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Evaluation of a Fluorescence Method for Quantifying Bioaerosol Concentrations on Air Quality Filter Samples

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EVALUATION OF A FLUORESCENCE METHOD FOR QUANTIFYING
BIOAEROSOL CONCENTRATIONS ON AIR QUALITY FILTER SAMPLES

By

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Bachelor of Science – Microbiology

University of Arizona

2010

A thesis submitted in partial fulfillment of the requirements for the

Master of Public Health

Department of Environmental and Occupational Health

School of Community Health Sciences

Division of Health Sciences

The Graduate College

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ABSTRACT

Evaluation of a fluorescence method for quantifying bioaerosol concentrations on air quality filter samples

By

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Airborne particulate matter (PM) in outdoor environments contains many components that cause adverse human health effects. The size of the particulates determine in what manner the particles would bypass the body's defense mechanisms to enter the respiratory system and is directly related to their health impacts. Currently the United States Environmental Protection Agency is enforcing the National Ambient Air Quality Standards (NAAQS) to regulate the annual and 24-hour average concentrations of PM_{2.5} and PM₁₀ in the air. PM_{2.5} are fine particles with aerodynamic diameter <2.5µm, small enough to reach the deepest parts of the bronchi and lungs. PM₁₀ include PM_{2.5} and larger particles with aerodynamic diameter of 2.5-10µm. Both PM_{2.5} and PM₁₀ contain multiple components from multiple sources. Bioaerosols are an important component of PM, but there is limited knowledge about how bioaerosols contribute to PM_{2.5} and PM₁₀ concentrations. There is also a lack of research about the incidence and prevalence of disease caused by bioaerosols and about the limits of exposure to bioaerosol

particulates. The main barrier to assess bioaerosol concentrations and health-related effects is the absence of quick and inexpensive methodology for quantifying bioaerosols. This study explored the feasibility of using fluorescence microscopy to quickly quantify bioaerosols in PM_{2.5} and PM₁₀ collected on polycarbonate filters. Bioaerosols were stained with a DNA marker directly on a filter, followed by fixation, microscopic imaging, and automatic counting. The method was first validated using reference samples prepared by depositing different known concentrations of *E. coli* onto blank polycarbonate filters. The results indicated a linear response over two orders of magnitude ($R^2 = 0.9$) and an accuracy within $\pm 25\%$. *E. coli* were also deposited onto selected ambient PM₁₀ and PM_{2.5} filter samples to determine if pre-loaded particles would interfere with bioaerosol imaging and counting. It was found that despite an increase in uncertainty (variability), the calibration slope remained within $\pm 10\%$ of unity for both PM_{2.5} and PM₁₀ samples. Bioaerosol concentrations in ambient samples, as quantified by this method, were on average 14% higher for PM₁₀ than for PM_{2.5} acquired concurrently in a desert environment of Las Vegas, Nevada. The application of this method to other types of compliance filters, such as Teflon filters and tapes of a Beta Attenuation Monitor (BAM) were also explored in this study. By means of a high-yield approach this method is expected to facilitate bioaerosol research, support exposure and health assessments, and help refine NAAQS for PM_{2.5} and PM₁₀.

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CHAPTER 1

INTRODUCTION

Airborne particulate matter (PM) in the outdoor environment contains many components that cause adverse human health effects. Most commonly, ambient PM is a mixture of nitrates, sulfates, organics, dust, trace elements, and liquid droplet-particles resulting from man-made or natural sources (Neri et al., 2016). The size of the particulates is directly related to their human health impacts and determine in what manner the particles would bypass the body's defense mechanisms to enter the respiratory system. The depth at which particles travel into the lungs correlates with health matters such as pulmonary, cardiovascular disease, and death (Neri et al., 2016). Many of the PM constituents are fine particles with sizes small enough to reach the deepest parts of the bronchi and lungs causing acute and/or chronic damage. The finest particles (e.g., nanoparticles) can reach the blood stream causing illnesses including cancer in various parts of the body (Buzea, et. al., 2007).

PM size is measured using aerodynamic diameter, with PM_{10} ($<10\mu m$), $PM_{2.5}$ ($<2.5\mu m$), and PM_{coarse} ($2.5\mu m - 10\mu m$) being the most commonly measured PM (Franklin, Brook, & Pope, 2015). The capability of entering the deepest parts of the lungs and blood stream because of their small size has led to some airborne PM being designated as a Group 1 carcinogen (Erratum, 2014). There is mounting evidence suggesting the causal relationship of $PM_{2.5}$ exposure with morbidity and mortality because it increases the risks of DNA mutations, lung and other cancers, heart attacks and other cardiovascular diseases, COPD, and other respiratory diseases (Raaschou-Nielsen, et. al., 2016). On the other hand, PM_{course} has been suggested to cause illnesses including allergenic and asthmatic effects (Seggev, plunkett, Sword, & symmonds, 2008).

Elevated PM also causes haze to cover the skyline impairing visibility and diminishing aesthetics. In addition to the particle size, chemical composition of PM may be as important in

determining its health and other effects. However, this area is not fully explored partly due to complex nature of PM and difficulties to analyze all its components.

Currently, the United States Environmental Protection Agency (U.S. EPA) is enforcing the National Ambient Air Quality Standards (NAAQS) for PM_{2.5} and PM₁₀ to regulate their annual and 24-hour average concentration in the air (EPA, 2016). NAAQS assume all PM_{2.5} and PM₁₀ are the same regardless of their sources. However, PM chemical composition can change diurnally and seasonally. In urban areas, such as Las Vegas, human causes of PM, specifically fossil fuel combustion, are responsible for the peak concentrations during high traffic hours. There is more wildfire related PM during the summer months while more ammonium nitrate aerosols during the winter months (Hand, Schichtel, Pitchford, Malm, & Frank, 2012). Dust particles are often abundant in the arid desert or urban areas with lots of construction (Boreson, J et. al., 2004).

Bioaerosols are one of the least known among all PM components, with respect to their abundance and exposure limits. However, bioaerosols can be a core issue to PM health effects. Bioaerosol particles that contribute to PM₁₀ and PM_{2.5} consist of airborne biological organisms including virus, bacteria, fungal spores, pollen, and plant debris (Després et al., 2012). Those with known health effects include bacteria, fungi, mycotoxins, endotoxins, and pollen allergens (Chen Q., 2009).

Bioaerosol exposure has been recognized as causing adverse health effects in occupational and residential indoor air and has been a major public health concern. It is related to allergies, acute toxic effects, contagious disease, and even cancer (Boreson, J., et. al., 2004). However, there are less studies about the impact on human health in regards to elevated bioaerosol fractions in ambient PM pollution. There is also a lack of research about the

incidence and prevalence of disease caused by bioaerosol exposure and about the limits of the exposure (Chen, Q., & Hildemann, L.M., 2009). Knowing the bioaerosol concentrations in PM will help determine the role bioaerosols have in developing PM-related diseases. This research will also evaluate current PM standards and exposure limits in outdoor or indoor environments where bioaerosols are important.

U.S. EPA measures PM_{2.5} and PM₁₀ mass and chemical composition routinely from filter-based samples acquired at > 200 air quality compliance sites across the country. Chemical components including sulfate, nitrate, ammonium, organic and elemental carbon, water soluble potassium, sodium, and chloride, and > 40 elements are quantified on filter samples every 3rd day but neither bioaerosols nor any of their surrogates are quantified. There has not been a standardized, validated method for quantifying bioaerosol concentration in PM_{2.5} or PM₁₀. If there is such a method, it has to be cost effective with a quick turnaround time so that a large number of samples can be analyzed in a timely manner. Standard culture methods are time-consuming and do not measure nonviable bioaerosols. There is not a sufficient method of non-culturable quantification, and this absence is preventing an accurate determination of threshold values and dose-response relationships of bioaerosols (Perrino, C., & Marcovecchio, F., 2016). There are many limitations to existing methods including high variation in concentration assessments between researchers using the same method (Perrino, C., 2016). Therefore, non-culturable methods should be explored further to more precisely quantify and assess bioaerosols in PM.

The advantage of non-culturable techniques for quantifying bioaerosols is that not all hazardous biological pathogens are culturable and culturable techniques take days to analyze and are very specific. Some pathogenic bacteria can cause infectious disease at very low levels, but

other organisms may require high concentrations of exposure to become a human health hazard.

Recent scares of bioterrorism threats have ignited interest in quantifying specific microorganisms using PCR and other DNA based tools. There is also a rise in infectious diseases outbreaks, which has led to a need for a rapid, near real-time assessment of bioaerosols. On the other hand, lack of thorough knowledge on hazardous bioaerosols and about dose-response levels calls for a method that is able to quantify all organisms rather than a specific microbe that is a causative for health risks (Chen, 2009).

Microorganisms' vast variety of structures limit the effectiveness of quantification methods. There are methods that are designed to test for specific properties of bioaerosols rather than quantifying bioaerosol mass as a whole. Fungal spores' characteristics make them easy to distinguish and quantify using light microscopy alone. However, this method does not apply to all characteristics of all bioaerosols (Huffman, J., 2010). Another limitation to methods that are used to quantify organisms is that some biologicals are fragile and degrade easily. A method that can quantify bioaerosols quickly with little requirement on sample preparation and handling is preferred.

Fluorescence microscopy is a non-culturable method used to determine whether microorganisms are contained in a sample. Fluorescence microscopy uses fluorochromes to attach and dye DNA or other specific parts of cells that are characteristic of nearly all organisms. This method detects fluorescent properties of biological molecules and uses them as labelers (Ishikawa-Ankerhold, 2012). In practice, the sample is imaged with specific incident light(s) and the cell of an organism will fluoresce at a particular wavelength if it contains DNA or RNA (Després et al., 2012).

Fluorescence microscopy is not often applied and has not been validated to quantify bioaerosols, particularly those on filter-based PM samples. However, fluorescence microscopy could be an efficient method for analyzing bulk bioaerosol samples since advancements with fluorochrome dye have improved the sensitivity of this technique and allowed for minimization of sample preparation. The sample filters can now be directly analyzed by applying fluorochrome dye. Further speciation based on fluorescence spectroscopy is possible making this method a candidate for identifying and quantifying bioaerosols (Grimm et al., 2015). Applying this method to U.S. EPA's PM measurements may give a good indication about the level and trends of bioaerosols that can be related to PM concentration, composition, and human health implications.

Previous studies have used fluorescence stain methods to quantify bioaerosol concentrations in indoor and outdoor air. A study looking at concentrations of viruses and bacteria in air quality samples used a similar staining technique as the method being evaluated for this current study (Prussin, A., Garcia, E., and Marr L., 2015). The experiment performed by Aaron Prussin (2015) looks at indoor and outdoor air to evaluate total concentrations of virus-like particles and bacteria-like particles by staining each filter with SYBRGold fluorescent dye. The filters were soaked in the stain and relied on the stain traveling from the bottom of the filter through the top where the particles were to be evaluated. This staining method also required the samples to be incubated in the dark. The data for quantification of virus and bacteria particles on the filters were averaged, over 25 fields of view, per slide by fluorescence microscopy, and ImageJ, an open source image processing software for scientific analysis, was used for total counts (Prussin, A., et. al., 2015).

A second study compared a cultivating method to a fluorescence microscopy method using AO and DAPI fluorochrome stains (Li, C., and Huang, T., 2006). This study found that the bioaerosol concentrations measured were up to 5200 times higher with the fluorescence method than with the cultivation method (Li, C., and Huang, T., 2006). This study used the bioaerosol collection to create a liquid suspension. The solution was dyed with AO and DAPI and was filtered through a black isopore membrane filter using vacuum. The filter was placed on a slide and counted between 15 and 100 fields of view per slide were counted and analyzed for bioaerosol concentrations. The results were compared to the culture results (Li, C., and Huang, T., 2006).

These studies and others have a variety of drawbacks including using fluorochrome dyes that are sensitive to light or temperature. The current study used NucBlue fluorescence dye, which can be used to directly stain particles on a filter. Previous studies, such as in the Li and Huang (2006) study, take extra steps before using a stain to dye the PM. The Li and Huang (2006) method created an aliquot solution of the collected PM before the particles were dyed causing their method to be time-consuming and had a risk of losing bioaerosols from pulling the PM off of the filter. The loss of potential particles to a wash method, such as in the Li and Huang (2006) study, has been addressed in this current study by directly dyeing the filter the particles were collected on to decrease particle loss. The NucBlue stain can be stored at room temperature in any type of light. The Prussin et. al. (2015) study used a stain that was light sensitive and needed to be incubated and stored in the dark for proper use and optimal attachment to DNA of bioaerosols. Another potential drawback to previous studies was the lack of reference standards to evaluate the accuracy of bioaerosol concentration measurements and lack of evaluation of PM components' interference with bioaerosol counting. The current

method addresses this issue by using a reference *E. coli* standard to compare theoretical and observed concentrations. The method evaluated in this study recognizes the drawbacks in previous studies and builds from the various techniques to get a more accurate quantification method using fluorescence microscopy.

The occupational and environmental health fields will greatly benefit from having better methods for quantification of bioaerosols. More importantly, research into exposure limits, dose-response levels, and the health effects on humans and the environment will be better conducted with more convenient techniques to identify and quantify what we are breathing every day. Better methods can also lead to more research into other potential health effects caused by bioaerosols, such as neurological symptoms, pre-term births, or cancers. The benefits to using a quick and repeatable method are countless and crucial to improve ambient air quality management.

Objective

The objective of this research is to evaluate fluorescence microscopy for quantifying concentrations of bioaerosols. We sought to develop a method that is readily adaptable to filter-based PM_{2.5} and PM₁₀ samples, and to validate it by using reference samples without and with pre-loaded airborne particles. We based our method on polycarbonate filters, taking advantage of their relatively smooth surface and low fluorescence background (Hobbie, J. E., Daley, R. J., and Jasper, S., 1977). In addition, we evaluated the method on Teflon filters and tapes of the Beta Attenuation Monitor (BAM) that are commonly used in U.S. EPA's compliance monitors.

Research Questions

- 1) Can the fluorescence microscopy method quantify reference bioaerosols deposited on blank polycarbonate filters?

- 2) Can the fluorescence microscopy method quantify reference bioaerosols deposited on PM-loaded polycarbonate filters?
- 3) Can the fluorescence microscopy method be used to assess bioaerosols in PM_{2.5} and PM₁₀ samples in a desert environment of Las Vegas, NV?
- 4) Can the fluorescence microscopy method be adapted to Teflon filter and BAM tape from compliance PM monitors?

Hypotheses

H^1_0 : There is no difference between the concentrations of reference bioaerosols deposited on blank polycarbonate filters and those quantified by the fluorescence method.

H^1_a : There is a difference between the concentrations of reference bioaerosols deposited on blank polycarbonate filters and those quantified by the fluorescence method.

H^2_0 : There is no difference between the concentrations of reference bioaerosols deposited on PM-loaded polycarbonate filters and those quantified by the fluorescence method.

H^2_a : There is a difference between the concentrations of reference bioaerosols deposited on PM-loaded polycarbonate filters and those quantified by the fluorescence method.

H^3_0 : The fluorescence method does not quantify variations of bioaerosol concentrations in Las Vegas, NV PM_{2.5} and PM₁₀ samples.

H^3_a : The fluorescence method quantifies variations of bioaerosol concentrations in Las Vegas, NV PM_{2.5} and PM₁₀ samples.

H^4_0 : There is no difference in the performance of the fluorescence method for Teflon filter and BAM tape as for polycarbonate filter.

H^4_a : The performance of the fluorescence method is lower for Teflon filter and BAM tape than for polycarbonate filter.

CHAPTER 2

MATERIALS AND METHODS

The research method included preparation of standard reference and ambient bioaerosol samples on polycarbonate and other filter substrates. These samples were subsequently stained and imaged by a fluorescence microscope for counting bioaerosol particles. Replicate experiments were conducted to evaluate the precision, accuracy, and detection range of the bioaerosol concentrations.

Materials and Reagents

Materials and Reagents used in the study include:

1. Olympus BX51 fluorescence microscope with DP70 imaging camera (Olympus Corporation, Tokyo, Japan)
2. UV excitation – blue emission fluorescence filter was used for specimen detection
3. Poretics polycarbonate (PC) track etched black 13mm diameter, 0.2 μm pore size, membrane filters (GVS life sciences, USA)
4. Swinnex filter holders, 13mm diameter (Millipore Corporation, Bedford, Massachusetts, USA)
5. Single use syringes (1ml) (HSW Soft-Ject, Nörten-Hardenberg, Germany)
6. 13 mm diameter filter punch
7. Cover glass slides 22x22mm, 0.13-0.17mm (Sail Brand, Jiangsu, China)
8. Microscope slides with fine ground edge (Premiere Scientific, Grand Prairie, Texas, USA)
9. NucBlue live cells stain ready probes reagent (Life Technologies Corp., Eugene, Oregon, USA)

10. Fluoromount-G for immunofluorescence (Electron Microscopy Sciences, Hatfield, Pennsylvania, USA)
11. Ethyl alcohol (Pharmco products Inc., Brookfield, Connecticut, USA)
12. DI water (UAQ Lab, Las Vegas, Nevada, USA)
13. *Escherichia coli* 25922 (American Type Culture Collection, Manassas, VA, USA)

Preparation of the Reference *E. coli* Solution and Concentrations

A stock solution of *E. coli* 25922 was prepared with a determined concentration of 7.0×10^9 cells/ml. *E. coli* 25922 strain was cultivated in Tryptic Soy Broth (TSB) agar which was incubated at 35°C for 24hrs to harvest 40ml of culture. The harvested culture was used to inoculate a working culture and was harvested in late-log phase by centrifugation. The culture was washed with Phosphate Buffer with 0.05% Tween (PBT) and the supernatant was removed. The concentration of the suspension was determined by performing serial dilutions with PBT and spread plating the dilutions on replicate trypticase soy agar (TSA) plates. The TSA plates were incubated for 24hrs at 35°C . The plates were counted to determine average CFUs. The suspension was stored at 4°C for 24hrs to deter bacterial growth.

Subsequently, the stock *E. coli* solution was diluted with distilled (DI) water to a final concentration of: 1) 1.7×10^6 cells/ml, 2) 7.0×10^5 cells/ml, 3) 3.6×10^5 cells/ml, and 4) 7.0×10^4 cells/ml to establish four different quantifiable reference concentrations. The detection range of *E. coli* concentrations (i.e., $7.0 \times 10^4 - 2.0 \times 10^6$ cells/ml) were determined based on the optimal counting efficiency under a 40X objective of the fluorescence microscope. The lower limit of detection range is $\sim 5.0 \times 10^4$ cells/ml. The upper limit of detection is $\sim 2.0 \times 10^6$ cells/ml due to counting error from aggregation of *E. coli* cells.

Preparation of the Reference *E. coli* Samples

For preparation of reference *E. coli* filter samples, the process was performed separately for each *E. coli* concentration as follows. An individual dark polycarbonate filter punch of 13mm diameter was placed into a filter holder (i.e., Swinnex filter holder). Next, 0.1ml of one of the four *E. coli* concentrations was inoculated onto the filter membrane. To stain the *E. coli* a drop NucBlue reagent was placed over the *E. coli* loaded filter punch, along with 0.1ml of DI water to disperse the NucBlue. The filter was set aside for 20 minutes for the stain to react with the *E. coli* DNA. NucBlue when coupled to DNA, produces a blue fluorescence under UV excitation.

After staining, the filter was washed using a 1ml syringe with 1ml 70% ethyl alcohol and 3ml of DI water while still in the filter apparatus. Finally, air (1ml) was pushed through to remove the remainder of the solution, to dry the filter, and create homogeneity. In addition to the four *E. coli* loaded samples, two controls without *E. coli* deposits were also prepared: one was stained with NucBlue while the other is a blank without *E. coli* or NucBlue reagent. The controls were used to test for possible contamination or background fluorescence.

The samples and controls were then mounted on slides with a fluorescence enhancing reagent (Fluoromount-G) for microscopic analysis. Eight (8) sets of reference samples, each of which contains four *E. coli* concentrations and two controls were prepared on blank polycarbonate substrates to establish a calibration curve. The four reference resolutions correspond to four *E. coli* concentrations on the reference filters: 1.7×10^6 , 7.0×10^5 , 3.6×10^5 , and 7.0×10^4 cells/ml. A similar procedure was applied to environmental PM collected on polycarbonate as well as, on Teflon or BAM-tape substrates to prepare them for analysis of bioaerosol content.

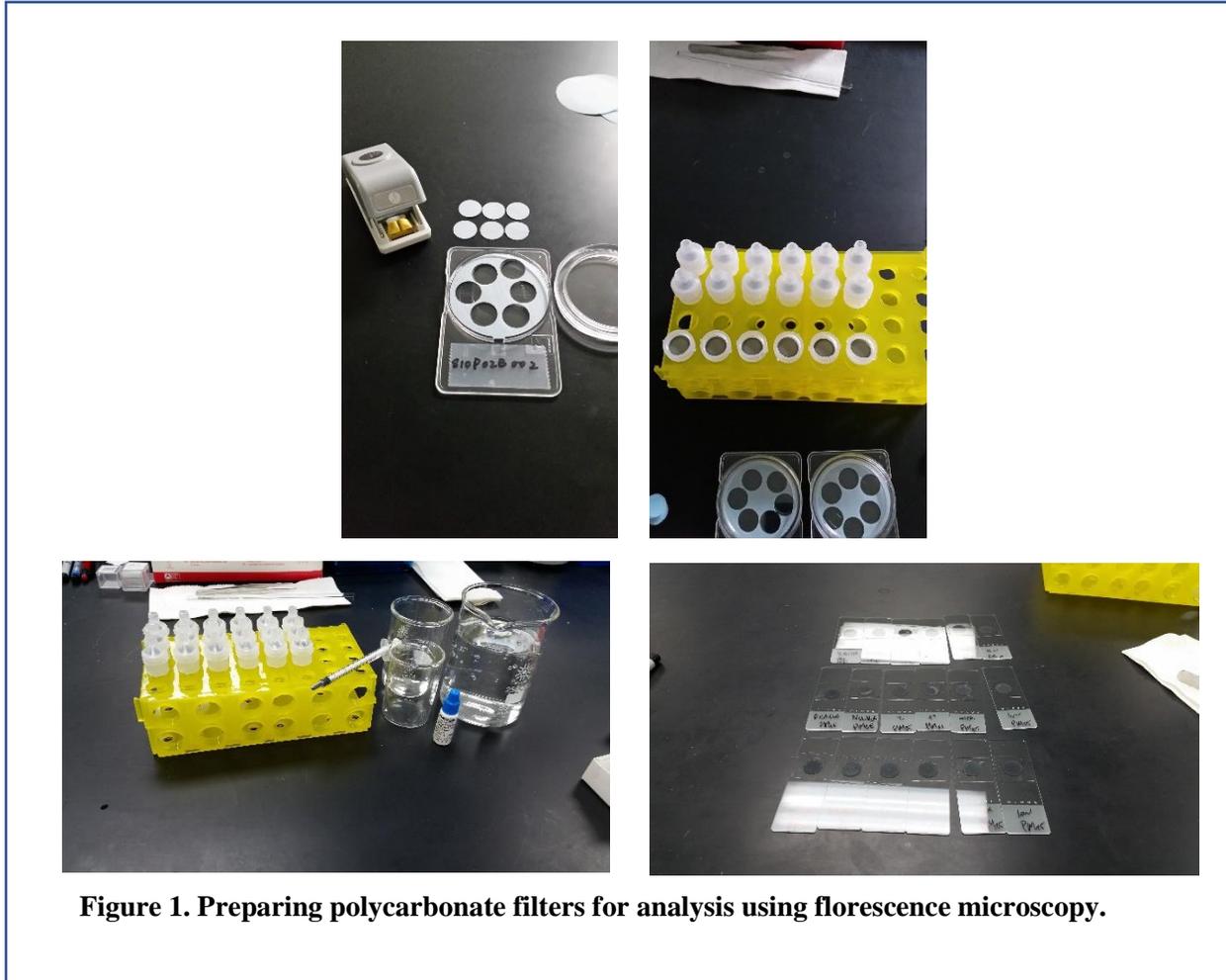
Preparation of Environmental PM Sample Filters

Six pairs of PM₁₀ and PM_{2.5} samples were collected at the University of Nevada, Las Vegas from February 15-20, 2017 and March 13-15, 2017. Each PM sample was acquired by a MiniVol sampler (Airmetrics, Springfield, Oregon, USA) sampling at 4 lpm for 24hrs. The sampling site was located on the roof of a three-story building (WHI) on University of Nevada, Las Vegas campus. The PM sampling was performed on dark polycarbonate 47mm filters with a 0.2µm pore size, and the same filters were used for reference samples. The samples were collected and prepared for analysis within 48 hrs.

Each filter was used to obtain six circular samples (Figure 1 top left panel) using a 13mm diameter punch. Previous studies indicated distribution of airborne elements are relatively uniform (within $\pm 15\%$ of the mean value) throughout a filter sample (Marrero, J., et. al., 2005). Therefore, we assumed that PM and bioaerosol loadings have no significant difference among the six 13mm punches from the same sample filter. Each punch was placed into a filter holder apparatus (Figure 1 top right panel). The method used previously for preparing reference filters with four *E. coli* concentrations and two controls was applied to these PM loaded filters. The purpose of the PM loaded experiment is to test whether environmental PM on a filter would interfere with the *E. coli* or bioaerosol fluorescence (i.e., detectability). The control filters with no *E. coli* deposits but stained with NucBlue reagent was used to detect background environmental bioaerosol concentrations (Figure 1 bottom panels).

Compliance sample filters were also prepared for analysis using the same method. This includes a pair of PM₁₀ and PM_{2.5} sampled on 45mm Teflon filters on March 16, 2017 and a BAM-filter tape sampled for PM₁₀ during February 23, 2017. These experiments were used to

test the applicability of the method for quantifying bioaerosols on various types of PM filters.



Imaging Data Analysis

For each prepared filter (or filter punch) ten random fields were observed through a blue fluorescence filter using the Olympus BX51 microscope at 40x magnification (Hobbie, Daley, & Jasper, 1976). Each of the ten fields were captured through imaging using the Olympus DP70 microscope camera, and the ten images were used to represent the entire 13 mm area. NucBlue stained cells were characteristically found to stand out brighter than previous tested stains such

as AO and DAPI fluorochrome stains, appearing blue against a darker background (Figure 2 top panel).

Numerical quantification of fluorescence cells was achieved using ImageJ data analysis software (Figure 2 bottom panel). The images were processed using a similar technique as described by Perrino, and Marcovecchio (2016). As in the study, this method applied grey-scale to amplify the blue color and applied a threshold value of 255 (Figure 2 middle panel). The image was then converted to a binary, black-and-white image with the particles in white and the background in black (Figure 2 bottom panel). The particles were enumerated and summarized using the software to determine bioaerosol concentration (Perrino & Marcovecchio, 2016). The particle counts observed were converted to yield bioaerosol concentrations in $\#/cm^2$ and also in cells/ml for comparing with the original *E. coli* solutions used to make reference samples. The calculation used to determine cells/ml from analyzing ten images was performed by the following steps: the number of cells counted on the image divided by the area of field of view multiplied by the size of the filter. The total cell count determined from the equation were then divided by the volume of standard solution added to the filter, ($\#$ of cells/ $(375\mu m^2)$ $(13.27mm^2)$)/0.1ml.

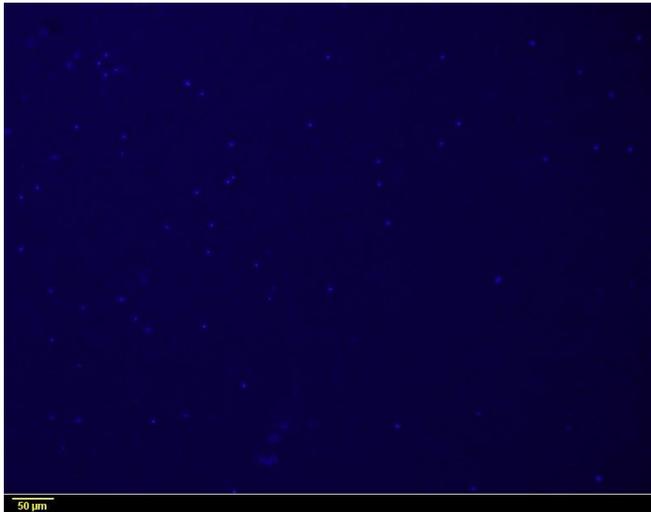
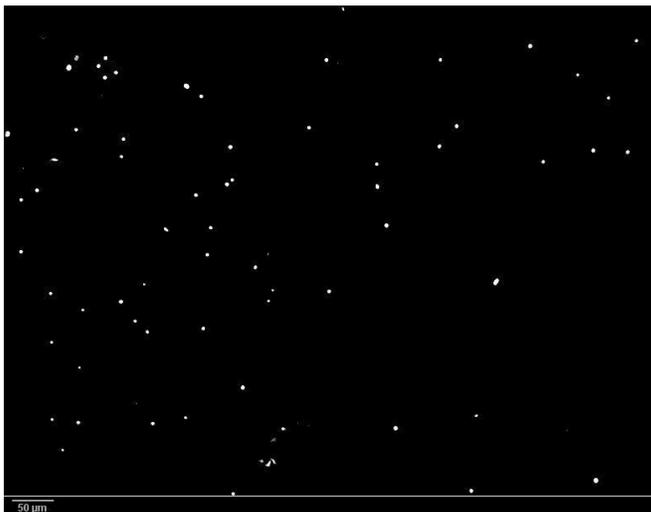
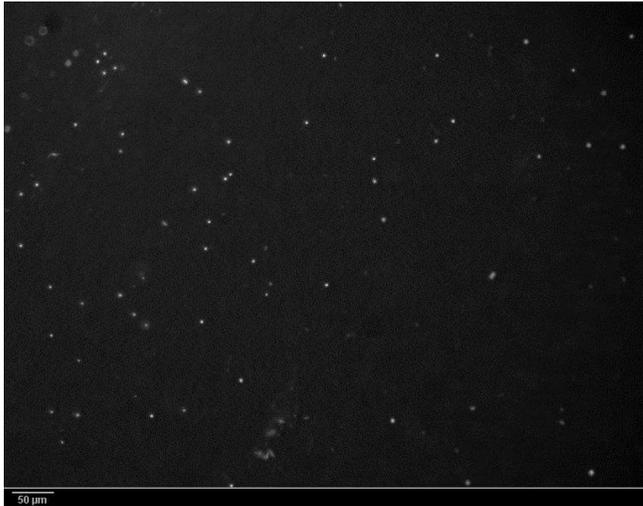


Figure2. *E. coli* cells on a dark polycarbonate filter at 40X magnification.

The top panel represents the original image. The middle panel represents greyscale image of the original image. The bottom panel represents the binary image of the original image.



Statistical analysis

The objective of the statistical analysis was to determine precision and accuracy around the mean. The calculations used to determine precision were performed in several separate calculations. The first step was to determine the average counts of 10 fields of view in bacterial counts/cm². Second the average standard error (standard deviation of the mean) of the 10 fields of view in bacterial counts/cm² was determined. Finally, precision was determined by dividing the mean count by the standard error and then multiplying it by 100% to report the data percent precision. This precision mainly reflects the bioaerosol deposit uniformity. Accuracy is determined from the deviation between observed and expected (actual) *E. coli* concentrations (i.e., 1 - %bias). The t-test was also used to measure significance of the differences between observed and actual *E. coli* concentrations. Their correlations were also determined. Similar statistics would be applied to environmental PM samples.

This study acquired replicate experiments to estimate uncertainty of the measured bioaerosol counts on both reference and ambient samples. The coefficient of variation (COV), ratio of standard deviation over the mean of the replicate measurements, was used to evaluate measurement uncertainty beyond the deposit uniformity such as sample handling, particle loss, and contamination (Taylor, 1997). The COV was calculated in Excel, and the COVs of the total quantifications were compared with the percent precision estimated from 10 images of individual samples.

CHAPTER 3

RESULTS

Bioaerosol Counting Precision and Accuracy

Precision and accuracy of bioaerosol quantifications were measured by comparing our particle counting with known *E. coli* concentrations from the standard suspension. Figure 3 illustrates the distribution of *E. coli* on a reference sample. Each panel in Figure 3 represents a different *E. coli* concentration from high (Figure 3 top left panel) to low (Figure 3 bottom right panel).

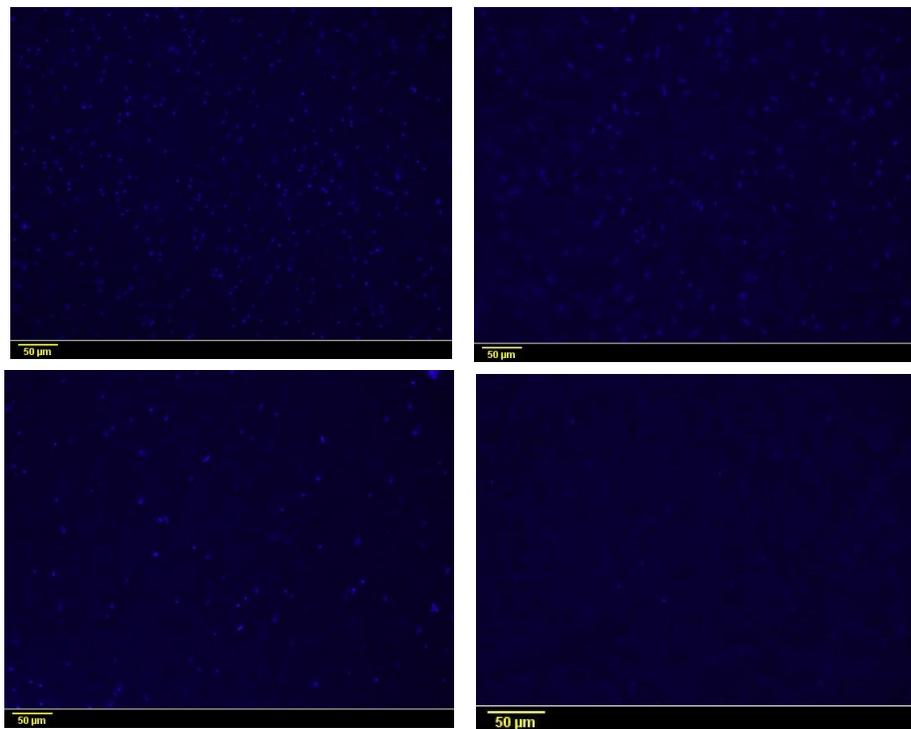


Figure 3. *E. coli* NucBlue stained dispersed onto blank polycarbonate filters observed at 40X magnification.

The images show homogenous deposit of four different concentrations. The top left image has a high concentration of 1.68×10^6 cells/ml, the top right image has a theoretical concentration of 7.0×10^5 cells/ml, the bottom left has a concentration of 3.6×10^5 , and the bottom right image has a concentration of 7.0×10^4 cells/ml.

Results determined by analyzing 10 different images (fields of view) on a sample show that the non-uniformity of *E. coli* distribution varies with *E. coli* concentration level. The average and standard error (standard deviation of the mean) of bioaerosol counting over the 10 images were used to report bioaerosol concentration and uncertainty, respectively. The uncertainty/concentration ratio determined the precision of each measurement, which is summarized in Figure 4. In general, the measurement of precision is within 20%, with the greatest precision (0.02% - 12%, average 7%) found for Conc1, the highest *E. coli* concentration prepared. Conc2 had a precision of 0.05% - 32%, (average of 16%), Conc3 had a precision of 0.06% - 22%, (average of 15%), and Conc4 had a precision of 0.06% - 20%, (average of 12%).

Measurement precision are estimated from the 8 replicate experiments by calculating the coefficient of variance (COV). In this case, the precision includes not only deposition non-uniformity but also variability in particle loss and contamination when preparing the reference samples. Table 1 compares the two precision estimates for each *E. coli* concentration level. Among the 4 concentration levels, COV varied from 10% - 24% and, as expected, is larger than the precision based on deposit non-uniformity (7-16%). The highest concentration level, Conc1, has the lowest COV (i.e., best precision) at 10%. The blank filters have the highest COV (i.e., lowest precision) because their bioaerosol concentrations are close to or below the MDL. Bioaerosols on blank filters without *E. coli* deposit indicate contaminants from the regents, sample handling, and/or environment.

The accuracy of bioaerosol counting can be determined from the % bias between the actual (expected) concentrations, i.e., accuracy = 1 - %bias. Accuracy ranges from 76% - 93%, as %bias ranges between 7% - 24% (Table1). For Conc1, the observed concentrations are significantly lower than the actual concentration (p=0.001), for Conc2 and Conc3, the

differences are not significant ($p=0.26$ and 0.34), and for Conc4, the observed concentrations are significantly higher than the actual concentration ($p=0.009$).

Regression analysis was conducted to establish the association between observed and actual bioaerosol concentrations, using data from the 8 replicate experiments. Figure 5 shows a linear relationship with R^2 of 0.97 indicating that the fluorescence method is able to predict bioaerosol concentration on reference samples and, with proper calibration, can be useful for quantifying bioaerosol concentration in ambient PM samples. The current calibration has a slope of 1.23 and an intercept of $\sim 2000 \text{ \#/cm}^2$. The results determined the null hypothesis to be true H^1_0 : There is no difference between the concentrations of reference bioaerosols deposited on blank polycarbonate filters and those quantified by the fluorescence method.

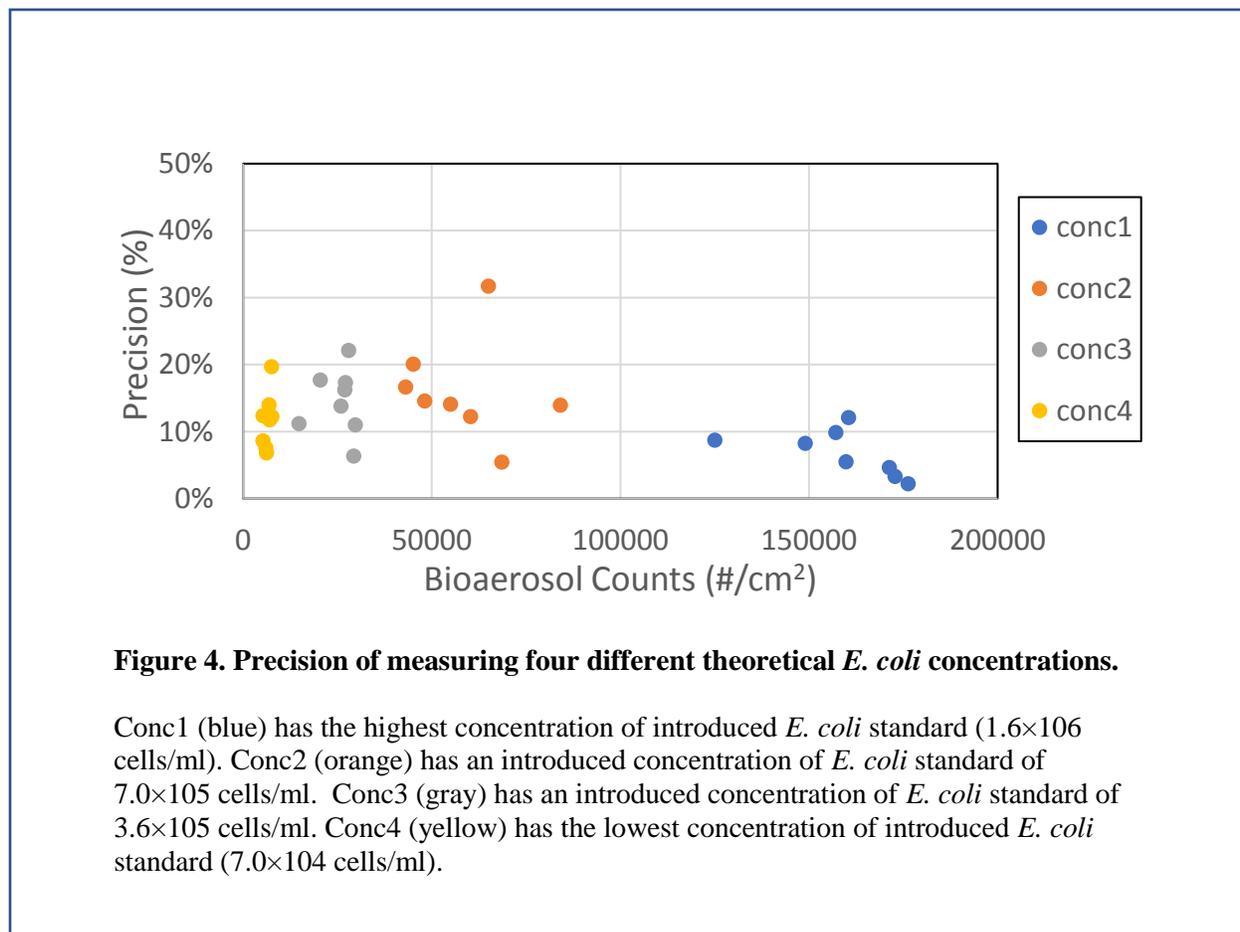


Table 1. Overview of measurement precision and accuracy for bioaerosol counts on the reference samples.

Expected bioaerosol concentrations are based on *E. coli* deposit. Observed bioaerosol concentration and COV are based on 8 replicate experiments for each concentration level, while precision is based on deposit non-uniformity (see text). Accuracy is the deviation between observed and expected (actual) concentration (i.e., 1 - %bias). Also compared are the blanks with only NucBlue added and the blanks with only DI water added (no stain).

Concentration Level	Total Samples	Expected bioaerosol concentration (#/cm ²)	Observed bioaerosol Average (#/cm ²)	COV Precision (%)	Non-uniformity Precision (%)	Accuracy of Observed <i>E. coli</i> (%)
1	8	128109	158955	10	7	76
2	8	52751	58632	24	16	89
3	8	27129	25275	20	15	93
4	8	5275	6449	14	12	78
NucBlue Blank	8	0	1573	26		
DI Blank	8	0	52	46		

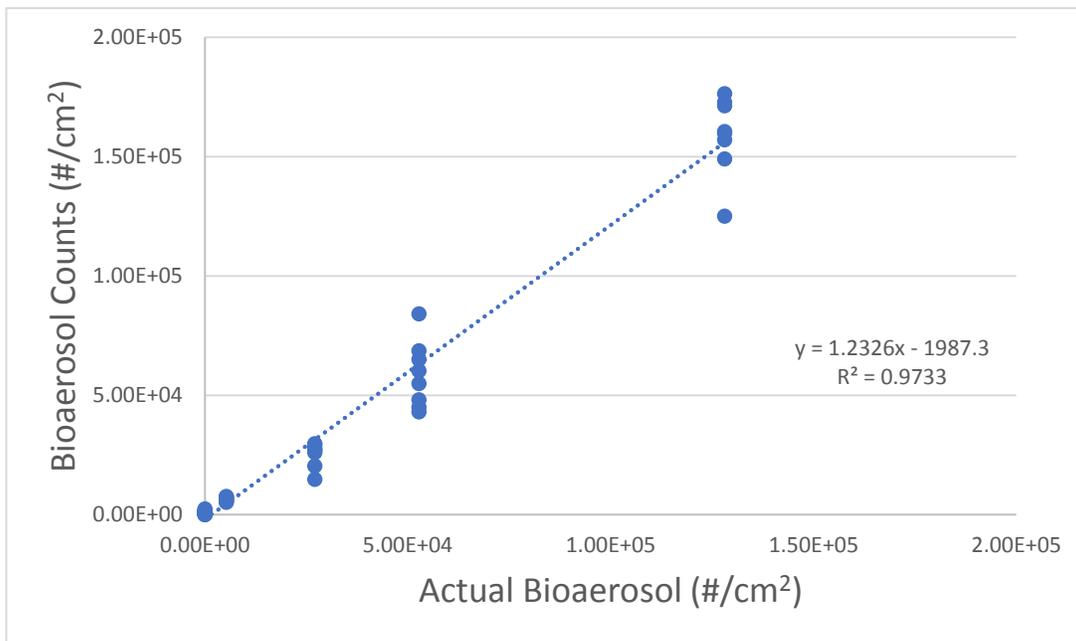


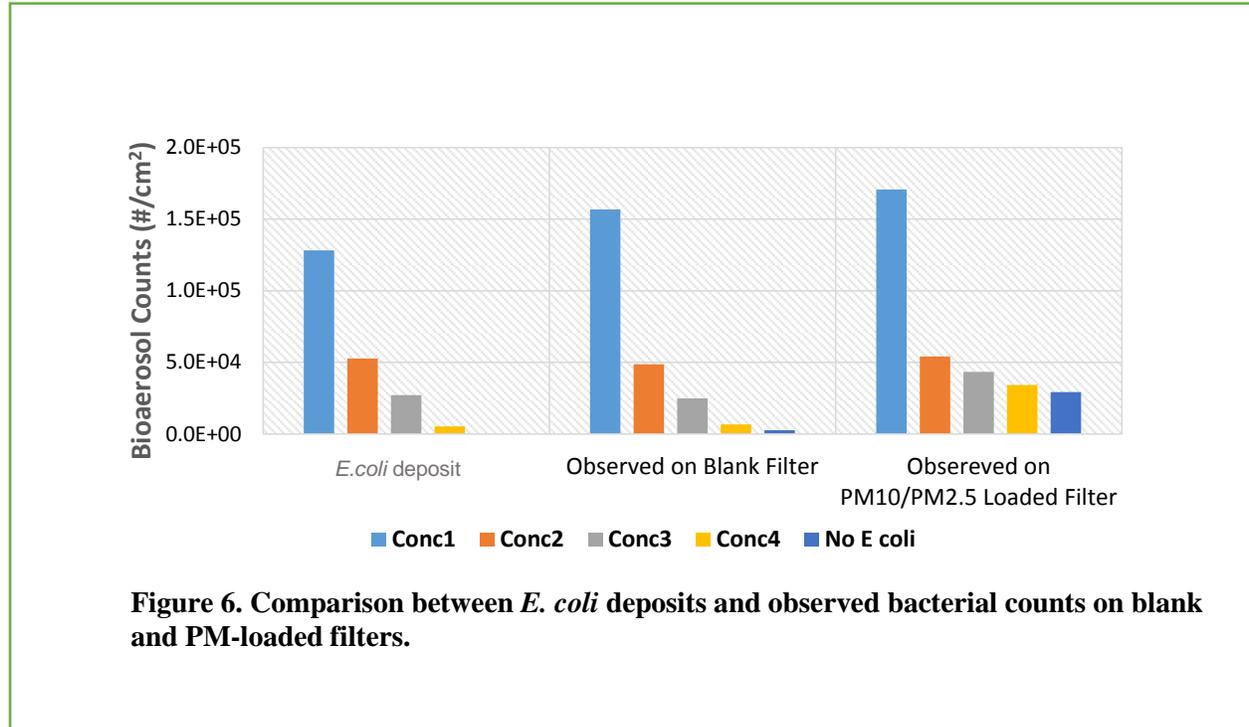
Figure 5. Accuracy of each polycarbonate experiment.

Observed bioaerosol counts by fluorescence method, compared with actual bioaerosol concentrations (i.e., *E. coli* deposit) for reference samples. Results are based on 8 replicate experiments.

Particulate Matter Samples

PM_{2.5} and PM₁₀ samples were collected on polycarbonate filters over a period of three different days during March 13-15, 2017. For each of the 6 samples (3 PM_{2.5} and 3 PM₁₀), 4 punches were taken for additional *E. coli* deposition at 4 concentration levels (1.68×10^6 cells/ml, 7×10^5 cells/ml, 3.6×10^5 cells/ml, and 7×10^4 cells/ml), and 2 punches were taken to prepare samples without *E. coli* deposits. Figure 6 compares averages of observed bioaerosol counts on the PM loaded filters versus those on PM free (blank) polycarbonate filters as well as the expected *E. coli* concentrations. Results showed that *E. coli* deposits associated with PM substantially increased the total bioaerosol counts.

The average bioaerosol concentration reported for six PM samples with no addition of *E. coli* was 29,202 #/cm², while the average concentration observed for eight blank filters was only 2,560 #/cm². The difference indicates contributions of ambient bioaerosols.



The measurement precision based on deposit inhomogeneity from 10 fluorescence images was higher with PM-loaded samples than with PM-free filters. The precision ranged from 9% to 37% for PM_{2.5} (Table 2) and from 7% to 39% for PM₁₀ (Table3). This suggests that PM deposits on filter were more non-uniform than the additional *E. coli* deposit. Even for ambient PM samples without *E. coli* deposit, the measurement precision is better than 40% (average ~25%). The COV across the three PM_{2.5} and PM₁₀ samples is no longer a good measure of precision since it is influenced by different bioaerosol levels associated with PM on different days. For both PM_{2.5} and PM₁₀, COV is smaller when *E. coli* deposit dominates the bioaerosol count at the 1st and 2nd concentration levels (Table 2 and Table 3).

Table 2. Overview of measurement precision for bioaerosol counts on PM2.5-loaded samples with additional E coli deposits (Concentration level 1-4).

Observed bioaerosol concentration and COV are based on 3 samples taken on different days, while precision (%) is based on deposit non-uniformity on the filter (see text for details). NucBlue indicates samples without *E coli* deposit (only stain).

Concentration Level	Total Samples (PM ₁₀)	<i>E. coli</i> Deposit (#/cm ²)	Observed Bioaerosol Count (#/cm ²)	COV (%)	Range of Non-uniformity Precision (%)	Average of Non-uniformity Precision (%)
1	3	128109	168405	6	9 - 12	10
2	3	52751	60068	13	14-16	15
3	3	27129	44864	38	11-17	13
4	3	5275	32838	44	18-29	22
NucBlue	3	0	28092	57	15-37	29

Table 3. Overview of measurement precision for bioaerosol counts on PM10-loaded samples with additional E coli deposits (Concentration level 1-4).

Observed bioaerosol concentration and COV are based on 3 samples taken on different days, while precision (%) is based on deposit non-uniformity on the filter (see text for details). NucBlue indicates samples without *E coli* deposit (only stain).

Concentration Level	Total # of Samples (PM ₁₀)	<i>E. coli</i> Deposit (#/cm ²)	Observed Bioaerosol Count (#/cm ²)	COV (%)	Range of Non-uniformity Precision (%)	Average of Non-uniformity Precision (%)
1	3	128109	172854	2	7 - 12	9
2	3	52751	48068	7	11-12	12
3	3	27129	41764	40	13-18	15
4	3	5275	35450	46	13-17	15
NucBlue	3	0	30313	56	10-39	21

The observed bioaerosol concentrations ($\#/cm^2$) from six PM samples are compared with the level of *E. coli* deposits in Figure 7. Note that each experiment contains five different concentration levels (Conc1-4 and NucBlue only). Figure 7 demonstrates a linear response with a R^2 of 0.85 – 0.94 for PM_{10} and a R^2 of 0.94 – 0.97 for $PM_{2.5}$ supporting that the added *E. coli* can be quantified effectively even with the interference of PM preloaded on the filter. The linear regression has a slope of 0.92 – 1.24 for PM_{10} and $PM_{2.5}$, respectively varying from the slope of 1.23 from PM-free filters but overlapping the 95% confidence interval (1.17 – 1.29). This proves the null hypothesis which states H^2_0 : There is no difference between the concentrations of reference bioaerosols deposited on PM-loaded polycarbonate filters and those quantified by the fluorescence method.

Figure 8 and Figure 9 display the collection of bioaerosols on polycarbonate filters for $PM_{2.5}$ and PM_{10} , respectively. The samples were acquired on February 23, 2017 for 24 hrs. There is a higher concentration of fluorescence particles in PM_{10} than there is for $PM_{2.5}$ and the images also show less homogeneous distribution than dispersal of *E. coli* on reference samples (Figure 8 and Figure 9).

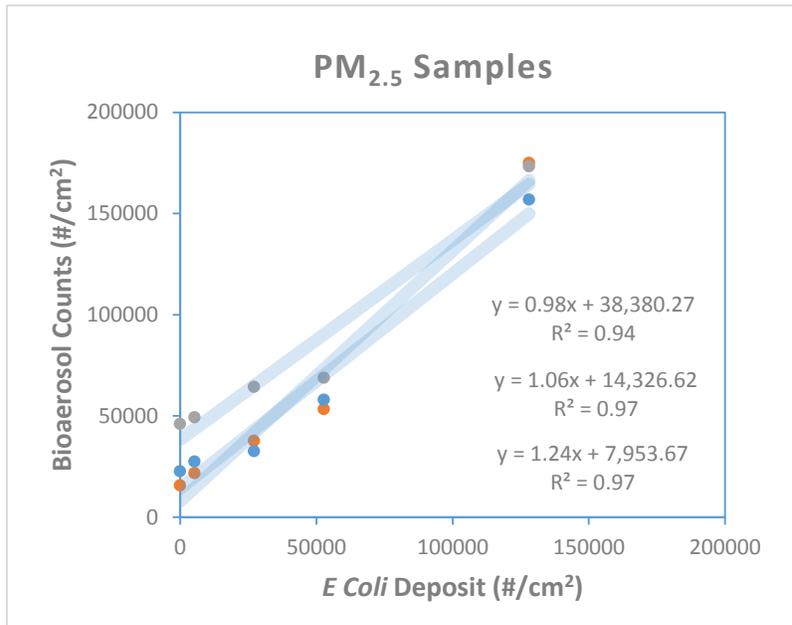
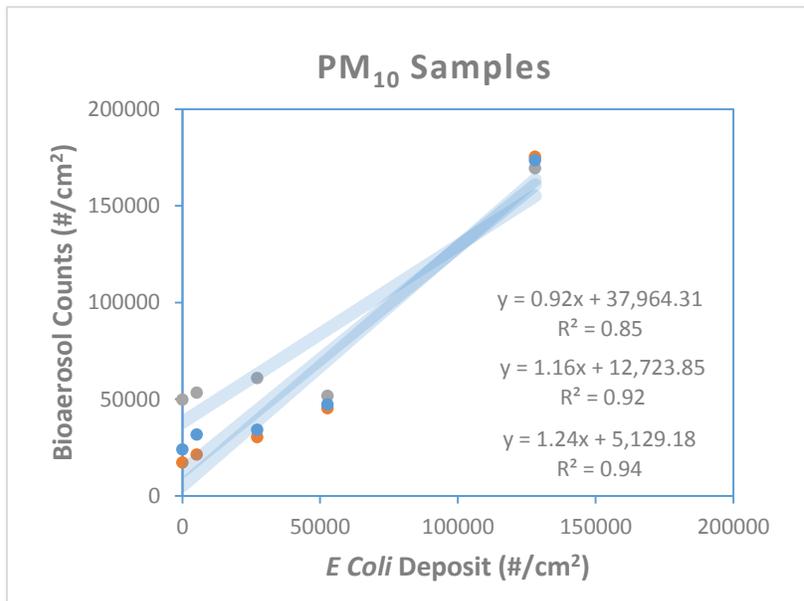


Figure 7. Fluorescence-observed bioaerosol counts on 3 PM₁₀ (upper panel) and 3 PM_{2.5} (lower panel).

Loaded samples in comparison with amounts of *E coli* deposit added to the samples.

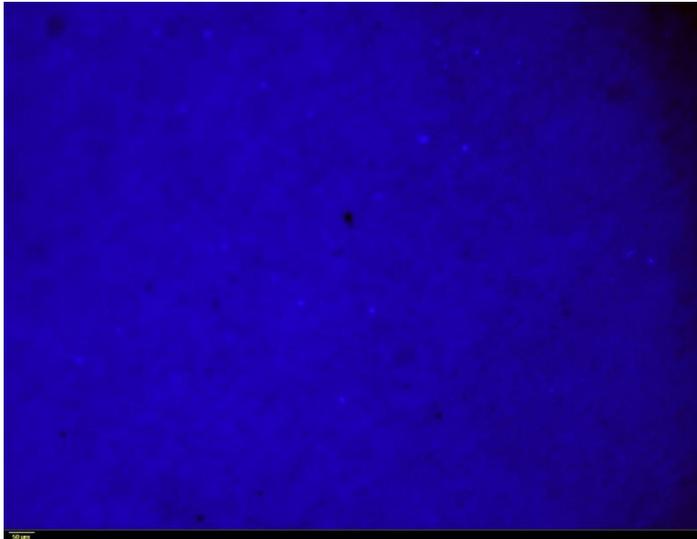
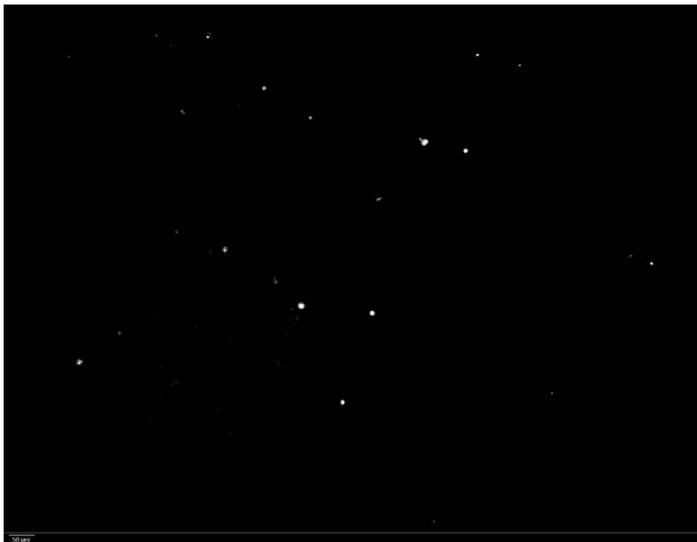


Figure 8. PM_{2.5} concentrations without *E. coli* reference standard.

The top panel is the original image. The middle panel is contrasted to black and white image. The bottom panel represents the binary image in which ImageJ uses the analysis tool and quantifies the bioaerosols.



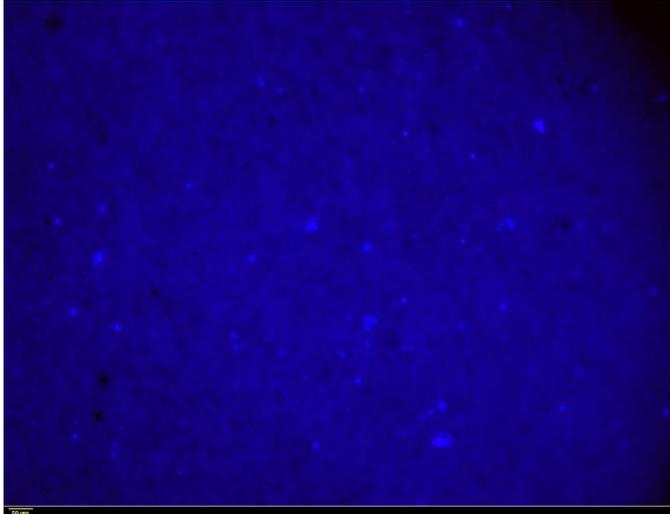


Figure 9. PM₁₀ concentrations without *E. coli* reference standard.

The top panel is the original image. The middle panel is contrasted to black and white image. The bottom panel represents the binary image in which ImageJ uses the analysis tool and quantifies the bioaerosols.

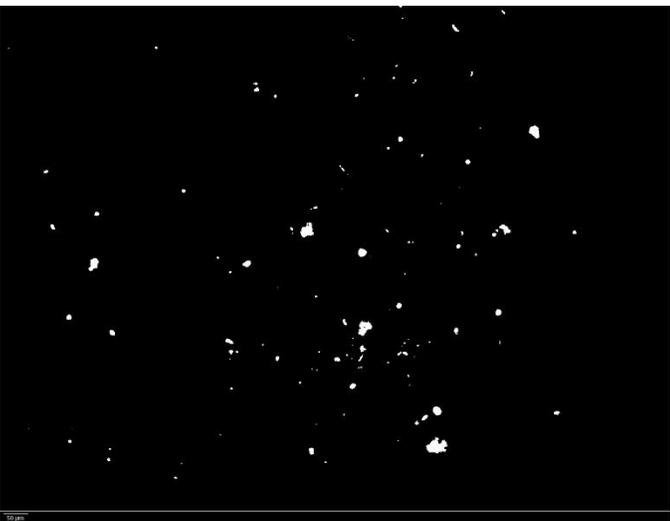
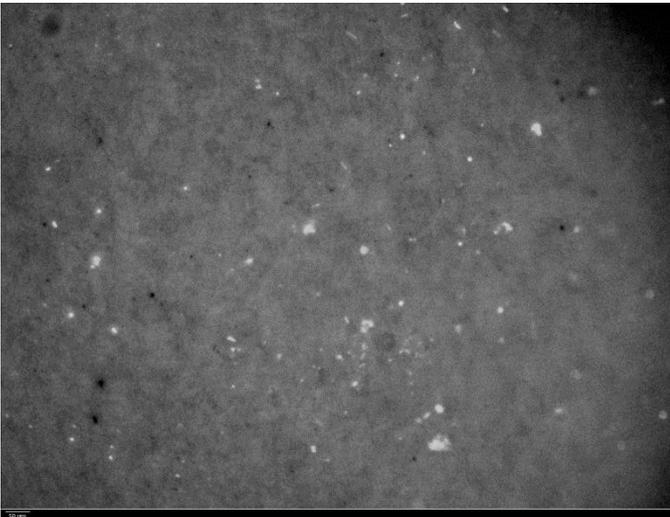


Figure 10 displays the dates of sampling and quantified bioaerosol associated with PM_{2.5} and PM₁₀ (without additional *E. coli*). The concentrations in #/cm² were also converted to ambient concentrations in bioaerosols/m³ using an aerosol deposition area of 13.85 cm² and 25hr sampling volume of 5.76 m³. PM₁₀ contained more elevated bioaerosol concentrations than PM_{2.5}. Two PM₁₀ samples were acquired on February 15, 2017 and showed similar quantifications of bioaerosol with 24,687 #/cm² (59,360 bioaerosols/m³) and 22,885 #/cm² (55,027 bioaerosols/m³), and this supports consistency of the measurement method used. PM_{2.5} sampled on February 20, 2017 showed a higher bioaerosol concentration though PM₁₀ data was not available for that day. A pair of PM₁₀ and PM_{2.5} samples were acquired on February 23, 2017 with a higher bioaerosol concentration found in PM₁₀ at 7,916 #/cm² (19,034 bioaerosols/m³) than in PM_{2.5} at 3,736 #/cm² (8,983 bioaerosols/m³).

The three pairs of PM₁₀ and PM_{2.5} samples acquired during a three-day period in March showed that PM₁₀ and PM_{2.5} had similar bioaerosol concentrations though the PM₁₀ was slightly higher than PM_{2.5} each day (Figure 10). March 15, 2017 had elevated bioaerosol counts of 49,661 #/cm² (119,410 bioaerosols/m³) in PM₁₀ and 46,083 #/cm² (110,807 bioaerosols/m³) in PM_{2.5}. These levels of bioaerosol concentrations have been reported in the literature (Toprak and Schnaiter, 2013). On average, only ~8% of bioaerosol are associated with PM_{coarse} with aerodynamic diameter between 2.5 and 10 μm. However, when considering all the PM₁₀ and PM_{2.5} samples available in Figure 10, bioaerosol concentrations were on average 14% higher in PM₁₀ than in PM_{2.5}.

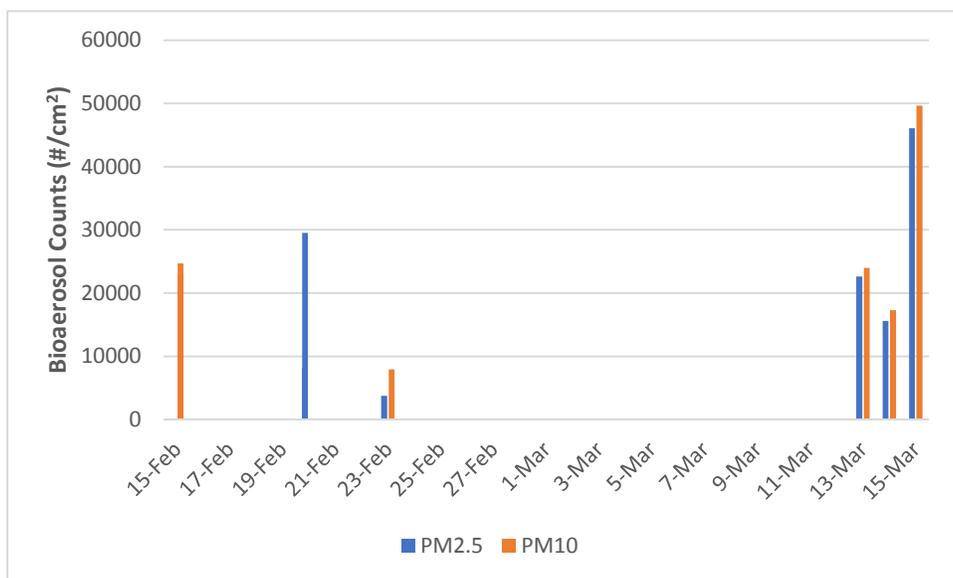


Figure 10. Bioaerosol concentrations in PM_{2.5} and PM₁₀ on each sampling date.

The observed bioaerosol concentrations are between 4×10^3 and 5×10^4 #/cm² (9.6×10^3 and 1.2×10^5 bioaerosols/m³) well above the MDL of 10^3 #/cm². The COV of bioaerosol counts are >50% for both PM₁₀ and PM_{2.5}, well above the measurement precision of 20-30% (see the “NucBlue” line in Table 2 and 3). Therefore, the method is capable of assessing bioaerosol variations in ambient PM_{2.5} and PM₁₀. This study found bioaerosol concentrations in ambient samples, as quantified by this method, were on average 14% higher for PM₁₀ than for PM_{2.5} acquired concurrently in a desert environment of Las Vegas, Nevada. Proving the alternative hypothesis stating H³_a: The fluorescence method quantifies variations of bioaerosol concentrations in Las Vegas, NV PM_{2.5} and PM₁₀ samples.

Compliance samples

The method was also applied to MetOne BAM 1020 filter tape and Teflon filters that are commonly used to collect compliance PM samples. Both polycarbonate and Teflon filters were

deployed in size-selective samplers to acquire PM_{2.5} and/or PM₁₀. The BAM projects a beta radiation onto the filter tape, and based on the attenuation of Beta rays over sampling time will quantify the hourly particulate matter concentration in $\mu\text{g}/\text{m}^3$ (Gobeli et. al., 2008). This method allows rapid analysis of bioaerosols and streamlines bioaerosol monitoring. The results indicated that both filter materials are not compatible to use the method without adjustment. The method did not produce any meaningful results for quantification of bioaerosol in PM.

Presented in Figure 11 are four panels of a Teflon filter field of view representing deposits of four different *E. coli* reference solutions (7×10^5 cells/ml, 7×10^4 cells/ml, 7×10^3 cells/ml, and 7×10^2 cells/ml). The figures show a bright and uneven background that may interfere with bioaerosol counting.

Presented in Figure 12 are four panels of a BAM filter tape field of view representing deposits of four different *E. coli* reference solutions (7×10^5 cells/ml, 7×10^4 cells/ml, 7×10^3 cells/ml, and 7×10^2 cells/ml). A BAM uses a dedicated inlet that selects PM₁₀ or PM_{2.5}, and collects PM on a glass fiber filter tape. The figures show that the background is bright and has many layers of cellulose structures that would interfere with accurate counts of bioaerosol particles. The results found the alternative hypothesis was true for compliance filter testing with states H⁴_a: The performance of the fluorescence method is lower for Teflon filter and BAM tape than for polycarbonate filter.

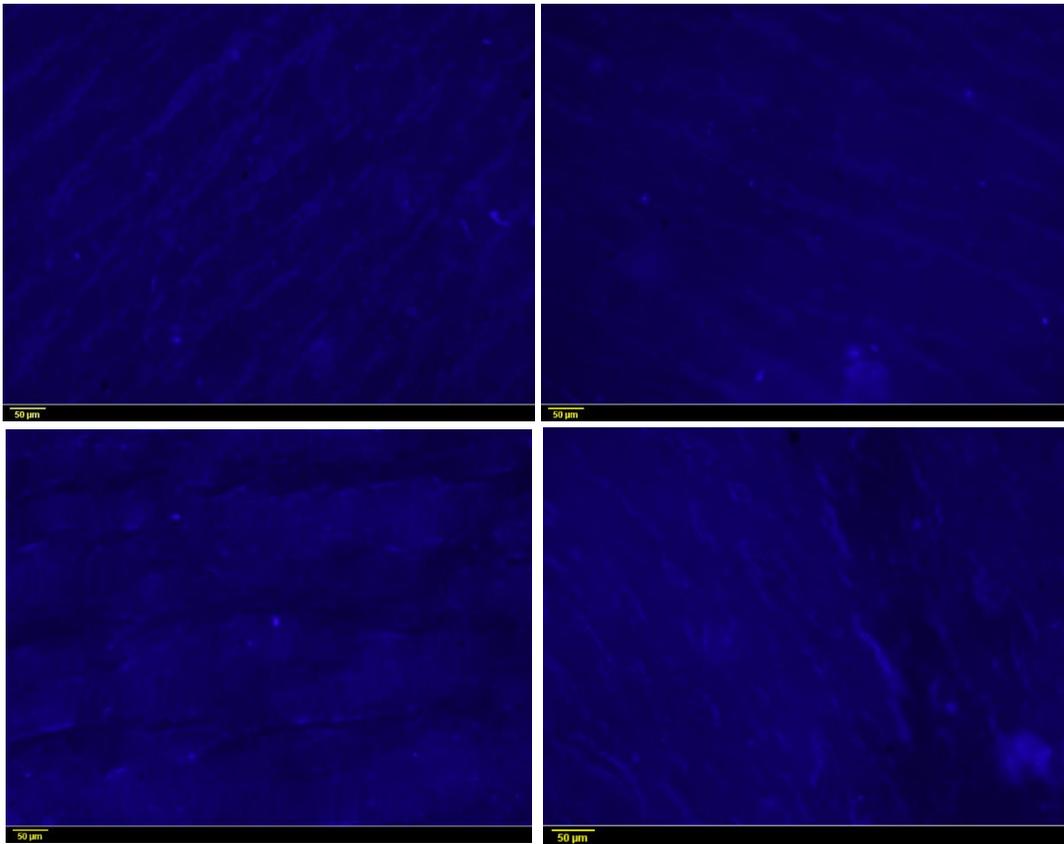


Figure 11. PM samples using Teflon 45mm filter with deposited *E. coli* reference.

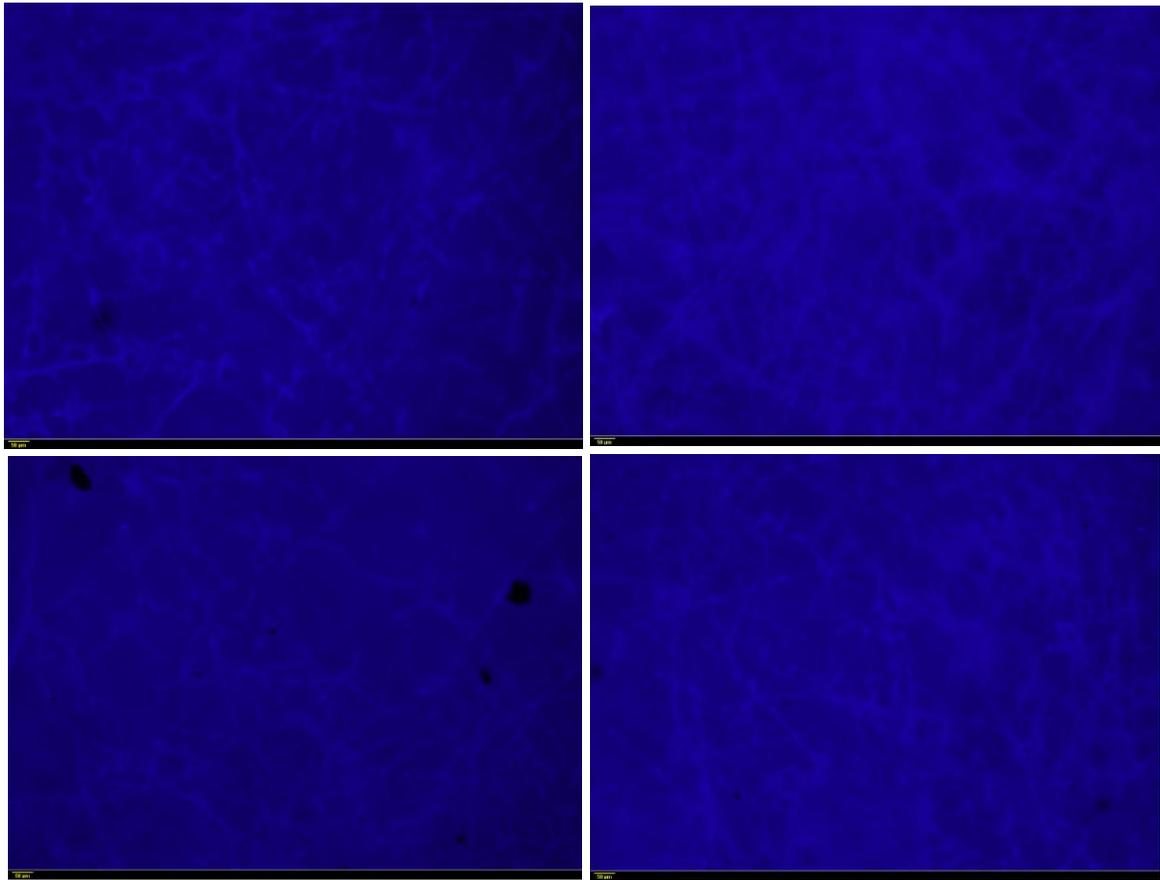


Figure 12. PM samples using BAM MetOne filter tape with deposited *E. coli* reference.

CHAPTER 4

DISCUSSION

A fluorescence microscopy method was established to quantify bioaerosol concentrations in PM in a quick, inexpensive, and consistent manner. *E. coli* was used as a reference for bioaerosols on blank polycarbonate filters, sampled polycarbonate filters, and common compliance filters to determine accuracy and versatility of the method. ImageJ was used to analyze each field of view observed for counting bioaerosol particles. The results indicated that this method is both accurate and precise within 25% uncertainty for the bioaerosol concentration range applicable to ambient samples. This method is best used with polycarbonate filters.

Counting differences and uncertainties could be attributed to a variety of reasons including non-uniform bioaerosol deposits, interference of dust or other non-biological particles that fluoresce, and the ImageJ counting errors. However, the observed bioaerosol counts correlated well with *E. coli* deposits on both blank and PM-loaded polycarbonate filters. Therefore, the method serves to predict bioaerosol concentrations. The uncertainty appears to decrease with the increase in *E. coli* loading. This may be because there is more non-uniformity in the PM deposits that is more noticeable with less *E. coli* standard added.

To reduce the uncertainty in quantifying concentrations of bioaerosol particles using this method, it would be optimal to increase the number of fields to be analyzed at the microscope. However, it is important to note that increasing the fields of view to be analyzed would also increase the time it would take to complete the measurement.

The method was validated by using the fluorescent dye reagent NucBlue which binds to DNA and fluoresces blue when introduced to UV light under a blue optical filter. The NucBlue stain was tested in comparison to DAPI reagent and it was found that the NucBlue fluorescent intensity was comparable in color of DAPI fluorescence. The NucBlue provided a more

convenient use for the method because of the vessel itself was a dropper bottle and there was no need to prepare the solution. The NucBlue reagent does not need to have a perfect measurement using a pipette and could be kept at room temperature without degradation of strength in fluorescence.

The standard concentrations of *E. coli* dilutions used to validate the quantification method were chosen to have high concentrations, medium concentrations, and low concentrations. This was chosen as optimal for analysis of fields of view. The lowest *E. coli* concentration chosen for testing on polycarbonate filters was 7×10^4 cells/ml or 5.28×10^3 #/cm² which was optimal for counting during analysis and closely matched the atmospheric bioaerosol concentrations on the sampled PM filters. The concentration of 7×10^4 cells/ml *E. coli* reference solution was used to validate the ImageJ software by hand counting the images and comparing the counts with the software counts which never varied more than 2%.

The small number of PM_{2.5} and PM₁₀ samples does not provide a full representation of the variability and uncertainty of the bioaerosol concentrations quantified using the applied method. The values observed establish an estimate of bioaerosol contribution to the PM mass during only a few days in February, 2017, and a few days in March, 2017. The results established did show PM₁₀ generally had higher concentrations of bioaerosol. It is necessary to analyze more PM_{2.5} and PM₁₀ filters to establish a more accurate quantification of bioaerosols in the Las Vegas area. With more ambient PM studies and quantifications of bioaerosols the more likely it will be able to convert the bioaerosol counts to ambient concentrations.

A study on seasonal concentrations of bioaerosol in PM found that ambient concentrations during summer months were much higher than concentrations found during winter months (Menetrez, M. Y., et. al., 2007). Following the findings of the study allowed for

speculation that the bioaerosol concentrations in PM collected in Las Vegas would be lower than if collected during the summer. A second study conducted examined atmospheric bioaerosol concentrations which contribute to PM throughout the seasons in the state of Ohio (Menetrez, M. Y., et. al., 2007). This study was performed during a two-year period and found that bioaerosol concentrations were higher during the summer and fall months than during the winter months (Menetrez, M. Y., et. al., 2007).

This small set of convenience samples established the method that could be used to quantify bioaerosol concentration in PM sampled on polycarbonate filters at a flow rate 4 lpm for 24hrs. The particulate matter and debris that did not contribute to bioaerosol concentrations did not obscure the ability to count the individual bioaerosol particles that were stained with NucBlue. These results may not be representative of bioaerosol concentrations throughout Las Vegas air quality. Further research needs to be conducted to determine the variability and uncertainty to the method used for detection of bioaerosols in PM. Findings in this study were consistent with a similar study that investigated fine and course PM and the presence of biological material in North Carolina (Menetrez, M. Y., et. al., 2007). The study found higher levels of biological material in the coarse PM speculating that more bioaerosol particles were able to settle onto the filter for coarse PM than for the fine PM.

The method used for this study did have several limitations with quantifying bioaerosol concentrations in PM. The *E. coli* standard may not be representative to all bioaerosols that exist in PM. This method could be tested with several different bioaerosols as standards that would represent a variety of sizes and speciation that would likely be found in ambient PM. The polycarbonate filters created a bright background under a blue filter that caused some difficulty to view all bioaerosol particles on the filter. This method would be more accurate with

quantification if the background would be darker and the particles brighter, and using a different type of polycarbonate filter may reduce the background.

The hypotheses of this study's findings found that the reference bioaerosols deposited on blank polycarbonate filters correlated well with measurements using the fluorescence method tested. The results also found there is no significant difference between the concentrations of reference *E. coli* deposited on PM-loaded polycarbonate filters compared to counts found using the fluorescence method. This current study also showed this method is able to find variation in ambient PM samples and is able to quantify bioaerosols accurately. The alternative hypothesis was true for testing compliance samples using this method as the Teflon filter and BAM tape tested did not hold up to testing of this method.

CHAPTER 5

CONCLUSIONS

This study found that quantification of bioaerosols using fluorescence microscopy by directly staining a filter from regular air quality samplers would be useful as a method for assessing bioaerosol in PM₁₀ or PM_{2.5}. The findings indicated that the best filter used for this method is polycarbonate filters with 0.2µm pores. This study also found that it would be best to analyze the filter for bioaerosol concentrations within 48hrs of sampling to get the best results for quantification. The NucBlue stain did not derogate from the intensity of color from differing bioaerosol and was useful for completing the analysis quickly and without issues.

Ambient samples of PM₁₀ and PM_{2.5} were collected and analyzed for quantification of bioaerosol concentrations, and reported as bioaerosol #/cm² which were also converted into bioaerosols/m³. The presence of bioaerosol were found in both PM size ranges with the PM₁₀ containing a higher concentration of bioaerosol than PM_{2.5}.

The results of verifying the method developed in this study support a relatively fast analysis that can be applied to understand the role bioaerosol performs in PM. Although this method was not compatible with commonly used compliance filters it did show there is room for improvement of this method with compliance samples. The method did work well on sampled polycarbonate filters and can be used as a means to directly detect and qualify atmospheric bioaerosols. This method needs minimum prep work, was fast at analysis (6 samples/3hrs), and required few resources. If adopted into U.S. EPA's air quality networks, it will support long-term health assessments and refinement of NAAQS. This method can lead to a clearer understanding of the health impacts airborne biologicals have on the respiratory system and exposure to allergens.

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