one milliliter of liquid was added to each of the two microcentrifuge tubes (1.5 mL) for sample analysis.

3.3.3 Tested Aerosol Generators

Four bioaerosol generators representing different operating principles were compared (Figure 3.1). When the Collison nebulizer (4) (Figure 3.1A) is in operation, a highly pressurized air steam, Q_A, is pushed through several nozzles (versions ranging from 1 to 24 nozzles are available) in its stem. Due to high velocity of the produced air jets, air pressure in the vertical channels of the stem becomes lower than the ambient air pressure and pulls up fluid from the liquid reservoir. Thereafter, the highly accelerated air jet breaks up the fluid and disperses the droplets into the air. Larger droplets impact onto the inner wall of the jar and are broken up. The resulting smaller droplets as well as initially produced small droplets and material within them are carried away by the aerosolization air flow while the larger ones returning to the liquid reservoir are reaerosolized. In order to reduce the damage to bacterial cell integrity, a polycarbonate jar instead of a glass jar was used for each test with a three-jet Collison nebulizer (13).

The Single-Pass Aerosolizer (Figure 3.1B) is cylindrical in shape (approximately 0.64 m in length and 3.8 cm in diameter) with a gradually narrowing outlet (1.6 cm in diameter). Compressed aerosolization air, Q_B, is forced through two centered and overlapping nozzles, while the liquid with particles is delivered into a space between them at a desired flow rate, Q_{BL}, using a syringe pump. While traveling through the two nozzles, the accelerated air jet breaks up the liquid and disperses liquid droplets into a chamber. Here, the cloud of liquid droplets is picked up by an additional air flow, Q_{DIL},

entering the chamber. As a result, smaller droplets are desiccated and carried away by the combined air stream Q_{BL} + Q_{DIL}, while larger droplets settle inside and are drained. Preliminary experiments showed that an increase in the liquid supply rate Q_{BL} would increase particle output but would also result in a larger amount of drained fluid. Eventually, an optimum delivery rate of Q_{BL} = 0.2 ml/min was determined and used in our experiments.

A C-Flow nebulizer (Figure 3.1C) is a microflow nebulizer. As described by the manufacturer, the nebulizer consists of a Teflon® PFA (perfluoroalkoxy) outer body and a Teflon® PTFE (polytetrafluoroethylene) capillary which is positioned centrally within the outer body with an inner support. The aerosolization air flow, Q_C, is accelerated and flows around the inner support, forming an annular gas stream around the end of the capillary. As the liquid exits the capillary, the annular gas stream shears the liquid into a thin film. After travelling through the C-Flow tip, gas flow then converts the liquid into an aerosol due the venturi effect. In the original design of the C-Flow concentric nebulizer, the liquid suspension, Q_{CL}, is self-aspirated through the capillary and into the PFA body. In our experiment, to control the liquid flow, the bacterial suspension was supplied by a syringe pump (Kent Scientific Corp., Torrington, CT) at a steady delivery rate of Q_{CL} = 0.2 ml/min, which is the maximum uptake rate of liquid as suggested by the manufacturer.

The LSA (Figure 3.1D) is described in detail in a previous study (3). Briefly, it utilizes a bubbling mechanism, which mimics the naturally occurring phenomenon of bubble bursting, to generate particles. The liquid is delivered onto a porous disk at a flow rate, Q_{DL}. The aerosolization air Q_D is forced from underneath the disk and forms

multiple jets. The air jets break up the liquid film into droplets carrying particles, and the small droplets ultimately escape the device at a flow rate Q_D. Larger droplets return to a reservoir and are not re-circulated. As suggested by our earlier study (3), here we used a disk with porosity of 2.0 μm and delivered the bacterial suspension at a steady rate of Q_{DL} = 2.0 ml/min by a syringe pump (Kent Scientific Corp.).

3.3.4 Generator Test Parameters

Our first set of experiments investigated changes in particle concentration as a function of aerosolization pressure for each device. We started at 5 psi, a minimum pressure at which each aerosol generator produced a stable particle output and then increased the pressure in 5 psi increments until we reached the highest tested pressure of 40 psi. The aerosolization air flow rates were in the ranges of 2.7-10.6 L/min, 1.2-4.2 L/min, 0.3-1.5 L/min and 2.5-17.7 L/min for the Collison nebulizer, Single-Pass Aerosolizer, C-Flow nebulizer and LSA, respectively. The *E. coli* culture suspension from the same master batch was supplied to the four devices, and the suspension supply rates were 0.2, 0.2 and 2.0 ml/min for the Single-Pass Aerosolizer, C-Flow nebulizer and LSA, respectively. For the Collison nebulizer, 20 ml liquid suspension from the same batch of bacterial culture was used. Measurements were performed in triplicate for all devices under each test condition. The duration of each test was 5 minutes.

In the second set of experiments, we focused on the performance of the four aerosol generators when each one was operated at three different pressures. For the Collison nebulizer, Single-Pass Aerosolizer and C-Flow nebulizer, the three tested pressures were 5, 15 and 40 psi. For the LSA, the three selected air pressures were 5, 15

and 25 psi. The operational air flow rates corresponding to the three tested aerosolization pressures, from the lowest to highest, were as follows: 2.7, 5.3 and 10.6 L/min for the Collison nebulizer; 1.2, 2.2, 4.2 L/min for the Single-Pass Aerosolizer; 0.3, 0.7, 1.5 L/min for the C-Flow nebulizer; and 2.5, 7.8, 11.9 L/min for the LSA. The duration of each test was 5 minutes. Bioaerosols generated at each tested pressure were sampled using a Biosampler as described above. Each device was tested under three aerosolization pressures using the same batch of *E. coli* cell suspension on the same day. The tests for Collison nebulizer and C-Flow nebulizer had four replicates on different days, while the tests with Single-Pass Aerosolizer and LSA had three replicates on different days. The RH measurements at three tested pressures (from lower to higher) were 30, 34, and 44% for Collison nebulizer, 41%, 43%, 44% for Single-Pass Aerosolizer, 30%, 32% and 33% for C-Flow nebulizer, and 31%, 36% and 39% for LSA, respectively. For *E. coli* bioaerosols generated at each set of conditions, we determined the Fragment Fraction (F_F), Culturability Reduction (C_R), and Cell Membrane Damage Index (I_D) by using methods described below.

3.3.5 Fragment Fraction

For generated *E. coli* bioaerosols, the Fragment Fraction (F_F) was calculated as follows:

$$F_F = \frac{C_1}{C_2} \times 100\% \qquad (1)$$

Where C_1 is the number concentration (#/cm^3) of particles in the size range from 0.37 μm to 0.523 μm as measured by the APS in summing mode (20) and C_2 is the total number concentration (#/cm^3) of all size particles as measured by the APS.

3.3.6 Analysis of Bacterial Culturability

The total and culturable number of bacteria in each sample were determined using the same 1 ml aliquot which was diluted as needed. 100 µl at each used dilution factor were plated in triplicate onto Tryptic Soy Agar using a sterile plastic spreader and incubated at 37°C for 16 hours. The colonies were counted, and the number was used to calculate the concentration of culturable cells in the original sample using the following equation:

$$C_{culturable} = \frac{N_1 \times f_1}{V_1} \quad (2)$$

Where $C_{culturable}$ is the number concentration (#/ml) of culturable *E. coli* in a sample; N_1 is the average number of colonies that grew on agar plates; f_1 is the sample dilution factor relative to the original sample; and V_1 is the volume of liquid sample (100 µl) plated on agar.

The total number of bacterial cells in each sample was determined by epifluorescence microscopy using the Axioskop 20 (Carl Zeiss Inc. Thornwood, NY) according to a previously published procedure (16). Depending on the initial concentration of bacteria in a sample, it was diluted to observe 10 to 40 stained bacteria per microscope view field. At least 40 microscopic fields were counted and the counts were averaged. The total number concentration of bacteria (#/ml), C_{total}, was calculated as follows:

$$C_{total} = \frac{N_2 \times X \times f_2}{V_2} \quad (3)$$

Where N_2 is the average count of bacteria per microscope view field; X is the number of view fields for the entire filter: 6125 in our case; f_2 is the sample dilution factor used to

prepare the microscope slide; and V_2 is the sample volume used to prepare the microscope slide (ml).

The culturability of *E. coli* in liquid suspension, η, was calculated as follows:

$$\eta = \frac{C_{culturable}}{C_{total}} \times 100\% \quad (4)$$

3.3.7 Culturability Reduction

The bacterial Culturability Reduction, C_R (%), in a collected bioaerosol sample compared to fresh culture was calculated as follows:

$$C_R = (1 - \frac{\eta_1}{\eta_2}) \times 100\% \quad (5)$$

Where η_1 is the culturability of *E. coli* that were aerosolized and collected by a BioSampler, η_2 is the culturability of fresh *E. coli* culture (determined in the same manner) before aerosolization.

3.3.8 Cell Membrane Damage Index

Previously we showed that bacterial membranes could be structurally damaged during aerosolization and sampling, resulting in the release of DNA as extracellular molecules, and introduced the Cell Membrane Damage Index (I_D) to quantify the damage (13). I_D is defined as the ratio of 16S rRNA genes released from membrane-damaged bacterial cells to the entire amount of 16S rRNA genes in a sample and can vary from "0" (no damage) to "1" (all genomic DNA from bacteria has been released).

In this work, I_D was used as a metric to compare the magnitude of stress exerted on bacteria during aerosolization by the different aerosol generators. *E. coli* aerosolized

by the four aerosol generators were collected by a BioSampler using the same sampling condition, and the I_D was determined as previously described (13) with a slight modification. Briefly, the second set of 1-ml aliquot from BioSampler was centrifuged at 16,100×g for 10 minutes at 4 °C. Then 950 µl of the supernatant liquid was carefully transferred into a new sterile 1.5 ml microcentrifuge tube, while the remaining 50 µl liquid containing the cell pellet was subjected to DNA extraction. We have demonstrated previously that in each sample after centrifugation, the DNA in the supernatant liquid originated from bacterial cells that lost membrane integrity, while the DNA in the pellet were from cells that maintained membrane integrity (13). The I_D was calculated using the following equation:

$$I_D = \frac{N_S}{N_S + N_P} \quad (6)$$

Where N_S (#/ml) is the number of target 16S rRNA gene copies in the supernatant liquid sample; N_P (#/ml) is the number of target 16S rRNA gene copies in the cell pellet. In our previous study (13), N_P was calculated using cell counts from epifluorescence microscopy and multiplying them by the number of 16S rRNA genes for *E. coli* genome; in this study, N_P was determined by quantifying the 16S rRNA genes copies directly from the extracted DNA from the pellet sample. N_P determined by the two methods were found to correspond well with each other (r = 0.96, data not shown).

3.3.9 DNA Extraction and Quantitative PCR

The 16S rRNA gene in the supernatant liquid and pellet were extracted and purified using a DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's

protocol. Thereafter, the number of target DNA copies was determined by qPCR as described below.

Quantitative PCR was performed on an iCycler iQ5 RT-PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The universal PCR primer pair (forward: 5'-TCCTACGGGAGGCAGCAGT-3'; reverse: 5'-GGACTACCAGGGTATCTAATCCTGTT-3') for bacteria were utilized and were expected to yield a target amplicon size of 466 bp according to previous studies (16, 21). Reaction mixtures were prepared by combining 10 µl of 2×SYBR Green supermix (Bio-Rad Laboratories, Hercules, CA), 2 µl of each 2.5 µM primer, 5 µl of template DNA and 1 µl PCR-grade water to a total volume of 20 µl for each reaction. The amplification reaction was performed with iCycler iQ thermal cycler (Bio-Rad Laboratories, Hercules, CA) using the following temperature program: 10 min of denaturation at 95°C; 40 cycles of 15 s of denaturation at 95°C and 1 min of annealing/extension at 60 °C. Data analysis was performed using iCycler iQ Real-Time detection system software. After completion of the PCR amplification in each reaction, a melt curve test was performed to check the purity of the amplicons generated.

To prepare standard curves for qPCR, a batch of freshly harvested *E. coli* cells was quantified by epifluorescence microscopy, genomic DNA was extracted from this known number of cells using the DNeasy Blood & Tissue Kit protocol (Qiagen, Valencia, CA), and the extracted DNA was serially diluted. Standard curves were made by plotting the Cycle threshold (C_t) value against the log of the corresponding target gene copy number, which was determined by multiplying the equivalent number of *E. coli* cells

extracted by the average 16S rRNA gene copy number (equal to seven) according to the whole genome sequence of *E. coli* (GenBank accession number NC_010473.1).

3.3.10 Statistical Analysis

Statistical analysis was performed using Statistica software version 10.0 (StatSoft Inc., Tulsa, OK). A two-way ANOVA was performed to test the main effects of air pressure and the day of testing on I_D or C_R values of *E. coli* bioaerosols for each device. Fisher's LSD was selected to examine the difference of I_D or C_R values between two pressure groups. A one-way ANOVA with Fisher's LSD test was applied to test the difference of F_F, I_D or C_R between *E. coli* aerosolized by different devices. Overall, a statistically significant difference was defined as $p<0.05$. Boxplots of F_F, I_D, and C_R values for all collected samples were prepared using Statistica software version 10.0. Outliers were defined as the data points lower (or higher) than 1.5 interquartile (the difference between the upper and lower quartiles) range of the lower (or higher) quartile; extreme values were defined as the datum lower (or higher) than 3 interquartile range of the lower (or higher) quartile.

3.4 Results

3.4.1 Effect of Aerosolization Pressure on Total Particle Output

In our first set of experiments, we investigated the effect of aerosolization pressure on the total number of *E. coli* bioaerosols produced by the four aerosol generators. It is clearly seen from Figure 3.2 that the total number concentration of *E. coli* particles gradually increased with increasing aerosolization pressure for the Collison nebulizer, Single-Pass Aerosolizer and C-Flow nebulizer. The output of the LSA increased with pressure up to 15 psi, then leveled off and began to gradually decrease when air pressure went beyond 25 psi. This pattern of particle output for the LSA was similar to our previous report (3). Based on these data, we considered the optimum operational air pressure for the LSA to be between 5 and 25 psi, and the maximum pressure at which the LSA was tested in later experiments was 25 psi. For any particular pressure between 5 and 25 psi, the highest total particle output was achieved with the Collison nebulizer, followed by the Single-Pass Aerosolizer, the LSA and the C-Flow nebulizer. For aerosolization pressures above 25 psi, the highest total particle output was achieved with the Collison nebulizer, followed by the Single-Pass Aerosolizer, and the C-Flow nebulizer.

3.4.2 Size Distribution of *E. coli* Aerosolized by the Four Devices

In the second set of experiments, we assessed the size distribution, cell membrane integrity and culturability of generated *E. coli* bioaerosol under three different aerosolization pressures for each device. Figure 3.3 presents size distributions of *E. coli* bioaerosols generated by the four devices under three aerosolization pressures. Both the Collison nebulizer (Figure 3.3A) and Single-Pass Aerosolizer (Figure 3.3B) produced

particles with a mode diameter of 0.723 μm at three different pressures. For the C-Flow nebulizer (Figure 3.3C) and the LSA (Figure 3.3D), the mode diameters of the bacterial size distribution were 0.965 μm at a pressure of 5 psi. When the pressure was elevated to 15 psi and higher, a second peak with a mode diameter at 0.723 μm was observed. In addition, especially for the C-Flow nebulizer, with the air pressure above 5 psi, the number concentration of particles with a 0.965 μm diameter decreased relative to that of particles with a 0.723 μm diameter.

One can also observe that size distribution produced by the Collison nebulizer contains particles with diameter less than 0.523 μm, the concentration of which increased dramatically with elevated aerosolization pressure. Bacterial particles less than 0.5 μm in size are considered fragments that have likely originated from damaged bacteria (14, 22). We calculated the fraction of those cell fragments (size from 0.37 μm which is the lower detection limit for APS to 0.523 μm) relative to the total number of particles (Table 3.1) and denoted as F_F. It can be clearly seen that the F_F value increased with increasing aerosolization pressure for all four devices. The highest increase was observed for the Collison nebulizer, where the F_F value increased from 2.2 ± 0.1% to 7.8 ± 1.1% when the aerosolization pressure was increased from 15 psi to 40 psi. When comparing the devices operating at the same air pressure, the F_F value of aerosolized bacteria was significantly higher for the Collison nebulizer than for the other three devices (p<0.05).

3.4.3 Cell Membrane Damage Index

Figure 3.4 presents the Cell Membrane Damage Index (I_D) for *E. coli* aerosolized by the four generators at different pressures and then sampled using a BioSampler. For the

Collison nebulizer (Figure 3.4A), the I_D values were 0.10 ± 0.04, 0.11 ± 0.04 and 0.36 ± 0.06 for aerosolization pressures of 5, 15 and 40 psi, respectively. A two-way ANOVA with Fisher's LSD test showed that the I_D for bacteria aerosolized at 40 psi was significantly higher than those for bacteria at 5 psi (p<0.001) and 15 psi (p<0.001), while no significant difference between 5 psi and 15 psi was found (p=0.241). For the Single-Pass Aerosolizer (Figure 3.4B), the I_D were 0.06 ± 0.02, 0.04 ± 0.00 and 0.08 ± 0.05 for bacteria aerosolized at 5, 15 and 40 psi, respectively and these values were not significantly different (p>0.05). A similar trend to that of the Collison nebulizer was observed for *E. coli* aerosolized by the C-Flow nebulizer (Figure 3.4C), where the I_D of bacteria aerosolized at 40 psi (0.09 ± 0.04) was significantly higher than that for 5 psi (0.03 ± 0.02, p=0.003) and 15 psi aerosolization pressures (0.03 ± 0.02, p=0.004), whereas no significant difference in I_D was found between the samples aerosolized at 5 psi and 15 psi (p=0.824). For the LSA (Figure 3.4D), the I_D values were 0.06 ± 0.04, 0.06 ± 0.03 and 0.06 ± 0.04 at 5, 15 and 25 psi, respectively and these values were not significantly different (p>0.05).

3.4.4 Culturability Reduction Compared to Fresh Culture

Figure 3.4 also presents a change in the culturability of *E. coli* when aerosolized and sampled by BioSampler. The change is expressed as Culturability Reduction (C_R, %) relative to the original culturability of bacteria in culture suspension prior to aerosolization. When the Collison nebulizer (Figure 3.4A) was operated at 5, 15 and 40 psi, the C_R of *E. coli* was 44.2 ± 9.9 %, 77.3 ± 11.7 % and 79.3 ± 10.7 %, respectively. The increased reduction in culturability when aerosolization pressure was increased from 5 to 15 psi (p<0.001) and from 5 to 40 psi (p<0.001) were statistically significant, but

there is no significant difference between C_R of *E. coli* aerosolized at 15 psi and 40 psi (p=0.600). For the Single-Pass Aerosolizer (Figure 3.4B), the C_R of *E. coli* were 23.3 ± 9.0 %, 42.2 ± 8.6 % and 53.7 ± 11.2 % at 5, 15 and 40 psi, respectively. The increased reduction in culturability were significant when the aerosolization pressure was increased from 5 to 15 psi (p=0.008), from 5 to 40 psi (p=0.001) and from 15 to 40 psi (p=0.042). As for the C-Flow nebulizer (Figure 3.4C), the C_R was 53.5 ± 7.2 %, 48.9 ± 10.9 % and 48.0 ± 8.7 % under 5 psi, 15 psi and 40 psi, respectively, while those values for the LSA (Figure 3.4D) at 5 psi, 15 psi and 25 psi were 40.6 ± 11.7 %, 47.2 ± 10.8 % and 52.0 ± 10.8 %, respectively. No statistically significant difference was observed (p>0.05) for samples aerosolized under three different pressures by the C-Flow nebulizer and the LSA.

3.4.5 Correlations between Fragment Fraction, Cell Membrane Damage Index and Culturability Reduction

We observed a positive correlation between I_D and F_F values for all *E. coli* bioaerosols generated by four devices (p<0.0001, r=0.92) as shown in Figure 3.5. Also, of the forty-two data points (samples), four points with the highest F_F values were classified either as outliers or extreme values as described in “Statistical Analysis” section, and they may have dominated the regression analysis obscuring contribution of other data points. However, a significant positive correlation between the two variables remained (p=0.043, r=0.33) even with those four values removed (insert in Figure 3.5).

Similar to the regression analysis between I_D and F_F, we also observed positive and significant correlation between C_R and I_D (p<0.0001, r=0.58) as shown in Figure 3.6. However, after removal of the four data points which were identified as either outliers or

extreme values (insert in Figure 3.6), the correlation remains positive, but not statistically significant (p=0.090, r=0.28).

3.4.6 Comparison of Bioaerosol Generators

Figure 3.7 presents the C_R and I_D as a function of number concentration of *E. coli* generated by the four devices. As observed earlier, the increase in output concentration by each device was driven by the increase in aerosolization pressure (Figure 3.2). As could be seen from Figure 3.7, in general, in order to produce a higher bioaerosol concentration, one pays a penalty in terms of the bacterial culturability and cell membrane integrity. When the Collison nebulizer, Single-Pass Aerosolizer, C-Flow nebulizer and LSA were operated at 5, 5, 40 and 15 psi, respectively, they produced bioaerosol concentrations of approximately 100 particles/cm^3 from a *E. coli* liquid suspension (~1×10^9 cells/ml): 81.2 ± 5.0, 70.5 ± 22.2, 106.9 ± 11.2 and 189.0 ± 58.5 particles/cm^3. Culturability Reduction for the Single-Pass Aerosolizer was significantly lower than that for the Collison nebulizer (p=0.02), C-Flow nebulizer (p=0.01) and LSA (p=0.01), while no significant differences among the other three devices were found. For the I_D at the same particle output of ~100 particles/cm^3, no significant difference was found between any pair of devices (p>0.05). Among these four devices, only the Collison nebulizer and Single-Pass Aerosolizer were able to achieve bioaerosol concentrations of approximately 1,000 particles/cm^3 from the same *E. coli* liquid suspension (~1×10^9 cells/ml). Our data show that for this high bioaerosol concentration, the Single-Pass Aerosolizer exhibited significant lower C_R (p=0.03) and I_D (p<0.001) values than the Collison nebulizer.

3.5 Discussion

The four aerosol generators tested in this study differ from each other in terms of their produced particle number concentration and size distribution, bacterial culturability loss and cell fragmentation. These differences seem to be largely determined by the physical aerosolization principle employed by each device.

Particles with two different sizes were distinctly observed in the size distributions of *E. coli* bioaerosols generated by the C-Flow nebulizer and the LSA. One possible reason for the occurrence of particles with larger diameters than typical single cells could be hygroscopic growth of bacteria cells inside the chamber due to high RH. However, previously reported RH resulting in hygroscopic growth of *E. coli* cells was above 85% (23). Since the RH in our tests reported here was below 50%, the effect of hygroscopic growth on cell size could be excluded. As a result, we speculate that particles with a larger size could be agglomerates of two or more single bacterial particles. Different from the C-Flow nebulizer and LSA, the peak representing bacterial agglomerates for the other two pneumatic nebulizers were much less distinct compared with the single particle-peak. While the double-particle peaks could still be observed in distributions at 5 and 15 psi for the Collison nebulizer, the second peak almost completely disappeared at 40 psi. The size distribution data for the Single-Pass Aerosolizer does contain particles of sizes consistent with doublets, but there was no clearly pronounced peak. One can also observe that normalized particle concentrations produced by these two devices are rather similar and about 3-fold higher that those produced by the C-Flow nebulizer and LSA. Thus, it could be concluded that two pneumatic nebulizers employed in our study are more efficient in terms of producing monodisperse bioaerosols than the other two devices.

The F_F values for *E. coli* bioaerosol as measured by APS were investigated and compared between devices. It should be noted that the APS 3321 used in our study was reported to have low detection efficiency for particles smaller than 0.5 μm in aerodynamic diameter (24). In addition, fragments smaller than the APS detection limit were also likely present according to our previous observation (unpublished data), but were not analyzed here. Thus, the F_F values for all samples were likely underestimated. However, even in such a case, it can be clearly seen that the F_F value increased with increasing aerosolization pressure for all four devices. The increase is likely due to the increase of mechanical stress on microorganisms to such an extent that it leads to fragmentation of bacterial cells. The Collison nebulizer also exhibited significant higher F_F values than other three devices at the same air pressure (p<0.05), which implied that bacteria aerosolized by the Collison nebulizer are subjected to greater stress and fragmentation compared to the other three generators. Thus, selection of the Collison nebulizer as a bioaerosol generator, especially at higher air pressures, may not be the best choice for studies where aerosolized bacteria with little injury are needed.

We applied an indirect method to assess the magnitude of cell membrane damage by calculating the I_D for each sample—the damage to cell membrane was not observed directly. The amount of extracellular DNA that was measured in a sample collected by the BioSampler may have resulted from the stress from both aerosolization and sampling, as we previously reported (13). Since the BioSampler was operated at the same conditions for all experiments, its contribution to I_D values should have been constant thus enabling us to discern the contribution of aerosolization stress alone to the presence of extracellular DNA in the collected samples. It was determined previously that when a

BioSampler aspirated particle-free air into sterile water spiked with a known concentration of *E. coli*, the *E. coli* sample exhibited I_D value of 0.014 ± 0.005. This number represents the contribution of BioSampler operation by motion of liquid alone (13). I_D values from some samples in our experiment were close to this number and indicated a negligible impact of aerosolization on the cell membrane integrity, while I_D values from other samples were higher (up to 30-fold) indicating an additional effect of aerosolization. For example, the significant increase of I_D values when the aerosolization pressure increases from 15 psi to 40 psi for the Collison and C-Flow nebulizers suggested a remarkable impact of aerosolization on the cell membrane integrity. It also should be emphasized that cell membrane damage and corresponding I_D values resulted from a 5-minute aerosolization using fresh bacterial liquid suspension. When the Collison is in operation, one would expect the I_D value to increase with longer aerosolization time due to the accumulated stress caused by recirculation of the liquid cellular suspension.

We also found culturability loss for *E. coli* bioaerosol generated by all four devices under the investigated air pressures. Since *E. coli* bioaerosols were sampled and cultivated under the same conditions for all experiments, the difference in culturability at three different pressures for each device was contributed not only by the aerosolization stress but also possibly by the desiccation stress while *E. coli* was in airborne phase. Airborne bacteria have been reported to survive better in environments with high RH level (25-28). For example, the survival rate of *Chlamydia pneumonia* at a RH of 95% was significantly higher than that of 50% RH during a 5.5-minute period after aerosolization (26); Rule *et al.* (2009) suggested that the increase in culturability of aerosolized *Pantoea agglomerans* followed a log-linear relationship with RH when RH

was above 15%. In our experiments, the RH levels varied in the range of 30-44% for all test conditions. In a previous study, the variation of RH in a similar range (30-50%) for aerosolized *Saccharomyces cerevisiae* did not have a significant impact on the culturability (28). It should additionally be noted that for studies reporting significant RH impact on bacterial survival, the bioaerosol residence time varied from 0.5 min (26) to 8 minutes (28). In contrast, in our tests the residence time of *E. coli* bioaerosols inside the mixing chamber was 2 seconds or less, far shorter than the aforementioned studies. Thus, it could be inferred that the variability of RH levels in our tests likely had a negligible impact on the observed significant difference in *E. coli* culturability for the Collison nebulizer and the Single-Pass Aerosolizer under different pressures. The observed differences for the two devices are likely attributed to the aerosolization stress alone. Similar to the I_D values, the C_R values for the Collison nebulizer are expected to increase with longer aerosolization times due to accumulated stress caused by suspension recirculation.

The variability in the significant correlation between I_D and F_F values could arise from two different sources. First, as mentioned earlier, the extracellular DNA that was measured in a sample collected by the BioSampler (I_D data) may have resulted from the stress from both aerosolization (F_F data) and sampling. Again, note that particles smaller than 0.37 μm in aerodynamic diameter, including possibly the extracellular DNA molecules, were not measured, and the number concentration of particles smaller than 0.5 μm was undercounted by the APS (24). Thus the total amount of fragments (F_F data) may have been underestimated. In consideration of these variability, the observed correlation between the airborne fragments of bacteria (F_F data) and the presence of

extracellular genomic DNA in the collected sample (I_D data) strongly suggests that the bacteria experience strong mechanical stress during aerosolization. When the stress becomes strong enough, cell membranes become ruptured and, as a result, cytoplasmic components including genomic DNA, are released and aerosolized as extracellular particles. In addition, our data imply that the Fragment Fraction determined easily by APS measurement could be used as a surrogate indicator of the cell membrane damage of aerosolized bacteria.

Previous studies have reported culturability loss (3, 12, 29) and damaged cell membranes (12, 13) for bacteria aerosolized by Collison nebulizer, but no quantitative relationship between these two factors has been investigated so far. Our data suggest that there is positive correlation between the loss of culturability by *E. coli* (C_R data) and the amount of bacteria with disintegrated cell membrane (I_D data), but experimental variability prevents showing statistical significance. In fact, depending on the stress airborne bacteria experience, they could be classified according to their physiological status as culturable, viable but not culturable, nonviable but maintaining membrane integrity, and cell fragments (30, 31). In our study, the bacteria losing culturability may enter into one of the other three statuses, while the I_D only represents the fraction of fragmented bacterial cells among the total cells. It is reasonable to assume that the stronger the mechanical stress due to aerosolization, the larger is the chance that the bacterial cells will break into fragments during aerosolization. When the C_R values of aerosolized *E. coli* as a function of I_D was stratified by aerosolization device (data not shown), we found a significant positive correlation only for Collison nebulizer (p=0.044), while correlation for other devices was not statistically significant: p=0.78 for Single-

Pass Aerosolizer, p=0.11 for C-Flow nebulizer and p=0.14 for LSA. For the Collison nebulizer, a strong correlation between the loss of culturability and the I_D demonstrated again that it imparts more stress than the other three devices and thus causes more injury to bacterial cells.

Previous studies comparing bioaerosol generators focused on physical properties of aerosolized bioaerosols (e.g. particle number concentration, size distribution), rather than their biological parameters (e.g. culturability and structural integrity) (1). In this study, it is possible to correlate the biological parameters with physical particle output for different generator types. Our data showed that for a similar produced bioaerosol concentration of approximately 100 particles/cm^3, the Single-Pass Aerosolizer most effectively preserved the culturability of the bacterial cells, while no difference were found among four devices in retaining the integrity of cell membranes during aerosolization. For a high bioaerosol concentration of approximately 1000 particles/cm^3, the Single-Pass Aerosolizer preserved the bacterial culturability and maintained the cell membrane integrity significantly better than the Collison nebulizer. Both generators are designed based on pneumatic nebulization, but bacteria in the Collison nebulizer are exposed to repeated stress of shear force and impaction due to liquid recirculation inside the vessel, while the bacteria aerosolized by the Single-Pass Aerosolizer experience the stress of shear force only once. Our findings here confirm conclusions of a previous study that repeated pneumatic dispersion of sensitive bacteria affects their structural integrity (14).

When selecting a bioaerosol generator, one needs to consider not only the accessibility of a device, but also its performance depending on the specific objective of

their study. In most cases, a high output concentration of biological particles with the least effect on viability and integrity of bacteria is desired. The Collison nebulizer is widely used and is capable of producing high particle concentrations, but resulting cell damage has been reported in multiple studies, including here. At the same time our data show that the Single-Pass Aerosolizer is capable of producing bioaerosol concentrations as high as those by the Collison nebulizer, while better maintaining cell culturability and preserving their membrane integrity. For lower airborne biological particle concentrations, one could refer to Figure 3.7 to select an instrument best suited for one's study.

Note that the gram-negative *E. coli* used in this study is considered to be more sensitive to mechanical stress when compared to gram-positive bacterial species as we reported in our previous study (13). Furthermore, due to different physical characteristics such as size and shape, other bioaerosol types, such as bacterial spores, fungi and viruses might behave differently in terms of their aerosolization efficiency, change in structure integrity and viability during aerosolization. Thus, investigations of various devices when aerosolizing different bioaerosol types are warranted in future studies.

3.6 Conclusions

Test results show that both the Collison nebulizer and Single-Pass Aerosolizer are able to produce higher bioaerosol concentrations compared to the C-Flow nebulizer and LSA at the same aerosolization pressures. We speculated that agglomerates of two or more bacteria were present in particle size distributions generated by the C-Flow nebulizer and the LSA. The Cell Membrane Damage Index, I_D, by the Collison nebulizer and C-Flow nebulizer at 40 psi were significantly higher than those at 5 and 15 psi, indicating that stress due to shear force is an important factor affecting bacteria. Reduction of *E. coli* culturability increased with aerosolization pressure when Collison nebulizer (from 5 to 15 psi) and Single-Pass Aerosolizer (from 5 to 40 psi) were used, while bacterial culturability was not significantly affected by increasing aerosolization pressure of the C-Flow nebulizer and LSA, likely due to their different operation principle compared to pneumatic nebulizers. In addition, the I_D for all samples showed positive correlation with the fraction of particles between 0.3 μm and 0.523 μm as measured by an APS, suggesting that an APS could serve as a surrogate to determine a bioaerosol generator's effect on bacterial integrity. When comparing the four generators at similar output concentrations, the Single-Pass Aerosolizer was found to preserve the culturability of bacteria better than other investigated devices. The Single-Pass Aerosolizer was the only generator matching the Collison nebulizer's output concentration at higher aerosolization pressures, while at the same time better preserving cell culturability and membrane integrity compared to the Collison. It is hoped that this comparison of different bioaerosol generators will help bioaerosol researchers to select a device that provides the best fit for their studies.

3.7 Acknowledgments

The publication was supported by Grant R01-OH009783 “Advanced Sampler for Measuring Exposure to Biological Aerosols” (CDC/NIOSH) and the New Jersey Agricultural Experiment Station (NJAES) at Rutgers, The State University of New Jersey. Its contents are solely the responsibility of the authors and do not necessarily represent the official view of the CDC/NIOSH or the NJAES.

References

1. **Simon, X., P. Duquenne, V. Koehler, C. Piernot, C. Coulais, and M. Faure.** 2011. Aerosolisation of *Escherichia coli* and associated endotoxin using an improved bubbling bioaerosol generator. Journal of Aerosol Science **42:**517-531.
2. **Ganan-Calvo, A. M., and A. Barrero.** 1999. A novel pneumatic technique to generate steady capillary microjets. Journal of Aerosol Science **30:**117-125.
3. **Mainelis, G., D. Berry, H. R. An, M. S. Yao, K. DeVoe, D. E. Fennell, and R. Jaeger.** 2005. Design and performance of a single-pass bubbling bioaerosol generator. Atmospheric Environment **39:**3521-3533.
4. **May, K. R.** 1973. The Collison nebulizer: description, performance and application. Journal of Aerosol Science **4:**235-243.
5. **Thomas, R. J., D. Webber, W. Sellors, A. Collinge, A. Frost, A. Stagg, S. Bailey, P. Jayasekera, R. Taylor, S. Eley, and R. Titball.** 2009. Generation of large droplet aerosols within microbiological containment using a novel flow-focussing technique. Aerobiologia **25:**75-84.
6. **Ulevicius, V., K. Willeke, S. A. Grinshpun, J. Donnelly, X. Lin, and G. Mainelis.** 1997. Aerosolization of particles from a bubbling liquid: characteristics and generator development. Aerosol Science and Technology **26:**175-190.
7. **Chen, S.-K., D. Vesley, L. M. Brosseau, and J. H. Vincent.** 1994. Evaluation of single-use masks and respirators for protection of health care workers against mycobacterial aerosols. American Journal of Infection Control **22:**65-74.
8. **Jensen, P. A., W. F. Todd, G. N. Davis, and P. V. Scarpino.** 1992. Evaluation of eight bioaerosol samplers challenged with aerosols of free bacteria. American Industrial Hygiene Association Journal **53:**660-667.
9. **Griffiths, W. D., and G. A. L. DeCosemo.** 1994. The assessment of bioaerosols: a critical review. Journal of Aerosol Science **25:**1425-1458.
10. **Reponen, T., K. Willeke, V. Ulevicius, S. A. Grinshpun, and J. Donnelly.** 1997. Techniques for dispersion of microorganisms into air. Aerosol Science and Technology **27:**405-421.
11. **Mainelis, G., K. Willeke, P. Baron, S. A. Grinshpun, and T. Reponen.** 2002. Induction charging and electrostatic classification of micrometer-size particles for investigating the electrobiological properties of airborne microorganisms. Aerosol Science and Technology **36:**479-491.
12. **Thomas, R. J., D. Webber, R. Hopkins, A. Frost, T. Laws, P. N. Jayasekera, and T. Atkins.** 2011. The cell membrane as a major site of damage during aerosolization of *Escherichia coli*. Applied and Environmental Microbiology **77:**920-925.
13. **Zhen, H. J., T. Han, D. E. Fennell, and G. Mainelis.** 2013. Release of free DNA by membrane-impaired bacterial aerosols due to aerosolization and air sampling. Applied and Environmental Microbiology **79:**7780-7789.
14. **Mainelis, G., K. Willeke, P. Baron, S. A. Grinshpun, T. Reponen, R. L. Górny, and S. Trakumas.** 2001. Electrical charges on airborne microorganisms. Journal of Aerosol Science **32:**1087-1110.
15. **Pierucci, O.** 1978. Dimensions of *Escherichia coli* at various growth rates: model for envelope growth. Journal of Bacteriology **135:**559-574.

16. **An, H. R., G. Mainelis, and L. White.** 2006. Development and calibration of real-time PCR for quantification of airborne microorganisms in air samples. Atmospheric Environment **40:**7924-7939.
17. **Chang, C.-W., and F.-C. Chou.** 2011. Assessment of bioaerosol sampling techniques for viable *Legionella pneumophila* by ethidium monoazide quantitative PCR. Aerosol Science and Technology **45:**343 - 351.
18. **Hospodsky, D., N. Yamamoto, and J. Peccia.** 2010. Accuracy, precision, and method detection limits of quantitative PCR for airborne bacteria and fungi. Applied and Environmental Microbiology **76:**7004-7012.
19. **Lee, B. U., and S. S. Kim.** 2003. Sampling *E. coli* and *B. subtilis* bacteria bioaerosols by a new type of impactor with a cooled impaction plate. Journal of Aerosol Science **34:**1097-1100.
20. **Peters, T. M., and D. Leith.** 2003. Concentration measurement and counting efficiency of the aerodynamic particle sizer 3321. Journal of Aerosol Science **34:**627-634.
21. **Nadkarni, M. A., F. E. Martin, N. A. Jacques, and N. Hunter.** 2002. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology and Molecular Biology Reviews **148**.
22. **Park, D., Y.-H. Kim, C. W. Park, J. Hwang, and Y.-J. Kim.** 2009. New bio-aerosol collector using a micromachined virtual impactor. Journal of Aerosol Science **40:**415-422.
23. **Lee, B. U., S. H. Kim, and S. S. Kim.** 2002. Hygroscopic growth of *E. coli* and *B. subtilis* bioaerosols. Journal of Aerosol Science **33:**1721-1723.
24. **Stein, S. W., P. B. Myrdal, B. J. Gabrio, D. Obereit, and T. J. Beck.** 2003. Evaluation of a new aerodynamic particle sizer spectrometer for size distribution measurements of solution metered dose inhalers. Journal of Aerosol Medicine **16:**107-119.
25. **Rule, A. M., K. J. Schwab, J. Kesavan, and T. J. Buckley.** 2009. Assessment of bioaerosol generation and sampling efficiency based on *Pantoea agglomerans.* Aerosol Science and Technology **43:**620-628.
26. **Theunissen, H. J. H., N. A. Lemmens-den Toom, A. Burggraaf, and M. F. Michel.** 1993. Influence of temperature and relative humidity on the survival of *Chlamydia pneumoniae* in aerosols. Applied and Environmental Microbiology **59:**2589-2593.
27. **Walter, M. V., B. Marthi, V. P. Fieland, and L. M. Ganio.** 1990. Effect of aerosolization on subsequent bacterial survival. Applied and Environmental Microbiology **56:**3468-3472.
28. **Stewart, I. W., G. Leaver, and S. J. Futter.** 1997. The enumeration of aerosolised *Saccharomyces cerevisiae* using bioluminescent assay of total adenylates. Journal of Aerosol Science **28:**511-523.
29. **Israeli, E.** 1973. Effect of aerosolization and lyophilization on macromolecular synthesis in *E. coli*, p. 110-113. *In* J. F. P. Hers, and Winkler, K. C. (ed.), Airbone transmissions and airborne infection. Oosthoek Publishing Company, Utrecht.
30. **Heidelberg, J. F., M. Shahamat, M. Levin, I. Rahman, G. Stelma, C. Grim, and R. R. Colwell.** 1997. Effect of aerosolization on culturability and viability of gram-negative bacteria. Applied and Environmental Microbiology **63:**3585-3588.

31. **Rule, A. M., J. Kesavan, K. J. Schwab, and T. J. Buckley.** 2007. Application of flow cytometry for the assessment of preservation and recovery efficiency of bioaerosol samplers spiked with Pantoea agglomerans. Environmental science & technology **41:**2467-2472.

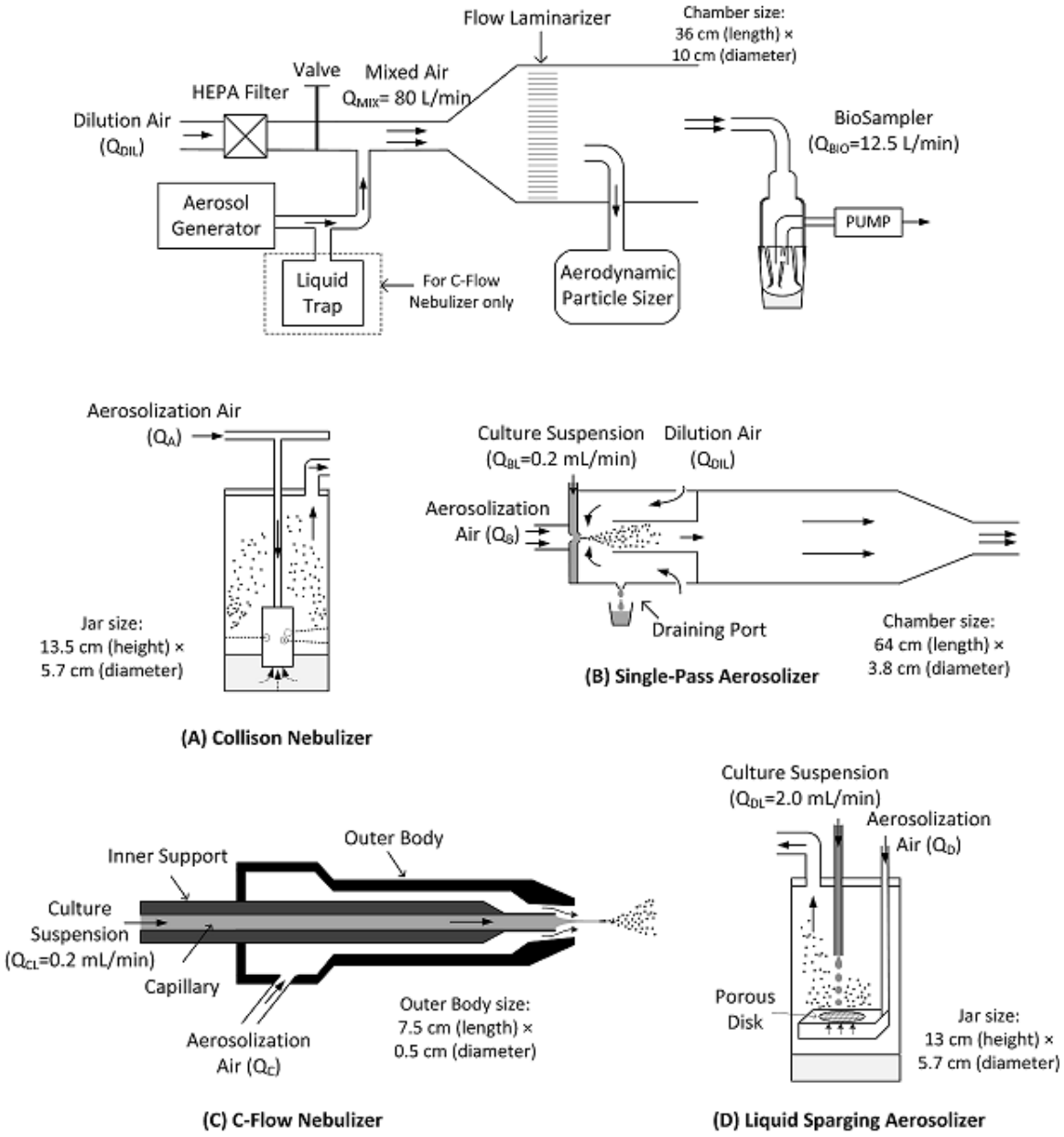

Figure 3.1 Experimental setup used to aerosolize *E. coli* with Collison nebulizer (A), Single-Pass Aerosolizer (B), C-Flow nebulizer (C), and Liquid Sparging Aerosolizer (LSA) (D) and collect *E. coli* bioaerosol with BioSampler.

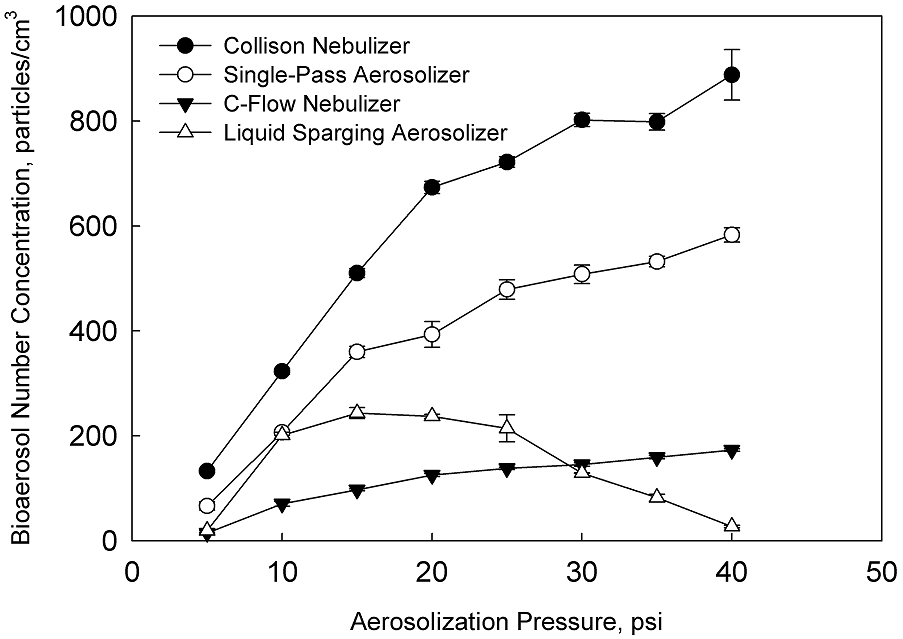

Figure 3.2 Number concentration of *E. coli* bacteria aerosolized by Collison nebulizer, Single-Pass Aerosolizer, C-Flow nebulizer, and Liquid Sparging Aerosolizer (LSA) under different aerosolization pressures. Symbols are averages of triplicates and error bars are one standard deviation.

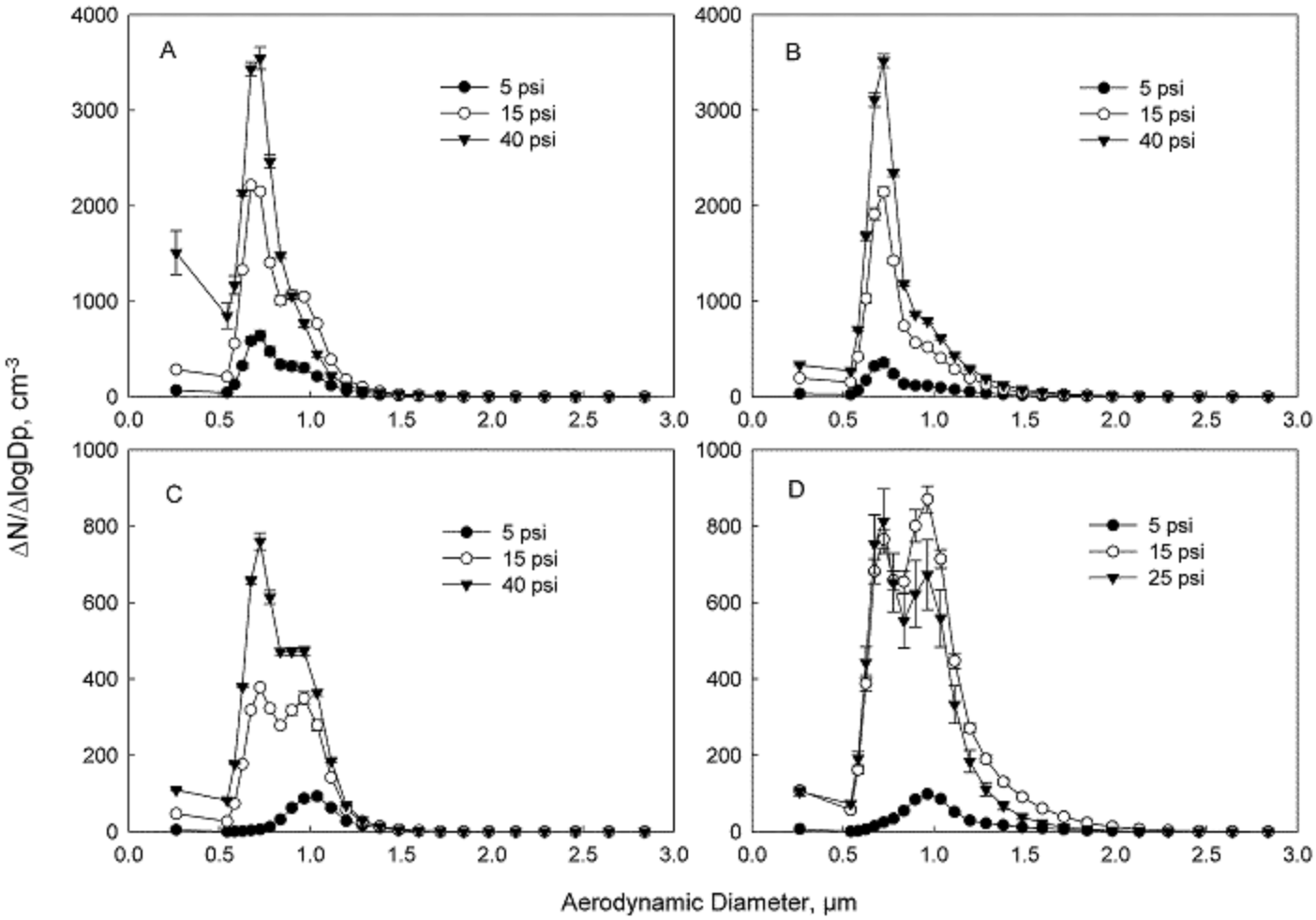

Figure 3.3 Size distribution of *E. coli* bioaerosols aerosolized by Collison nebulizer (A), Single-Pass Aerosolizer (B), C-Flow nebulizer (C), and Liquid Sparging Aerosolizer (LSA) (D) at different aerosolization pressures. Symbols are averages of triplicates and error bars are one standard deviation.

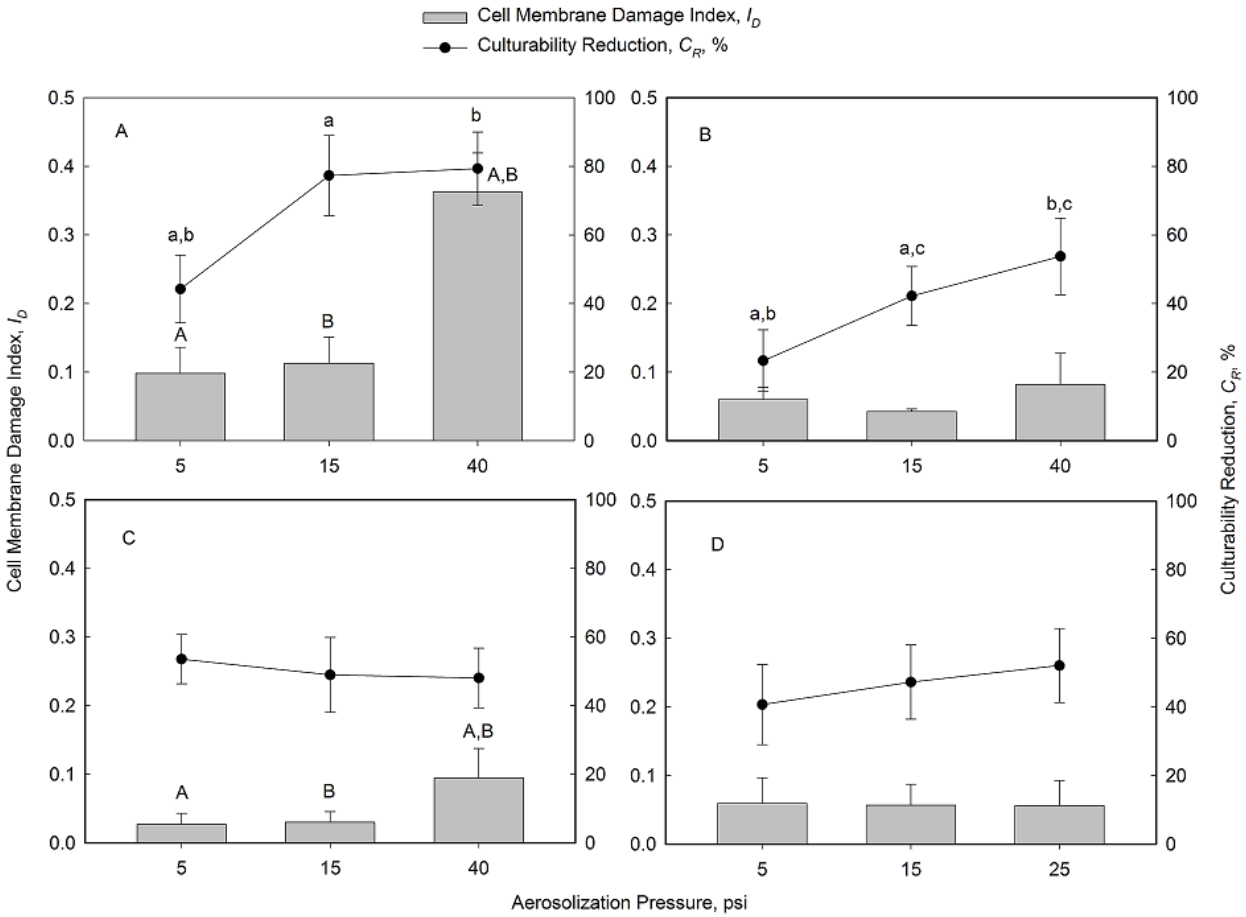

Figure 3.4 Cell Membrane Damage Index (I_D, indicated by bars, left y-axis) and Culturability Reduction (C_R, indicated by full circles, right y-axis) of *E. coli* bacteria aerosolized under different aerosolization pressures by Collison nebulizer (A), Single-Pass Aerosolizer (B), C-Flow nebulizer (C), and Liquid Sparging Aerosolizer (LSA) (D), and then collected by a BioSampler. Symbols and bars are averages of triplicates and error bars are one standard deviation. Capitalized symbol pairs (A or B) on bars indicate statistical difference (p<0.05) of I_D values at different aerosolization pressures. Lower-case symbol pairs (a, b, or c) indicates statistical difference of C_R values at different aerosolization pressures.

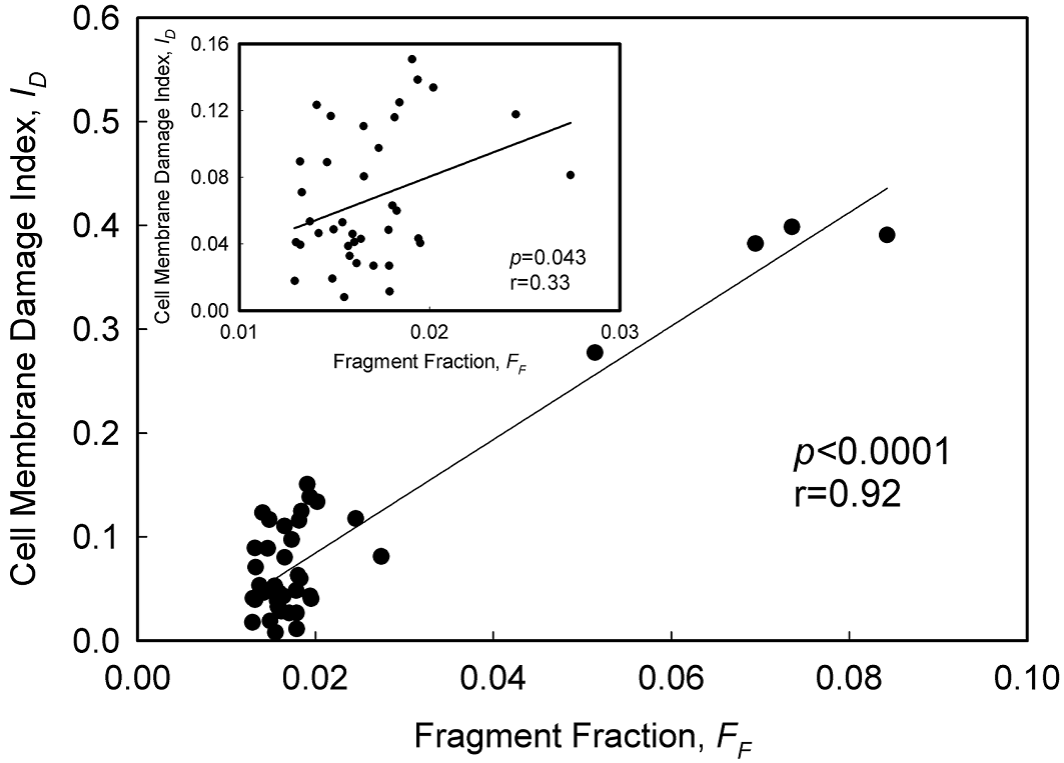

Figure 3.5 The Cell Membrane Damage Index (I_D) as a function of the Fragment Fraction (F_F) as measured by the APS.

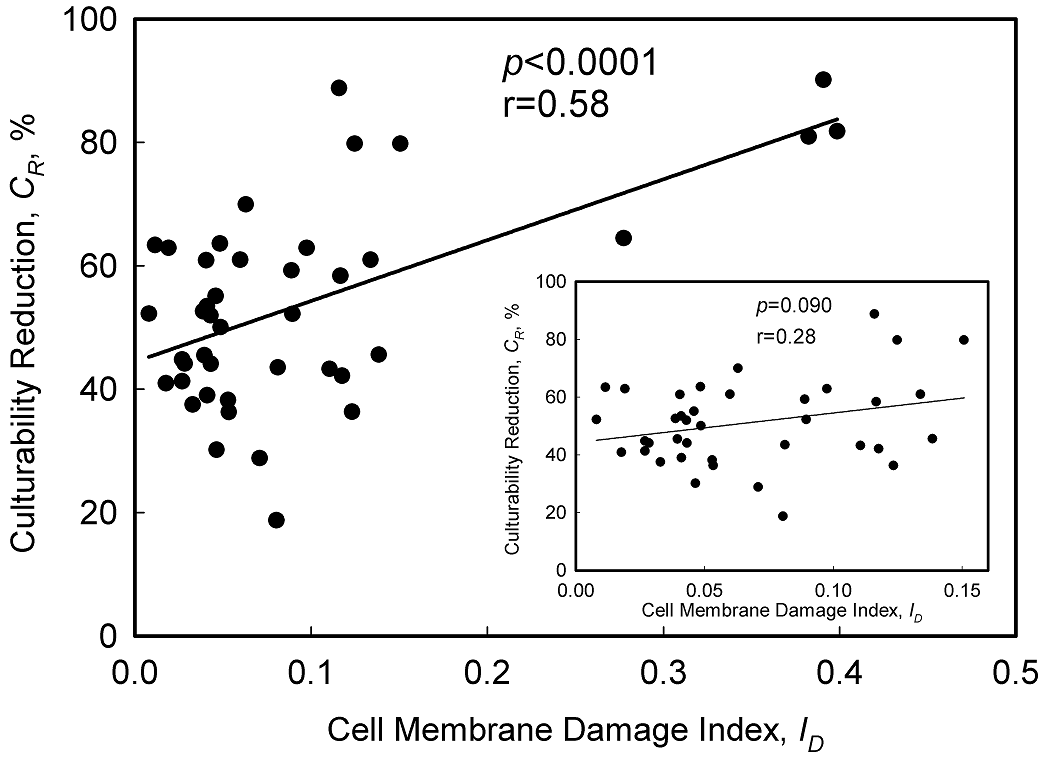

Figure 3.6 The correlation between the Cell Membrane Damage Index (I_D) and Culturability Reduction (C_R) for *E. coli* aerosolized by different methods.

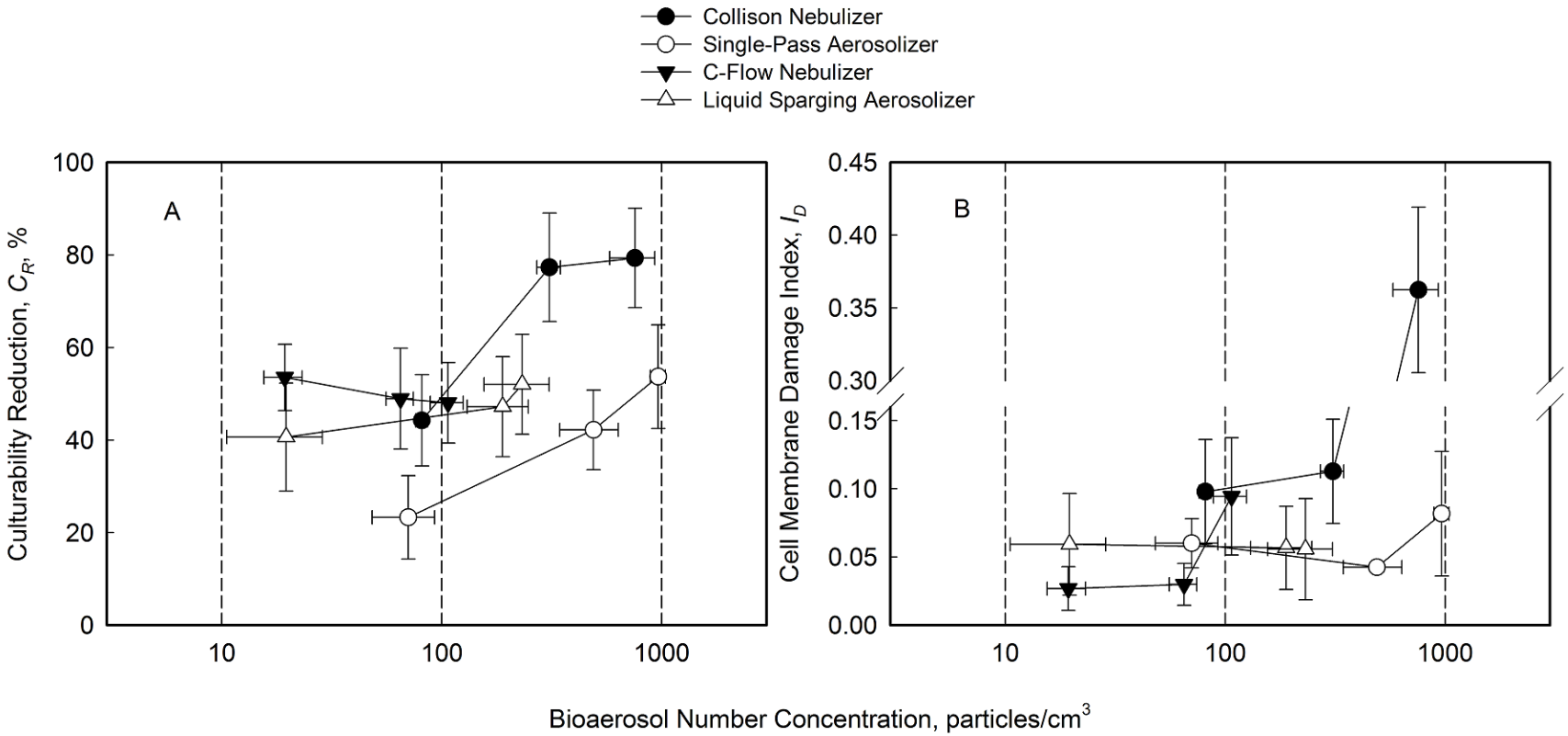

Figure 3.7 Culturability Reduction (C_R, left panel) and Cell Membrane Damage Index (I_D, right panel) of *E. coli* samples as a function of their airborne concentration when aerosolized by different methods. The airborne concentration was determined by the APS. Symbols are averages of triplicates and error bars are one standard deviation.

Table 3.1 The Fragment Fraction (F_F, fraction of the cell fragments with size from 0.37 µm to 0.523 µm relative to the total number of particles as measured by APS) as a function of bioaerosol generators and aerosolization pressures. The measurements were performed by the APS.

Aerosolization pressure (psi)	Collison nebulizer	Single-Pass Aerosolizer	C-Flow nebulizer	Liquid Sparging Aerosolizer
5	1.83 ± 0.04%	1.70 ± 0.02%	1.20 ± 0.02%	1.27 ± 0.02%
15	2.22 ± 0.07%	1.89 ± 0.01%	1.68 ± 0.01%	1.51 ± 0.01%
25	ND[a]	ND	ND	1.69 ± 0.03%
40	7.78 ± 1.14%	2.01 ± 0.00%	2.25 ± 0.02%	ND

a) Not Determined.

Chapter 4: Development of a Dual-internal-reference Technique to Improve Accuracy in Determination of Bacterial 16S rRNA: rRNA Gene Ratio with Application to *Escherichia coli* Aerosol Samples[3]

[3] This chapter is modified from the manuscript by Huajun Zhen, Valdis Krumins, Donna E. Fennell, and Gediminas Mainelis. Development of a dual-internal-reference technique to improve accuracy in determination of bacterial 16S rRNA: rRNA gene ratio with application to *Escherichia coli* aerosol samples, to be submitted.

4.1 Abstract

Accurate enumeration of rRNA level in microbial cells, e.g. by using the 16S rRNA: rRNA gene ratio, is critical to properly understand its relationship to microbial activities, but few studies have considered the possible methodological artifacts which may contribute to the variability of rRNA analysis results. In this study, a technique was developed by utilizing the genomic DNA and 16S rRNA from an exogenous species (*Pseudomonas fluorescens*) as dual internal references to improve accuracy in enumerating 16S rRNA: rRNA gene ratio of *Escherichia coli*. Results showed that this technique was able to adequately control the variability in sample processing and analysis procedures due to sample (DNA and RNA) losses, inefficient reverse transcription, and inefficient PCR amplification. The ratios of *E. coli* samples were found to increase by 2-3 fold after normalizing *E. coli* 16S rRNA gene and 16S rRNA quantities to the sample-specific fractional recoveries of reference (*P. fluorescens*) 16S rRNA gene and 16S rRNA, respectively. Besides, the intra-sample variation of this ratio, represented by coefficients of variation from replicate samples, decreased significantly after normalization. This technique was applied to investigate the temporal variation of 16S rRNA: rRNA gene ratio for *E. coli* during unsteady growth in complex liquid medium and *E. coli* aerosols in exposure to particle-free air after collection on filter. And it was found that this technique could greatly enhance our ability to detect the change of ratio across different physiological states of microbial samples. Another interesting finding was that *E. coli* bioaerosols produced more rRNA in response to air sampling stress of filtration, which indicates the potential cellular activities during bioaerosol collection.

4.2 Introduction

Small subunit ribosomal RNA (rRNA) gene, e.g. prokaryote 16S rRNA gene, has been commonly used to assess the phylogenetic structure of microbial community in various ecosystems, because it is ubiquitous and has low mutation rates throughout prokaryotic evolution (1). On the other hand, its transcription product, the 16S rRNA, is directly involved in protein synthesis; thus its relative abundance, often presented as the rRNA: rRNA gene ratio (herein referred to as "rRNA level"), is considered to be an indicator of microbial activity (2-7). Unlike the rapid turnover of messenger RNA (mRNA) within the cell, rRNA is perceived as a relatively stable RNA type (8). As a result, the 16S rRNA has been frequently used as an endogenous reference to normalize the mRNA quantities when studying specific gene expression of microbial sample (9-11). However, the rRNA level within the cell also exhibited some changes depending on the physiological state of the cell. For example, a number of studies have shown positive correlation between rRNA concentrations and growth rates of specific bacterial species in pure cultures (12-15), although this correlation was not linear under non-steady-state growth conditions (13, 16, 17). A more recent study also reported elevated 16S rRNA level for *Sphingomonas aerolata* aerosols on supply of gaseous growth substrates in a rotating bioreactor (17). In addition, Blazewicz *et al*. indicated in a review paper that little information is available regarding the relationship between non-growth activities and bacterial rRNA concentration (18). For example, one study mentioned that the rRNA level in *Lactococcus lactis* cell decreased by 50% when exposed to heat shock at 43°C for 30 min (19). Thus, it is apparent that the relationship between rRNA content and microbial activity is still inconclusive and needs further exploration.

More recently, the application of reverse transcription (RT) in conjunction with real-time PCR (qPCR) has become the primary method for quantifying specific rRNA and mRNA from microbial samples. A number of studies have applied both qPCR and RT-qPCR to examine the 16S rRNA: rRNA gene ratio for microbes from a variety of environmental systems, including plant leaves (5), marine surface waters (2, 6), biofilms (3), nitrification reactor (7) and water from sewage treatment plants (4). However, none of these studies have revealed any information regarding the DNA and RNA losses during sample processing, thus the accuracies of those reported ratios, which spanned almost 5 orders of magnitude from ~0.1 (7) to ~10^4 (3), were largely unknown. In addition, high intra-sample variation, as indicated by the large coefficient of variation (COV) from replicate samples, has also been observed in these studies (3-7). The large intra-sample variation could reduce the statistical power of detecting difference of 16S rRNA: rRNA gene ratios among multiple samples. For example, when the variation in rRNA level across different physiological states of bacterial cell is only minimal, the large intra-sample variation might prevent from showing statistical significance in ratios between samples. To overcome these issues, a robust analytical method is needed to determine 16S rRNA: rRNA gene ratio with improved accuracy and reduced intra-sample variation so that a link between the variation in rRNA abundance and bacterial activity could be examined.

In most gene expression studies, the variation among individual samples comes from two different sources, the biological variability and technical variability (20-22). The biological variability is the inherent difference in RNA transcription level between different samples including sample replicates (20, 21), while the technical variability is

mainly influenced by the experimental artifacts (20, 22), and thus could be controlled and reduced by modification of experimental procedures and parameters. For studies on 16S rRNA: rRNA gene ratio, the technical variability can stem from multiple sources, e.g. sample losses during DNA/RNA isolation and DNase treatment for RNA sample, inefficient reverse transcription of 16S rRNA, and biased quantification of 16S rRNA gene or reverse transcribed 16S rRNA during qPCR amplification. In order to control for this variability, an exogenous internal reference technique has been applied in a number of previous studies to improve the accuracy of RNA quantification by RT-qPCR (23-25). The main assumption is that the reference RNA, a known amount of which is spiked prior to RNA extraction, behaves similarly to the target RNA analyte through the entire analysis process. The quantity of the target RNA analyte is then obtained by normalizing the recovered RNA quantity to the fractional recovery of the reference RNA. In these studies, the most commonly used reference RNA was the firefly luciferase *luc* mRNA (3, 24, 26). In addition to its application in RNA quantification, this technique has also been used to enumerate DNA in environmental samples, where the exogenous references included plant DNA from *Arabidopsis thaliana* (27), or whole genetically modified *Escherichia coli* cell (28, 29).

In this study, we developed an exogenous dual-internal-reference technique to determine the 16S rRNA: rRNA gene ratio with improved accuracy for bacterial samples, including bioaerosol samples. Specifically, known amounts of 16S rRNA and genomic DNA from a reference bacterial species (*Pseudomonas fluorescens*) were spiked into the target *E. coli* sample prior to DNA and RNA co-extraction. The quantities of extracted16S rRNA and 16S rRNA gene for both bacteria were measured by a multiplex

qPCR method independently. This technique was then applied to *E. coli* from both liquid culture and bioaerosol samples to investigate the variation of 16S rRNA: rRNA gene ratio as a function of both growth and non-growth activities. To the best of our knowledge, this is the first study to apply dual internal references simultaneously to accurately quantify RNA and DNA from a microbial sample. It is hoped that this technique will have a wide application when investigating rRNA abundance and its relationship to bacterial activity.

4.3 Materials and Methods

4.3.1 Bacterial Culture

Two bacterial species, *E. coli* (ATCC 15597, Manassas,VA) and *P. fluorescens* (ATCC 13525) were cultivated on Tryptic Soy Agar and Nutrient Agar (Becton, Dickinson and Company, Sparks, MD), respectively, and stored at 4 °C. Prior to experiments, *E. coli* and *P. fluorescens* were precultured in Tryptic Soy Broth (TSB) and Nutrient Broth (Becton, Dickinson and Company, Sparks, MD) at 37°C and 26°C for 16 hr, respectively. An individual aliquot of bacterial culture was harvested by centrifugation and the pelleted cells were saved at -80 °C for subsequent experiments. If needed, the bacterial cell numbers were quantified by epifluorescence microscopy using Axioskop 20 microscope (Carl Zeiss Inc. Thornwood, NY) according to a previously published procedure (30).

4.3.2 Extraction of Nucleic Acids

A phenol-chloroform protocol was applied to extract the total genomic DNA and RNA from *E. coli* samples either in cell pellets or on filters (12, 16). Prior to the extraction, *E. coli* samples were spiked with dual internal references: 5 µl of genomic DNA from *P. fluorescens* (1.1×10^6 copies of 16S rRNA gene/µl) and 5 µl 16S rRNA extracts (8.6×10^9 copies/µl) from *P. fluorescens*. The samples were suspended in 50 µl buffer A (50 mM glucose, 10 mM EDTA and 25 mM pH=8.0 Tris), and treated with five quick freeze/thaw cycles of freezing with liquid nitrogen and thawing with water bath at 55 °C, respectively. Thereafter, 200 µl of buffer A, 100 µl of 4 mg/ml lysozyme in buffer A and 50 µl of 500 mM EDTA were added in sequence into the sample. After incubation on a rotator at room temperature for 5-10 minutes, a 50 µl 10% sodium dodecyl sulfate (SDS) solution was

added into the sample solution. The cell lysate was then extracted twice with 800 µl phenol-chloroform-isoamyl alcohol mixture (25:24:1, PH=6.7). After that, the aqueous phase was mixed with 50 µl of 3.0 M sodium acetate, 2 µl of glycogen (20 mg/ml), and 1 ml 100% ice-cold ethanol. The nucleic acids were pelleted by centrifugation at 16,100×g at 4°C for 15 minutes and then washed once with 400 µl 70% ethanol solution. The retrieved pellets were first dried under a laminar flow hood for 10 minutes and then dissolved in 100 µl diethylpyrocarbonate (DEPC)-treated water. DNA was quantified by qPCR from the extracted final product, while a separate aliquot from the sample was immediately saved at -80°C for subsequent RNA analysis.

4.3.3 DNA Removal and Reverse Transcription

The RNA samples were subjected to DNA removal and reverse transcription before qPCR quantification. DNA was removed using the Ambion TURBO DNA-free DNase kit (Life Technologies, Grand Island, NY) following manufacturer's instructions. The DNA-free RNA sample was reverse transcribed into complementary DNA (cDNA) using SuperScript® VILO cDNA synthesis kit (Life Technologies), and then cDNA was diluted and stored at -20°C prior to qPCR quantification.

4.3.4 Preparation of Dual Internal References

To prepare the genomic DNA reference from *P. fluorescens*, 4×1 ml freshly grown pure culture ($7.6\times10^8\pm2.3\times10^8$ cells/ml) were harvested and subjected to nucleic acids extraction by a phenol-chloroform protocol described above with the following modification: after addition of SDS solution, 1µl of 10 unit RNase ONE™ ribonuclease (Promega, Madison, WI) was applied to each crude extract for 10 mins at 37°C; the cell

lysate were extracted by phenol-chloroform solution three times instead of twice; the total nucleic acids dissolved in DEPC-water after ethanol precipitation was treated further with 2.5 unit of RNase ONE™ ribonuclease for 30 minutes at 37°C, and then RNase was inactivated by addition of 5 mM Dithiothreitol (DTT) and incubation at 70°C for 15 minutes. The complete degradation of RNA in DNA/RNA mixture was verified with agarose gel electrophoresis on a 1.2% gel (110 V, 45 minutes). In the final step, the genomic DNA extracts were purified by using a DNeasy blood & tissue kit (Qiagen, Valencia,CA) according to the manufacturer's protocol.

To prepare the 16S rRNA reference, total nucleic acids from 8×1 ml *P. fluorescens* pure culture ($7.6\times10^8\pm2.3\times10^8$ cells/ml) were extracted according to the unmodified phenol-chloroform protocol described above. The final products were dissolved in 600 µl DEPC-water and then the entire sample was loaded in small quantities into individual wells on a 1.2% agarose gel, and the 16S rRNA was separated from other nucleic acids by electrophoresis. The volume of loaded sample after mixing with loading dye was 10 µl (<5 µg nucleic acids) per well, and thus a total of ~70 wells on three individual agarose gels were used. The gels were run at 110 V for 45 mins in cold 1×TAE buffer. The 16S rRNA bands on each gel were cut and collected into a total of 10 microcentrifuge tubes, each of which contained ~400 mg gel material. The agarose gel pieces containing 16S rRNA bands were then extracted and purified with a Zymoclean™ Gel RNA Recovery Kit (Zymo Research Corp., Irvine, CA) according to the manufacturer's protocol. The integrity of extracted 16S rRNA was checked by agarose gel electrophoresis (1.2%, 110 V for 45 min). The genomic DNA and 16S rRNA for *E. coli* were prepared in a similar way to that of *P. fluorescens*. The integrity of

purified nucleic acids for both bacteria were checked by agarose gel electrophoresis (1.2%, 110 V for 45 min) with 1000-bp DNA ladders (Thermo Scientific, Waltham, MA) for genomic DNA and with a Lambda DNA/HindIII marker (Thermo Scientific) for 16S rRNA.

4.3.5 Quantification of Genomic DNA and 16S rRNA Standards

The purified genomic DNA and 16S rRNA extracts from both bacteria were quantified individually by a Qubit dsDNA HS assay kit and RNA HS assay kit (Life technologies, Grand Island, NY) with a Qubit 2.0 fluorometer (Life technologies). After quantification, the DNA/RNA extracts were divided into several microcentrifuge tubes and stored in -80 °C prior to use.

To convert the concentrations of genomic DNA from a mass basis to a 16S rRNA gene copy number basis, the following equation was applied:

$$C_{16S\ rRNA\ Gene} = \frac{C_{mass} \times f \times n}{N} \qquad (1)$$

where $C_{16S\ rRNA\ gene}$ is the number concentration of 16S rRNA gene in the sample (copies/ml); C_{mass} is the mass concentration of genomic DNA in the same sample (ng/ml); *f* is the average number of base pairs per unit mass of DNA, which is equal to 0.978×10^{12} bp/ng (31); *n* is the average number of 16S rRNA gene copy in the bacterial genome, which are 7 and 5 for *E. coli* (32) and *P. fluorescens* (33), respectively; *N* is the number of base pairs in the bacteria genome: 4,639,221 and 6,845,832 bp for *E. coli* (32) and *P. fluorescens* (33), respectively. The number concentration of 16S rRNA was determined by the following equation:

$$C_{16S\ rRNA} = \frac{C_{mass} \times K}{M} \times 10^{-9} \quad (2)$$

where $C_{16S\ rRNA}$ is the number concentration of 16S rRNA in the sample (copies/ml); C_{mass} is the mass concentration of 16S rRNA in the same sample (ng/ml); *K* is the Avogadro's Constant ($6.02214129 \times 10^{23}$/mol); *M* is the molecular weight of target 16S rRNA (g/mol), which can be calculated based on the equation:

$$M = 320.5 \times N + 159 \quad (3)$$

where M is the molecular weight of 16S rRNA (g/mol); *N* is the number of nucleotides for a complete16S rRNA sequence, which are 1542 and 1529 for *E. coli* (32) and *P. fluorescens* (33), respectively.

4.3.6 Primers and Probes

Taqman primer-probes sets were purchased from Sigma-Aldrich (Woodlands, TX). The primer-probes sets were designed by Beacon Designer's program (Premier Biosoft, Palo Alto, CA) to target the 16S rRNA gene sequence of *E. coli* and *P. fluorescens*, respectively. For *E. coli* 16S rRNA gene, they include forward primer: GGGAGTAAAGTTAATACCTTTG, reverse primer: CCAGTATCAGATGCAGTTC, and probe: TCACATCTGACTTAACAAACCGCCT-FAM. The primer-probe sets for *P. fluorescens* 16S rRNA gene are forward primer: CCTTGTCCTTAGTTACCAG, reverse primer: CTCTGTACCGACCATTGTA, and probe: CACTCTAAGGAGACTGCCGGTGAC. When single-stranded cDNA samples after reverse transcription were quantified, the theoretical copy number of double-stranded DNA standard (genomic DNA extracts) was multiplied by two.

4.3.7 Quantitative PCR

Multiplex quantitative PCR was applied using the iCycler iQ5 RT-PCR detection system (Bio-Rad Laboratories, Hercules, CA) to quantify *E. coli* and *P. fluorescens* 16S rRNA gene or reverse transcribed 16S rRNA, simultaneously. To optimize the qPCR condition, the primer and probe concentrations were tested with initial concentrations in the range of 250-750 and 150-300 nM, respectively, and the annealing temperatures were tested from 58 to 62°C. The determined optimal reaction conditions in a 20 µl mixture included 10 µl of 2× TaqMan® universal PCR master mix (Life technologies), 650 nM of each forward and reverse primer, 200 nM of each probe, 0.04 mg/ml bovine serum albumin (Sigma, St. Louis, MO) and 4 µl sample DNA or cDNA. The amplification reaction was performed using the following temperature program: 10 min of denaturation at 95°C and 40 cycles of 15 s of denaturation at 95°C and 1 min of annealing/extension at 58°C and 30 s further extension at 72°C. Data analysis was performed using iCycler iQ real-time detection system software. A 10-fold serial dilution of mixture containing *E. coli* and *P. fluorescens* genomic DNA standards [with similar 16S rRNA gene copy numbers for each organism] were performed in multiplex qPCR along with samples in each plate. Standard curves for individual amplicons (*E. coli* or *P. fluorescens* 16S rRNA gene sequence) were prepared by plotting each cycle threshold (C_T) value against the log of target gene copy number contained in the mixture. Each sample was measured by multiplex qPCR in triplicate.

4.3.8 Reverse Transcription Efficiency

The reverse transcription efficiencies for *E. coli* and *P. fluorescens 1*6S rRNA were examined separately. Specifically, serially diluted 16S rRNA standards for individual bacteria were reverse transcribed according to RT protocols described in section 4.3.3. The reverse transcribed 16S rRNA standards were then analyzed in parallel with serially diluted 16S rRNA gene standards by singleplex qPCR assay. Each 20 µl reaction mixture for singleplex assay included 10 µl of 2× TaqMan® universal PCR master mix, 650 nM of forward and reverse primer, 200 nM of probe, 0.04 mg/ml bovine serum albumin and 4 µl sample DNA or cDNA. The temperature program was the same as in multiplex qPCR assay (section 4.3.7). The threshold cycle values were automatically determined by iCycler iQ real-time detection system software. For a fixed threshold cycle value, the 16S rRNA gene copy number was first multiplied by a factor of two. This number was then divided the number of 16S rRNA copies for the same threshold cycle, and the resulting ratio was defined as reverse transcription efficiency. The threshold cycle, 20, was selected as the fixed cycle value due to its proximity to the midpoint on both standard curves.

4.3.9 Methods Used to Isolate DNA and RNA

A typical DNA and RNA co-extraction protocol often includes two steps, e.g. cell lysis and DNA/RNA isolation. Previously, it was suggested that the loss of RNA during isolation in a phenol-chloroform protocol (50%) was significantly higher than that in the cell lysis step (24). The sample loss due to incomplete lysis of cells can not be controlled by the exogenous reference method and thus should be carefully optimized (24). Here, in order to test the effectiveness of the internal reference technique in quantifying the sample loss during isolations of DNA and RNA by different methods, we used a single

batch of lysed *E. coli* cells with dual internal references, and then subjected the lysed cells to different isolation methods from three commonly used DNA/RNA co-extraction protocols, which included the aforementioned phenol-chloroform protocol (section 4.3.2), a TRI-Reagent (Molecular Research Center, Cincinnati, OH) method and an Allprep DNA/RNA mini Kit (Qiagen, Valencia, CA). After isolation, the DNA and RNA were processed and quantified in the same way as described in sections 4.3.3 and 4.3.5, and the determined 16S rRNA: rRNA gene ratios from the three isolation methods were compared.

Specifically, in the phenol-chloroform protocol, the cell lysis was completed after adding the SDS solution, and DNA and RNA were isolated from other cell components from this step forward. For TRI-Reagent method, isolation of DNA and RNA from the cell lysate was performed as described by Perez-Osorio and Franklin (3). Briefly, 500 µl TRI Reagent, 3 µl PolyAcryl carrier, and 50 µl 1-bromo-3-chloropropane (BCP) were added into cell lysate; the mixture was vortexed thoroughly and separated into organic phase and aqueous phase after centrifugation. A 250 µl isopropanol was added into the aqueous phase and the RNA sample was precipitated by centrifugation. The organic phase containing DNA sample was mixed with 3 µl PolyAcryl carrier and 150 µl ethanol, and then the DNA was precipitated by centrifugation. The precipitated DNA and RNA samples were further washed with 75% ethanol twice and subjected to the treatment procedures described in sections 4.3.3 and 4.3.5.The third DNA/RNA isolation method used the Allprep DNA/RNA mini Kit according to the manufacturer's instructions. This method separated and purified the nucleic acids on silica membrane spin columns (4).

The detailed steps for DNA and RNA isolation for the three protocols are illustrated in Figure 4.1.

4.3.10 Change in 16S rRNA: rRNA Gene Ratio for *E. coli* Growing in Liquid Culture

A few *E. coli* colonies from a TSA agar plate were randomly picked and inoculated into 50 ml freshly made TSB medium under strict aseptic conditions. The inoculated medium was incubated at 37°C on a mechanical shaker at 120 rpm for over 16 hrs. 100 µl homogenized culture samples were taken at 1, 2, 3, 4, 5, 6, 7, 9, 12 and 16 hours after inoculation and stored at -80°C prior to analyzing their16S rRNA: rRNA gene ratio. The absorbance of growth medium was measured every hour after inoculation until 16 hrs with a spectrophotometer at 600 nm wavelength (PerkinElmer, Shelton, CT). The relative growth rate, V_t, of *E. coli* in liquid culture each hour was calculated by the following equation:

$$V_t = (Abs_{t+1} - Abs_t)/Abs_t \qquad (4)$$

where Abs_t is the absorbance at time *t*; Abs_{t+1} is the absorbance at t+1 hour; is the relative growth rate of *E. coli* culture at time t.

4.3.11 Aerosolization of *E. coli*

The precultured *E. coli* cells were first pelleted and then washed twice with 1×PBS solution by centrifugation at 6,000×g for 5 min at 25 °C (BR4; Jouan, Winchester, VA) and refilled with 50 ml 1×PBS solution after the second wash. The bacterial suspensions were aerosolized using a Single-Pass Aerosolizer (CH Technologies Inc., Westwood, NJ)

which was previously shown to induce minimal stress to bacteria due to aerosolization compared to other commonly used bioaerosol generators (34). The culture was supplied by a syringe pump (Kent Scientific Corp., Torrington, CT) at a rate of 0.1 ml/min, and the aerosolization air flow rate was 1.2 L/min. The aerosolized *E. coli* was diluted with a particle-free airflow at 80 L/min and introduced into an open cylinder-shaped chamber (36 cm in length × 10 cm in diameter). The aerosolization time was one minute. Five Button aerosol samplers (SKC Inc., Eighty Four, PA) were placed inside the chamber and simultaneously operated to sample *E. coli* bioaerosol (for 1 minute during aerosolization) at a flow rate of 17 L/min with a 0.8 µm-pore-size and 22 mm-diameter polyethersulfone (PES) membrane filter (SUPOR filter, Pall Corp., Port Washington, NY). Once *E. coli* aerosolization stopped, all Button samplers continued with operation and sampled particle-free air (relative humidity at 25-30%) for additional times of 0, 2, 4, 6 and 10 hours, respectively. After that, filters from each sampler were removed and cut into 1cm×1cm pieces with sterile scissors and stored at -80°C. Prior to each test, a 100 µl liquid *E. coli* culture from the same suspension used for aerosolization was also saved at -80°C to serve as reference cells for analysis of 16S rRNA: rRNA gene ratio. The entire experiment was conducted inside a Class II Biosafety cabinet (NUAIRE Inc., Plymouth, MN) to prevent the escape of bioaerosols into the laboratory environment.

4.3.12 Statistical Analysis

Statistical analysis was performed using SPSS v.18.0 (IBM Corp., Armonk, NY). The specific statistical tests and investigated variables are described in more detail in Table 4.1. Overall, a statistically significant difference was assumed for $p<0.05$.

4.4 Results

4.4.1 Quality of Genomic DNA and 16S rRNA Extracted from *E. coli* and *P. fluorescens*

The quality of extracted genomic DNA and 16S RNA was assessed by agarose gel electrophoresis as shown in Figure 4.2. The lack of smearing in the bands of extracted 16S rRNA (Figure 4.2a) and genomic DNA (Figure 4.2b) for both bacterial species indicated integrity of both nucleic acids. It was estimated that 6 µg and 3 µg of genomic DNA and 16S rRNA, respectively, were extracted and purified per ml of *E. coli* ($1.6\pm0.3\times10^9$ cells/ml), and 3 µg and 2 µg genomic DNA and 16S rRNA, respectively, were harvested per ml of *P. fluorescens* ($7.6\pm2.3\times10^8$ cells/ml).

4.4.2 Examination of Reverse Transcription Efficiencies of *E. coli* and *P. fluorescens* 16S rRNA

Inefficient reverse transcription is a major source of data variability when analyzing RNA. The exogenous reference RNA should have satisfactory and similar reverse transcription efficiencies to the target RNA so that the loss of target RNA due to inefficient reverse transcription can be estimated by the reference RNA. In order to examine the transcription efficiencies for 16S rRNA of both species, the 10-fold diluted 16S rRNA extracts from each species were reverse transcribed and analyzed in parallel with the 16S rRNA gene standards from the same species.The average reverse transcription efficiencies for *E. coli* (see Figure 4.3a) and *P. fluorescens* (Figure 4.3b) were determined to be 77±11% and 70±8% (N=3, calculated at threshold cycle of 20), respectively, which

is indicative of similar efficiency for both target and reference 16S rRNA during reverse transcription.

4.4.3 Development of Multiplex qPCR Assay for Determination of 16S rRNA Genes of *E. coli* and *P. fluorescens*

Prior to multiplex qPCR assay, the validity of designed primers and probe sets for each bacteria were tested by singleplex qPCR assays using *E. coli* and *P. fluorescens* genomic DNA. Linear amplifications with quantification ranging from 10^2 to 10^7 copies of 16S rRNA gene per reaction were obtained for both assays (Figure 4.4a and 4.4b). No amplification was observed in simplex assays using *E. coli* primer and probe set with *P. fluorescens* genomic DNA or *P. fluorescens* primer and probe set with *E. coli* genomic DNA (data not shown). The amplification efficiencies for *E. coli* and *P. fluorescens* singleplex assays were 93% and 90%, respectively, when corresponding genomic DNA was applied for each assay. In multiplex assay, when approximately equal quantities of DNA from the two species were mixed to make a serial dilution, linear amplifications were achieved in the range from 10^2 to 10^7 copies of 16S rRNA gene per assay (Figure 4.4a and 4.4b). The slopes of the amplification curves did not exhibit significant difference between two target genes in the multiplex assay (p=0.659), indicating similar amplification efficiencies for both targets. Similarly, the difference in amplification efficiencies between the multiplex and singleplex assay for *E. coli* and *P. fluorescens* 16S rRNA genes were not statistically significant: p=0.08 in both cases.

One common issue in application of multiplex qPCR is that when two target amplicons are present in the same sample at greatly different quantities, the amplification

signals are often distorted especially for the less abundant amplicon. This effect could be due to multiple reasons, such as reagent limitation and amplification inhibition. Thus, in order to determine the conditions when such experimental errors could be avoided, a suite of 10-fold dilutions (10^2-10^7 copies per sample) of *E. coli* 16S rRNA gene was mixed with similar dilutions of *P. fluorescens* 16S rRNA gene (10^2-10^7 copies per sample) in reverse order, e.g. ~10^7 copies of *E. coli* amplicon mixed with ~10^2 copies of *P. fluorescens* 16S rRNA gene, ~10^6 copies of *E. coli* amplicon mixed with ~10^3 copies of *P. fluorescens* 16S rRNA gene, *etc*. According to the results presented in Figure 4.5a, when the 16S rRNA gene quantity of *E. coli* was greater than that of *P. fluorescens* by a factor of 1000:1 or higher, the threshold cycle numbers of *P. fluorescens* increased compared with singleplex assay. Similarly, when *P. fluorescens* 16S rRNA gene quantities exceeded *E. coli* 16S rRNA gene quantities by a factor of 1000:1 or higher (Figure 4.5b), the threshold cycle numbers of *E. coli* decreased dramatically compared with the singleplex assay. Thus, in order to achieve reliable quantification of both target genes when performing multiplex assay, the difference in relative initial quantities of *E. coli* and *P. fluorescens* 16S rRNA genes should not exceed a factor of 10. As a result, an initial estimation of the target *E. coli* 16S rRNA gene quantity relative to that of *P. fluorescens* in each sample was required prior to qPCR reaction.

4.4.4 Effects of Initial Cell Quantities on Determination of 16S rRNA: rRNA Gene Ratio with Dual-internal-reference Technique

The data in section 4.4.3 and Figure 4.5 show that a substantial difference in initial quantities of 16S rRNA gene from *E. coli* and *P. fluorescens* affected the quantification of both analytes when performing multiplex-qPCR. Likewise, when different amounts of

E. coli are analyzed together with a fixed quantity of *P. fluorescens* 16S rRNA and rRNA gene, the initial quantity of *E. coli* cells could have an effect on the amplification efficiency of both species and thus on the accuracy of the dual-internal-reference technique. To investigate this possibility, aliquots with a fixed quantitity of *P. fluorescens* genomic DNA and RNA (4.3×10^{10} and 5.5×10^{6} copies of 16S rRNA gene and rRNA, respectively) were mixed with different numbers of *E. coli* cells (2.0×10^{4} to 2.0×10^{6}) prior to DNA/RNA co-extraction. The *E. coli* cells were prepared by making serial dilutions from the same batch of pure culture, and three separate batches were evaluated.

The results presented in Figure 4.6a show that the recovered copy number of *E. coli* 16S rRNA gene increased linearly with increasing cell quantity. The recovery of *P. fluorescens* 16S rRNA gene was on average 52±28% across different initial *E. coli* cell quantities. When *E. coli* 16S rRNA gene quantities are corrected using the *P. fluorescens* reference, the quantities increase by nearly a factor of two, and the difference between slopes of regression lines before and after normalization to the recovery of the reference 16S rRNA gene is insignificant (p=0.143). A similar normalization approach was performed with *E. coli* 16S rRNA (Figure 4.6b). The average recovery for *P. fluorescens* 16S rRNA (a reference) was 19±6% across all test sample groups with different initial cell quantities, and the quantities of *E. coli* 16S rRNA increased by approximately a factor of five after taking into account recovery of *P. fluorescens* 16S rRNA. Likewise, the difference between slopes of regression lines before and after normalization to the recovery of the reference 16S rRNA was insignificant (p=0.150).

Figures 4.6a and 4.6b clearly indicate that accounting for the recoveries of dual references (*P. fluorescens* 16S rRNA gene and 16S rRNA) substantially affects the

amount of recovered *E. coli* 16S rRNA gene and 16S rRNA. Using this information, one can calculate 16S rRNA: rRNA gene ratios for *E. coli* samples of different initial cell quantities and the results are presented in Figure 4.6c. When normalization to recoveries of dual references (*P. fluorescens* 16S rRNA and 16S rRNA gene) was not applied, the average ratio was $1.6\times10^{3}\pm9\times10^{2}$ (n=15). When the recoveries of the references were taken into account, the average ratio increased to $4.3\times10^{3}\pm1.1\times10^{3}$ (n=15), and the difference was statistically significant ($p<0.001$). In addition, the *E. coli* 16S rRNA: rRNA gene ratio is no longer dependent on the initial cell quantity after normalization of *E. coli* 16S rRNA and rRNA gene to recoveries of dual references.

4.4.5 Effect of DNA/RNA Isolation Protocol on Determination of 16S rRNA: rRNA Gene Ratio with Dual-internal-reference Technique

It was shown previously that a large fraction of mRNA was lost during the isolation steps in a RNA extraction protocol (24). To test the ability of the dual-internal-reference technique to control sample loss during the DNA/RNA isolation, we used and compared three common extraction protocols (Figure 4.1): 1) a traditional liquid-liquid extraction method with Phenol/Chloroform followed by nucleic acid precipitation, 2) a liquid-liquid extraction method with TRI-Reagent followed by nucleic acid precipitation and 3) a silica gel membrane-based adsorption and elution by a commercial Allprep DNA/RNA mini Kit.

Here, a batch of lysed *E. coli* cells was divided into three equal aliquots and each aliquot was spiked with known quantities of references (*P. fluorescens* 16S rRNA and rRNA gene) and then subjected to sample preparation according to the procedures of the

individual protocols. The recovery of *P. fluorescens* 16S rRNA (reference) was 16±1%, 18±6% and 17±5% for Phenol/Chloroform, TRI-Reagent and Allprep kit methods, respectively, and the difference was not significantly different. Conversely, the recovery of *P. fluorescens* (reference) 16S rRNA gene was 76±11%, 21±3% and 16±14% for Phenol/Chloroform, TRI-Reagent and Allprep kit methods, respectively, and the Phenol/Chloroform method exhibited significantly higher recovery than the other two protocols ($p<0.05$). Figure 4.7 illustrates the 16S rRNA: rRNA gene ratios for the same *E. coli* sample extracted with three different methods before and after normalization based on the recoveries of reference (*P. fluorescens*) rRNA and rRNA gene. It can be clearly seen that without normalization with dual references, the ratios showed distinct differences among the three methods. Also high COV (up to 90%) were observed for triplicate samples extracted with the same method. However, after normalizing the *E. coli* 16S rRNA and rRNA gene quantities based on the recoveries of dual references, nearly identical ratios were obtained for three extraction protocols. In addition, the COV decreased substantially for determined ratios from each protocol.

4.4.6 Application of Dual-internal-reference Technique with *E. coli* Liquid Culture at Different Growth Stage

The dual-internal-reference technique was applied to explore the temporal variation of 16S rRNA: rRNA gene ratio for *E. coli* growing in TSB at 37 °C. As shown in Figure 4.8, the bacteria manifested rapid growth during the exponential growth stage at approximately 3-5 hours after inoculation, as measured by optical density of the liquid at 600 nm. The 16S rRNA: rRNA gene ratio (normalized to the recoveries of dual references) increased rapidly during the initial three hours after inoculation: from 2.4×10^{3}

to 1.1×10^4, then decreased to ~5.0×10^3 during 3-6 hours, and finally remained stable during 6-16 after inoculation. The ratio at 3 hours was significantly higher ($p<0.05$) than the ratios at other times except for the 4-hour sample ($p=0.372$). The ratio of the 4-hour sample was higher than the rest of ratios ($p<0.05$) except for the 5-hour sample ($p=0.074$). It was also found that the 16S rRNA: rRNA gene ratio peaked at the same time when the relative growth rate of bacteria reached maximum. However, the 16S rRNA: rRNA gene ratio calculated without normalization of DNA and RNA quantities to recoveries of dual references did not exhibit any distinct temporal trend, and the only statistical difference was found for the ratios determined at 7 hours and 1 hour ($p=0.026$). The COV for ratios at each time point with normalization were also lower compared to those without normalization ($p=0.003$).

4.4.7 Application of Dual-internal-reference Technique with *E. coli* Aerosol Samples

Figure 4.9 illustrates the application of the dual-internal-reference technique to investigate the potential change in 16S rRNA: rRNA gene ratio for non-growth activities of bacteria in response to environmental stress, e.g. stress to *E. coli* aerosols when collected on a filter and then subjected to particle-free air for prolonged periods of time. As could be seen in Figure 4.9, the ratio for *E. coli* collected on a filter and not subjected to additional stress (t = 0 hr) was 4.0×10^3, nearly identical to the ratio of *E. coli* in liquid suspension (3.8×10^3). Interestingly, when sampling time was extended to 2 hours, the ratio increased slightly to 4.4×10^3 but not significantly ($p=0.305$). As the sampling of particle free air continued for another 2 hours (4 hours total), this ratio reached at ~6.5×10^3 and then leveled off for the remaining exposure time. The ratios for 4, 6 and 10

hours were all significantly higher (p<0.05) than those at 0 and 2 hours and also *E. coli* in liquid suspension. In contrast, if the ratios are determined without normalization with dual references, they do not show any significant temporal trend (p>0.05). Likewise, the COV for ratios determined after normalization of DNA and RNA quantities by recoveries of dual references were significantly lower compared to those before normalization (p=0.02).

4.5 Discussion

In this study, a dual-internal-reference technique was developed to improve the accuracy and reduce variability in determination of 16S rRNA: rRNA gene ratio by taking into account samples losses due to sample processing and analysis procedures. Specifically, two exogenous references (*P. fluorescens* 16S rRNA gene and 16S rRNA in this study) were added into microbial samples prior to DNA/RNA co-extraction to act as surrogates for the target (*E. coli* sample in our case) DNA and rRNA, respectively. The absolute quantities of recovered target DNA and rRNA were normalized to the recoveries of respective internal reference, and then the 16S rRNA: rRNA gene ratio was calculated.

Analysis of 16S rRNA: rRNA gene ratio using qPCR and RT-qPCR often involves multiple procedures in DNA and RNA co-extraction from a microbial sample, e.g. cell lysis, DNA/RNA isolation, and DNA removal and reverse transcription for RNA sample. In these steps, sample losses could result from incomplete cell lysis, incomplete volume transfer and phase separation, enzymatic and abiotic degradation of RNA, and inefficient reverse transcription of RNA (24). Presumably, the exogenous references behave similarly to the target analyte due to their similar physical and chemical properties. Thus, the percentage loss of the target analyte in each step should be the same as the percentage loss of corresponding reference. For example, a similar RT efficiency of RNA for both reference (*P. fluorescens* 16S rRNA) and target analyte (*E. coli* 16S rRNA) have been verified (Figure 4.3). One limitation, however, of this technique is that the loss of target DNA and RNA due to incomplete cell lysis cannot be controlled by the dual references (24). Thus, depending on a specific cell lysis method, e.g. mechanical disruption, enzyme digestion, freeze-thaw cycles, or a combination of these, the

determined 16S rRNA: rRNA gene ratios from the same sample could display some variation. Nonetheless, the sample loss during extraction steps other than cell lysis should be controlled by this technique (see Figure 4.7).

In order to quantify two gene sequences (*E. coli* and *P. fluorescens* 16S rRNA gene), singleplex qPCR could be performed for each gene on two separate reaction plates. Alternatively, a multiplex qPCR targeting both gene sequences could be performed on a single plate to avoid the variability caused by inconsistent pipetting and amplification when using two plates, which should further improve the accuracy of results. Thus, for such purpose, a multiplex qPCR method was developed to amplify 16S rRNA gene sequences for both bacteria simultaneously in a single reaction. However, it is critical to determine the optimal conditions for multiplex qPCR in order to avoid any potential amplification bias for each amplicon (28). In our case, when *E. coli* 16S rRNA gene is present at or over 1000× of the *P. fluorescens* 16S rRNA gene quantity, the *P. fluorescens* sequence showed distorted amplification and the threshold cycle was delayed in comparison to singleplex reaction. This could be due to limited availability of PCR reagents for *P. fluorescens* amplification when it competes with more abundant of *E. coli*. On the other hand, when *P. fluorescens* 16S rRNA gene is at or over 1000× of the *E. coli* 16S rRNA gene quantity, the threshold cycle number of *E. coli* sequence decreased dramatically compared to the Ct value obtained in singleplex reaction. This was mainly caused by the cross-talk effect of HEX fluorophore (*P. fluorescens* probe) on FAM fluorophore (*E. coli* probe) (Data not shown). In addition, the effect of initial cell quantities on the accuracy of this technique was determined by mixing different amounts of cells with a fixed quantity of DNA and rRNA reference. The experiment allowed

determining an optimal range of initial cell quantities where 16S rRNA: rRNA gene ratios were independent of the initial cell quantity (Figure 4.6).

Normalization of *E. coli* DNA and rRNA quantities to the recoveries of reference *P. fluorescens* DNA and rRNA, respectively, changed the results in two ways. First, the numerical value of 16S rRNA: rRNA gene ratio exhibited an approximately a 2-3 fold increase after normalization. For instance, the ratio of *E. coli* growing in TSB at 37 °C for 16 hrs (stationary phase) was measured in multiple tests at around 4.0×10^3 after normalization with the references. Since seven copies of 16S rRNA gene are present per *E. coli* genome (32), the 16S rRNA level of *E. coli* in the stationary phase was estimated to be around 3×10^4 copies/cell. This number is in good agreement with the previously reported average number of ribosomes ($1\text{-}7\times10^4$) per *E. coli* cell (35). Second, the intra-sample variability, represented by the COV of triplicate samples, decreased significantly in all investigated *E. coli* samples from the liquid culture (Figure 4.8) and aerosol samples (Figure 4.9) once the data were normalized based on the recoveries of dual references, thus showing improved precision of the results once the normalization is applied.

In our experiments, *P. fluorescens* 16S rRNA and rRNA gene were selected as dual references to investigate the 16S rRNA: rRNA gene ratios of pure *E. coli* samples. However, the use of dual references from *P. fluorescens* for environmental samples may not work because *P. fluorescens* has been detected in samples from different environments (36-38). Therefore, a new set of exogenous internal references may be needed to investigate the 16S rRNA: rRNA gene ratio for specific bacterial taxa in complex microbial communities by applying dual-internal-reference technique. For

example, the firefly luciferase *luc* mRNA and DNA might be good candidates for dual internal references due to several reasons (24, 28). First, they are not expected in environmental microbial samples. Second, a primer-probe set which does not target any bacterial or archaeal genes can be designed. Third, luciferase mRNA and a *luc* gene-containing DNA vector are commercially available. The use of luciferase mRNA to control for the loss of target RNA in the application of RT-qPCR has been reported in earlier studies (3, 24, 26, 28). However, when applying luciferase mRNA and DNA with dual-internal-reference technique one has to consider the following: first, discrepancies may exist between the luciferase and target bacteria, e.g. *E. coli* in this study, including the DNA size (5 Kbp v.s. 4 Mbp) and RNA stability (mRNA v.s. rRNA), thus the use of *luc* mRNA and DNA vector as dual references should be evaluated in detail. Second, a multiplex qPCR assay should be carefully designed to ensure amplification of both target and reference gene with good and similar efficiencies. If there are conditions where multiplex qPCR fails to produce unbiased amplification, one could apply singleplex qPCR for two amplicons separately.

We successfully applied the dual-internal-reference technique to investigate the variation of 16S rRNA: rRNA gene ratio for *E. coli* during its growth at 37 °C in TSB. The results showed that the ratio increased dramatically during the first few hours when *E. coli* was in early exponential growth stage. After that, the ratio first decreased and then leveled off as *E. coli* entered in stationary phase in growth. This observed pattern corresponded well with previous findings for a number of bacterial species in unsteady growth conditions (13, 16, 17). These studies including ours strongly implied that the cellular rRNA level and bacterial growth rate are not always simply correlated (18).

This technique has also been applied to investigate the potential change of *E. coli* 16S rRNA: rRNA gene ratio due to a non-growth activity, e.g. cellular activities in response to air sampling stress. It was found that the 16S rRNA: rRNA gene ratio of *E. coli* bioaerosols collected on a filter increased by more than 50% after exposure to particle-free air for an additional 4 hours. This increase in 16S rRNA: rRNA gene ratio was apparently due to the production of 16S rRNA by *E. coli* when the cells remained on filters: we observed increased quantities of 16S rRNA but no change of 16S rRNA gene quantities during the 4-hour period (data not shown). Thus, our data imply that bacteria could produce more rRNA in reaction to the stress associated with air sampling, e.g. osmotic and dessication stress, thus reflecting the potential cellular activities during bioaerosol collection. In addition, the results also show that to analyze 16S rRNA sequences from airborne bacteria, one has to carefully evaluate the sampling protocols and their potential bias effect on the accuracy of analysis.

For application of the developed technique in both experiments, no significant temporal trend in *E. coli* 16S rRNA: rRNA gene ratio was observed when determined ratios are not normalized based on the recoveries of dual references. Under such condition, the variation of the ratio between different samples was likely obscured by the large intra-sample variation as represented by the COV of individual samples. On the other hand, this intra-sample variation was greatly reduced after normalization of results to the recoveries of dual references. Thus, the dual-internal-reference technique could enhance our ability to detect the change in rRNA level of a microbial sample.

4.6 Conclusions

A dual-internal-reference technique was successfully developed to examine the 16S rRNA: rRNA gene ratio of *E. coli* samples by using multiplex qPCR. Dual exogenous internal references were introduced into samples prior to nucleic acids extraction to control the technical variability resulting from DNA/RNA loss and inefficient reverse transcription and PCR amplification. This technique was shown to improve the accuracy of results and substantially reduce intra-sample variation. Application of this technique with *E. coli* cells in liquid culture and in bioaerosols samples demonstrated its ability to successfully identify the change in 16S rRNA: rRNA gene ratios in samples at different physiological states. It is hoped that this technique can facilitate future investigation of the relationship between rRNA abundance and microbial activities, especially non-growth related activities.

4.7 Acknowledgments

The publication was supported by Grant R01-OH009783 “Advanced Sampler for Measuring Exposure to Biological Aerosols” from CDC/NIOSH, Grant IOS-1022254 “Air as an Active Bacterial Ecosystem” from the National Science Foundation (NSF), and Project 07160 funded by the New Jersey Agricultural Experiment Station (NJAES) at Rutgers, The State University of New Jersey. Its contents are solely the responsibility of the authors and do not necessarily represent the official view of the CDC/NIOSH, the NSF or the NJAES.

References

1. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:**221-271.
2. **Campbell, B. J., L. Y. Yu, J. F. Heidelberg, and D. L. Kirchman.** 2011. Activity of abundant and rare bacteria in a coastal ocean. Proc. Natl. Acad. Sci. U.S.A. **108:**12776-12781.
3. **Perez-Osorio, A. C., K. S. Williamson, and M. J. Franklin.** 2010. Heterogeneous rpoS and rhlR mRNA Levels and 16S rRNA/rDNA (rRNA Gene) Ratios within Pseudomonas aeruginosa Biofilms, Sampled by Laser Capture Microdissection. J. Bacteriol. **192:**2991-3000.
4. **Pitkanen, T., H. Ryu, M. Elk, A. M. Hokajarvi, S. Siponen, A. Vepsalainen, P. Rasanen, and J. W. S. Domingo.** 2013. Detection of Fecal Bacteria and Source Tracking Identifiers in Environmental Waters Using rRNA-Based RT-qPCR and rDNA-Based qPCR Assays. Environ. Sci. Technol. **47:**13611-13620.
5. **Kim, J. S., and N. Wang.** 2009. Characterization of copy numbers of 16S rDNA and 16S rRNA of *Candidatus Liberibacter* asiaticus and the implication in detection *in planta* using quantitative PCR. BMC Research Notes **2:**37.
6. **Campbell, B. J., L. Yu, T. R. A. Straza, and D. L. Kirchman.** 2009. Temporal changes in bacterial rRNA and rRNA genies in Delaware (USA) coastal waters. Aquatic Microbial Ecology **57:**123-135.
7. **Hawkins, S., K. Robinson, A. Layton, and G. Sayler.** 2012. Molecular indicators of Nitrobacter spp. population and growth activity during an induced inhibition event in a bench scale nitrification reactor. Water Res. **46:**1793-1802.
8. **Deutscher, M. P.** 2003. Degradation of stable RNA in bacteria. J. Biol. Chem. **278:**45041-45044.
9. **Takle, G. W., I. K. Toth, and M. B. Brurberg.** 2007. Evaluation of reference genes for real-time RT-PCR expression studies in the plant pathogen Pectobacterium atrosepticum. Bmc Plant Biology **7**.
10. **Arunasri, K., M. Adil, P. A. A. Khan, and S. Shivaji.** 2014. Global Gene Expression Analysis of Long-Term Stationary Phase Effects in E. coli K12 MG1655. Plos One **9**.
11. **Fey, A., S. Eichler, S. Flavier, R. Christen, M. G. Hofle, and C. A. Guzman.** 2004. Establishment of a real-time PCR-based approach for accurate quantification of bacterial RNA targets in water, using Salmonella as a model organism. Appl. Environ. Microbiol. **70:**3618-3623.
12. **Kerkhof, L., and B. B. Ward.** 1993. Comparison of Nucleic-Acid Hybridization and Fluorometry for Measurement of the Relationship between Rna/DNA Ratio and Growth-Rate in a Marine Bacterium. Appl. Environ. Microbiol. **59:**1303-1309.
13. **Muttray, A. F., Z. T. Yu, and W. W. Mohn.** 2001. Population dynamics and metabolic activity of Pseudomonas abietaniphila BKME-9 within pulp mill wastewater microbial communities assayed by competitive PCR and RT-PCR. FEMS Microbiol. Ecol. **38:**21-31.
14. **Dortch, Q., T. L. Roberts, J. R. Clayton, and S. I. Ahmed.** 1983. Rna DNA Ratios and DNA Concentrations as Indicators of Growth-Rate and Biomass in Planktonic Marine Organisms. Marine Ecology Progress Series **13:**61-71.

15. **Poulsen, L. K., G. ballard, and D. A. Stahl.** 1993. Use of rRNA fluorescence in situ hybridization for measuring the activity of single cells in young and established biofilms. Appl. Environ. Microbiol. **59:**1354-1360.
16. **Kerkhof, L., and P. Kemp.** 1999. Small ribosomal RNA content in marine Proteobacteria during non-steady-state growth. FEMS Microbiol. Ecol. **30:**253-260.
17. **Krumins, V., G. Mainelis, L. J. Kerkhof, and D. E. Fennell.** 2014. Substrate-dependent rRNA production in an airborne bacterium. Environ. Sci. Technol. Letters **1:**376-381.
18. **Blazewicz, S. J., R. L. Barnard, R. A. Daly, and M. K. Firestone.** 2013. Evaluating rRNA as an indicator of microbial activity in environmental communities: limitations and uses. ISME J. **7:**2061-2068.
19. **Hansen, M. C., A. K. Nielsen, S. Molin, K. Hammer, and M. Kilstrup.** 2001. Changes in rRNA levels during stress invalidates results from mRNA blotting: Fluorescence in situ rRNA hybridization permits renormalization for estimation of cellular mRNA levels. J. Bacteriol. **183:**4747-4751.
20. **Cheng, W. C., W. Y. Shu, C. Y. Li, M. L. Tsai, C. W. Chang, C. R. Chen, H. T. Cheng, T. H. Wang, and I. C. Hsu.** 2012. Intra- and Inter-Individual Variance of Gene Expression in Clinical Studies. Plos One **7**.
21. **Oberg, A. L., B. M. Bot, D. E. Grill, G. A. Poland, and T. M. Therneau.** 2012. Technical and biological variance structure in mRNA-Seq data: life in the real world. Bmc Genomics **13:**2-14.
22. **Whitehead, A., and D. L. Crawford.** 2006. Variation within and among species in gene expression: raw material for evolution. Molecular Ecology **15:**1197-1211.
23. **Smith, R. D., B. Brown, P. Ikonomi, and A. N. Schechter.** 2003. Exogenous reference RNA for normalization of real-time quantitative PCR. Biotechniques **34:**88-91.
24. **Johnson, D. R., P. K. H. Lee, V. F. Holmes, and L. Alvarez-Cohen.** 2005. An internal reference technique for accurately quantifying specific mRNAs by real-time PCR with a application to the tceA reductive dehalogenase gene. Appl. Environ. Microbiol. **71:**3866-3871.
25. **McMaugh, S. J., and B. R. Lyon.** 2003. Real-time quantitative RT-PCR assay of gene expression in plant roots during fungal pathogenesis. Biotechniques **34:**982-+.
26. **Wagner, A., L. Adrian, S. Kleinsteuber, J. R. Andreesen, and U. Lechner.** 2009. Transcription Analysis of Genes Encoding Homologues of Reductive Dehalogenases in "Dehalococcoides" sp Strain CBDB1 by Using Terminal Restriction Fragment Length Polymorphism and Quantitative PCR. Appl. Environ. Microbiol. **75:**1876-1884.
27. **Luciano, P., C. M. Bertea, G. Temporale, and M. E. Maffei.** 2007. DNA internal standard for the quantitative determination of hallucinogenic plants in plant mixtures. Forensic Science International-Genetics **1:**262-266.
28. **Hatt, J. K., K. M. Ritalahti, D. M. Ogles, C. A. Lebron, and F. E. Loffler.** 2013. Design and Application of an Internal Amplification Control to Improve Dehalococcoides mccartyi 165 rRNA Gene Enumeration by qPCR. Environ. Sci. Technol. **47:**11131-11138.

29. **Sen, K., N. A. Schable, and D. J. Lye.** 2007. Development of an internal control for evaluation and standardization of a quantitative PCR assay for detection of Helicobacter pylori in drinking water. Appl. Environ. Microbiol. **73:**7380-7387.
30. **An, H. R., G. Mainelis, and L. White.** 2006. Development and calibration of real-time PCR for quantification of airborne microorganisms in air samples. Atmos. Environ. **40:**7924-7939.
31. **Dolezel, J., J. Bartos, H. Voglmayr, and J. Greilhuber.** 2003. Nuclear DNA content and genome size of trout and human. Cytometry Part A **51A:**127-128.
32. **Blattner, F. R., G. Plunkett, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. ColladoVides, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau, and Y. Shao.** 1997. The complete genome sequence of Escherichia coli K-12. Science **277:**1453-&.
33. **Redondo-Nieto, M., M. Barret, J. P. Morrisey, K. Germaine, F. Martinez-Granero, E. Barahona, A. Navazo, M. Sanchez-Contreras, J. A. Moynihan, S. R. Giddens, E. R. Coppoolse, C. Muriel, W. J. Stiekema, P. B. Rainey, D. Dowling, F. O'Gara, M. Martin, and R. Rivilla.** 2012. Genome Sequence of the Biocontrol Strain Pseudomonas fluorescens F113. J. Bacteriol. **194:**1273-1274.
34. **Zhen, H. J., T. W. Han, D. E. Fennell, and G. Mainelis.** 2014. A systematic comparison of four bioaerosol generators: Affect on culturability and cell membrane integrity when aerosolizing Escherichia coli bacteria. J. Aerosol Sci. **70:**67-79.
35. **Bremer, H., and P. P. Dennis.** 1996. Modulation of chemical composition and other parameters of the cell by growth rate. ASM Press, Washington D.C.
36. **Vanderkooij, D., A. Visser, and J. P. Oranje.** 1982. Multiplication of Fluorescent Pseudomonads at Low Substrate Concentrations in Tap Water. A. Van. Leeuw. J. Microb. **48:**229-243.
37. **Delille, A., F. Quiles, and F. Humbert.** 2007. In situ monitoring of the nascent Pseudomonas fluorescens Biofilm response to variations in the dissolved organic carbon level in low-nutrient water by attenuated total reflectance-fourier transform infrared spectroscopy. Appl. Environ. Microbiol. **73:**5782-5788.
38. **Wong, V., K. Levi, B. Baddal, J. Turton, and T. C. Boswell.** 2011. Spread of Pseudomonas fluorescens Due to Contaminated Drinking Water in a Bone Marrow Transplant Unit. Journal of Clinical Microbiology **49:**2093-2096.

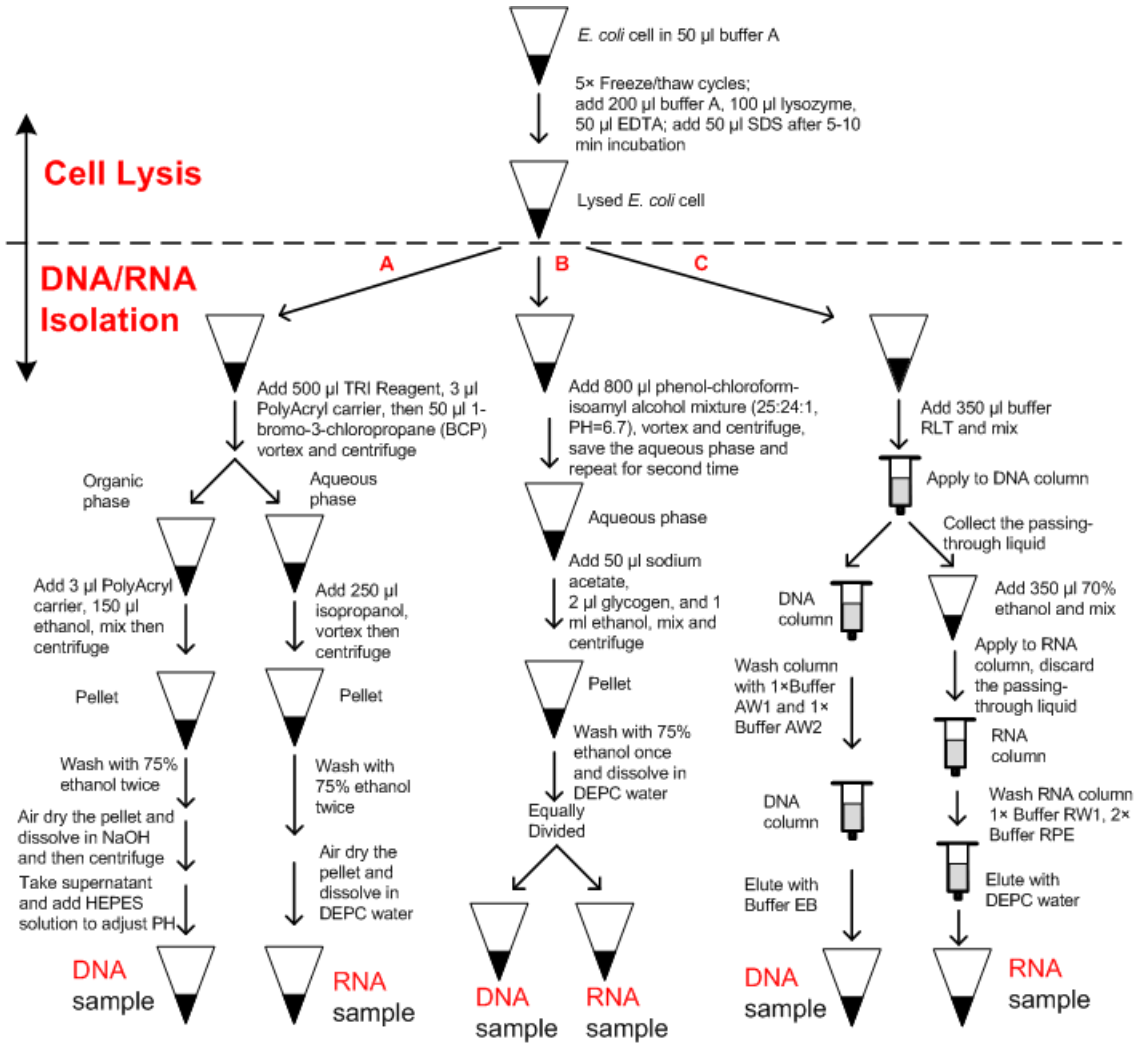

Figure 4.1 Schematic of three DNA/RNA co-extraction protocols (A: TRI-Reagent, B: Phenol-Chloroform, C: Allprep DNA/RNA mini Kit) used in this study.

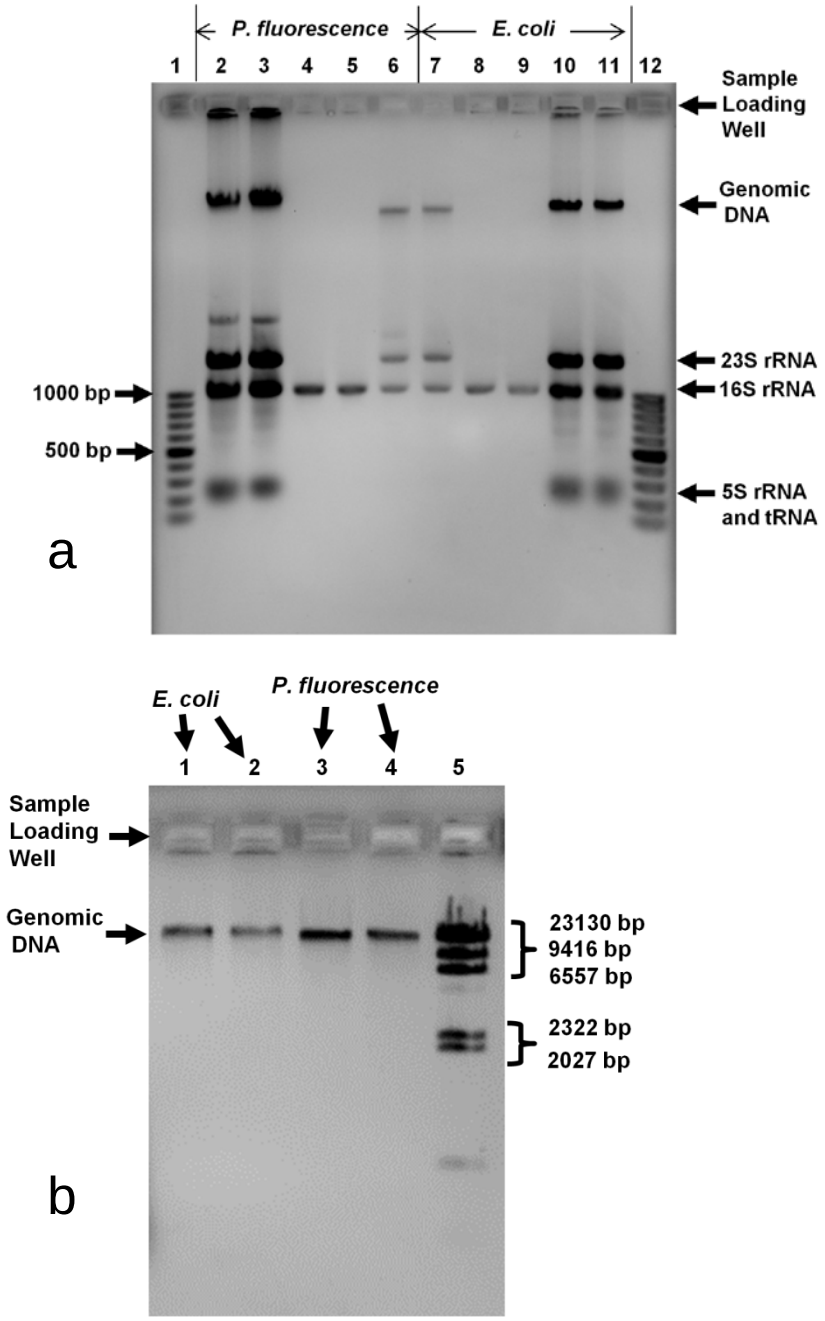

Figure 4.2 Agarose gel electrophoresis of genomic DNA and 16S rRNA extracted from *E. coli* and *P. fluorescens* cells. a) Agarose gel electrophoresis of nucleic acids extracted from *P. fluorescens* (lanes 2 to 6) and *E. coli* (lanes 7 to 11). The lanes contained two 1000-bp DNA ladders (lanes 1 and 12), total extracted nucleic acids by phenol-chloroform protocol for *P. fluorescens* (lanes 2 and 3) and *E. coli* (lanes 10 and 11), by DNeasy blood & tissue kit for *P. fluorescens* (lane 6) and *E. coli* (lane 7), and the 16S rRNA reference for *P. fluorescens* (lanes 4 and 5) and *E. coli* (lanes 8 and 9) extracted from the freshly grown bacterial cells. b) Agarose gel electrophoresis of genomic DNA extracted from *E. coli* (lanes 1 to 2) and *P. fluorescens* (lanes 3 to 4). Lane 5 was the electrophoresis of a Lambda DNA/HindIII marker.

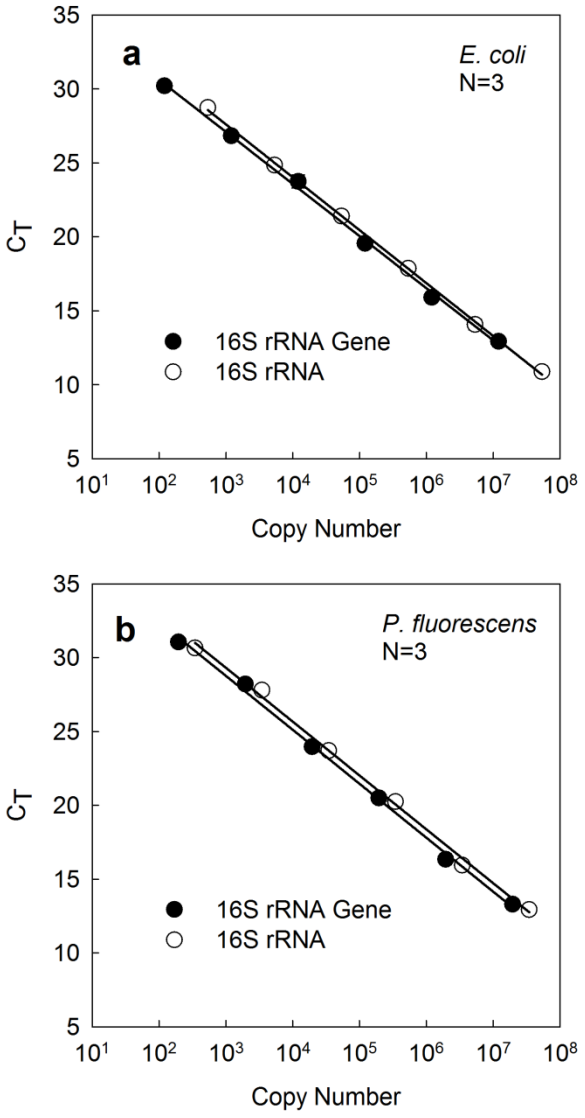

Figure 4.3 Standard curves of 16S rRNA gene (closed circle) and reverse transcribed 16S rRNA (open circle) with qPCR quantification of *E. coli* (a) and *P. fluorescens* (b). All data points are averages of triplicate samples, and error bars are 1 standard deviation. Where not visible, error bars are smaller than the symbol.

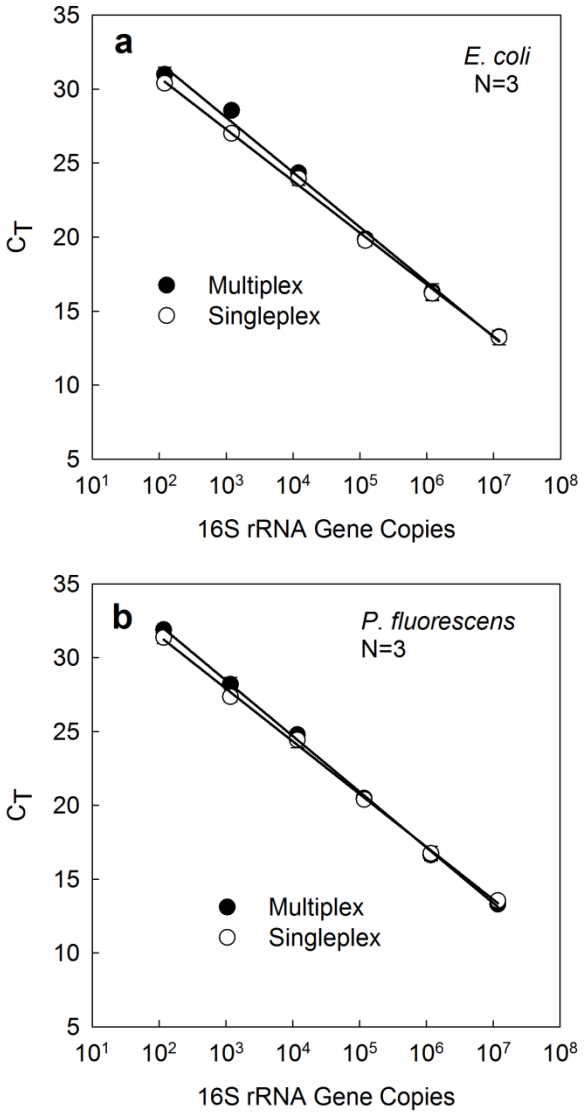

Figure 4.4 Standard curves for *E. coli* (a) and *P. fluorescens* (b) 16S rRNA gene in multiplex (closed circle) and singleplex (open circle) qPCR reactions. All data points are averages of triplicate samples, and error bars are 1 standard deviation. Where not visible, error bars are smaller than the symbol.

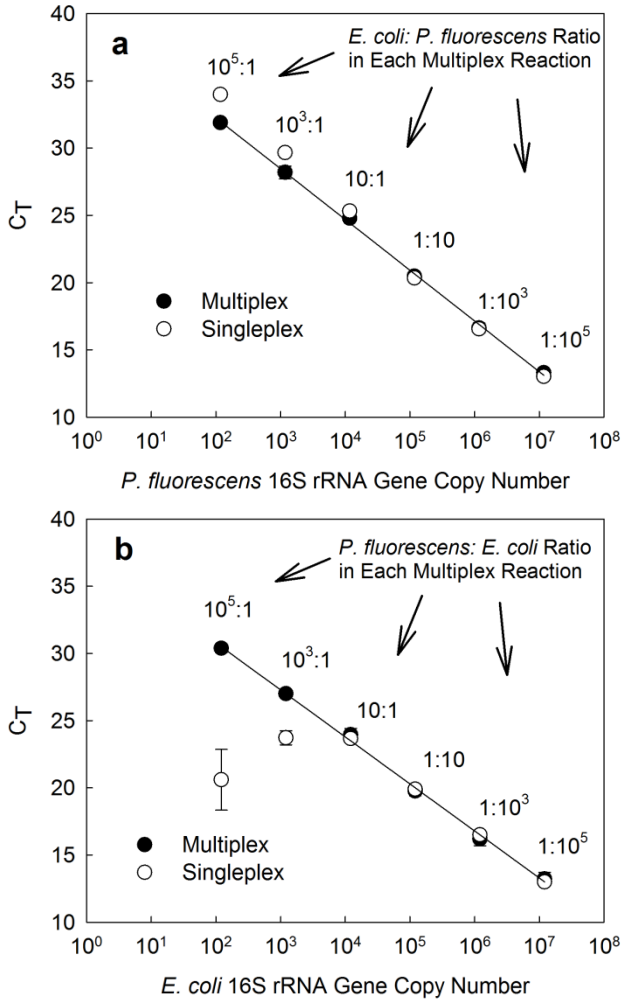

Figure 4.5 Effects of relative quantities between *E. coli* and *P. fluorescens* 16S rRNA genes in each multiplex qPCR reaction on amplification curves of both 16S rRNA genes. a) biased amplification of *P. fluorescens* 16S rRNA gene (compared with singleplex amplification) when *P. fluorescens* exceeded *E. coli* in 16S rRNA gene quantities by a factor of 1000:1 or higher. b) biased amplification of *E. coli* 16S rRNA gene (compared with singleplex amplification) when *E. coli* exceeded *P. fluorescens* in 16S rRNA gene quantities by a factor of 1000:1 or higher. All data points are averages of triplicate samples, and error bars are 1 standard deviation. Where not visible, error bars are smaller than the symbol.

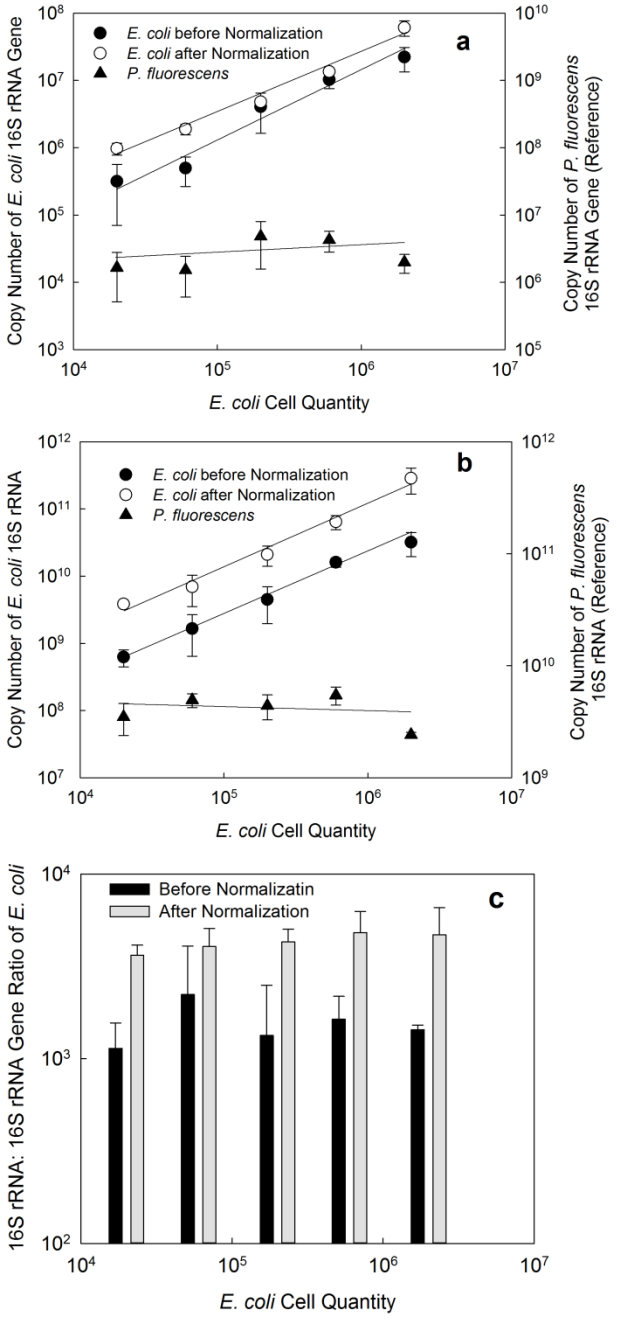

Figure 4.6 Effect of initial *E. coli* cell quantity on determination of 16S rRNA: rRNA gene ratio with dual-internal-reference technique. a) The quantities of *E. coli* 16S rRNA gene before (closed circle) and after normalization (open circle) with the recovered quantity of *P. fluorescens* 16S rRNA gene (triangle) for different initial cell quantities. b) The quantities of *E. coli* 16S rRNA before (closed circle) and after normalization (open circles) with the recovered quantity of *P. fluorescens* 16S rRNA (triangle) for different initial cell quantities. c) The determined 16S rRNA: rRNA gene ratio for *E. coli* with different initial cell quantities before and after normalization with dual internal references. All data points are averages of triplicate samples, and error bars are 1 standard deviation.

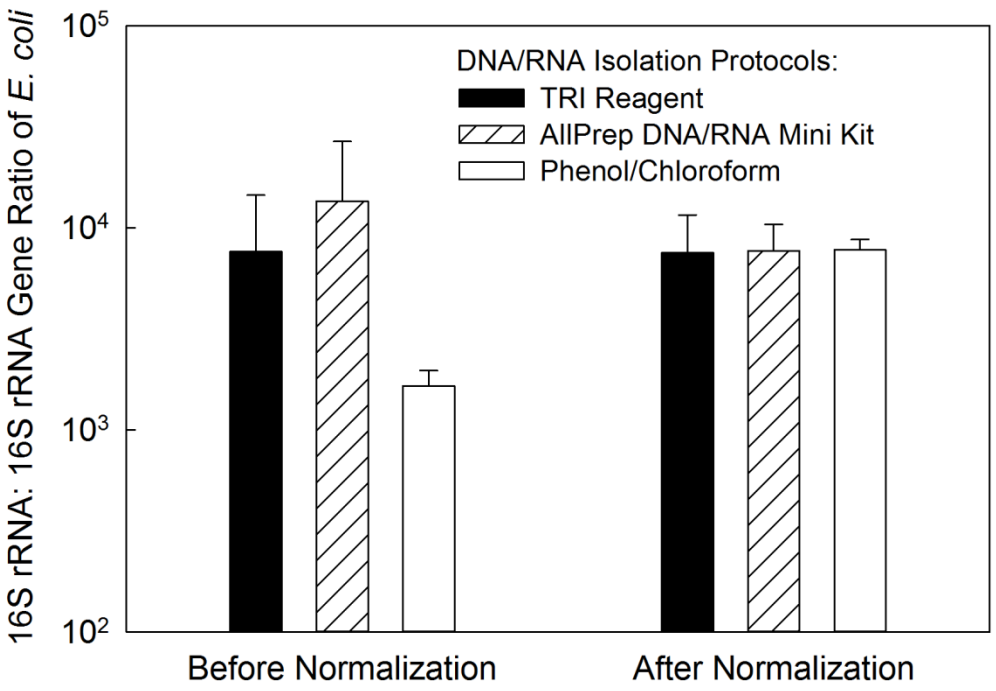

Figure 4.7 The effects of different DNA/RNA isolation protocols on the determined 16S rRNA: rRNA gene ratio before and after normalization with dual internal references. All data points are averages of triplicate samples, and error bars are 1 standard deviation.

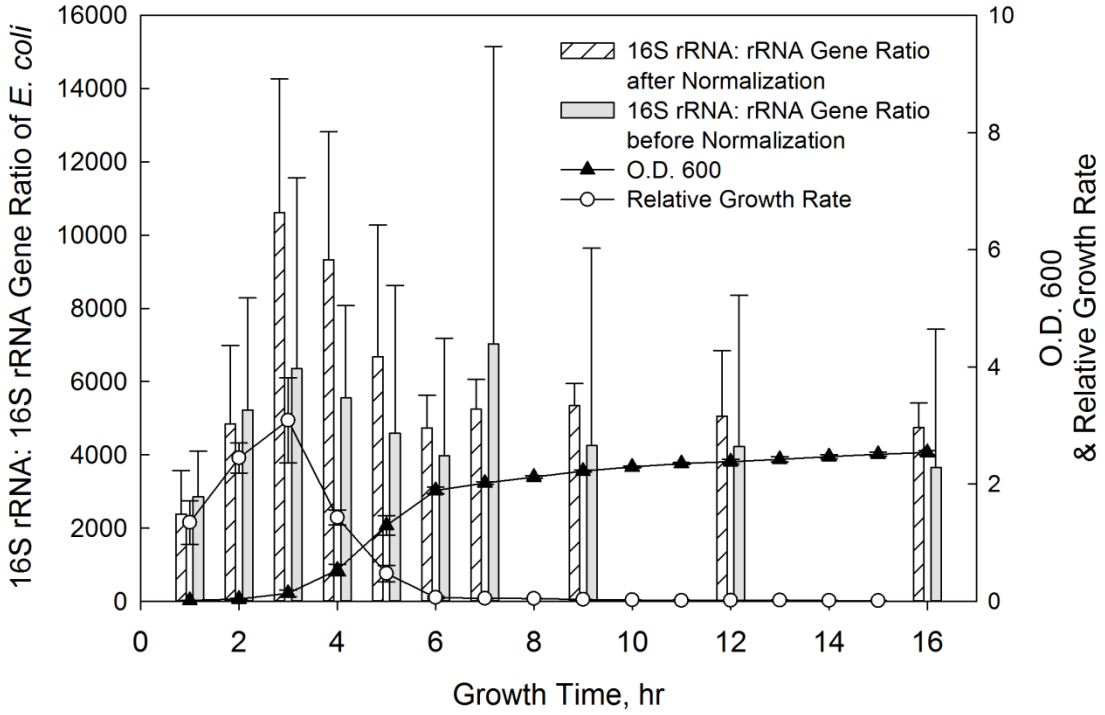

Figure 4.8 Temporal variation of 16S rRNA: rRNA gene ratio (before and after normalization with dual internal references) for *E. coli* growing in Tryptic Soy Broth at 37°C and its associations with the optical density measurements at 600 nm (O.D. 600) and the relative growth rates of bacterial culture. All data points are averages of triplicate samples, and error bars are 1 standard deviation.

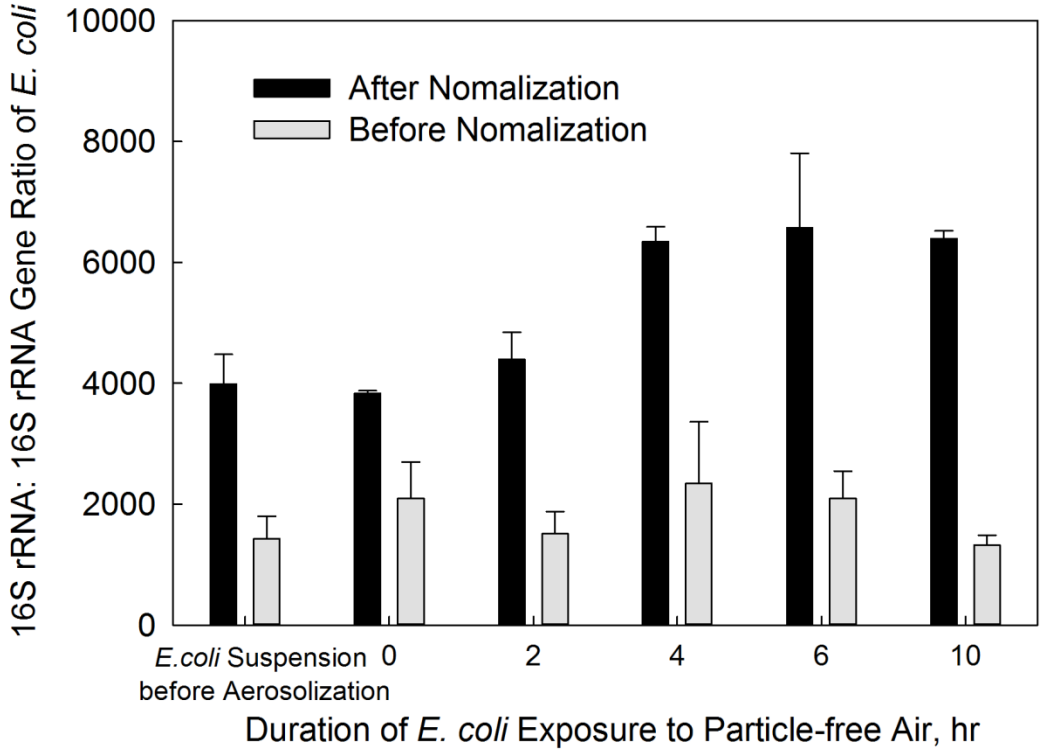

Figure 4.9 Effect of extended air sampling time on the change of 16S rRNA: rRNA gene ratio for *E. coli* aerosols collected on filters. The determined ratios before and after normalization with dual internal references were shown. All data points are averages of triplicate samples, and error bars are 1 standard deviation.

Table 4.1 List of statistical analyses performed in this study

Subsection in "Results"	Dependent Variable	Independent Variable	Statistical Test
Development of multiplex qPCR assay for determination of 16S rRNA genes of *E. coli* and *P. fluorescens*	Slope of standard curve in multiplex qPCR test	*E. coli v.s. P. fluorescens*	Student's *t* test
	Slope of qPCR standard curve for *E. coli*; Slope of qPCR standard curve for *P. fluorescens*	Multiplex *v.s.* singleplex qPCR	
Effects of initial cell quantities on determination of 16S rRNA: rRNA gene ratio with dual-internal-reference technique	Slope of linear regression curve between copy number of *E. coli* 16S rRNA gene (or 16S rRNA) and *E. coli* cell quantity	Before *v.s.* after normalization with dual *P. fluorescens* references	Student's *t* test
	16S rRNA: rRNA gene ratios	Before *v.s.* after normalization with dual *P. fluorescens* references paired by initial cell quantities	Paired Student's *t* test
	16S rRNA: rRNA gene ratios before normalization with dual *P. fluorescens* references; 16S rRNA: rRNA gene ratios after normalization with dual *P. fluorescens* references	Initial cell quantities	One-way ANOVA with Fisher's LSD
Effect of DNA/RNA isolation protocol on determination of 16S rRNA: rRNA gene ratio with dual-internal-reference technique	Recoveries of 16S rRNA gene; Recoveries of 16S rRNA	Three DNA/RNA co-extraction protocols	One-way ANOVA with Fisher's LSD
Application of dual-internal-reference technique with *E. coli* liquid culture at different growth stage	16S rRNA: rRNA gene ratios before normalization with dual *P. fluorescens* references; 16S rRNA: rRNA gene ratios after normalization with dual *P. fluorescens* references	Growth times; Triplicate test on three different days	Two-way ANOVA with Fisher's LSD
	Coefficient of variation for 16S rRNA: rRNA gene ratios	Before *v.s.* after normalization with dual *P. fluorescens* references paired by growth times	Paired Student's *t* test
Application of dual-internal-reference technique with *E. coli* aerosol samples	16S rRNA: rRNA gene ratios before normalization with dual *P. fluorescens* references; 16S rRNA: rRNA gene ratios after normalization with dual *P. fluorescens* references	Extended sampling time for filters collected with *E. coli* aerosols; Triplicate test on three different days	Two-way ANOVA with Fisher's LSD
	Coefficient of variation for 16S rRNA: rRNA gene ratios	Before *v.s.* after normalization with dual *P. fluorescens* references paired by extended sampling time	Paired Student's *t* test

Chapter 5: Effect of Sampling Stress on Measurement and Analysis of 16S Ribosomal RNA of Airborne Bacteria[4]

[4] This chapter is modified from the manuscript by Huajun Zhen, Valdis Krumins, Taewon Han, Donna E. Fennell, and Gediminas Mainelis. Effect of sampling stress on measurement and analysis of 16S ribosomal RNA of airborne bacteria, to be submitted.

5.1 Abstract

Analysis of ribosomal RNA (rRNA) is frequently employed to identify currently or potentially active microbes in environmental samples. However, few studies have been conducted on bioaerosol samples using this approach, and there is still a big knowledge gap regarding whether the bioaerosol rRNA levels stays unchanged during the bioaerosol collection process. In this study we investigated the effect of air sampling stress on the measurement and analysis of 16S rRNA for bioaerosols in laboratory and field experiments. In a laboratory study, freshly grown *Escherichia coli* cells were added to Button aerosol sampler, BioSampler and SpinCon wet cyclone portable air sampler and then exposed to active sampling stress when the samplers pulled particle-free air or indoor air for 2 hours. We found that the recovered cellular 16S rRNA level depended on a particular sampler, i.e. the sampling method biased recovered 16S rRNA quantity. Further, two devices which exhibited different efficiency in preserving 16S rRNA were employed in an outdoor environment to collect bioaerosols simultaneously on three different days, and the recovered microbial communities were examined by pyrosequencing of paired 16S rRNA and rRNA gene for each sample. The abundance of 16S rRNA in the outdoor air sample (1.1×10^6-1.8×10^7 copies/m^3) was about two orders of magnitude higher than that of 16S rRNA gene (6.9×10^3-1.5×10^5 copies/m^3). Statistical analysis revealed that the microbial communities were dependent on sampling day, while sampler and sequence type had no significant impact on the community composition. No interaction effect between sampler and sequence type was observed when examining individual bacterial taxa, which indicated that the sampler-dependent biased effect on analysis of 16S rRNA sequences was minimal in investigated outdoor bioaerosols. In

addition, a number of bacterial taxa exhibited higher abundance in the 16S rRNA gene sequences than 16S rRNA sequences, which suggests the potential activities of certain microbes in airborne phase.

5.2 Introduction

The ribosomal RNA (rRNA) gene, particularly the prokaryotic 16S rRNA gene, has been widely utilized in environmental microbiology studies, because it is ubiquitous and has low mutation rates throughout prokaryotic evolution (1), and also encompasses hypervariable regions which can be used to distinguish between bacterial taxa (2). However, microbial samples often contain16S rRNA genes from dormant cells (3, 4) or lysed cells (5, 6), and thus the applicability of 16S rRNA gene for community analysis could be limited if one is interested in active microbial community members. In contrast, the rRNA is directly linked to cell physiology, e.g. the synthesis of rRNA was found to be growth-rate dependent for a number of bacterial species (7-9). Thus, analysis of 16S rRNA sequences can help revealing those members who are or have recently been active within a complex microbial community (10, 11). Previously, this approach has been employed to analyze microbial samples from a variety of environments, such as natural waters, soil, sediments and biofilm (10-17). However, there has been a limited number of studies conducted on rRNA measurement for bioaerosols (18) and to the best of our knowledge, there is no published study of rRNA-based community analysis in bioaerosol samples.

Due to a relatively low abundance of biomass in the air compared to soil and natural waters (19, 20), a typical bioaerosol sampling protocol often requires operating a particular bioaerosol sampler for a long period of time which could cause stress on the collected sample (21-23). Thus, one major concern when studying rRNA in bioaerosols is whether the rRNA level in collected cells stays unchanged during a long sampling time period. Although rRNA is a relatively stable RNA type, it could also exhibit significant

variation within a cell under certain changes in environmental conditions (24). For example, it has been reported that the rRNA concentrations increased during early exponential growth of several bacterial species (7), and rRNA underwent degradation under conditions that lead to slow cell growth, such as depletion of nutrients and glucose starvation (24). A more recent study showed that the 16S rRNA level in *Sphingomonas aerolata* aerosols in a rotating bioreactor increased when the bacteria were supplied with gaseous growth substrates (18). In addition, bacteria in non-growth state also displayed varying rRNA content in response to changing environmental conditions, i.e. the rRNA level per *Lactococcus lactis* cell was found to decrease by 50% when exposed to heat shock at 43°C for 30 min (25). Previously, it has been shown that air sampling stress such as impaction, impingement and desiccation can significantly alter the physiological status of collected bioaerosols, and result in loss of viability and impaired cell membrane integrity of bacterial cells (21-23, 26). Thus it is of great interest to determine whether the rRNA level of bioaerosol samples undergoes change in response to sampling stress, and if so, whether changes in rRNA level depend on particular bioaerosol collection methods or devices.

Historically, rRNA analyses have been used to investigate the potential of bacterial growth or metabolic activity by measuring the change of 16S rRNA: rRNA gene ratio for particular bacterial taxa (7-9, 27). More recently, the application of rRNA has shifted towards the more qualitative approach to identify recently or potentially active members within a microbial population by analyzing the 16S rRNA sequences from complex microbial samples (11-16). However, if the sampling stress leads to changes in the rRNA content of specific bacterial taxa, i.e. they either produce more rRNA or show

rRNA degradation during sample collection, then their relative abundance within a mixed community could be overestimated or underestimated when the 16S rRNA sequences are used to analyze the bacterial community. Additionally, this potential effect on the sequence abundance of active microbial community members may be device-dependent, and thus one particular sampling device may possibly reveal distinctively different active bacterial members compared to another device.

In this study, we investigated the effect of air sampling stress on measurement and analysis of 16S rRNA for bioaerosols. In the first part of the study, we used a laboratory setting to investigate the change of 16S rRNA: rRNA gene ratio of *Escherichia coli* in response to bioaerosol sampling stress during two hours of simulated active sampling by three collection devices, which included a Button aerosol sampler (SKC Inc., Eighty Four, PA), a BioSampler (SKC Inc.) and a SpinCon Wet Cyclone Portable Air Sampler (PAS 450-10A, InnovaPrep LLC., Drexel, MO). In the second part of the study, we analyzed microbial communities simultaneously collected from outdoor air by same devices on three different days. Particularly, the 16S rRNA gene and 16S rRNA-based pyrosequencing analyses were applied in an attempt to identify active microbial population within the outdoor microbial community. The objectives of this study included: 1) to study whether the rRNA content of bioaerosol samples is subjected to change due to air sampling stress; 2) to assess whether this effect of sampling stress on bioaerosol rRNA is device-dependent; 3) to investigate how this effect impacts the analysis of 16S rRNA sequences from bioaerosols collected in the field. To the best of our knowledge, this is the first study to investigate the potential effect (bias) of sampling stress on the quantification and characterization of 16S rRNA from bioaerosol samples.

5.3 Materials and Methods

5.3.1 Bacterial Culture in Laboratory Experiments

The Gram-negative bacterium *E. coli* (ATCC 15597, Manassas,VA) was pre-cultured in 50 ml Tryptic Soy broth (Becton, Dickinson and Company, Sparks, MD) at 37 °C for ~16 hours prior to each test. The bacteria was harvested and washed once with 1×phosphate buffer solution (1×PBS, 10 g/L NaCl, 0.25 g/L KCl, 1.43 g/L Na_2HPO_4, 0.25 g/L KH_2PO_4) by centrifugation at 7000×g (Jouan Inc., Winchester, VA) for 5 min at room temperature (25 °C) and resuspended in 1×PBS solution. The cell numbers in the final bacterial solution were determined by epifluorescence microscopy using the Axioskop 20 (Carl Zeiss Inc. Thornwood, NY) as reported previously (22).

5.3.2 Experimental Setup for Laboratory Experiment

Three bioaerosol samplers were tested to investigate the effect of two-hour sampling on the change of *E. coli* 16S rRNA. They included a Button aerosol sampler (SKC Inc., Eighty Four, PA), a BioSampler (SKC Inc.) and a SpinCon Wet Cyclone Portable Air Sampler (PAS 450-10A, InnovaPrep LLC., Drexel, MO). The Button aerosol sampler was selected as a filter holder and used with a 0.8-µm-pore-size polyethersulfone (PES) membrane filter (SUPOR, Pall Life Sciences, Port Washington, NY). The Button aerosol sampler is designed to operate at 4 liters/min (28), but in this study it was deliberately operated at a flow rate of 18 liters/min to exacerbate a potential effect of filtration stress on bacterial cells. The two other devices are liquid-based bioaerosol samplers which collected airborne biological particle by liquid impingement. 1×PBS solution was used as collection fluid for both devices. The SKC BioSampler with a 5-ml collection cup was

operated at a flow rate of 12.5 liters/min. Due to the evaporation of liquid, the BioSampler cup was refilled with pure water approximately every 15 minutes during its operation. The SpinCon air sampler was operated at a flow rate of 450 L/min, and the liquid loss due to evaporation was compensated by an automatic injection of sterile water to maintain the total sample volume of approximately 10 ml inside the collection chamber.

In order to simulate bioaerosol sampling conditions where bacterial cells have already been collected but continue to be exposed to sampling stress as the sampling continues, a known amount of freshly grown *E. coli* cells were loaded onto the filter (~5×10^5 cells) or spiked into the collection fluid inside the BioSampler cup (~3×10^8 cells) or SpinCon collection chamber (~1×10^9 cells) prior sampling. The spiked amount was different due to different amount of collection media in the devices. A separate aliquot of *E. coli* cells was concurrently saved at -80°C to serve as a reference for subsequence analysis of 16S rRNA: rRNA gene ratio. Thereafter, each device was operated for 2 hours at room temperature by aspirating particle-free air inside a class II biosafety cabinet (NuAire Inc., Plymouth, MN). The cabinet was disinfected with 70% ethanol and ultraviolet radiation prior to each experiment. The air temperature and relative humidity (RH) for tested conditions were 25 °C and between 25-30%, respectively.

[It should be noted that since the operational sampling flow rate of SpinCon (450 L/min) exceeded the upper limit of particle-free air flow in the biosafety cabinet, SpinCon test was conducted by aspirating the indoor air (RH between 50-55%) from the laboratory. Our preliminary experiments showed that the concentration of indoor bacteria from laboratory indoor air was below 1×10^4 cells/m^3, and *E. coli* was non-detectable by

using qPCR with primer and probe set targeting the *E. coli* 16S rRNA gene (22). Thus, 2 hours continuous sampling with SpinCon collected less than 10^6 cells in total: a quantity at least three orders of magnitude less than that of spiked *E. coli* cells (~10^9). Thus, we considered the impact of background indoor bioaerosol on SpinCon test to be minimal.]

After the 2-hour sampling, the filter was removed from the Button aerosol sampler, immediately placed into a sterile 1.5 ml microcentrifuge tube and stored at -80°C. For liquid samples recovered from BioSampler and SpinCon air sampler, 1 ml homogenized liquid was taken from each device and 1% β-mercaptoethanol (β-ME) was added to inhibit the potential RNase activity associated with RNA degradation. Our previous study showed that the supernatant liquid after centrifugation of liquid-based bioaerosol samples could have a substantial quantity of extracellular DNA (eDNA), and thus should not be discarded, but should be analyzed for presence of eDNA (22). Therefore, a 1-ml liquid sample from each device was centrifuged at 16,100×g for 10 min at 4°C, after which 950 µl of supernatant liquid was transferred into a new 1.5 ml centrifuge tube, and the rest 50 µl of sample containing pellet cells was saved. Both supernatant liquid and pellet cells were stored at -80°C instantly after separation.

5.3.3 Experimental Setup for Sampling Outdoors

Air samples were collected on the campus of Rutgers University in New Brunswick, NJ (40.48°N, 74.44°W). The sampling location was on a grass field and about 10 meters from the border of a building. One Button Aerosol Sampler, 2 BioSamplers and 1 SpinCon air sampler were collocated approximately 1 m above the ground and operated simultaneously for two hours on three different days (Aug. 6th, Sep. 15th and 17th of 2014).

Since the BioSampler has lower flow rate (12.5 L/min) compared with two other samplers (18 L/min for Button aerosol sampler and 450 L/min for SpinCon) when they were in operation, thus two BioSamplers were employed to collect more bioaerosols for subsequent sample analysis. The Button aerosol sampler was used with a 0.8-μm-pore-size PES membrane filter. 1×PBS solution was used as collection fluid for BioSamplers and SpinCon air sampler. The liquid volume for each BioSampler was 5 ml and BioSampler sampling cup was refilled approximately every 15 minutes to compensate for water loss due to evaporation. The SpinCon air sampler automatically maintained the liquid volume at approximately 10 ml by refilling with pure water. The temperature of outdoor air varied between 21-26°C during the three sampling periods. The RH was between 60-70% during the first sampling period and between 40-45% during the two other sampling periods.

Upon completion of each sampling event, the filter was immediately placed into a sterile centrifuge tube and stored at -80°C. The collection liquid from the two BioSamplers was combined and transferred into a 50-ml centrifuge tube. Then two BioSamplers were refilled with 2 ml sterile water each and shaken vigorously to wash the residual particles off the inner wall. The washed liquid was then combined with the initial sample reaching a total volume of ~15 ml. For SpinCon air sampler, the 10 ml final solution was transferred into a 50-ml centrifuge tube. A 1% β-mercaptoethanol (β-ME) was added into both liquid samples to prohibit the potential RNase activities. Thereafter, both liquid samples were centrifuged at 16,100×g for 10 min at 4°C. After centrifugation, the pellet sample was immediately saved at -80°C. The supernatant liquid was first extracted with *sec*-butanol (Acros Organics, Somerset, NJ) to reduce the volume to ~400

µl and then stored at -80°C. A blank filter for Button aerosol sampler and sterile liquid solutions for two liquid samplers were saved.

5.3.4 Nucleic Acids Extraction

We previously developed a dual-internal-reference technique to improve the accuracy when quantifying bacterial 16S rRNA: rRNA gene ratio by introducing two exogenous DNA and RNA references (29). In this study, the technique was applied to quantify the 16S rRNA and 16S rRNA gene for *E. coli* samples from laboratory experiment. Thus, prior to DNA/RNA co-extraction, *E. coli* samples were spiked with dual internal references which included 5 µl of genomic DNA (1.1×10^6 copies of 16S rRNA gene/µl) and 5 µl 16S rRNA extracts (8.6×10^9 copies/µl) from *Pseudomonas fluorescens*.

The total genomic DNA and RNA from *E. coli* samples in laboratory experiment and outdoor bioaerosol samples in field test were extracted by a phenol-chloroform method. Briefly, the whole filter from Button aerosol sampler and pelleted samples collected by BioSampler and SpinCon air samplers were suspended in 50 µl buffer A (50 mM glucose, 10 mM EDTA and 25 mM pH=8.0 Tris). The solutions were subjected to five freeze/thaw cycles of freezing with liquid nitrogen and thawing in a 55 °C water batch. Then, 200 µl of buffer A, 100 µl of 4 mg/ml lysozyme in buffer A and 50 µl of 500 mM EDTA were added to the liquid. After incubation at room temperature for 10 minutes, a 50 µl 10% sodium dodecyl sulfate (SDS) solution was added followed by extraction with 800 µl phenol-chloroform-isoamyl alcohol mixture (25:24:1, PH=6.7) twice. The aqueous phase was then transferred to a separate 1.5 ml microcentrifuge tube and the total nucleic acids were precipitated by mixing the liquid with 50 µl of 3.0 M

sodium acetate, 2 µl of glycogen (20 mg/ml), and 1 ml 100% ice-cold ethanol. The nucleic acids were pelleted by centrifugation at 16,100×g at 4°C for 15 minutes and washed once with 400 µl cold 70% ethanol solutions. The recovered pellets were first dried under a laminar flow hood for 10 minutes and then dissolved in 100 µl diethylpyrocarbonate (DEPC)-treated water for subsequent analysis.

The extracellular nucleic acids (DNA and RNA) in supernatant liquids from BioSampler and SpinCon samples were extracted by a modified phenol-chloroform protocol as mentioned above by removal of the cell lysis procedures. Briefly, the liquid was extracted twice with 800 µl phenol-chloroform-isoamyl alcohol mixture (25:24:1, PH=6.7) and the aqueous phase was transferred to a separate 1.5 ml microcentrifuge tube after extraction. The total nucleic acids were precipitated by mixing the liquid with 50 µl of 3.0 M sodium acetate, 2 µl of glycogen (20 mg/ml), and 1 ml 100% ice-cold ethanol, pelleted by centrifugation at 16,100×g at 4°C for 15 minutes, and then washed once with 400 µl cold 70% ethanol solutions. The recovered pellets were first dried under a laminar flow hood for 10 minutes and then dissolved in 100 µl diethylpyrocarbonate (DEPC)-treated water for subsequent analysis. The pellet and supernatant extracts were combined into one sample and then subjected to subsequence analysis.

5.3.5 DNA Removal and Reverse Transcription

The total nucleic acids extracts from *E. coli* samples in laboratory experiment were diluted by a factor of 10, and then 45 µl of diluted sample was treated with DNase using Ambion TURBO DNA-free DNase kit (Life Technologies, Grand Island, NY) by following the manufacturer's instructions. The treated samples were then subjected to

PCR reaction with primer sets targeting general bacterial 16S rRNA gene sequences to verify the efficient removal of DNA from RNA sample. Then 5 µl RNA sample was reverse transcribed into complementary DNA (cDNA) using SuperScript® VILO cDNA synthesis kit (Life Technologies, Grand Island, NY). The cDNA was further diluted by a factor of 10 and then stored at -20°C. The cDNA and remaining DNA/RNA mixed samples before DNase treatment were saved for subsequent qPCR and sequencing analysis.

For outdoor air samples, the extracted nucleic acids were processed with the Qiagen AllPrep DNA/RNA Mini kit following the manufacturer's instructions (Qiagen, Valencia, CA). DNA and RNA were separated from the initial 100 µl mixed solution, and eluted in 100 µl TE buffer and 60 µl RNase-free water, respectively. PCR reaction with RNA sample did not produce amplicon when using primer sets targeting general bacterial 16S rRNA gene sequences, which demonstrated efficient separation of DNA from RNA with the kit. A 10 µl of RNA sample was used to make cDNA by using SuperScript® VILO cDNA synthesis kit (Life Technologies, Grand Island, NY). The DNA and cDNA samples were saved at -20°C for subsequent qPCR and sequencing analysis.

5.3.6 Quantitative PCR

A multiplex qPCR method was developed to quantify the 16S rRNA gene and reverse transcribe 16S rRNA for both sample (*E. coli)* and reference (*P. fluorescens)* (29). The primer-probe sets for *E. coli* 16S rRNA gene included forward primer: GGGAGTAAAGTTAATACCTTTG, reverse primer: CCAGTATCAGATGCAGTTC, and probe: TCACATCTGACTTAACAAACCGCCT-FAM. For *P. fluorescens* 16S

rRNA gene, they included forward primer: CCTTGTCCTTAGTTACCAG, reverse primer: CTCTGTACCGACCATTGTA, and probe: CACTCTAAGGAGACTGCCGGTGAC-HEX. Each multiplex qPCR reaction included 10 µl of 2× TaqMan® universal PCR master mix (Life technologies, Grand Island, NY), 650 nM of each forward and reverse primer, 200 nM of each probe, 0.04 mg/ml bovine serum albumin (Sigma, St. Louis, MO) and 4 µl sample DNA or cDNA. The amplification was performed on an iCycler iQ5 RT-PCR detection system (Bio-Rad Laboratories, Hercules, CA) using the following temperature program: 10 min of denaturation at 95°C and 40 cycles of 15 s of denaturation at 95°C and 1 min of annealing/extension at 58°C and 30 s further extension at 72°C. Standard curves were prepared by performing PCR amplification with a 10-fold serial dilution of *E. coli* and *P. fluorescens* genomic DNA mixture with similar 16S rRNA gene copy numbers across five orders of magnitude. The qPCR standards were prepared with the genomic DNA extracted from pure *E. coli* and *P. fluorescens* bacterial cultures. Detailed protocols including extraction, purification and quantification of DNA standards are reported elsewhere (29). PCR amplification for each sample and standard were performed in triplicates.

To quantify the 16S rRNA and 16S rRNA gene from bioaerosols collected outdoors, a SYBR-Green based qPCR assay was developed and performed on iCycler iQ5 RT-PCR detection system (Bio-Rad Laboratories, Hercules, CA). The universal primer sets targeting bacterial 16S rRNA gene included forward primer: 5'-TCCTACGGGAGGCAGCAGT-3' and reverse primer: 5'-GGACT ACCAGGGTATCTAATCCTGTT-3' with an amplicon size of 466 bp on the reference *E.*

coli genome. Each 20 µl reaction contained 10 µl of 2× SYBR green supermix (Bio-Rad Laboratories, Hercules, CA), 2 µl of each 2.5 µM primer, 5 µl of template DNA, and 1 µl PCR-grade water. The thermo-cycler was programmed for 10 min of denaturation at 95°C and 40 cycles of 15 s of denaturation at 95°C and 1 min of annealing/extension at 60°C. Upon completion of PCR amplification, a melting curve test was performed to check the purity of generated amplicons. A 10-fold serial dilution of *E. coli* genomic DNA was amplified with samples to serve as standard curve on each reaction plate.

5.3.7 Sequence Analysis

To characterize the microbial communities in samples collected outdoors by each device, multiplex barcoded 16S rRNA amplicon pyrosequencing was performed in an external laboratory (Molecular Research LP, Shallowater, TX). Prior to sequencing, bacterial 16S rRNA gene sequences and reversed transcribed 16S rRNA sequences in each sample were amplified by PCR with universal primers sets 515f/909r. All amplicon products from different samples were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). The pyrosequencing was performed on a Roche 454 FLX+ titanium instrument (454 Life Sciences, Branford, CT) following manufacturer's guidelines and reagents.

All sequences analysis was conducted utilizing the Quantitative Insights Into Microbial Ecology (QIIME) software package (30). Quality filtering were performed by removing any sequence which had less than 200 base pairs in length, had a machine quality score lower than 25, contained any mismatches in the barcode or primer sequence, or had any ambiguous bases. The chimeras were removed by UCHIME (31). A total of

58 336 sequences passed the quality control checks and were subjected to subsequent analyses. Sequences were clustered into operational taxonomic units (OTUs) by UCLUST (32) using minimal 97% sequence similarity and representative sequence was aligned with PyNAST (33) against the greengenes core set from July 2012 (34). Taxonomic assignment was conducted using the Ribosomal Database Project classifier (35). To correct for different sequencing depth, all samples were rarefied to 700 sequences prior to downstream analyses of diversity and community composition. The phylogenetic distance in microbial community between paired samples was analyzed using weighted UniFrac algorithm (36) and the results were presented in principal coordinate analyses (PCoA) plots.

When analyzing the sequences of outdoor bioaerosols, we found >98% of total bacterial sequences were from the genus *Sphingomonas* for samples collected outdoors on day Two and day Three. This result was completely different from the diverse microbial communities observed in samples collected by two other samplers, which suggested a contamination issue with the samples collected by SpinCon. We suspected the contamination might come from the liquid supply system of the device. Thus, the sequencing results for SpinCon were removed from subsequent analyses and are not presented in Results section.

5.3.8 Statistical Analysis

Paired Student's *t* test was performed to compare the change of 16S rRNA: rRNA gene ratio for *E. coli* samples from each device after two hours active sampling relative to the ratio of spiked *E. coli* cells. Likewise, paired Student's t test was employed to compare

the number of unique phylotypes from paired 16S rRNA and 16S rRNA gene sequences. Permutational ANOVA (PERMANOVA) was performed in R (ADONIS function in VEGAN package) (37) to test the effects of sampling device, sequence type (16S rRNA or rRNA gene) and sampling day on the weighted-UniFrac pairwise distances of bacterial communities. We performed ANOVA using SPSS (verison20, IBM Corp., Armonk, NY) to test the effects of three aforementioned factors on the relative abundance of individual bacterial order and genus. For both PERMANOVA and ANOVA, the interaction effect between collection device and sequence type were also included in the model. Overall, a statistically significant difference was assumed for $p<0.05$.

5.4 Results

5.4.1 Effect of Sampling Device on Change of 16S rRNA: rRNA Gene Ratio of Spiked *E. coli*

After two hours of sampling as described in Methods, the abundance of 16S rRNA gene relative to the quantity of spiked *E. coli* cells were found to be 81.7±3.1%, 89.2±14.6 % and 84.0±8.2% (Figure 5.1a) for Button aerosol sampler, SpinCon air sampler and BioSampler, respectively, indicating relatively low and similar loss of *E. coli* cells during sample collection with three devices. However, the relative abundances of 16S rRNA for recovered *E. coli* to the quantities of spiked cells were 108.5±12.0%, 82.3±2.1% and 52.8±9.7% for Button aerosol sampler, SpinCon air sampler and BioSampler, respectively. The distinct difference in the relative abundances of 16S rRNA among devices is also reflected in the 16S rRNA: rRNA gene ratios (Figure 5.1b). As shown in Figure 5.1b, the variation pattern for the ratio in response to active air sampling stress for two hours was dependent on particular sampler. For Button aerosol sampler, the ratio increased significantly (p=0.034) from ~4300 to ~5900 (Figure 5.1b) after two hours of sampling. When *E. coli* was spiked into liquid-based samplers, it was found that the ratio associated with SpinCon air sampler remained steady at ~4000 with no significant change (p=0.44), however, two hours active sampling with BioSampler of particle-free air resulted in approximately one-half decrease (p=0.026) in the 16S rRNA: rRNA gene ratio relative to that of initially spiked *E. coli* cells: from 4330 to 2500.

5.4.2 Quantification of Outdoor Bioaerosols

The abundance of 16S rRNA gene was in the range from 6.9×10^3 to 1.2×10^5 copies/m^3 (6.5×10^4 copies/m^3 on average, Figure 5.2) and from 1.0×10^4 to 1.5×10^5 copies/m^3 (6.9×10^4 copies/m^3, on average) for outdoor bioaerosols collected by Button aerosol sampler and BioSampler, respectively. In general, the 16S rRNA level was about two orders of magnitude higher than the corresponding number of 16S rRNA gene copies. For example, an average of 7.3×10^6 copies/m^3 (1.1×10^6-1.8×10^7 copies/m^3) and 5.6×10^6 copies/m^3 (1.2×10^6-1.4×10^7 copies/m^3) of 16S rRNA were detected in outdoor air samples collected by Button aerosol sampler and BioSampler, respectively. Statistical analysis did not show any significant difference in quantities of 16S rRNA gene or 16S rRNA between the two devices. No amplicons were detected in qPCR reaction with DNA extracted from blank samples in outdoor air sampling by using primer sets targeting the general bacterial 16S rRNA gene sequences. As explained in the Methods, the results on bioaerosols collected by SpinCon were removed due to sample contamination and not considered for subsequent analysis.

5.4.3 General Characteristics of Outdoor Bioaerosols

The paired 16S rRNA gene and 16S rRNA sequences from six bioaerosol samples collected by two devices on three days were analyzed. In general, the outdoor air samples were highly diverse in bacterial community composition and the total number of unique phylotypes (with equal or higher than 97% in sequence similarity) was in the range from 273 to 441 in each DNA or cDNA pool prior to pyrosequencing. The average number of unique phylotypes yielded from 16S rRNA sequences (375±27) was higher than that from 16S rRNA gene sequences (334±59), but this difference was not statistically different (p=0.102). Besides, the number of unique phylotypes revealed by both

sequencing method showed positive but insignificant correlation (p=0.258) (Figure 5.3). The *Proteobacteria* was the most abundant bacterial phyla on average, which accounted for 25.6%, 4.8% and 9.3% of all reads for α-, β- and γ- subgroups (Figure 5.4), respectively. Other dominant phyla included the *Actinobacteria* (20.6%), *Bacteroidetes* (12.5%), *Cyanobacteria* (11.3%) and *Firmicutes* (9.3%).

5.4.4 Comparison in Airborne Microbial Community

The bacterial communities (16S rRNA gene and 16S rRNA analyses) from six samples collected by two devices on three different days were compared by a weighted-UniFrac-based analysis and depicted in principle coordinate analysis plots (PCoA) as shown in Figure 5.5. It was found that the communities recovered on the same sampling day were clustered close with each other irrespective of the collection device and sequencing method. The PC1 that accounted for 38% of the overall data variability could be partially attributed by the factor of sampling day. By considering data from each sampling day separately, it was observed that the bacterial community showed great similarity as revealed by the paired 16S rRNA gene (closed triangles and squares in Figure 5.5) and 16S rRNA sequences (open triangles and squares in Figure 5.5) irrespective of the collection device. Likewise, the data points represented the bacterial community recovered by Button Sampler (closed and open triangles) and BioSampler (closed and open squares) on each day resided closely with each other on the plot regardless of the sequence types (16S rRNA gene or 16S rRNA). In a further step, permutational ANOVA (PERMANOVA) analysis was performed with the multivariate data set containing the relative abundances of individual bacterial phylotype (Table 5.1). The result from PERMANOVA was in agreement with the findings from PCoA plots: the bacterial

communities exhibited statistically significant difference in samples collected on different days (p=0.002), while no statistical difference was observed in bacterial communities collected by the two devices (p=0.234) and analyzed by the two types of sequences (p=0.315).

Although the sampling day was the only factor that affected airborne bacterial community in outdoor environment with statistical significance, it was still of great interest to investigate the influence of all three factors (sampling day, collection device and sequence type), as well as the interaction effect between collection device and sequence type, on the relative abundance of individual bacterial taxa across all collected samples. Figure 5.6 shows the major bacterial orders (>0.5% in average abundance for all samples) that exhibited significant difference in abundances between the collection devices (Button aerosol sampler or BioSampler) or two types of sequences. It can be seen that bacterial orders *Bacillales* and *Rhizobiales* exhibited higher (p<0.05) percentages in samples collected by BioSampler than those by Button aerosol sampler. Besides, another group of bacterial orders that showed higher (p<0.05) abundance in 16S rRNA sequences than 16S rRNA gene sequences included the *Caulobacterales*, *Myxococcales*, *Rhizobiales* and *Xanthomonadales*. No interaction effect between the collection device and sequence type was found with abundance data for individual bacterial order.

Similarly, the data on relative abundance of individual major bacterial genus (>0.3% in average abundance for all samples) was analyzed in three-way ANOVA while considering the three main factors and also the interaction between the collection device and the sequence type. Of the 53 bacterial genus, only the *Methylobacterium* exhibited higher percentage in presence of samples collected by BioSampler than Button aerosol

sampler (p<0.05). A total of 12 bacterial genera varied in the relative abundance between three collection days. Further, Fisher's LSD test revealed that 9 out of those 12 genera exhibited significant difference in their abundance between day One and Two, 7 out of 12 genera differed in their abundance between day One and Three, and 4 out of 12 genera were different in their abundance between day Two and Three. Six out of 53 genera were detected with higher abundance in 16S rRNA sequences than 16S rRNA gene sequences (p<0.05). No interaction effect between the collection device and sequence type was found with abundance data for individual bacterial genus.

5.4.5 Comparison between Relative Abundance of 16S rRNA and 16S rRNA Gene Sequences in Outdoor Bioaerosols

Figure 5.7 shows the relationship between paired 16S rRNA and 16S rRNA gene sequences in relative abundance for all bacterial genera from six samples collected on three days; we observed a positive linear correlation between the two numbers (p<0.001, R^2=0.627). Six bacterial genera were identified with higher abundance of 16S rRNA than 16S rRNA gene sequences, and thus they have higher ratios of 16S rRNA: rRNA gene than the average ratio for all bacterial genera. These six genera were highlighted in Figure 5.7, and they include the *Rhodanobacter*, *Methylobacterium*, *Roseomonas* and three other unclassified genera belonging to the families of *Acetobacteraceae*, *Ellin6075* and *Isosphaeraceae*, respectively. On average, these six genera were present at frequencies of 0.5%, 2.2%, 0.2%, 0.1%, 0.1% and 0.1% respectively in 16S rRNA gene sequences, but at much higher frequencies of 4.5%, 4.1%, 0.9%, 0.6%, 0.6% and 0.3% respectively in 16S rRNA sequences.

Comparison of 16S rRNA: rRNA gene ratios among multiple bacterial taxa might be inadequent to reveal the difference in their cellular rRNA abundance. This is especially true considering the fact that the copy number of 16S rRNA genes in a bacterial genome varies among different species; previous studies have reported an average of 4 copies of 16S rRNA genes per bacterial genome (38). For a specific bacterial species, the 16S rRNA abundance on a per-cell basis could be determined by multiplying the 16S rRNA: rRNA gene ratio with the copy number of 16S rRNA gene per genome. After searching in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) for complete genome from bacterial genera *Rhodanobacter*, *Methylobacterium* and *Roseomonas*, we found one, six and one representative species for these three genera, respectively. The average copy number of 16S rRNA genes per bacterial genome were found to be 2, 5.2 (average of six species having a range of copy numbers beteeen 4 and7) and 1 for genera *Rhodanobacter*, *Methylobacterium* and *Roseomonas*, respectively. Among the three genera, *Methylobacterium* was the only one that possessed a higher number of 16S rRNA gene copies in its genome compared to the average copy number per bacterial genome (38). Thus, it could be inferred that *Methylobacterium* exhibited higher 16S rRNA abundance on a per-cell basis than the average abundance of 16S rRNA for all bacterial genera in collected outdoor bioaerosols.

5.5 Discussion

A number of studies have investigated the variation of rRNA level within bacterial cells in response to the changes in environment conditions with particular focus on growth-related cell activities (7, 11, 27, 39). For example, the total RNA content per bacterial cell was found to be positively correlated with growth rates in liquid culture (7-9), and degradation of rRNA was frequently associated with conditions of starvation, transition into stationary phase or slow cell growth (24). However, a limited amount of published work is available regarding the relationship between non-growth activities and rRNA concentration (11, 25), although it was hypothesized that microbes under certain stress may shift towards the non-growth maintenance activities (11, 40). In our test with Button aerosol samplers, *E. coli* cells exposed to osmotic and desiccation stress failed to manifest any growth, which was supported by the relatively stable genomic DNA level during the experiment. Previously, it has been reported that *E. coli* was able to actively respond to desiccation condition by changing the membrane phase behavior (41-43), e.g. increasing in the percent composition of saturated fatty acids (41), and synthesizing more intracellular compatible organic solutes including trehalose, proline and glutamine (42, 43). Thus in our study, the non-growth activities of *E. coli* may partially explain the elevated 16S rRNA level under air drying condition.

In our tests with two liquid-based samplers, a significant degradation of 16S rRNA was observed in *E. coli* samples after two hours of sampling with BioSampler but not with SpinCon sampler, although both devices operate using liquid impingement. It is worth mentioning that we observed a greater temperature drop in collection liquid of BioSampler (25 °C to 12 °C) than that of SpinCon (25 °C to 22 °C) after two hours test.

Cold shock to *E. coli* has been known to elevate the *RNase R* activity within *E. coli* cells (44, 45), e.g. more than 10-fold increase was observed for a temperature drop from 37°C to 10°C (46). Thus, we hypothesized that the difference in collection liquid temperature drop during sampler's operation might be the main factor responsible for the different rRNA levels in samples from two devices, In fact, when both devices were in operation, the air was pulled through either three nozzles (BioSampler) or a thin slot (SpinCon) at high flow rates driven by an active working pump and then impinged into the liquid inside the collection vessel. Due to the venturi effect, air pressure inside the collection vessels exhibited a significant drop, which resulted in rapid evaporation of water and thus the evaporative cooling effect on the remaining collection liquid (47). However, the BioSampler was specially designed to be operated at a high pressure drop ($\geq$0.5 atmospheric pressure) with the air stream passing each nozzle at a sonic speed (~340 m/s) (48). In contrast, the pressure drop inside of SpinCon was much lower than that of BioSampler and we estimated that the air speed was ~30 m/s when passing through the thin slot on cyclone wall. Thus, it is likely that the heat loss rate of collection liquid in BioSampler was much higher than that of SpinCon, which may partially explain the discrepancy in temperature drop of collection liquids from two devices.

The paired DNA and RNA samples collected by two bioaerosol samplers on three different days were analyzed with pyrosequencing to assess the composition of microbial communities in an outdoor air environment. In general, the major identified bacterial phyla exhibited great similarity to those described in previous studies of outdoor air (49-52). To investigate the difference in bacterial populations between the samples, three potential factors were considered: the sampling day, collection device and analysis

sequence type. Overall, the results showed that air samples collected at the same outdoor location but on different days harbored distinctly different bacterial communities irrespective of the sampling device and analysis sequence type (Figure 5.5 and Table 5.1). This finding was similar to the conclusions from other studies, where temporal variation in airborne bacterial population has been reported across several consecutive days (53) as well as across multiple different seasons (51, 52, 54). A three-way ANOVA with Fisher's LSD test further indicated that the samples collected on day One were less similar to those from day Two and Three. Here, days Two and Tree were more than one month apart from the first sampling day. This observation was in accordance with a previous finding that airborne bacterial communities become less similar to one another with more elapsed time between samples (51). The temporal variation in airborne microbial communities could be driven by the change in contribution from individual sources, e.g. soils, water bodies, plant surfaces, animal and human activities (50-52), and meteorological conditions also play a role in shaping the outdoor airborne microbial community (55).

The bacterial communities between samples collected by the two devices did not show significant difference according to PERANOVA test. Nonetheless, further examination with individual bacterial taxa at the order and genus levels revealed that two bacterial orders (*Rhizobiales* and *Xanthomonadales*) and one genus (*Methylobacterium*, belonging to the *Rhizobiales* order) exhibited higher abundance in samples collected by the BioSampler than by Button aerosol sampler. Similarly, a recent study suggested that the bacterial community collected from the air was affected by the selection of particular

sampler (56), which might be contributed by the different sampler design factors such as cut-off particle size (48), collection efficiency (57) or other unidentified parameters (56).

It was also found that when assessing the composition of airborne microbial communities, the use of 16S rRNA sequences was at least as equal to, if no better than, the 16S rRNA gene sequences with respective to the number of unique phylotypes being detected at similar sequencing depth. While the total number of unique phylotypes determined with 16S rRNA and 16S rRNA gene sequences exhibited positive but insignificant correlation, the 16S rRNA sequences returned a somewhat higher number of unique phylotypes compared to 16S rRNA gene (p=0.102). Results from PERMANOVA (Table 5.1) and PCoA plots (Figure 5.5) suggested that there was no significant difference in microbial community composition between paired 16S rRNA gene and 16S rRNA sequences for all collected outdoor bioaerosol samples. However, the absolute quantity of 16S rRNA in outdoor bioaerosols was generally almost two orders of magnitude higher than that of 16S rRNA gene (Figure 5.2). The relatively higher abundance of rRNA sequence greatly increases the sensitivity of detection method especially for those rare species in a particular environment (10), which thus allows the reduction of sample size needed for analysis. As a result, it also reduces sampling time and efforts needed to achieve a required sample size. However, the hypothesis of using 16S rRNA sequence rather than 16S rRNA gene sequences to analyze the airborne bacteria still needs to be validated with a large number of samples and from other air environments.

One advantage of analyzing 16S rRNA sequences in combination with 16S rRNA gene sequences is the ability to identify the potentially active bacterial populations in

environments of interest (10, 11, 13). With the six outdoor air samples collected in this study across three different days, we were able to identify six bacterial genera that exhibited higher abundance in 16S rRNA sequences than in 16S rRNA gene sequences. They included the genus *Rhodanobacter*, *Methylobacterium*, and *Roseomonas*, and three other phylotypes that could not be classified at the genus level. Within the same bacterial species, increased ratio of 16S rRNA: rRNA gene was often considered to represent higher current metabolic activity or greater potential of becoming more metabolically active (7-9). However, it might not be possible to compare this ratio between different bacterial species, because different species could have different numbers of 16S rRNA gene copies on their genomes (38). When analyzing the 16S rRNA abundance on a per-cell basis, only the genus *Methylobacterium* exhibited higher abundance of 16S rRNA compared to the average abundance of 16S rRNA from all bacterial genera in the collected samples; this observation might suggest a greater current or potential cellular activity of *Methylobacterium* spp. Bacteria from the genus *Methylobacterium* can grow on one-carbon compounds as sole source of carbon and energy, and they have been reported in a variety of habitats including soil, dust, leaf surfaces and air, *etc.* (58) As common airborne microorganisms, *Methylobacterium* spp. are capable of resisting desiccation to a certain degree and scarvenging trace amounts of nitrogen and carbon which makes them well suited to survive in stressful environments (59). Due to the lack of information on the residence time of these bacteria in the air, however, we are unable to conclude that the elevated cellular rRNA level in genus *Methylobacterium* compared to the average abundance from other genera is attributed to their potential activities when airborne. Thus, future investigation on the activity of environmentally-relevant bacterial

species in airborne phase is warranted (18) and our data suggested *Methylobacterium* spp. could be potential candidates for such investigation.

Considering the sampler-dependent variation of 16S rRNA level as suggested by our laboratory experiment, we also investigated the interaction between sampling devices and sequence type in ANOVA test of three main factors with respect to their effect on the abundance of individual bacterial taxa. Presumably, inclusion of this interaction effect in data analysis would help to identify those bacterial taxa whose 16S rRNA sequences were up-regulated or down-regulated when collected with a particular device. However, no significant interaction was observed between the two factors. A number of potential factors may contribute to such difference between the laboratory and field test. One such factor could be the specific physiological characteristics of bacteria that might be associated with the abilities of different bacteria types to withstand the environmental stress. The atmosphere has been commonly considered a hostile environment for airborne bacteria due to adverse environmental conditions such as such as desiccation, low nutrients, temperature variation, ultraviolet radiation, *etc.* (59-61) Thus, some airborne bacterial populations in the outdoor environment could simply be non-active when staying aloft (59-61). On the other hand, those surviving bacteria might be well adapted to various environmental stress (62, 63) prior to being aerosolized or while staying airborne. Thus they may be less sensitive to the sampling stress, such as cold shock and desiccation, and exert lower level of cellular activity than *E. coli* grown in laboratory conditions. Nonetheless, the impact of sampler selection on the biased analysis results with rRNA sequences warrants further investigation with more sampler types and different bacteria.

5.6 Conclusions

The stress during long-term air sampling resulted in different rRNA levels for *E. coli* cells in laboratory experiments. It was also shown that a particular sampling device might affect the recovered 16S rRNA quantity, i.e. the sampling device might introduce bias to the determined 16S rRNA quantity. However, such bias effect did not play a significant role when analyzing the outdoor airborne bacterial communities with paired DNA and RNA samples from six bioaerosols collected by Button aerosol sampler and BioSampler on three different days. A significant difference in bacterial community composition was observed between bioaerosols collected on different days. In general, 16S rRNA copies of outdoor bioaerosols were two orders of magnitude higher in abundance than that of 16S rRNA gene copies, while analysis two types of sequences provided similar results regarding the composition of bacterial community. Bacteria from genus *Methylobacterium* exhibited higher abundance of 16S rRNA sequences on a per-cell basis compared to average 16S rRNA abundance of all collected bacterial genera. This warrants future investigation of potential activity of environmentally relevant bacterial taxa, e.g. genus *Methylobacterium*, in the airborne phase.

5.7 Acknowledgments

The publication was supported by Grant R01-OH009783 “Advanced Sampler for Measuring Exposure to Biological Aerosols” from CDC/NIOSH, Grant IOS-1022254 “Air as an Active Bacterial Ecosystem” from the National Science Foundation (NSF), and Project 07160 funded by the New Jersey Agricultural Experiment Station (NJAES) at Rutgers, The State University of New Jersey. Its contents are solely the responsibility of the authors and do not necessarily represent the official view of the CDC/NIOSH, the NSF, or the NJAES.

References

1. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:**221-271.
2. **Van de Peer, Y., S. Chapelle, and R. De Wachter.** 1996. A quantitative map of nucleotide substitution rates in bacterial rRNA. Nucleic Acids Res. **24:**3381-3391.
3. **Keer, J. T., and L. Birch.** 2003. Molecular methods for the assessment of bacterial viability. J. Microbiol. Methods **53:**175-183.
4. **Josephson, K. L., C. P. Gerba, and I. L. Pepper.** 1993. Polymerase chain reaction detection of nonviable bacterial pathogens. Appl. Environ. Microbiol. **59:**3513-3515.
5. **England, L. S., S. B. Holmes, and J. T. Trevors.** 1998. Persistence of viruses and DNA in soil. Would Journal of Microbiology & Biotechnology **14:**163-169.
6. **Cai, P., Q. Y. Huang, and X. W. Zhang.** 2006. Interactions of DNA with clay minerals and soil colloidal particles and protection against degradation by DNase. Environ. Sci. Technol. **40:**2971-2976.
7. **Kerkhof, L., and B. Ward.** 1993. Comparison of nucleic acid hybridization and fluorometry for measurement of the relationship between RNA/DNA ratio and growth rate in a marine bacterium. Appl. Environ. Microbiol. **59:**1303-1309.
8. **Poulsen, L. K., G. Ballard, and D. A. Stahl.** 1993. Use of rRNA fluorescence in situ hybridization for measuring the activity of single cells in young and established biofilms. Appl. Environ. Microbiol. **59:**1354-1360.
9. **Kemp, P. F., S. Lee, and J. LaRoche.** 1993. Estimating the growth rate of slowly growing marine bacteria from RNA content. Appl. Environ. Microbiol. **59:**2594-2601.
10. **Pitkänen, T., H. Ryu, and M. Elk, *et al.*** 2013. Detection of fecal bacteria and source tracking identifiers in environmental waters using rRNA-based RT-qPCR and rDNA-based qPCR assays. Environ. Sci. Technol. **47:**13611-13620.
11. **Blazewicz, S. J., R. L. Barnard, R. A. Daly, and M. K. Firestone.** 2013. Evaluating rRNA as an indicator of microbial activity in environmental communities: limitations and uses. ISME J. **7:**2061-2068.
12. **DeAngelis, K. M., C. H. Wu, H. R. Beller, and E. L. Brodie, *et al.*** 2011. PCR amplification-independent methods for detection of microbial communities by the high-density microarray PhyloChip. Appl. Environ. Microbiol. **77:**6313-6322.
13. **Campbell, B. J., L. Yu, J. F. Heidelberg, and D. L. Kirchman.** 2011. Activity of abundant and rare bacteria in a coastal ocean. Proc. Natl. Acad. Sci. U.S.A. **108:**12776-12781.
14. **DeAngelis, K. M., W. Silver, A. W. Thompson, and M. K. Firestone.** 2010. Microbial communities acclimate to recurring changes in soil redox potential status. Environ. Microbiol. **12:**3137-3149.
15. **Gentile, G., L. Giuliano, G. D'Auria, F. Smedile, and M. Azzaro, *et al.*** 2006. Study of bacterial communities in Antarctic coastal waters by a combination of 16S rRNA and 16S rDNA sequencing. Environ. Microbiol. **8:**2150-2161.
16. **Yarwood, S., E. Brewer, R. Yarwood, K. Lajtha, and D. Myrold.** 2013. Soil microbe active community composition and capability of responding to litter addition after 12 years of no inputs. Appl. Environ. Microbiol. **79:**1385-1392.

17. **Perez-Osorio, A. C., K. S. Williamson, and M. J. Franklin.** 2010. Heterogeneous *rpoS* and *rhlR* mRNA levels and 16S rRNA/rDNA (rRNA gene) ratios within *Pseudomonas aeruginosa* biofilms sampled by laser capture microdissection. Journal of Bacteriology **192:**2991-3000.
18. **Krumins, V., G. Mainelis, L. J. Kerkhof, and D. E. Fennell.** 2014. Substrate-dependent rRNA production in an airborne bacterium. Environ. Sci. Technol. Letters **1:**376-381.
19. **Lighthart, B.** 2000. Minireview of the concentration variations found in the alfresco atmospheric bacterial populations. Aerobiologia **16:**7-16.
20. **Bauer, H., H. Giebl, and R. Hitzenberger, et al.** 2003. Airborne bacteria as cloud condensation nuclei. J. Geophys. Res. **108:**4658.
21. **Mainelis, G., and M. Tabayoyong.** 2010. The effect of sampling time and the overall performance of portable microbial impactors. Aerosol Sci. Technol. **44:**75-82.
22. **Zhen, H. J., T. Han, D. E. Fennell, and G. Mainelis.** 2013. Release of free DNA by membrane-impaired bacterial aerosols due to aerosolization and air sampling. Appl. Environ. Microbiol. **79:**7780-7789.
23. **Wang, Z., T. Reponen, S. A. Grinshpun, R. I. Gorny, and K. Willeke.** 2001. Effect of sampling time and air humidity on the bioefficiency of filter samplers for bioaerosol collection. J. Aerosol Sci. **32:**661-674.
24. **Deutscher, M. P.** 2003. Degradation of stable RNA in bacteria. J. Biol. Chem. **278:**45041-45044.
25. **Hansen, M. C., A. K. Nielsen, S. Molin, K. Hammer, and M. Kilstrup.** 2001. Changes in rRNA levels during stress invalidates results from mRNA blotting: Fluorescence in situ rRNA hybridization permits renormalization for estimation of cellular mRNA levels. J. Bacteriol. **183:**4747-4751.
26. **Chang, C. W., and F. C. Chou.** 2011. Assessment of bioaerosol sampling techniques for viable *Legionella pneumophila* by ethidium monoazide quantitative PCR. Aerosol Sci. Technol. **45:**343-351.
27. **Rosset, R., J. Julien, and R. Monier.** 1966. Ribonucleic acid composition of bacteria as a function of growth rate. J. Mol. Biol. **18:**308-320.
28. **Aizenberg, V., S. A. Grinshpun, K. Willeke, J. P. Smith, and P. A. Baron.** 2000. Performance characteristics of the button personal inhalable aerosol sampler. Am. Ind. Hyg. Assoc. J. **61:**398-404.
29. **Zhen, H. J., V. Krumins, D. E. Fennell, and G. Mainelis.** *In preparation*. Improved accuracy in determination of bacterial 16s rRNA: rRNA gene ratio by a dual-internal-reference technique with application to *Escherichia coli* aerosol samples.
30. **Caporaso, J. G., J. Kuczynski, J. Stombaugh, and K. Bittinger, *et al.*** 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods. **7:**335-336.
31. **Edgar, R. C., B. J. Haas, J. C. Clemente, C. Quince, and R. Knight.** 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics **27:**2194-2200.
32. **Edgar, R. C.** 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics **26:**2460-2461.

33. **Caporaso, J. G., K. Bittinger, F. D. Bushman, T. Z. DeSantis, G. L. Andersen, and R. Knight.** 2010. PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics **26:**266-267.
34. **DeSantis, T. Z., P. Hugenholtz, N. Larsen, M. Rojas, and E. L. Brodie, et al.** 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl. Environ. Microbiol. **72:**5069-5072.
35. **Wang, Q., G. M. Garrity, J. M. Tiedje, and J. R. Cole.** 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol. **73:**5261-5267.
36. **Lozupone, C. A., M. Hamady, S. T. Kelley, and R. Knight.** 2007. Quantitative and qualitative beta diversity measures lead to different insights into factors that structure microbial communities. Appl. Environ. Microbiol. **73:**1576-1585.
37. **Oksanen, J., F. G. Blanchet, R. Kindt, P. Legendre, and P. R. Minchin, *et al.*** 2014. Vegan: community ecology package. http://cran.r-project.org/web/packages/vegan/.
38. **Lee, Z.-P., B. C, and T. M. Schmidt.** 2009. rrnDB: documenting the number of rRNA and tRNA genes in bacteria and archaea. Nucleic Acids Research **37:**D489-D493.
39. **Neidhardt, F. C., and B. Magasanik.** 1960. Studies on the role of ribonucleic acid in the growth of bacteria. Biochim. Biophys. Acta **42:**99-116.
40. **Schimel, J., T. Balser, and W. M.** 2007. Microbial stress-response physiology and its implicatiosn for ecosystem function. Ecology **88:**1386-1394.
41. **Scherber, C. M., J. L. Schottel, and A. Aksan.** 2009. Membrane phase behavior of *Escherichia coli* during desiccation, rehydration, and growth recovery. Biochim. Biophys. Acta **1788:**2427-2435.
42. **Welsh, D. T., and R. A. Herbert.** 1999. Osmotically induced intracellular trehalose, but not glycine betaine accumulation promotes desiccation tolerance in *Escherichia coli*. FEMS Microbiol. Lett. **174:**57-63.
43. **Zhang, Q., and T. Yan.** 2012. Correlation of intracellular trehalose concentration with desiccation resistance of soil *Escherichia coli* populations. Appl. Environ. Microbiol. **78:**7407-7413.
44. **Purusharth, R. I., B. Madhuri, and M. K. Ray.** 2007. Exoribonuclease R in *Pseudomonas syringae* is essential for growth at low temperature and plays a novel role in the 3' end processing of 16 and 5 S ribosomal RNA. J. Biol. Chem. **282:**16267-16277.
45. **Cairrao, F., A. Cruz, H. Mori, and C. M. Arraiano.** 2003. Cold shock induction of RNase R and its role in the maturation of the quality control mediator SsrA/tmRNA. Mol. Microbiol. **50:**1349-1360.
46. **Chen, C. L., and M. P. Deutscher.** 2005. Elevation of RNase R in response to multiple stress conditions. J. Biol. Chem. **280:**34393-34396.
47. **Springorum, A. C., M. Clauß, and J. Hartung.** 2011. A temperature-controlled AGI-30 impinger for sampling of bioaerosols. Aerosol Sci. Technol. **45:**1231-1239.
48. **Willeke, K., X. J. Lin, and S. A. Grinshpun.** 1998. Improved aerosol collection by combined impaction and centrifugal motion. Aerosol Sci. Technol. **28:**439-456.

49. **DeLeon-Rodriguez, N., T. L. Lathem, L. M. Rodriguez-R, J. M. Barazesh, and B. E. Anderson, *et al.*** 2013. Microbiome of the upper troposphere: Species composition and prevalence, effects of tropical storms, and atmospheric implications. Proc. Natl. Acad. Sci. U.S.A. **110:**2575-2580.
50. **Bowers, R. M., S. McLetchie, R. Knight, and N. Fierer.** 2011. Spatial variability in airborne bacterial communities across land-use types and their relationship to the bacterial communities of potential source environments. ISME J. **5:**601-612.
51. **Bowers, R. M., N. Clements, J. B. Emerson, C. Wiedinmyer, M. P. Hannigan, and N. Fierer.** 2013. Seasonal variability in bacterial and fungal diversity of the near-surface atmosphere. Environ. Sci. Technol. **47:**12097-12106.
52. **Bowers, R. M., A. P. Sullivan, E. K. Costello, J. L. Collett Jr., R. Knight, and N. Fierer.** 2011. Sources of bacteria in outdoor air across cities in the midwestern United States. Appl. Environ. Microbiol. **77:**6350-6356.
53. **Fierer, N., Z. Z. Liu, M. Rodriguez-Hernandez, R. Knight, M. Henn, and M. T. Hernandez.** 2008. Short-term temporal variability in airborne bacterial and fungal populations. Appl. Environ. Microbiol. **74:**200-207.
54. **Bowers, R. M., I. B. McCubbin, A. G. Hallar, and N. Fierer.** 2012. Seasonal variability in airborne bacterial communities at a high-elevation site. Atmos. Environ. **50:**41-49.
55. **Jones, M., and R. M. Harrison.** 2004. The effects of meteorological factors on atmospheric bioaerosol concentrations - a review. Sci. Total Environ. **326:**151-180.
56. **Hoisington, A. J., J. P. Maestre, M. D. King, and J. A. Siegel.** 2014. Impact of sampler selection on the characterization of the indoor microbiome via high-throughput sequencing. Build Environ **80:**274-282.
57. **Burton, N. C., S. A. Grinshpun, and T. Reponen.** 2007. Physical collection efficiency of filter materials for bacteria and viruses. Ann. Occup. Hyg. **51:**143-151.
58. **Dworkin, M., S. Falkow, E. Rosenberg, K. H. Schleifer, and E. Stackebrandt.** 2006. The prokaryotes: A handbook on the biology of bacteria, p. 257-265. *In* P. N. Green (ed.), *Methylobacterium*, vol. 5. Spinger, New York, NY.
59. **Handley, B. A., and A. J. F. Webster.** 1995. Some factors affecting the airborne survival of bacteria outdoors. J. Appl. Bacteriol. **79:**368-378.
60. **Tang, J. W.** 2009. The effect of environmental parameters on the survival of airborne infectious agents. J. R. Sco. Interface **6:**S697-S702.
61. **Tong, T., and B. Lighthart.** 1997. Solar radiation has a lethal effect on natural populations of culturable outdoor atmospheric bacteria. Atmos. Environ. **31:**897-900.
62. **Beales, N.** 2003. Adaptation of microorganisms to cold temperatures, weak acid preservatives, low pH, and osmotic stress: A Review. Compr Rev Food Sci F **3:**1-20.
63. **Csonka, L. N.** 1989. Physiological ang genetic responses of bacteria to osmotic stress. Microbiol. Rev. **53:**121-147.

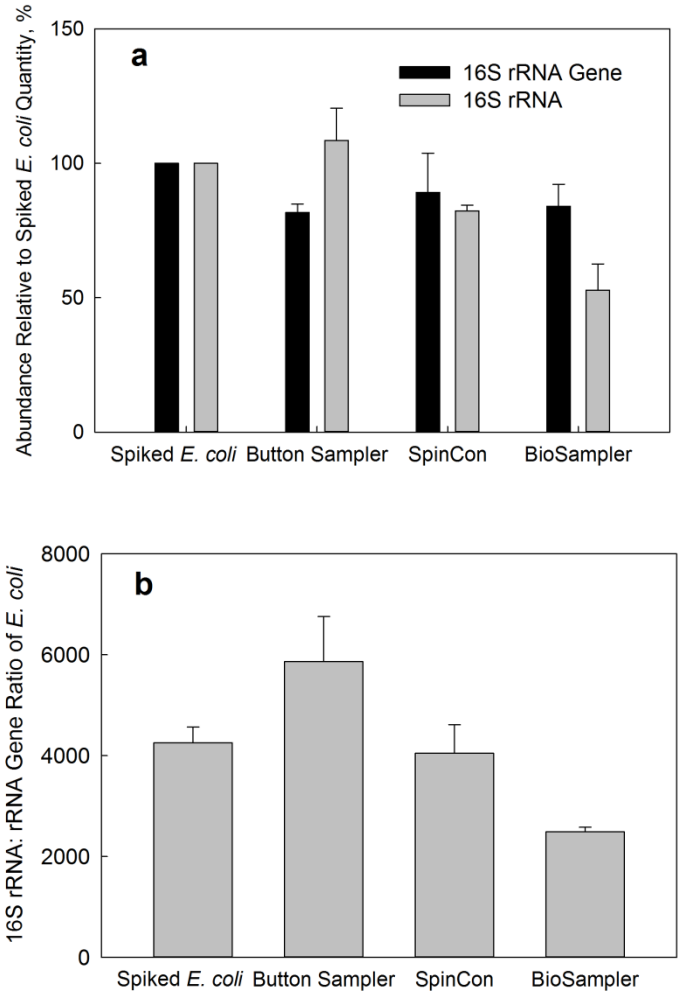

Figure 5.1 Effect of sampling device on changes of 16S rRNA: rRNA gene ratio for *E. coli* cells spiked into three samplers and recovered after two hours of active air sampling. a) Abundance of 16S rRNA gene and 16S rRNA in *E. coli* cells spiked into three samplers (Button aerosol sampler, SpinCon air sampler and BioSampler) and recovered after two hours of active air sampling relative to the spiked *E. coli* quantities. Each bar shows the average from triplicate samples, and error bars show 1 standard deviation. b) The 16S rRNA: rRNA gene ratio of *E. coli* cells spiked into three samplers and recovered after two hours of active air sampling compared with the ratio of spiked *E. coli* cells. Each bar from the left to right shows the average for 10, 4, 3 and 3 samples respectively, and error bars show 1 standard deviation.

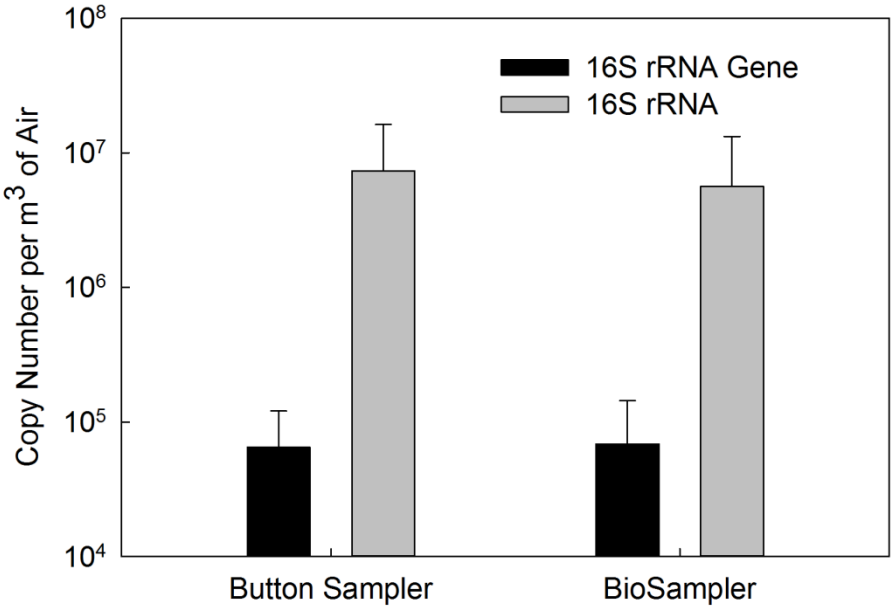

Figure 5.2 The abundance of bacterial 16S rRNA gene and 16S rRNA in air samples collected by Button aerosol sampler and BioSampler simultaneously in an outdoor environment for two hours. Each bar shows the average for triplicate samples, and error bars show 1 standard deviation.

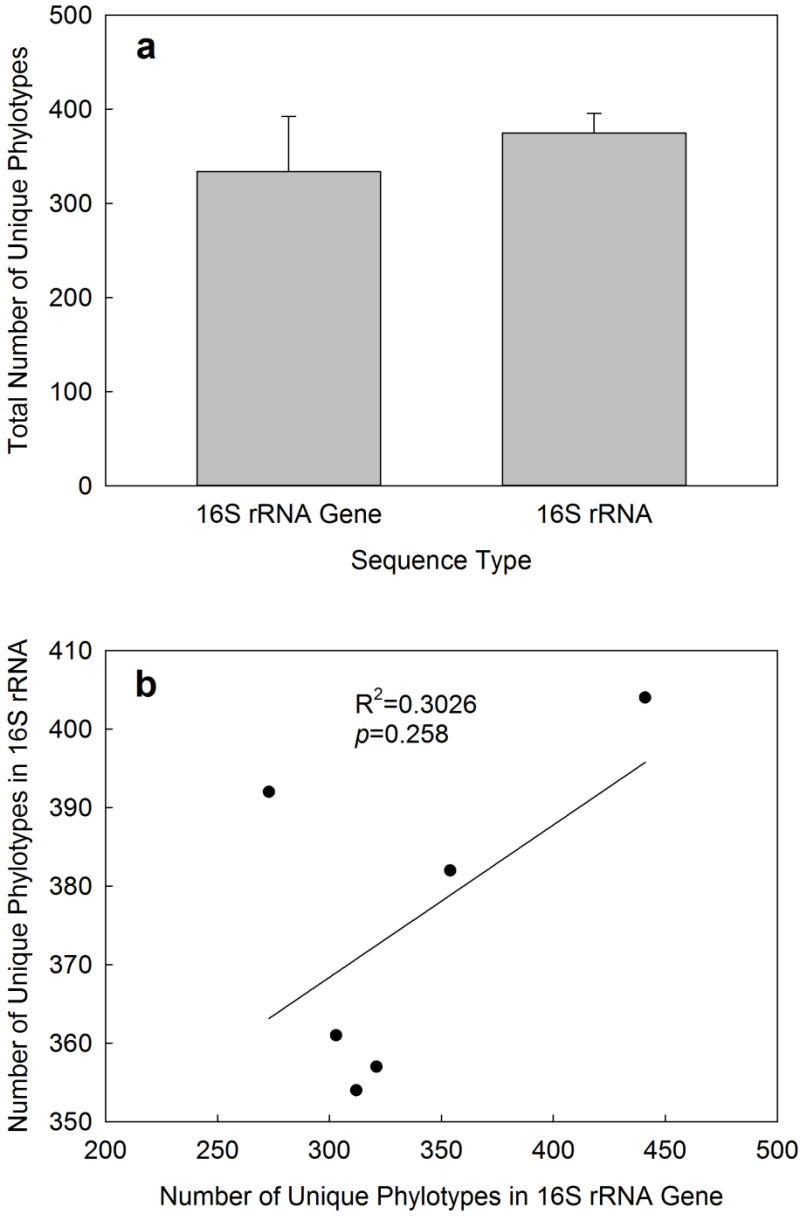

Figure 5.3 Investigation on the number of unique phylotypes from paired 16S rRNA and 16S rRNA gene sequences after normalizing to the similar sequencing depth from all collected bioaerosols. a) Comparison of the total number of unique phylotypes in paired 16S rRNA and 16S rRNA gene sequences after normalizing to the similar sequencing depth from all collected bioaerosols. b) Linear correlation between the number of unique phylotypes in paired 16S rRNA and 16S rRNA gene sequences after normalizing to the similar sequencing depth from all collected bioaerosols.

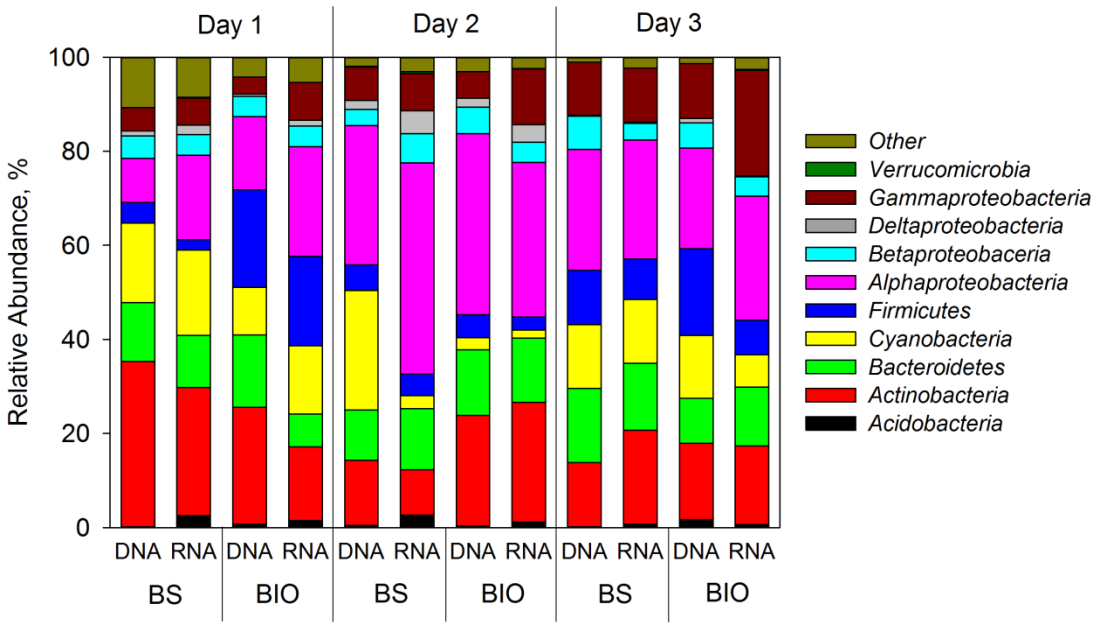

Figure 5.4 Relative abundance of dominant bacterial phyla for all samples (N=6) collected by two devices (BS: Button aerosol sampler; BIO: BioSampler) on three different days and analyzed based on 16S rRNA gene and 16S rRNA sequences.

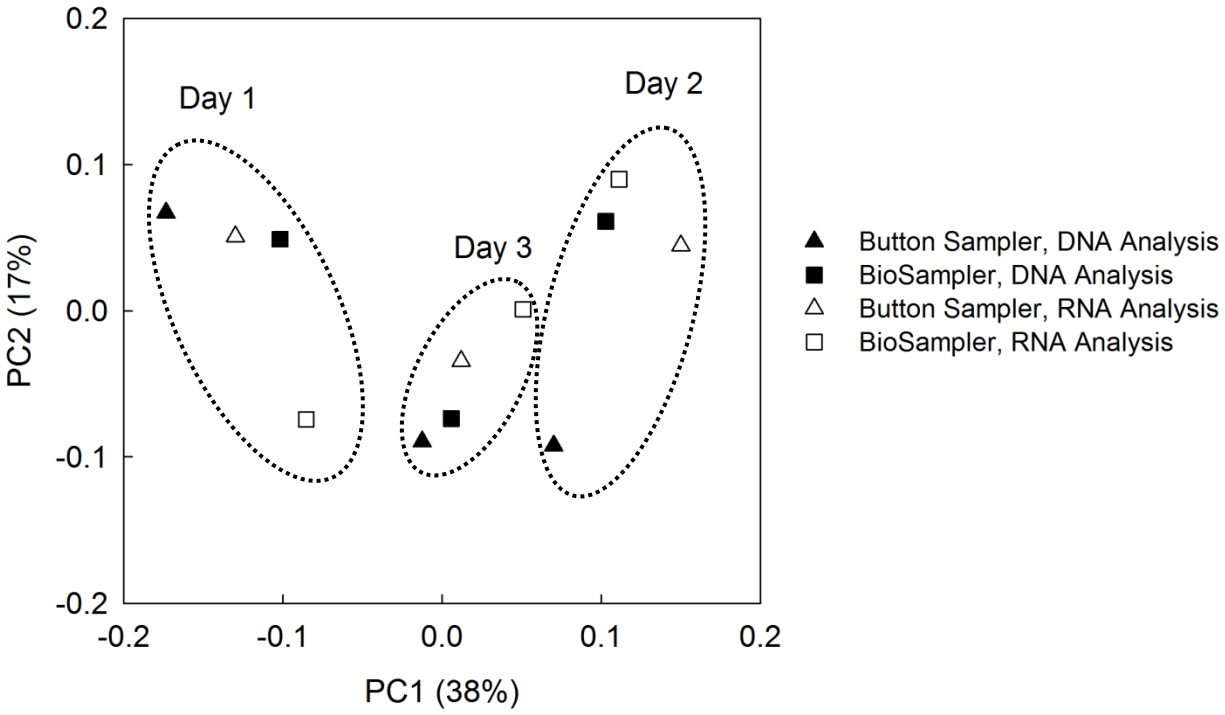

Figure 5.5 Weighted UniFrac-based bacterial diversity principal coordinate analysis of outdoor air samples collected by two devices (Button aerosol sampler and BioSampler) on three different days and analyzed based on 16S rRNA gene and 16S rRNA sequences.

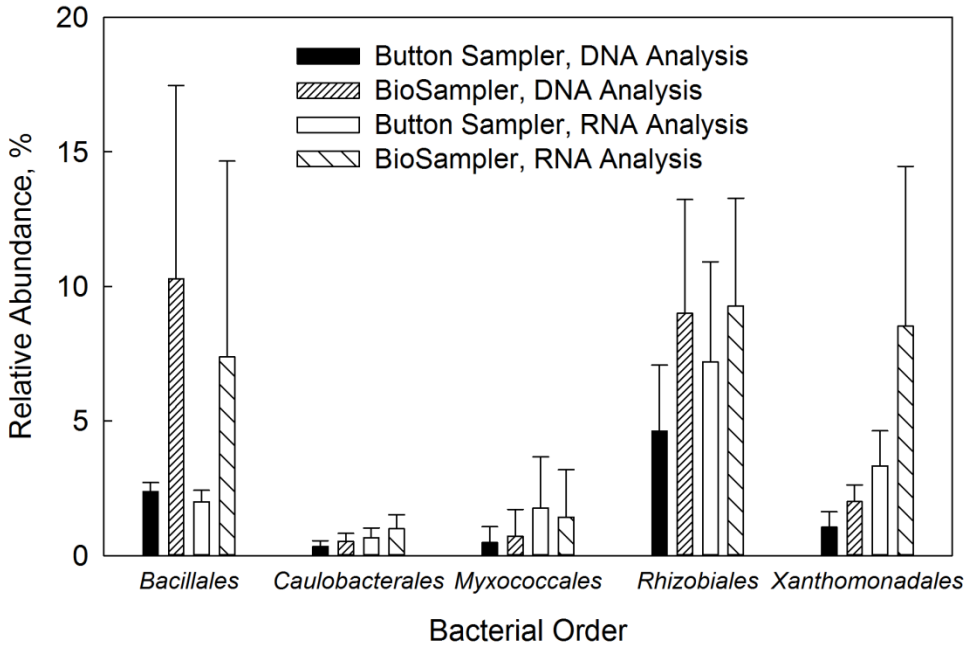

Figure 5.6 Bacterial orders that were found to have significantly different relative abundance between samplers (Button aerosol sampler and BioSampler) and/or between sequence types (16S rRNA and 16S rRNA gene). Each bar shows the average for triplicate samples, and error bars show 1 standard deviation.

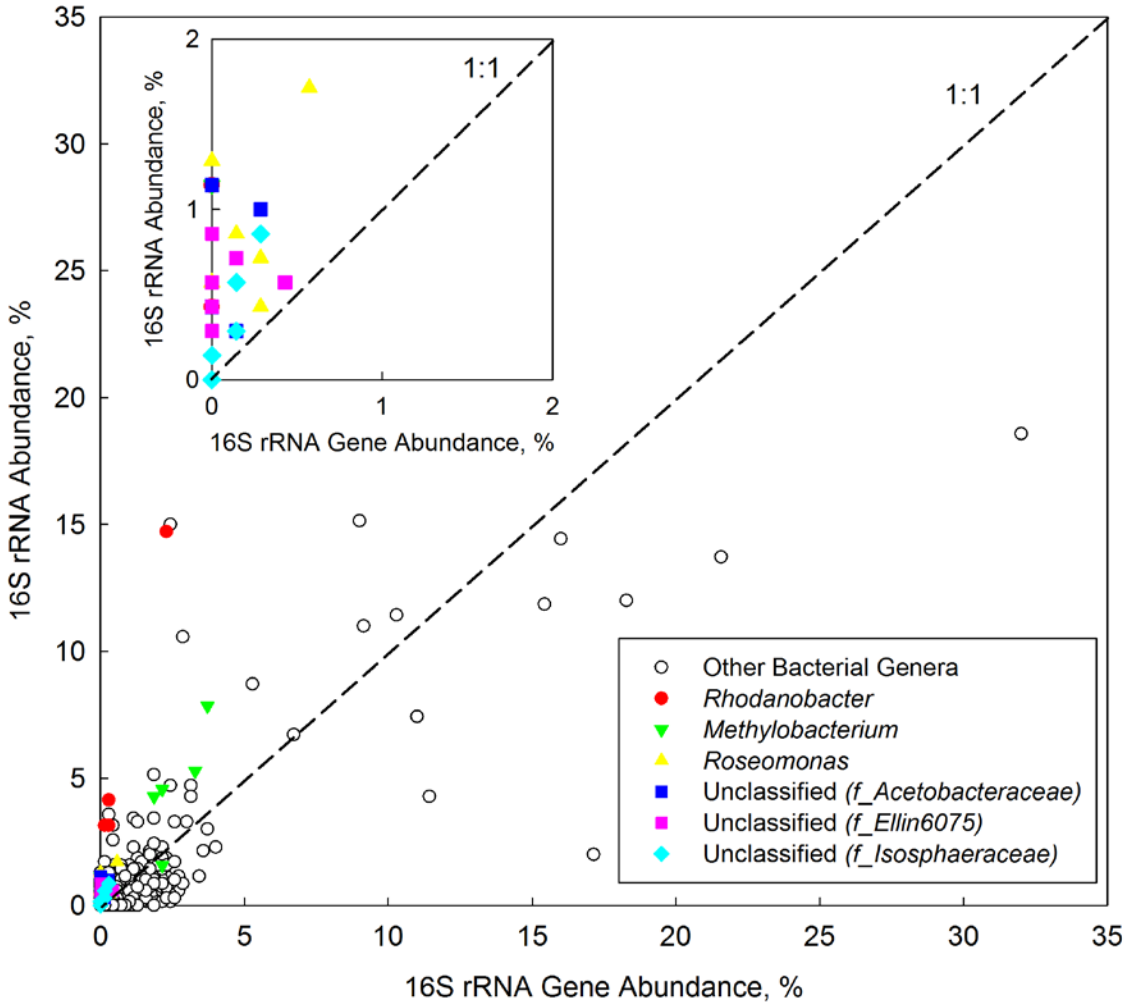

Figure 5.7 The relationship between paired 16S rRNA and 16S rRNA gene sequences based on relative abundance of individual bacterial genus from all samples collected by two devices on three different days. The dotted line is the 1:1 line. The six bacterial genera which exhibited significantly higher relative abundances in 16S rRNA sequences than 16S rRNA gene sequences are presented in color. For clarity, the data points for other bacterial genera are not shown in the insert at top left corner.

Table 5.1 PERMANOVA tests on the effects of sampling day, sampling device, sequence type, as well as the interaction between sampling device and sequence type on the bacterial community weighted-UniFrac pairwise distances. Bold text indicated that $p < 0.05$.

Variables	**df**	**SS**	**MS**	**Pseudo-F**	***p***
Sampling Day	2	0.132	0.066	6.848	**0.002**
Sampling Device	1	0.014	0.014	1.402	0.234
Sequence Type	1	0.012	0.012	1.192	0.315
Sampling Device × Sequence Type	1	0.007	0.007	0.740	0.625

Chapter 6: Summary and Research Outlook

6.1 Summary

Accurate quantification and characterization of airborne microorganisms is a critical first step in most bioaerosol studies. The concentration of biological particles in the atmosphere is typically rather low. For example, the abundance of bacteria in the ambient air was estimated to be in the range of 10^4 to 10^6 cells/m^3 (1, 2), while their abundance is much higher in soils (~10^9 bacteria/gram) and natural waters (~10^6 cells/ml) (3). A large volume of air often needs to be collected in order to harvest sufficient biomass for bioaerosol analysis. As a result, a typical bioaerosol sampling protocol often requires operating different types of sampling devices for long sampling periods. Unlike the inorganic aerosols, the biological characteristics of microorganisms may vary constantly due to the changing environmental conditions (4), e.g. the stress imposed on biological cells by a particular sampler. Thus, it is highly likely that the bioaerosols collected after long-term sampling will not be representative of those airborne microorganisms prior to their sampling and, therefore, the analysis results from the collected samples could be biased (4, 5). For example, a previous study has shown a decrease in bioaerosol culturability when sampling airborne bacteria for extended time periods (6); thus analysis of a bioaerosol from long-term filtration sample would underestimate the abundance of culturable bacteria. In this dissertation, we focused on the potential bioaerosol investigation bias due to a variety of sampling stressors. Several commonly used bioaerosol samplers were selected in such investigations. Specifically, the bioaerosol samples collected by each device were analyzed for extracellular DNA (eDNA, Chapter 2) and the 16S ribosomal RNA (rRNA) content (Chapter 4&5) by using qPCR and pyrosequencing techniques.

In Chapter 2, we compared the sampling stressors induced by four sampling devices: the Button Aerosol Sampler (SKC Inc., Eighty Four, PA), an Anderson-type impactor (BioStage, SKC Inc.), the BioSampler (SKC Inc.), and a newly developed Electrostatic Precipitator with Superhydrophobic Surface (EPSS) (7-9). Each device employs the mechanisms of filtration, impaction, impingement and electrostatic precipitation, respectively. The fraction of eDNA in the total extracted DNA, calculated as cell membrane damage index (I_D), represents the percent of bacterial cells which had damaged cell membranes and present as cell fragments in the sample. Our results revealed that eDNA was detected in samples from all four devices after 5 minutes collection of freshly aerosolized *E. coli*. The sampler-dependent difference was also discovered when comparing the I_D values from samples collected by different samplers at identical conditions. Particularly, collection of bioaerosols by EPSS exhibited the least amount of cell membrane damage compared to the other three devices. Thus, the mechanism of electrostatic precipitation is favored over other three techniques in bioaerosol collection in terms of lessening the sampling stress. Our results also show that EPSS is better suited than other devices to analyze the actual fraction of eDNA in environmental bioaerosols after sample collection. In addition, we found that the air sampling stress varied by adjusting operational parameters for each device, such as the jet-to-plate distance and jet velocity for impactor and collection liquid selection for BioSampler; which was reflected by the changing I_D values under different sampling condition. However, it should be noted that the collection efficiency of a particular sampler could also vary according to the changing sampler operation parameters (10, 11) and it should be considered when designing a sampling protocol.

Another important finding in Chapter 2 was the ubiquitous occurrence of eDNA in environmental bioaerosol samples. In environmental waters and aquatic sediments, eDNA has been reported to comprise up to 90% of the overall DNA pool (12). However, there has been no study reporting the occurrence of eDNA in bioaerosols. In previous studies, the employed DNA extraction protocols failed to fully recover eDNA from the liquid-based bioaerosol samples. For example, one common practice in DNA extraction is to centrifuge the liquid and save the pellets; however, in such case most eDNA stays in the supernatant phase and is inevitably overlooked (5, 13, 14). The other approach is to filtrate the liquid through a filter which was then subject to nucleic acids extraction; however, it was reported previously that filter could only retain less than 20% of total eDNA (15). As a result, the reported DNA concentrations from liquid-based bioaerosol samples in previous studies might be substantially underestimated (5). In Chapter 2, we developed a sample processing protocol for liquid bioaerosol samples to separate the eDNA from intracellular DNA (iDNA), after which both DNA samples were analyzed separately. Our results showed that the sampling stress facilitated the release of genomic DNA (iDNA) from membrane-impaired cells to become eDNA after sample collection. Moreover, our study also implies that a bioaerosol sample collected in a real environment contains a substantial amount of eDNA prior sampling, i.e., in the airborne phase; however, our sampling protocol did not separately determine what fraction of eDNA was captured directly from air as eDNA and what fraction was released by membrane damaged cells due to sampling stress.

In Chapter 4 &5, changes in 16S rRNA level, defined as 16S rRNA: rRNA gene ratio, in bioaerosol samples attributed to long-term sampling effects were investigated for

three sampling devices: the Button Aerosol Sampler (SKC Inc., Eighty Four, PA), the BioSampler (SKC Inc.) and the SpinCon Wet Cyclone Portable Air Sampler (PAS 450-10A, InnovaPrep LLC., Drexel, MO). The 16S rRNA level in bacterial cells has been utilized to represent the current or potential activity of cells with respect to new proteins synthesis (16-18). Thus, quantification of the 16S rRNA: rRNA gene ratio could reveal information regarding the change of bacterial activities (16, 17, 19) while characterizing microbial community by 16S rRNA sequences could discover those active members within a complex microbial community (20, 21). Despite those possibilities, there is still a lack of studies analyzing the rRNA from bioaerosol samples. More importantly, we hypothesized that long-term sampling stress might produce bias in the quantity of recovered rRNA content, and the potential bias could become an obstacle for performing bioaerosols studies based on 16S rRNA analysis. The results from our study indicated that such bias does indeed exist and that this effect depends on a particular sampling device. For example, *E. coli* held on a filter produced more rRNA when they were exposed to active sampling stress, while the rRNA level of *E. coli* decreased by 50% when collected by a BioSampler. Even so, no significant difference in the community composition was revealed among outdoor air samples simultaneously collected by Button aerosol sampler and BioSampler using a 16S rRNA-based pyrosequencing technique. It should be noted that the bacterial community in the outdoor environment is typically much more complex than pure *E. coli* tested in the lab with respect to the present species. Moreover, the microbes in the outdoor air may be well acclimated to the hostile conditions such as desiccation and nutrient limitation in natural environment (22-25),

thus they might be more resistant to the sampling stress than *E. coli* growing in a complex medium under laboratory conditions.

In Chapters 2 and 5, our findings strongly suggest that sampling stress results in the change of biological characteristics of bioaerosols, and thus introduces bias to the final analysis results. In Chapter 3, we focused on the effect of aerosolization stress on microorganisms and tested the impairment of microbes with several commonly used aerosolization devices. It was found that the pneumatic nebulization was the most efficient way of generating large quantity of bioaerosols, although a representative device, the Collison nebulizer, exhibited a significant damage to the cell membrane and resulted in a great culturability loss. In contrast, a modified pneumatic nebulizer, the Single-Pass aerosolizer, showed great improvement over the Collison nebulizer with respect to reduction in cell impairment while retaining similar aerosolization efficiency to Collison nebulizer. The major change in design of Single-Pass aerosolizer with respect to the Collison nebulizer is that the bacterial liquid suspension is not recirculated when subjected to aerosolization and thus the aerosolization stress does not accumulate (26, 27). The Single-Pass aerosolizer could serve in a wide variety of applications in future bioaerosol studies.

Overall, in this dissertation, we systematically investigated the potential impact of variety of stressors on microorganisms during bioaerosol sample collection and aerosolization with different devices. Our results indicated that each device inevitably produced change in the biological characteristics of microorganisms. Two major outcomes due to this impact include: 1) the results obtained by analyzing collected bioaerosols are most likely biased, e.g. the exclusion of extracellular nucleic acids from

bioaerosol analysis would underestimate the quantity of biological cells, and collection of bioaerosols by Button Sampler and BioSampler are likely to produce bias in rRNA quantity , although the effect of this bias was minimal when analyzing outdoor air bacterial community with 16S rRNA sequences; 2) the microorganisms aerosolized from the same liquid suspension batch by multiple aerosolization devices exhibit different changes in their biological properties including damage to cell membrane and loss of culturability. Here, the novel findings of this dissertation are: First, a method for quantifying eDNA from bioaerosol samples was developed and for the first time it was applied to investigate the occurrence of eDNA in environmental bioaerosol samples; Second, we evaluated several different sampling devices for their use to collect bioaerosols without producing bias to the 16S rRNA analysis results of microbial samples, thus this research fills a knowledge gap in measurement and analysis of 16S rRNA in environmental bioaerosols. Based on the results of this dissertation, Table 6.1 summarizes the best practices for sampling and aerosolizing bioaerosols for different purposes.

Table 6.1 The suggested best practices for sampling and aerosolizing bioaerosols for different purposes.

Method	**Purpose**		
	Analysis of ribosomal RNA	Minimal Cell Membrane Integrity	Minimal Culturability Loss
Aerosolization	*Not investigated*	Single-Pass Aerosolizer	Single-Pass Aerosolizer
Sample Collection	SpinCon or Filters (reduce the sampling time)	BioSampler (with pure water as collection fluid) or Filters (reduce the sampling time)	*Not investigated*

6.2 Implications for Future Research

The research conducted in this dissertation answers several important questions for bioaerosols scientists regarding the change of biological characteristics of microorganisms during sampling and aerosolization. The results from this dissertation also provide guidance for bioaerosol researchers in selecting suitable research protocols in terms of collection, characterization, and aerosolization of airborne microorganisms. Moreover, it presents interesting new questions that could be explored and answered in future studies.

We believe that the investigation of eDNA in bioaerosols samples in this research is truly novel. A number of previous studies have described the ubiquitous occurrence of eDNA in natural waters and sediment samples (12, 15, 28-30). However, the ecological significance for the widely distributed eDNA is largely unknown (12). It was suggested that the presence and persistence of large amounts of eDNA in the deep sediment layer might provide a source of nitrogen and phosphorous and/or exogenous nucleotides for bacterial activity (12, 31-33). The eDNA may also serve as an important source of exogenous genes for horizontal gene transfer through natural transformation (12, 28, 30, 34). As a result, it was recommended by another study that the investigation of two DNA fractions, iDNA and eDNA, in natural environment is essential and should be studied simultaneously (12).

In our initial investigation of bioaerosols, the eDNA was released by damaged bacterial cells due to sampling stress. When further investigating the air samples collected inside an equine facility, we were rather surprised to find that the fraction of

eDNA in total DNA extracts could reach as high as 50%. But our sampling protocol did not separately determine what eDNA fraction was already airborne prior to sampling and what fraction was released due to sampling stress; thus there is a great need for future investigation of this question. However, the eDNA attributed to sampling stress cannot be circumvented since a sampler always has to be used to capture bioaerosols. Thus a sampler showing the least damage to cell membrane is preferable in future research on this topic. The results from our study showed that the EPSS has such potential. In addition, samplers with passive sampling mechanism such as producing only a minimal disturbance of bacteria, e.g. natural settling, could be a promising tool to study the presence of eDNA in the air, although the collection efficiency for such device needs to be assessed separately. In addition, in our study we separated eDNA from iDNA in intact cells simply by centrifuging the sample liquid. We didn't consider the effect of sample matrix on efficient separation of eDNA from iDNA because the studied bioaerosols were pure bacterial cells. However, earlier studies with sediment and water sample from the environment strongly suggested that eDNA could easily bound with the complex environmental matrix (12, 15, 29, 30), and thus an improved sample pretreatment protocol is needed.

The analysis of two DNA fractions in environmental samples could be carried out in two different ways: the relative abundance of eDNA versus total DNA could be quantified; also the community composition in each DNA fraction could be determined by high-throughput sequencing techniques. For the latter application, our preliminary result suggests that some bacterial species are preferentially present as eDNA in the supernatant liquid, and it is of great interest to verify this finding with more replicates

from variety of environments. The analysis of two bioaerosol DNA fractions could be conducted in multiple environments of interest. Only when the two DNA pools have been fully characterized with respect to their quantities and compositions, can a decisive conclusion be made regarding which component, or both, pose health risk due to exposure to bioaerosols.

In this dissertation, we investigated the potential bias in bioaerosol rRNA content caused by air sampling stress. This part of the research is forward-looking since the investigation into microbial activity in airborne phase has gained interest only recently, and the analysis of cellular rRNA could serve as a reliable method for exploring this topic (16-18). The results of our laboratory tests with fresh *E. coli* bioaerosols showed that the bias was dependent on a specific sampler. However, the 16S rRNA sequencing results from simultaneously collected outdoor air sample collected did not show such difference between devices. It was hypothesized that two factors might have contributed to difference in results between the lab test and field test. One is that the outdoor environment contained bacteria from a variety of taxonomic groups, and they exhibited distinct and different characteristics. For example, some species may be resistant to the sampling stress such as desiccation and cold shock while other may be more sensitive to stress (5). It is also reasonable to hypothesize that some bacterial species may have evolved to endure outdoor environment as a survival strategy (22-25). The other reason could be that most airborne microorganisms exhibit a limited amount of activity or typically are inactive in airborne phase. In our lab tests, we did not investigate the variation of rRNA level during collection of bioaerosols with low or no activities prior to sampling. However, the constant 16S rRNA level of *E. coli* bioaerosols collected on

filters for 4 hours or longer suggested that there was lack of variability of rRNA in cells with low or no activity in response to filtration sampling stress.

Future studies could focus on exploring the two aforementioned potential reasons. To be more specific, a similar experiment as designed in this dissertation could be performed to investigate the bias on cellular rRNA due to other environmental bacterial species. It also should be noted that the test bacteria should be pre-cultured in a medium where limited nutrients is available for bacterial growth (or starved cells). The purpose of this design is to more accurately simulate the real environmental conditions where there is a lack of nutrients. Besides, the selected bacteria species should also be tested when having different initial activities, including viable and non-viable cells. Upon completion of this experiment, a further step in testing our hypothesis could be performed using field air sampling when employing multiple devices simultaneously in an environment where a significant fraction of microorganisms are presumably to be active, e.g. in wastewater treatment plants (35, 36) or animal feeding operation facilities (37). The collected samples could be analyzed with 16S rRNA pyrosequencing techniques, and the microbial communities could be compared between samples collected by different devices.

Another research direction beyond the work from this dissertation could be exploration of the active bacterial species in the air environment. In our study, we suggested a list of potential candidates which may exhibit metabolic activities when in airborne phase, and they included three bacterial genera *Rhodanobacter*, *Methylobacterium*, and *Roseomonas*, and three other phenotypes in families *Acetobacteraceae*, *Ellin6075* and *Isosphaeraceae* that could not be classified at the genus level. These bacterial genera were determined to have higher 16S rRNA: rRNA gene

ratios than the average ratio of all investigated bacterial genera. Specifically, the *Methylobacterium* genus exhibited higher 16S rRNA level on a per-cell basis than the average level of all investigated bacterial genera. Further exploration could be focused on identification at the species level of those potentially active airborne bacteria by isolation techniques and metagenomic analysis. Upon obtaining pure bacteria strains, further lab tests could be performed to verify their potential activities when staying aloft (38), and it would be of great interest to understand the physiology and ecological significance of these bacterial species when staying active in the airborne phase.

In addition to the aforementioned projects, our work conducted in Chapter 3 (aerosolization of microorganisms) could also be expanded further. For example, depending on specific research needs, other categories of microorganisms, such as gram-positive bacteria and fungi, could be tested using the same protocol developed in our study. In addition, in Chapter 3 we focused on the characteristics of aerosolized bacteria with respect to their culturability and cell membrane integrity. Further research could focus on the assessment of aerosolization stress on change in metabolic activity or other bioaerosol properties of interest. The new research could further facilitate the development of a bioaerosol generator which is versatile in applications with all types of microorganisms and in a broad range of research areas.

References

1. **Bowers, R. M., A. P. Sullivan, E. K. Costello, J. L. Collett Jr., R. Knight, and N. Fierer.** 2011. Sources of bacteria in outdoor air across cities in the midwestern United States. Appl. Environ. Microbiol. **77:**6350-6356.
2. **Lighthart, B.** 2000. Minireview of the concentration variations found in the alfresco atmospheric bacterial populations. Aerobiologia **16:**7-16.
3. **Whitman, W. B., D. C. Coleman, and W. J. Wiebe.** 1998. Prokaryotes: the unseen majority. Proc. Natl. Acad. Sci. U.S.A. **95:**6578-6583.
4. **Cox, C. S., and C. M. Wathes.** 1995. Editors' introduction, p. 3-4. *In* C. S. Cox and C. M. Wathes (ed.), Bioaerosols handbook. CRC Press, New York.
5. **Zhen, H. J., T. Han, D. E. Fennell, and G. Mainelis.** 2013. Release of free DNA by membrane-impaired bacterial aerosols due to aerosolization and air sampling. Appl. Environ. Microbiol. **79:**7780-7789.
6. **Wang, Z., T. Reponen, S. A. Grinshpun, R. I. Gorny, and K. Willeke.** 2001. Effect of sampling time and air humidity on the bioefficiency of filter samplers for bioaerosol collection. J. Aerosol Sci. **32:**661-674.
7. **Han, T., and G. Mainelis.** 2008. Design and development of an electrostatic sampler for bioaerosols with high concentration rate. J. Aerosol Sci. **39:**1066-1078.
8. **Han, T., H. R. An, and G. Mainelis.** 2010. Performance of an electrostatic precipitator with superhydrophobic surface when collecting airborne bacteria. Aerosol Sci. Technol. **44:**339-348.
9. **Han, T., P. J. Nazarenko, P. J. Lioy, and G. Mainelis.** 2011. Collection efficiencies of an electrostatic sampler with superhydrophobic surface for fungal bioaerosols. Indoor Air **21:**110-120.
10. **Yao, M. S., and G. Mainelis.** 2006. Investigation of cut-off sizes and collection efficiencies of portable microbial samplers. Aerosol Sci. Technol. **40:**595-606.
11. **Yao, M. S., and G. Mainelis.** 2007. Use of portable microbial samplers for estimating inhalation exposure to viable biological agents. J Expo Sci Environ Epidemiol. **17:**31-38.
12. **Corinaldesi, C., R. Danovaro, and A. Dell'Anno.** 2005. Simultaneous recovery of extracellular and intracellular DNA suitable for molecular studies from marine sediments. Appl. Environ. Microbiol. **71:**46-50.
13. **Chang, C. W., and F. C. Chou.** 2011. Assessment of bioaerosol sampling techniques for viable *Legionella pneumophila* by ethidium monoazide quantitative PCR. Aerosol Sci. Technol. **45:**343-351.
14. **Veillette, M., L. D. Knibbs, A. Pelletier, and R. Charlebois, *et al.*** 2013. Microbial contents of vacuum cleaner bag dust and emitted bioaerosols and their implications for human exposure indoors. Appl. Environ. Microbiol. **79:**6331-6336.
15. **Liang, Z. B., and A. Keeley.** 2013. Filtration recovery of extracellular DNA from environmental water samples Environ. Sci. Technol. **47:**9324-9331.
16. **Kemp, P. F., S. Lee, and J. LaRoche.** 1993. Estimating the growth rate of slowly growing marine bacteria from RNA content. Appl. Environ. Microbiol. **59:**2594-2601.

17. **Poulsen, L. K., G. Ballard, and D. A. Stahl.** 1993. Use of rRNA fluorescence in situ hybridization for measuring the activity of single cells in young and established biofilms. Appl. Environ. Microbiol. **59:**1354-1360.
18. **Blazewicz, S. J., R. L. Barnard, R. A. Daly, and M. K. Firestone.** 2013. Evaluating rRNA as an indicator of microbial activity in environmental communities: limitations and uses. ISME J. **7:**2061-2068.
19. **Kerkhof, L., and B. Ward.** 1993. Comparison of nucleic acid hybridization and fluorometry for measurement of the relationship between RNA/DNA ratio and growth rate in a marine bacterium. Appl. Environ. Microbiol. **59:**1303-1309.
20. **Campbell, B. J., L. Yu, J. F. Heidelberg, and D. L. Kirchman.** 2011. Activity of abundant and rare bacteria in a coastal ocean. Proc. Natl. Acad. Sci. U.S.A. **108:**12776-12781.
21. **Kamke, J., M. W. Taylor, and S. Schmitt.** 2010. Activity profiles for marine sponge-associated bacteria obtained by 16S rRNA vs 16S rRNA gene comparisons. ISME J. **4:**498-508.
22. **Womack, A. M., J. M. Bohannan, and J. L. Green.** 2010. Biodiversity and biogeography of the atmosphere. Phil. Trans, R. Soc. B **365:**3645-3653.
23. **Gregory, P. H.** 1971. Airborne Microbes: Their Significance and Distribution. Proc. R. Soc. Lond. B **177:**469-483.
24. **Handley, B. A., and A. J. F. Webster.** 1995. Some factors affecting the airborne survival of bacteria outdoors. J. Appl. Bacteriol. **79:**368-378.
25. **Hatch, M. T., and R. L. Dimmick.** 1966. Physiological responses of airborne bacteria to shifts in relative humidity. Bacteriol. Rev. **30:**597-602.
26. **Zhen, H. J., T. Han, D. E. Fennell, and G. Mainelis.** 2014. A systematic comparison of four bioaerosol generators: affect on culturability and cell membrane integrity when aerosolizing *Escherichia coli* bacteria. J. Aerosol Sci. **70:**67-79.
27. **May, K. R.** 1973. The Collison nebulizer: description, performance and application. J. Aerosol Sci. **4:**235-243.
28. **Mao, D. Q., Y. Luo, J. Mathieu, and Q. Wang, *et al.*** 2014. Persistence of extracellular DNA in river sediment facilitates antibiotic resistance gene propagation. Environ. Sci. Technol. **48:**71-78.
29. **Cheng, M., A. E. Cook, T. Fukushima, and P. L. Bond.** 2011. Evidence of compositional differences between the extracellular and intracellular DNA of a granular sludge biofilm. Letters in Applied Microbiology **53:**1-7.
30. **Zhang, Y. P., D. D. Snow, D. Parker, Z. Zhou, and L. Xu.** 2013. Intracellular and extracellular antimicrobial resistance genes in the sludge of livestock waste management structures. Environ. Sci. Technol. **47:**10203-10213.
31. **Jorgensen, N. O. G., and C. S. Jacobsen.** 1996. Bacterial uptake and utilization of dissolved DNA. Aquat. Microb. Ecol. **11:**263-270.
32. **Paul, J. H., A. W. David, M. F. DeFlaun, and L. H. Cazares.** 1989. Turnover of extracellular DNA in eutrophic and oligotrophic freshwater environments of southwest Florida. Appl. Environ. Microbiol. **55:**1823-1828.
33. **Turk, V., A. S. Rehnstam, E. Lundberg, and A. Hagstrom.** 1992. Release of bacterial DNA by marine nanoflagellates, an intermediate step in phosphorus regeneration. Appl. Environ. Microbiol. **58:**3744-3750.

34. **Ochman, H., J. G. Lawrence, and E. A. Groisman.** 2000. Lateral gene transfer and the nature of bacterial innovation. Nature **405:**299-304.
35. **Karra, S., and E. Katsivela.** 2007. Microorganisms in bioaerosol emissions from wastewater treatment plants during summer at a Mediterranean site. Water Research **41:**1355-1365.
36. **Sanchez-Monedero, M. A., M. I. Aguilar, R. Fenoll, and A. Roig.** 2008. Effect of the aeration system on the levels of airborne microorganisms generated at wastewater treatment plants. Water Research **42:**3739-3744.
37. **Wang-Li, L., Q. F. Li, and G. E. Byfield.** 2013. Identification of bioaerosols released from an egg production facility in the Southease United States. Environ. Eng. Sci. **30:**2-10.
38. **Krumins, V., G. Mainelis, L. J. Kerkhof, and D. E. Fennell.** 2014. Substrate-dependent rRNA production in an airborne bacterium. Environ. Sci. Technol. Letters **1:**376-381.