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Potential for human exposure to Legionella near Newtown Creek in Brooklyn, New York

Azlan Maqbool
Bard College

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Senior Project submitted to The Division of Social Studies

by Azlan Maqbool

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To my parents and siblings, thank you for all the love and support over the years. To all my friends, I cannot thank you all enough for all the emotional and physical support.

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Abstract

The air environment is known to contain a wide array of microbes. Urban coastal environment studies have shown plenty of interchange between land and sea bacteria. While there have been several studies analyzing the transfer between aquatic and terrestrial microbial communities, few have looked at the impact this transfer might have on individuals. Moreover, facilities like wastewater treatment plants situated in dense urban environments may play an important role in the proliferation of potentially pathogenic bacteria to nearby human populations. Wastewater treatment plants are known to be associated with a wide array of microbial communities, some of which when released into the environment can have detrimental effects to local ecology and human health. Of the many disease-carrying bacteria found in wastewater, *Legionella* is of specific importance given its context in New York City. In 2015, New York City experienced its worst outbreak of Legionnaires disease in history. While reports and studies confirm building top cooling towers to be breeding grounds for the bacteria, there are virtually no studies that explore how *Legionella* bacteria arrive there in the first place. This study seeks the plausibility of open-air discharge creeks such as Newtown Creek (Brooklyn, NY) acting as reservoirs for the bacteria. The ultimate question I will be attempting to answer is: How and what does an aerated urban waterway like Newtown Creek create harmful bacterial exposure for individual in and around it? In this project, I tested methods for use in pursuing this question. Specifically, I used a Sioutas five stage personal cascade impactor and a Coriolis C to characterize bacterial aerosols in different environments, including near-ground, building-top, and waterfront sites. I found that total culturable microbial aerosol counts were higher in the coarse fraction, >1.0um, (stages A and B) across all media types when compared to fine particle counts, <1.0um, (stages C and D). Finally, using metagenomic methods, I verified the presence of *Legionella* in sewage-contaminated waters.

Introduction

The air in urban settings has been studied and found to be the source of a wide array of pollutants and hazardous bacteria to humans (Lighthart, 1997; J. Douwes et al. 2002). The concentrations as well as the composition of the microbial community in the air can change based on a number of atmospheric factors such as temperature, wind velocity, smog, and specific humidity (W. Donderski et al 2005; Vantarakis et al., 2016) which influences the spread of aerosols as well as the ability of microbes to survive in the air environment. Bioaerosols can be comprised of several different disease-causing viruses and bacteria. *Legionella* is known for its association with bioaerosols due to its proliferation in engineered water systems (Addiss et al. 1986; Llewellyn et al. 2017; K.A. Hamilton et al. 2018). *Legionella* is widespread in water and soil (Prussian et al 2017). Previous studies have found that the bacteria pose a great risk to human health when water containing the bacteria becomes aerosolized (Chamberlain A Lehnert J Berkelman R 2017).

This ultimate goal of this study will be to further research the effects of wastewater treatment facilities proximity in dense urban environments like New York City. Air and water microbial communities will be analyzed for *Legionella* bacteria in the nearby neighborhoods of Newtown Creek located in Brooklyn, New York. Newtown Creek is a designated Superfund site with a history of over 140 years of industrial waste dumping, ongoing oil seepage from the Greenpoint Oil Spill, one of the largest underground oil spills in the country at approximately 1.7 $\times 10^6$ gallons oil (Dueker et al. 2012). The creek is currently being used for combined sewage overflow and is also in the process of being remediated via open-air aeration. A 2014 study of the site found a significant increase in near-surface coarse aerosol concentrations and microbial

fallout when aerators were on compared to when the aerators were off (Dueker and O'Mullan 2014). The ultimate aim of this study will build on the growing body of evidence of this phenomenon by exploring the impact of the open-air water reservoir and its microbial transport capacity to nearby buildings and people. This entails exploring the possibility of viable strains of *Legionella* being transported and settling at a site such as a cooling tower atop nearby buildings. To be able to ultimately study this phenomenon and detect personal exposures to *Legionella* in the urban environment, the current study aims to develop proper protocols for relatively new air sampling devices and the metagenomic exploration of the presence of the bacteria in the local environment.

Context of *Legionella* in New York

The relevance of *Legionella* is especially important in the state of New York. A 2017 case study found that the state of New York reports approximately 50% of all the cases of legionellosis in the entire country despite making up only 6% of the population (Prussin et al. 2017). The number of reported cases in NY rose from 47 in year 2000 to 438 in year 2015. In 2015, New York experienced its worst outbreak of Legionnaires disease in its history after the Department of Health and Mental Hygiene (DOHMH) reported a cluster of cases in the South Bronx area. The response to the July 2015 cluster began as a standard epidemiologic investigation as DOHMH staff interviewed case patients and close contacts in an attempt to determine the source of disease (ibid). Those patients who had spent any time in the 7 affected zip codes with symptom onset after July 2, 2015 were classified as part of the outbreak. By the time sampling and control methods were enacted, DOHMH officials had reports of more than 30 cases dispersed throughout the South Bronx. DOHMH began sampling cooling towers for

Legionella. Investigators took water and biofilm samples from each cooling tower. Towers that tested positive for *Legionella* were decontaminated. In total, 55 cooling towers in the South Bronx were screened. Samples were sent to the state laboratory where PCR was used as a rapid alternative to standard culture methods. On July 29, one cooling tower atop the Opera House Hotel was identified as the source of the outbreak.

Not soon thereafter, the DOHMH gained support for a citywide registry of cooling towers Chamberlin et al 2017 highlight, "Now that there was a large community outbreak with evidence pointing toward a cooling tower as the culprit, the concept of having immediate and up-to-date knowledge on all cooling towers in the city was particularly appealing and creation of the registry became a priority." Within weeks the NYC Council introduced legislation that required all cooling towers to be registered with the Department of Buildings (DOB) and inspected at least every 90 days during times of operation. The cooling tower legislation, officially known as Local Law 77, was enacted 8 days later on August 18 and subsequently NYC became the first large jurisdiction in the United States to take a regulatory approach to the management of cooling towers to prevent *Legionella* contamination. The outbreak was officially declared to have ended on August 20. A total of 138 cases and 16 deaths were attributed to this outbreak, making it the largest LD outbreak in NYC history. A notable fact about the 2015 outbreak was that there was found to be a strong association between LD and poverty in NYC; from 2002 to 2011, the rate of LD was 2.5 times higher in high-poverty areas than in low-poverty areas (Chamberlain et al. 2017).

Wastewater treatment plants

The quantity of municipal wastewater produced worldwide has drastically increased as a result of growing population numbers and an increased reliance on diminishing water resources. This coupled with the discharge of inefficiently treated wastewater into surrounding surface water sources serves as a direct threat to the macro- and microflora and fauna present (Naidoo and Olaniran 2013). Municipal WWTPs typically operate in four main stages: preliminary, primary, secondary, and tertiary stages. During the secondary stage, in particular, the activated sludge process used to remove dissolved compounds from wastewater ultimately involves aeration of primary treated wastewater in the activated sludge tank which promotes the growth of microorganisms (Caicedo et al. 2018). The combination of ideal temperatures and the availability of oxygen and organic nitrogen provide an ideal environment for the proliferation of many different types of bacteria including *Legionella*. In a 2018 study, Caicedo et al. did a critical review on current knowledge about *Legionella* in municipal and industrial WWTPs and found that these facilities may play an important role in local or community transmission of *Legionella*. Given the Centers for Disease Control and Prevention (CDC) definition of Legionnaires Disease outbreaks occurring when two or more people exposed to *Legionella* are sick at the same time and at the same place (CDC 2018), it can be assumed that WWTPs are more likely to be associated with *Legionella* outbreaks rather than sporadic cases. This provides an interesting perspective when considering the case of Newtown Creek, where the DEC is currently performing aeration of waters frequently contaminated with raw sewage in a public waterway, outside of the confines of a waste treatment facility. The immediate effects of exposure to aerosols from WWTPs can result in respiratory and digestive symptoms in humans and have been reported in workers exposed to particulate matter and bioaerosols working at these sites (Vantarakis et al., 2016). Similar health problems can occur in people living near such plants.

Bioaerosols, Transport and Public Health

Bioaerosols can contain different types of microorganisms such as viruses, pathogenic bacteria, and fungi, capable of causing many diseases and allergies in humans. Previous studies have shown evidence of sewage transfer from water to air at coastal environments with an enrichment of 12 times more sewage related coastal aerosols compared with off coast open sea measurements (Marks et al. 2001). In addition, past studies of microbial aerosols have been conducted in landlocked or remote geographical regions (Dueker et al. 2012). The importance of studying microbial aerosols in the context of urban settings is of great importance considering the public health in those settings.

Bacteria are often associated with larger particles, around 2.5 microns and above (Lighthart, 2000). The larger the particle size, the higher the potential for bacterial survival rates in the air (Dueker et al 2016). Additionally, the aerodynamic size of particles determines their atmospheric residence time and transport distance (Seinfeld & Pandis, 1998). Aerosols sized in the order of 1 mm have residence times from seconds to minutes and generally are deposited close to their original source, while those having diameters of 1μm can have residence times from days to months and reach worldwide distribution (Dueker et al 2016). Meteorological conditions are also an important factor that can influence bacterial aerosols. Temperature, wind velocity, smog, relative humidity can all influence the aerosol spread as well as the ability of certain bacterial organisms to survive in the air (Vantarakis et al., 2016). At very low humidity and high temperatures, for example, microbes face dehydration, whereas conditions of high humidity can give bacterial cells protection against the solar radiation (Vantarakis et al., 2016). In humans, aerosol particle sizes are directly linked to the probability of inhalation or ingestion and the likely deposition zones in the human body (Montero et al. 2016). The particle size range

of 1-5µm is optimal for deposition in the lung region, whereas particles greater than 5µm in size may still reach the lungs but at lower rates (Hines et al. 2014).

In 2012, Dueker et al. found that coarse aerosol concentrations at Newtown Creek pooled by day increased significantly ($p < 0.05$) when the aerator was on as opposed to when the aerator was off. The data presented in the study confirmed that the air at NTC supported high bacterial loads. Comparing NTC with two other sites, the wind speeds and relative humidity were comparably low and onshore during all sampling reported in this study. Thus, the differences in meteorological conditions between NTC and the comparison sites would not explain the difference in fallout rates, but the presence of high sewage content in surface waters of NTC would. This study also confirmed the overlap in bacterial community compositions between the terrestrial and aquatic environments. The NTC water and air isolate libraries shared dominant genera and OTU's. In contrast, the site produced a unique bacterial aerosol community demonstrated by the significant phylum-level differences between the NTC aerosols library and other published urban and nonurban aerosol libraries. The implications of this study could point to the historical pollution of the site as well as the nearby wastewater treatment plant.

Dueker and O'Mullan 2014 looks at how aeration remediation of NTC increases near-surface coarse and culturable microbial aerosols. Intuitively, the proximity of aquatic sources of these aerosolized bacteria would result in a higher probability of exposure to them. While vertical gradients of microbial concentrations have been substantively studied over terrestrial systems and to some degree wastewater treatment plants, there remains a gap in these studies in relationship to public waterways. To bridge this gap, Dueker and O'Mullan measured near-surface microbial aerosol deposition and coarse aerosol particles on small boaters along Newtown Creek. The findings were that culturable bacterial fallout was significantly greater

when measured at 0.6m above the water as compared with simultaneous bacterial fallout measurements at 2.5 m above the water surface. These results provide additional evidence for water being the source of bacterial transfer to the air. It also suggests that the highest probability of exposure to aerosolized bacteria is likely to occur very close to water level. This current study seeks to explore the probability of exposure farther away from the source and at the top of nearby buildings. Cooling towers, which are responsible for ventilating air throughout the insides of dozens of apartments, offices, recreational facilities, and other generally human inhabited environments, are located on top of buildings. The consequences of such design are what has been frequently studied and referred to as "sick building syndrome." (refs) Past studies have made a connection between Legionnaires disease and the sick building syndrome. For example, in 1985 in the United Kingdom, twenty-two outbreaks of legionnaires' disease were reported, five of which were associated with cooling towers of air-conditioning systems and the phenomenon of sick building syndrome (O'Mahony et al 1989). Of the many bacterial and viral agents responsible for getting people sick in buildings, *Legionella* in particular is a bacteria is of importance given its recent prominence in New York City.

Legionella

Legionella is a genus of gram-negative bacteria and it is known to cause a serious disease known as Legionnaires disease or legionellosis which is transmitted via inhalation of the pathogen in aerosol form. There are two forms of legionellosis: Legionnaires' disease, which causes pneumonia-like symptoms, and Pontiac fever, which causes influenza-like symptoms (Prussian et al. 2017). Of all reported cases in the US, approximately 5% to 15% are fatal. *Legionella* is the leading cause of deaths from waterborne outbreaks in the US and the rate of

reported cases of legionellosis in the US increased nearly 4-fold from 2000 to 2014 (Llewellyn et al 2017). Unfortunately, the number of reported cases of Legionnaires disease is most likely a fraction of actual cases due to underdiagnosis. This happens in large part to the fact that the disease usually can be treated with the same medicine recommended for community-acquired pneumonia, thus reducing the need for clinicians to order diagnostic tests for LD (Chamberlain et al 2017).

Legionella species are found widespread in water and soil. Humans are incidental hosts that acquire the organisms after exposure very commonly through engineered water systems in which conditions are optimal for growth and aerosol dispersal (Muder and Yu 2002; Buse et al. 2012). The bacteria pose great risk to human health when water containing the bacteria becomes aerosolized (Chamberlain et al 2017). *Legionella* can be aerosolized from various water sources such as showers, faucets, fountains, swimming pools, and cooling towers (Chamberlain et al 2017). Incidence of the disease is higher in the summertime, possibly because of increased use of cooling towers for air conditioning systems (Prussin et al 2011). According to the CDC, Legionnaires' disease and Pontiac fever are generally not contagious. Most healthy people exposed to *Legionella* do not get sick. People at increased risk of getting sick are: 50 years or older, current or former smokers, people with a chronic lung disease, people with weak immune systems, people with cancer, and with people underlying illnesses such as diabetes, kidney failure, or liver failure (CDC). There are currently more than 58 *Legionella* species that have been described in published articles (Prussin et al. 2017). Of these, approximately 25 are linked to disease, namely *Legionella* pneumophila species serogroup 1, 3, 4, and 6 (Muder et al 2002). *Legionella* pneumophila serogroup 1 is the most virulent strain causing the majority of infections

(Walser et al. 2013). The remaining non-pneumophila species (found in water and soil) are considered non-pathogenic until shown to cause disease (Muder and Yu 2002).

The common presence of *Legionella* in engineered systems can also be attributed to poor control methods. Buse et al. 2012 found that the persistence of *Legionella* bacteria in engineered water systems can be further exacerbated by bacterial regrowth in zones such as storage reservoirs or in hot water tanks that are not emptied or not regularly circulated. Furthermore, Hines et al. 2014 points out that epidemiological studies have struggled in distinguishing the importance of individual transmission sources when there is the high variability and plausibility for multiple and co-occurring exposure points (e.g., faucet, shower) from common originating sources such as contaminated water (Buse et al. 2012). This study seeks to further question the topic of transmission sources, particularly in relation to wastewater treatment facilities. The growth of *Legionella* species including *L. pneumophila* has been reported at temperatures ranging from 25 to 42 °C with an optimum growth at 35 °C (Falkinham et al., 2015), which happens to also be within the range at which aerated systems operate providing ideal conditions for *Legionella* proliferation (Caicedo et al. 2018).

Currently, detection methods of *Legionella* bacteria range from culture-based sampling to genetic based methods. Methods like qPCR detect and amplify a specific gene target known to be exclusive to a specific genus/species/serogroup. The qPCR technique, with the use of a standard curve, is far more effective and efficient at quantifying the presence of a specific microorganism than the traditional culture approach.

Donahue et al 2014 highlight that there are several qPCR assays for *Legionella* which have been developed: a genus-specific assay (targeting the 16S rRNA gene from *Legionella* spp.), a species-specific assay (targeting the mip gene, coding the macrophage infectivity-

potentiator. Of L. pneumophila), and a species/serogroup-specific assay (targeting the LPS-gene cluster from L. pneumophila Sg1). QPCR allows for rapid detection with high specificity and sensitivity. However, because DNA can persist in the environment for hours after cell death, qPCR cannot distinguish between viable and nonviable cells. This limitation can result in an overestimation of the potential exposure and associated risk due to false-positive results.

Relevance to Current Study

Although there has been extensive research related to *Legionella* transmission, many knowledge gaps remain. Few studies have grappled with the possibility of *Legionella* strains being aerosolized and transported throughout urban environments. Moreover, while there have been clear connections made between the source of cooling towers as breeding grounds for *Legionella* and other bacteria, there has not been extensive scientific inquiries into where and how these bacteria end up in cooling towers atop buildings, far away from the typical soil and water origins. The ultimate goal of this study is to begin to answer several questions pertaining to the local connection between a polluted urban waterway and the air and characteristics of certain bioaerosols present near a New York City wastewater treatment plant and along the waterfront.

To understand bioaerosols and ultimately detect personal exposures to *Legionella* in the urban environment, this study aims to develop proper protocols for relatively new air sampling devices. Protocols for a personal air sampler as well as a Coriolis air sampling device were developed over the course of a six-month period at Bard College. The goals of this study were to: (1) determine the size distribution of microbial aerosols; (2) examine the efficiency of devices by evaluation of the amount of bacterial growth on agar plates; (3) compare bacterial growth on plates by various locations on campus; and (4) to tweak device protocols to efficiently grow and

characterize bioaerosols. I hypothesized that culturable microbial aerosols would primarily be associated with particles in the first two size bins of the personal sampler (A: larger than 2.5um and B: 1.0um-2.5um); that higher growth rates would occur with the Coriolis C device due to its higher flow rate and design; and that sites with proximity to aerosol generating sources would see an increase in culturable bioaerosols.

Methods & Materials

Personal Cascade Impactor:

The Sioutas personal cascade impactor sampler or PCIS (SKC Inc., Eighty-Four, PA, USA) is used to measure personal exposures to bioaerosols (**Figure 1**). The device works by separating and collecting ultrafine, fine, and coarse airborne particles in five size bins (A: > 2.5, B: 1.0u-2.5u, C: 0.5u-1.0u, D: 0.25u-0.5u, After: < 0.25u) on 25-mm PTFE filters. These four impaction stages followed by an after-filter are shown in Fig. 1(a–d). The sampling flow rate is measured in liters per minute (LPM). The design of the impactor was based on impaction theory (Marple & Willeke, 1976). In each stage, the impacted particles are collected on a small surface area of about 1 cm^2 or less. Particles smaller than 0.25um are collected on an after filter.

The PCIS operates in conjunction with the Leland Legacy Personal Sample pump (SKC Inc., Eighty-Four, PA, USA) (**Figure 2**). The personal sample pump is a dual diaphragm pump, in which rotational energy from a small DC motor is converted to linear motion by a shaft mounted eccentric bearing and connecting rod assembly, to drive the two pumping diaphragms. Both the inlet and exhaust paths of the pump mechanism utilize dampener chambers to reduce pulsation in the airflow caused by the cyclic nature of the pump operation (Misra et al 2002).

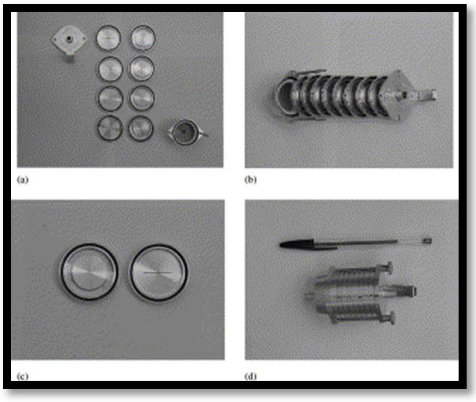


Figure 1: Personal Impactor

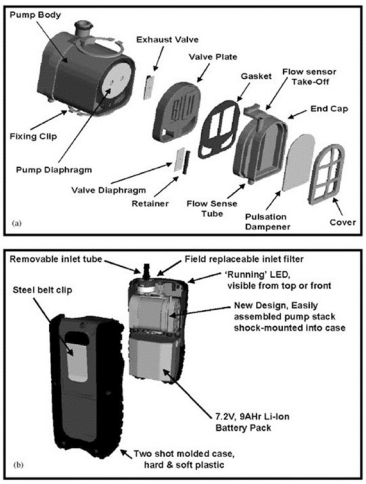


Figure 2: Personal Pump assembly

Coriolis C:

The Coriolis C (Bertin Technologies, Tarnos, France) is prototype and miniature version of the Coriolis Micro which is device for collecting physical and biological air particles based on dry cyclone technology. The Coriolis C, unlike the Coriolis Micro which has a much higher flow rate of 100 to 300 L/min, operates with a flow rate of 50 L/min.

Figure 3. Coriolis C

Sampling Sites:

A control study was set up at Bard College at 4 different sites: the rooftop of the Stevenson Library, the forested area behind the RKC, the open quad in front of the RKC, and along the banks of the outflow source of the Bard Wastewater treatment facility. Each site was sampled multiple times with the personal impactor device as well as the Coriolis C.

Site 1: Rooftop of Stevenson Library

The rooftop of the library was the first site sampled in this study. This site was chosen due to the similar conditions of the potential sampling site on building rooftops near Newtown Creek. Additionally, the presence of a cooling tower, a known aerosol generator and harbor of microbes, was ideal in relation to the scope of the study.

Figure 4. Aerial view of library rooftop (Map data: Google Maps, 42°01'20.8"N 73°54'23.2"W)

Sampling of the rooftop was conducted during October and November of 2018, resulting in 4 sampling events. Temperature, wind speed, and relative humidity were all recorded before sampling using data obtained through the local Red Hook, NY Weather Underground station (http://www.wunderground.com). During a sampling event, the PCIS was attached to my collar while the Coriolis C was placed in the middle of the rooftop. All four sides of the rooftop were covered by walking up and down each side for five minutes at a time.

Site 2: RKC Forest

The forested area parallel to the RKC was used as the second site to sample air. This site was chosen to examine if the forest environment would have a higher concentration of aerosols and subsequently produce more culturable bacteria once plated. Sampling of this site occurred in February 2019 on a single day for a total of two sampling events. The PCIS was attached to a branch and left running for 50 minutes. The device was then brought back to the lab disassembled for lab analysis and the brought back outside again for a 60-minute sampling period.

Site 3: RKC Quad

This site was chosen to simulate a ground level open-air environment similar to waterfront pathways in New York City. Sampling of this site occurred in March and April of 2019 for a total of four sampling events. Similar to the placement of the devices at the forest site, the PCIS was attached to a branch and the Coriolis C was placed on the surface nearby.

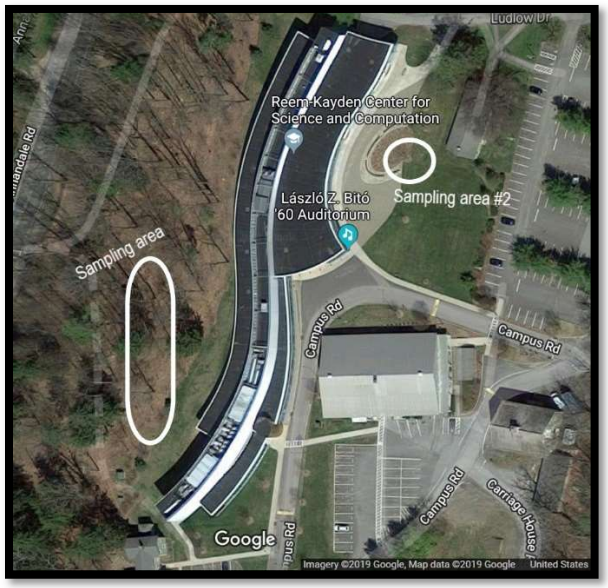


Figure 5. RKC Sampling Sites #1 and #2 (Map data: Google Maps, 42°01'09.5"N 73°54'29.5")

Site 4: Bard Waste Water Treatment Plant

The water plant was chosen to serve as a model for the potential conditions that could be encountered at Newtown Creek. Specifically, the presence of the dam served as a source for aerosolization to occur. As per Dueker et al. 2012, aeration of Newtown Creek was shown to increase near surface coarse and culturable bacteria. Therefore, this site was chosen to determine if coarse particles would similarly be more likely to be culturable using the PCIS.

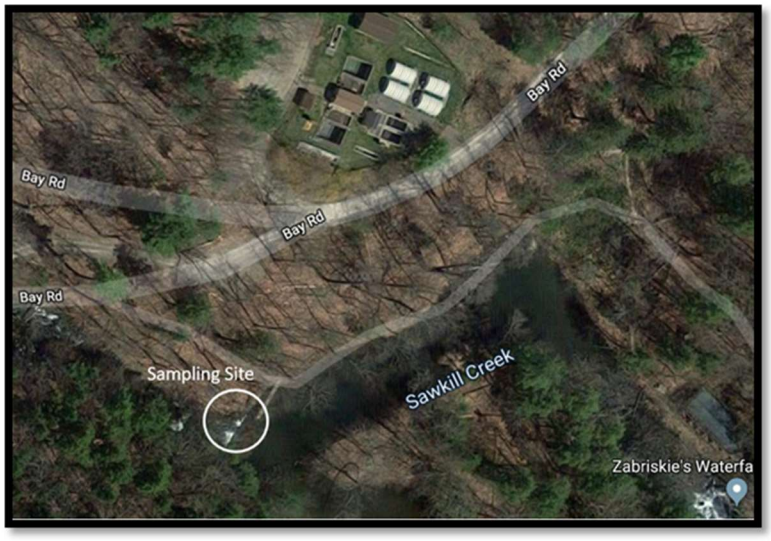


Figure 6. Bard Water Plant (Map data: Google Maps, 42°01'09.5"N 73°54'29.5")

PCIS Laboratory Analysis:

Before and after each sampling event, the PCIS was sterilized at each stage using 70% ethanol. After a sampling event the PCIS was transported back to the lab within an hour an analyzed. The first step was to wipe down the bench and to sanitize hands before putting on blue latex gloves. The PCIS was then disassembled stage by stage. Using a sterile tweezer, the O-ring retainers were removed, and the PTFE filters were carefully placed inside separate 10mL centrifuge tubes. For sampling events in the Fall (October, November), 4mL of Endotoxin free water was added to each tube with the filters inside. In the Spring (February, March, April), this amount was adjusted to 3mL of Endotoxin free water to increase the concentration factor of sampled air. Next each tube was labelled properly and then vortexed on a Vortex Mixer (Fisher-Scientific, Hampton, NH) at the 5-speed setting for a total of 60 seconds. The purpose of this step was to wash bacteria off the PTFE filters. Next, a micropipette was used to obtain 150ul of sample from each tube and subsequently pipetted on separate agar plates. Sterile beads were then used to spread the sample around the plates. It is worth noting here that in the fall sampling

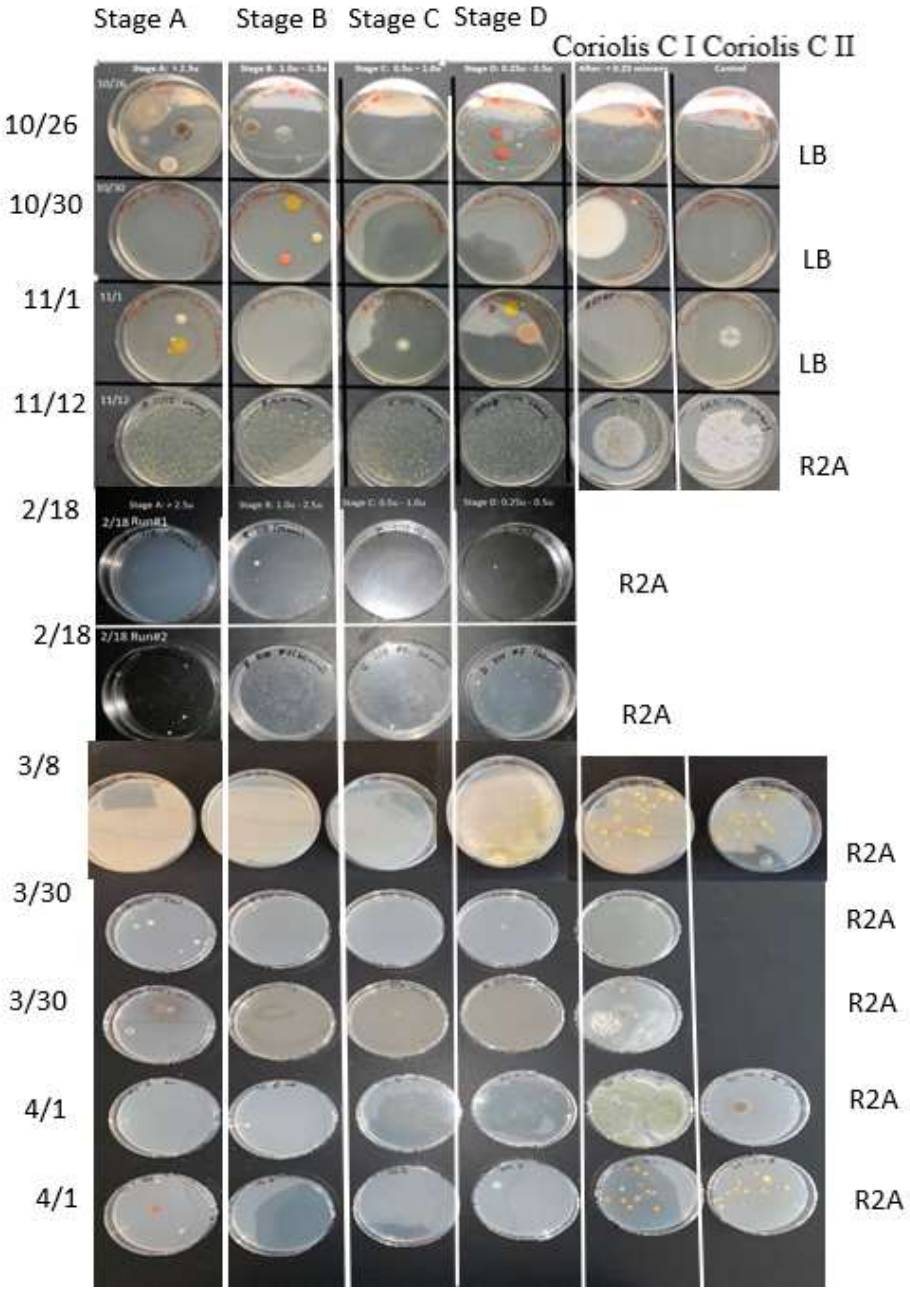
events Luria–Bertani (LB) agar plates were used and, in the spring, R2A agar plates were used in hopes of growing more bacteria.[1] Next, around 5-7 sterile glass beads were poured on to each plate and then stirred around seven times in each direction. Plates were left to sit for 2 minutes before throwing beads out. Each plate was properly labelled, stacked and inverted in the back of the bottom drawer next to the lab bench. Plates were left at room temperature (22° C) to incubate for 14 days. After the incubation period, bacterial colonies on each plate were enumerated based on colony morphology.

Coriolis Laboratory Analysis:

Before running the Coriolis C, the sampling cones were sterilized with 70% ethanol. After each sampling event, the device was brought back to the lab for further analysis. The first step was to detach the sampling cones from the Coriolis machine and then to pipette in 5mL of Tritan H20. Next, a sterile swab was used to swab down each side around 12 strokes and then spiral from bottom to top once. The contents of the cone were then decanted into a 10mL centrifuge tube and the tip of the swab was broken off and inserted into the tube as well. The next step was to vortex the tube for 60 seconds at the 6 speed setting. After using the Vortex, 150ul of sample was pipetted onto an agar plate (refer to footnote). Similar to the assay for the PCIS, the plates were left to sit for 2 minutes before throwing beads out. Then plates were labelled, stacked and inverted in the back of the bottom drawer next to the lab bench. After an incubation period of 14 days, bacterial colonies on each plate were enumerated. See **Figure 6**.

[1] The switch was because R2A media is commonly used in aerosol and surface water studies, including other studies in the New York region (Montero et al., 2016; Young, Juhl & O'Mullan, 2013; Dueker et al., 2012b). R2A is better for lower temperature (22°C) and longer incubation periods (14 days).

Figure 6. Array of all plates



Molecular Analysis

Metagenomic data from the Saw Kill was gathered in 2015 as a part of the Bard Summer Research Intensive program. During the project, water and sediment from multiple sites above, below and at the outflow point of a sewage discharge pipe in the Saw Kill tributary were collected and analyzed for bacterial sewage indicators. For the purpose of this study, metagenomic data obtained from the samples were used to examine the presence of *Legionella* in relationship to known sewage input.

Samples were collected by first flushing out a 2L bottle with sample water three times at each site. After flushing, the bottle was positioned sideways in the water with the mouth facing the current, allowing the water to flow directly into the bottle. After sample was collected, bottles were sealed and immediately stored on ice in an insulated cooler pack. Samples were then taken to the lab where they were put through the peristaltic pump using a Sterivex™ filter. Following this, DNA extraction occurred using the MOBIO Power Water DNA Isolation Kit.

Statistical Analysis:

An analysis of variance (ANOVA) test was performed to asses significant differences between total CFUs on Stage A(>2.5 μm), Stage B (1.0 μm-2.5 μm), Stage C (0.5 μm-1.0 μm), and Stage D (0.25 μm-0.5 μm) of the impactor. Significance was determined to be $\alpha=0.05$. A t-test was used to determine if there were significant differences between CFUs derived from the Coriolis C and the PCIS. A post-hoc Tukey test was used to assess if there was a significant difference over the four aerodynamic size fractions between four sampling sites. These statistical tests were performed using R statistical software (R Core Team, 2015). Sequences from the Saw Kill metagenomic samples were processed using a Dada2 pipeline developed in the Perron lab, as per (REF – find Dylan Dahan's published paper with Gabriel to use as ref). Once processed,

the resulting phyloseq object was searched at the genus level for the presence of *Legionella* above the outflow, at the outflow, and below the outflow of the Bard water plant. An ANOVA test was used to assess significant differences in percentage of *Legionella* found in samples between the three sites.

Results

There was no significant difference found between the average colony forming units (CFUs) per liter of air and impactor stage A-D (p-value= 0.481, F-statistic= 1.060) when LB media was used. An ANOVA test found that there was a slightly significant difference between bacterial and fungal CFUs (p-value= 0.06, F-statistic= 5.27). A post-hoc Tukey test compared stages and found there to be significant differences in mean bacterial CFUs between stage A (>2.5um) and stage C (0.5um-1.0um) (p-value = 0.008942) and stage B (1.0um-2.5um) and stage C (0.5um-1.0um) (p-value = 0.001) when grown on LB media (**Figure 7a**).

An ANOVA test on CFUs grown on R2A media found an insignificant overall difference between means of stages A-D (F-statistic = 0.277, p-value=0.84) (**Figure 7b**). There was found to be a significance between bacterial and fungal colonies grown (p-value=0.035). A post-hoc Tukey did find significant stage level differences. There was a significant difference between mean bacterial CFUs on Stage A compared to Stage D (p-value = 0.0008) and stage A compared with stage C (p-value = 0.0005). Though overall fungal CFUs were very low, there was growth in both stage A and stage D, where there happened to be to a significantly higher mean of CFUs in stage D than stage A (p-value = 0.0001).

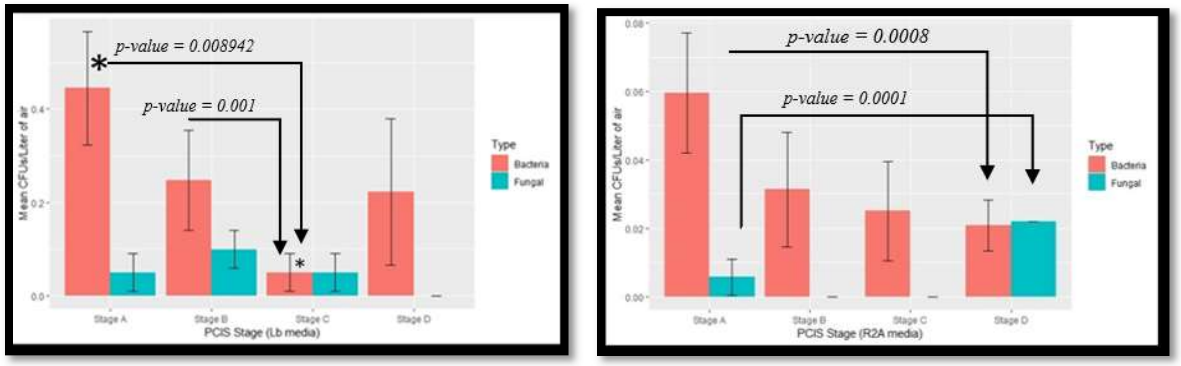


Figure 7a &7b. ***Distribution of particle size fractions by CFU type***. Particle sizes are binned by four stages. X-axis denotes stages A-D respectively. Y-axis is the mean amount of CFUs grown across all sampling events. Error bars represent standard error of mean. Asterisks denote significant differences.

Total culturable microbial aerosol counts were higher with the Coriolis C (mean = 0.489 ± 0.12 CFU/L of air) than the Impactor (mean = 0.146 ± 0.047 CFU/L of air). There was a significant difference found between the mean of bacterial CFUs between the two devices (p-value = 3.06e-05). There was not, however, a significant difference found between the fungal CFUs between the two devices (p-value = 0.9461). **Figure 8** depicts the results and differences of CFUs grown on R2A media between the PCIS and the Coriolis C.

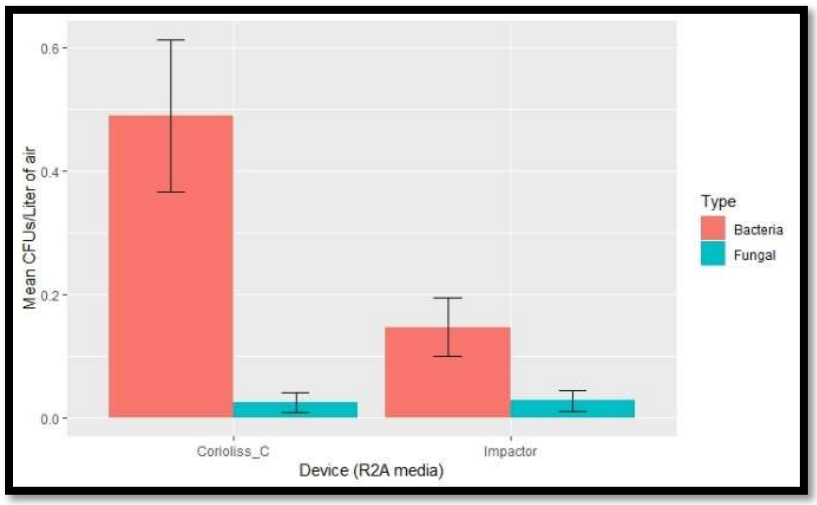


Figure 8. ***A comparison of CFUs by device.*** A comparison by device found a significant difference in bacterial CFUs (p-value=0.03216), but not fungal CFUs (p-value = 0.3131) between the PCIS and the Coriolis C.

Figure 9 depicts an analysis of pigmentation of CFUs on plates by size distribution for CFUs grown on both LB media and R2A media. A visual analysis shows that there was a diversity of pigmentations (orange, white, gray, yellow) that almost equally made up CFUs of stages A and B on LB media. Stage C and D, on the other hand, were less diverse in pigmentation being almost entirely made up of white and orange pigmentation. On R2A media, stage A similarly was the most diverse in pigmentation. However, diversity of pigmentation was higher across all stages with the fine particle stages (stages C & D) consisting of three out of five pigmentations.

The Coriolis C was also compared and showed a drastic difference in diversity between media types. On LB media, CFUs of the Coriolis C were entirely made up of gray pigment. On R2A media, the Coriolis C CFUSs were much more diverse consisting of all five observed pigments (orange, white, yellow, brown, and gray).

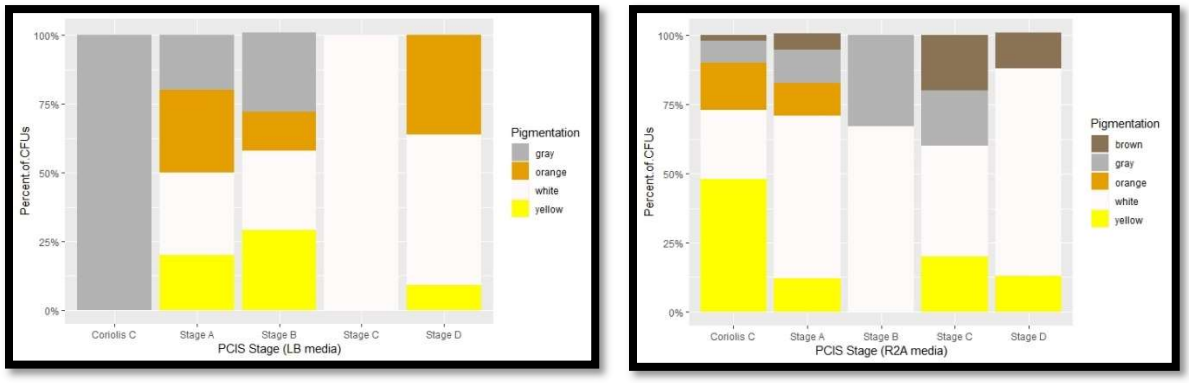


Figure 9. Percent of CFUs Particle Distribution and Pigmentation. A graphical representation of pigmentation of plates by stages A-D. The X-axis denotes the stages. The Y-axis denotes the percent of total CFUs made up of a certain pigment.

Differences in culturable bacterial aerosol counts from the PCIS by location were found to be significant through several stage level comparisons. On LB media, coarse particle sizes, >1um, (stages A & B) were both significantly higher than stage C (Welch t-test, stage A-stage C:

p-value = 0.0214, stage B-stage C: p-value), while there was found to be no significant differences when compared to stage D. On R2A media, differences in locations were found to be significant at almost every stage. At stage A, there was a significant difference between the RKC forest site and the wastewater plant site (p-value = 0.004); and a significant difference between the RKC quad and the wastewater plant site (p-value = 0.002). At stage B, there was a significant difference between the RKC forest site and the RKC quad (p-value=0.003) and there was a highly significant difference between the RKC forest site and the wastewater plant site (p-value= 1.42 x 10^{-5}). At stage C, there were significant differences between all sites (RKC forest-wastewater plant, p-value = 0.0241; RKC forest-RKC quad, p-value= 0.008; RKC quad-wastewater plant, p-value = 0.0003). Stage D also showed significant differences between each location (RKC forest-RKC quad, p-value= 0.001; RKC forest- wastewater plant, p-value = 0.022; RKC quad-wastewater plant, p-value = 0.047).

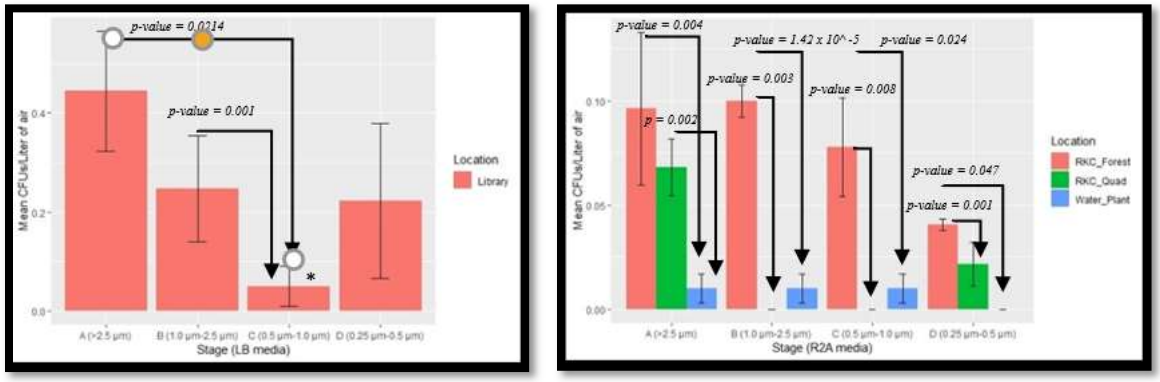


Figure 10a & 10b. Size distribution of bacterial colonies binned by location. The X-axis indicates stages A-D. The Y-axis indicates bacterial CFUs means.

Metagenomic data from 2015 Saw Kill water samples were loaded into R studio via the phyloseq object and then searched through for the presence of *Legionella* at the genus level. **Figure 11** shows the mean percentages of *Legionella* found at each sampling site along the Bard

wastewater outflow section of the Sawkill tributary. The outflow site boasted the highest mean percentage of *Legionella* found in water samples. There were significant differences found between the outflow and below the outflow site (p-value = 0.002874), as well as above and below the outflow (p-value = 9.76 x 10^{-5}).

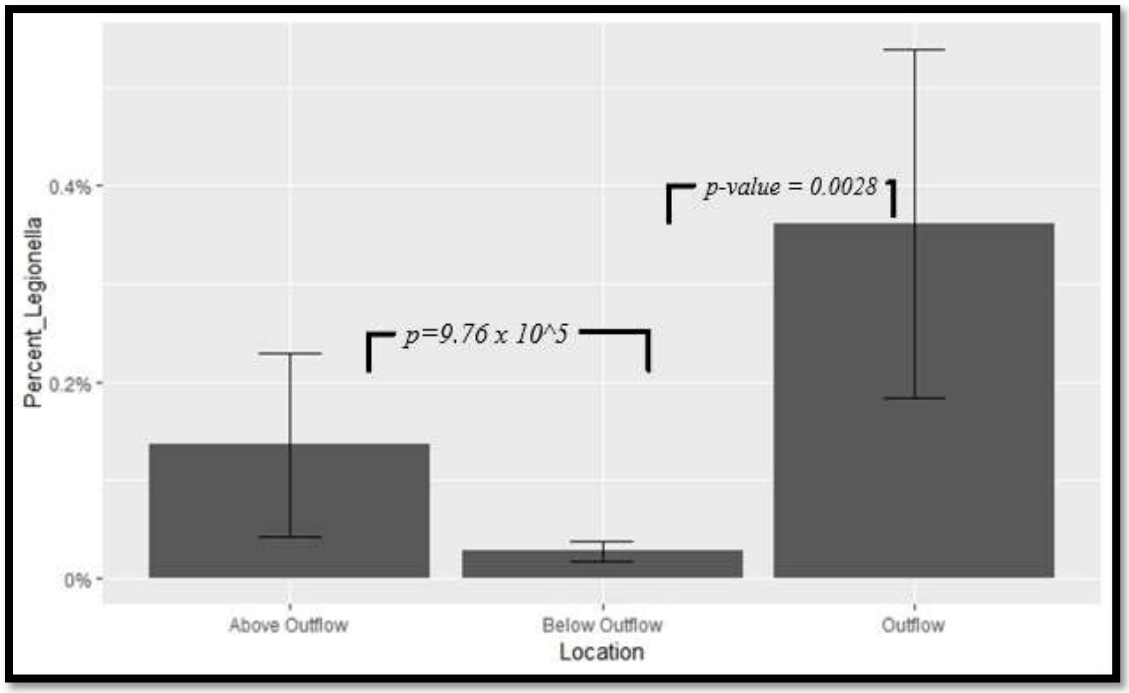


Figure 11. Presence of *Legionella* at three locations along the Saw Kill tributary. The percentage of *Legionella* found in 49 water samples. X-axis indicates location of water sampled. Y-axis indicates mean percent of genus *Legionella* found in water samples. Error bars represent standard error of the mean.

Discussion

Analysis of Particle Distributions

In both LB media and R2A media plates, mean CFUs/liter of air were significantly higher in the coarse particle stages, A and B (>2.5um and 1.0um-2.5um) than stage C (0.5um-1.0um). Coarse particles had higher overall CFUs in comparison to fine particle CFUs. Differences were not significant to stage D in LB media as results showed high standard errors relative to other

stages (stage D, LB media: s.e.m. = 0.16 CFU/liter of air). This was likely due to an outlier that occurred in one of the sampling events in which 9 CFUs were counted in stage D. A plausible explanation could be the altitude of the sampling event, being that sampling occurred atop a five-story building. Prior literature has shown that fine particles are commonly found at higher altitudes (Seinfeld & Pandis, 1998).

Overall trends of this stage level analysis are consistent with previous studies (Montero et al. 2016, insert more) that show culturable microbial aerosols are associated with coarse particles. The decision to use two different media types was intended to help increase CFUs, and thus after the fourth sampling event, the methods were adjusted to use R2A media for plating, which is a commonly used media type in aerosol and surface water studies (Montero et al. 2016; Dueker et al., 2012). The results of this experiment, however, showed that more CFUs grew on LB plates. This result could be attributed to be location-specific or due to an unequal amount of sampling events between the two media types.

The dominance of culturable bacteria within the coarse size fraction is strong evidence that most culturable bacterial aerosols are associated with cell-aggregating materials as has been observed in past studies (Aller et al., 2005). Moreover, the size distribution of bioaerosols can have an important impact on human health. In particular, different size particles can determine where in the respiratory system they ultimately deposit. According to Lee et al. 2010, a viable cell is likely to be deposited in the lung if it is within the range of 1–5 μm. The majority of the bacterial aerosols sampled in this study were present in the coarse particle size (>2.5μm), which subsequently means many aerosols could be deposited in the lungs. Thus, the viability of culturable microbial aerosols in the all stages of the personal impactor (>2.5um-0.5um) emphasize the potential impact on air quality from a public health perspective.

Comparison between PCIS and Coriolis C

A comparison of efficiency between the PCIS and the Coriolis C showed significant differences (p-value= 3.06e-05) in mean CFUs/liter of air. The mean of the Coriolis C was 0.49 ± 0.12 CFU/liter of air, while the mean of the PCIS was 0.15 ± 0.04 CFU/liter of air. This drastic difference is most likely due to a disparity in the lab procedures between the two devices. The Coriolis C lab protocol involved almost zero potential loss of microbial aerosol sample as the sampling apparatus to plate step required very few steps. The PCIS procedure, on the other hand, involved disassembly of device, removing and transferring of individual filters, inserting filters into test tubes, pipetting in endotoxin-free water, vortexing, and finally plating. The increased number of steps involved, therefore, increases the potential loss rate of sample as well as decreases efficiency of bacterial enumeration.

Analysis of Colony Pigmentations

An analysis of pigmentation of CFUs by stage and by colony type found coarse particles size (stages A and B) to be the most diverse particle fraction and found R2A media to boast the most variety of pigments. Pigments play an important role in the physiology and molecular process of microorganisms such as helping organisms adapt to environmental conditions and sunlight protection (Wiesbaden, Bergmann, 1884). Diversity of pigments are also directly related to diversity in bacterial and fungal types. The findings of this study further confirm the notion of coarse particles being more culturable as found by several studies (Montero et al. 2016; Aller et al., 2005; Maron et al., 2005). Moreover, the findings that showed higher diversity in terms of pigment on R2A media, at least initially, go against a previous study by Dueker et al. 2014 that found 159 near-surface microbial aerosol isolates, 126 of which came from LB media and only 33 of which came from R2A media. This comparison alone cannot serve to determine the

effectiveness of one media type over the other. Further analysis, including PCR amplification and sequencing would be required to make an equitable comparison.

Locational Influences

Comparing CFUs by location found significant differences between stages. The library site produced the highest microbial aerosols counts followed by the RKC forest site, then the RKC quad site, and finally the wastewater plant. Although not necessarily comparable, the microbial aerosol count of samples taken at the library were likely higher due to two factors supported by literature. The first plausible reason is due to higher relative humidity during sampling events on the library rooftop. The library sampling events experienced the highest relative humidity measurements of all sampling events (library relative humidity mean = 75%; mean of all other sites = 37%). Viable bacterial aerosols have been shown to increase with relative humidity; and one study showed the survival rate of the influenza virus in aerosols is highest with high relative humidity (Després et al., 2012, Hatch and Dimmick, 1966). Thus, the high overall number of CFUs found at the library site is consistent with prior research. Another possible reason microbial aerosols were higher on the library rooftop can be attributed to the presence of a cooling tower at the sampling site. Prior studies have found air around cooling towers to be associated with high bacterial concentrations; and moreover, cooling towers have been commonly associated with the aerosolization of *Legionella* (Prussin et al. 2017; Llewellyn et al. 2017; Addiss et al. 1989).

On R2A media, the CFUs at nearly every stage differed significantly by location. This finding again points towards previous studies that finds culturable microbial aerosols are most likely to be unique to local sources (Dueker et al. 2018, Dueker et al. 2012). The RKC forest site produced the highest mean CFUs/liter of air and was significantly higher in all but one stage

when compared to the RKC quad site and the wastewater site. The forested environment is known to produce aerosols (Pelwek et al. 2006) and therefore could be explained as a local source contributing to an increase in CFUs.

Presence of *Legionella* in Saw Kill

Being that the ultimate aim of this study is to connect the presence of *Legionella* in sewage-related water, metagenomic data from 2015 research conducted on the Saw Kill was used to search for the presence of *Legionella*. The results showed that the highest percentage of the genus-level *Legionella* were found at the outflow, followed by above the outflow, and lastly below the outflow. These findings indicate that the highest source for *Legionella* is the discharge point from the sewage treatment plant. Prior research has confirmed wastewater treatment plants to be a known source for *Legionella* (Caicedo et al. 2018; Buse et al. 2012). Moreover, one study found that protozoa and biofilms along pipe walls are considered to be one of the main reservoirs of *Legionella* (Lau and Ashbolt, 2009).

Looking ahead to the successive study at Newtown Creek, I expect to find the highest amount of *Legionella* near the combined sewer overflow pipes. Recently, a new culture method for the quantification of *Legionella* pneumophila called Legiolert® (IDEXX Labrotories, Westbrook, ME) was developed and granted for future study of *Legionella* in public waterways. The Legiolert method is based on a most probable number approach and differs significantly from traditional culture methods by providing rapid sample preparation and analysis, results within 7 days and objective interpretation of test results. (Rech et al. 2018). The use of this approach would significantly reduce the amount of time taken by traditional culture methods as well as qPCR approach.

Limitations

Limitations to the current study are primarily in relation to results produced over the course of the six-month sampling and protocol development phase. For much of this study, the protocols for both devices were being developed and adjusted. Therefore, much of the variance and shortage of sampling were factors in the analysis of this study. Optimization of this protocol was attempted through tweaking of elapsed sampling time, sampling amount of air, and lab methods. Due to the nature of the trial and errors in this process, it may be difficult to make definitive claims on the distribution of personal bioaerosol exposures as well as the presence of *Legionella* in bioaerosols.

The use of culturing plates has limitations in representing total microbial aerosol diversity and has been discussed in previous studies (Dueker & O'Mullan 2014; Young, Juhl & O'Mullan, 2013). Not all viable cells can be cultured on a single media type, and therefore can be a limitation. Viability plays an important role in aerosol production and transport and is expected to differ across aerosol size fractions (Montero et al. 2016).

Conclusion

This study was meant to serve as a pilot for further research into the study of *Legionella* in the urban environment. The results of this study found that a majority of enumerated bacterial and fungal colonies were in the coarse particle range of >2.5um. This is consistent with findings of Montero et al. 2016, which observed onshore, and offshore aerosols were associated with particles greater than 2.1um. The dominant presence of coarse microbial aerosols coupled by significant locational differences emphasizes the importance of local sources and the small-scale microbiological coupling of water, land, and air (Montero et al. 2016).

The presence of *Legionella* near a sewage outflow source further indicates a connection of the bacteria to wastewater treatment plants as cited in literature. This finding goes a step further in establishing a baseline understanding of the presence of *Legionella* in public waterways known for receiving raw sewage inputs. The successive study of this project will use a new detection method, Legiolert, to efficiently and quickly assess the presence of *Legionella* pneumophila, the specific strain linked to Legionnaires disease. This coupled with the use of the developed protocols for PCIS and the Coriolis C will attempt to identify and quantify the aerosolization of pathogens like *Legionella* from public waterways. The ultimate study will assess the likelihood of public waterways acting as *Legionella* sources and possibility of viable strains of *Legionella* being transported and settling at a site such as a cooling tower atop nearby buildings.

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Appendix

Spreadsheet of all data:

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
1	Date:	Site:	Minutes Sample	Sample Rate (L/min	Amount of Air (Liters):	Type (LilC/impactor):	ul wash	airperplate	B.cfu/Lair	F.cfu/Lair	Temp:	RH:	Wind Speed:	Days Incubated:	# Bacterial CFU	# Fungal CFUs	Colors:
2	26-Oct	Library	20	9	180	Impactor A	4000	6.75	0.592592593	0.148148148	43	70	7	14	4	1	off-white, light gray, dark gray, orange
3	26-Oct	Library	20	9	180	Impactor B	4000	6.75	0.296296296	0.148148148	43	70	7	14	2	1	dark gray, white
4	26-Oct	Library	20	9	180	Impactor C	4000	6.75	0	0	43	70	7	14	0	0	0
5	26-Oct	Library	20	9	180	Impactor D	4000	6.75	1.333333333	0	43	70	7	14	9	0	white, orange
6	26-Oct	Library	20	9	180	Impactor After	4000	6.75	0	0	43	70	7	14	0	0	0
7	30-Oct	Library	20	9	180	Impactor A	4000	6.75	0.148148148	0	45	61	6	14	1	0	orange
8	30-Oct	Library	20	9	180	Impactor B	4000	6.75	0.444444444	0	45	61	6	14	3	0	yellow, orange, white
9	30-Oct	Library	20	9	180	Impactor C	4000	6.75	0	0	45	61	6	14	0	0	0
10	30-Oct	Library	20	9	180	Impactor D	4000	6.75	0	0	45	61	6	14	0	0	0
11	30-Oct	Library	20	9	180	Impactor After	4000	6.75	0.148148148	0.148148148	45	61	6	14	1	1	white
12	1-Nov	Library	20	9	180	Impactor A	4000	6.75	0.592592593	0	50	94	6	14	4	0	white, yellow, orange
13	1-Nov	Library	20	9	180	Impactor B	4000	6.75	0	0.148148148	50	94	6	14	0	1	light gray
14	1-Nov	Library	20	9	180	Impactor C	4000	6.75	0.148148148	0.148148148	50	94	6	14	1	1	white
15	1-Nov	Library	20	9	180	Impactor D	4000	6.75	0.444444444	0	50	94	6	14	3	0	yellow, white, red-orange
16	1-Nov	Library	20	9	180	Impactor After	4000	6.75	0	0	50	94	6	14	0	0	0
17	12-Nov	Library	20	50	1000	LilC	5000	30	0.033333333	0	45	49	5	14	1	0	dark gray
18	18-Feb	RKC Forest Ri	50	9	450	Impactor A	3000	22.5	0.044444444	0	37	52	14	21	1	0	white
19	18-Feb	RKC Forest Ri	50	9	450	Impactor B	3000	22.5	0.088888889	0	37	52	14	21	2	0	white
20	18-Feb	RKC Forest Ri	50	9	450	Impactor C	3000	22.5	0.044444444	0	37	52	14	21	1	0	light gray
21	18-Feb	RKC Forest Ri	50	9	450	Impactor D	3000	22.5	0.044444444	0	37	52	14	21	1	0	white
22	18-Feb	RKC Forest Ri	60	9	540	Impactor A	3000	27	0.148148148	0	31	54	14	21	4	0	white, yellow
23	18-Feb	RKC Forest Ri	60	9	540	Impactor B	3000	27	0.111111111	0	31	54	14	21	3	0	white, light gray
24	18-Feb	RKC Forest Ri	60	9	540	Impactor C	3000	27	0.111111111	0	31	54	14	21	3	0	white, yellow
25	18-Feb	RKC Forest Ri	60	9	540	Impactor D	3000	27	0.037037037	0	31	54	14	14	1	0	white
26	8-Mar	RKC Quad	50	9	450	Impactor A	3000	22.5	0.044444444	0	31	38	7	14	1	0	white, orange
27	8-Mar	RKC Quad	50	9	450	Impactor B	3000	22.5	0	0	31	38	7	14	0	0	0
28	8-Mar	RKC Quad	50	9	450	Impactor C	3000	22.5	0	0	31	38	7	14	0	0	0
29	8-Mar	RKC Quad	50	9	450	Impactor D	3000	22.5	0.044444444	0.133333333	31	38	7	14	1	3	white, yellow
30	8-Mar	RKC Quad	50	50	2500	LilC	5000	75	0.506666667	0.013333333	31	38	7	14	38	1	white, yellow, orange
31	8-Mar	RKC Quad	50	50	2500	LilC	5000	75	0.48	0.013333333	31	38	7	14	36	1	yellow, orange, white
32	30-Mar	RKC Quad	111.1	9	999.9	Impactor A	3000	49.995	0.100010001	0	67	48	10	14	5	0	white
33	30-Mar	RKC Quad	111.1	9	999.9	Impactor B	3000	49.995	0	0	67	48	10	14	0	0	0
34	30-Mar	RKC Quad	111.1	9	999.9	Impactor C	3000	49.995	0	0	67	48	10	14	0	0	0
35	30-Mar	RKC Quad	111.1	9	999.9	Impactor D	3000	49.995	0	0.020002	67	48	10	14	0	1	white, dark brown
36	30-Mar	RKC Quad	20	50	1000	LilC	5000	30	0.033333333	0.033333333	67	48	10	14	1	1	white
37	30-Mar	Water Plant	111.1	9	999.9	Impactor A	3000	49.995	0.020002	0.040004	70	35	15	14	1	2	white, brown
38	30-Mar	Water Plant	111.1	9	999.9	Impactor B	3000	49.995	0	0	70	35	15	14	0	0	0
39	30-Mar	Water Plant	111.1	9	999.9	Impactor C	3000	49.995	0.020002	0	70	35	15	14	1	0	brown, cream
40	30-Mar	Water Plant	111.1	9	999.9	Impactor D	3000	49.995	0	0	70	35	15	14	0	0	0
41	30-Mar	Water Plant	20	50	1000	LilC	5000	30	0.166666667	0.133333333	70	35	15	14	5	4	yellow, white, brown
42	1-Apr	Water Plant	111.1	9	999.9	Impactor A	3000	49.995	0	0	52	24	14	14	0	0	0
43	1-Apr	Water Plant	111.1	9	999.9	Impactor B	3000	49.995	0.020002	0	52	24	14	14	1	0	white
44	1-Apr	Water Plant	111.1	9	999.9	Impactor C	3000	49.995	0	0	52	24	14	14	0	0	0
45	1-Apr	Water Plant	111.1	9	999.9	Impactor D	3000	49.995	0	0	52	24	14	14	0	0	0
46	1-Apr	Water Plant	20	50	1000	LilC I	5000	30	0.3	0	52	24	14	14	9	0	green, white
47	1-Apr	Water Plant	20	50	1000	LilC II	5000	30	0.066666667	0	52	24	14	14	2	0	yellow, dark brown
48	1-Apr	RKC Quad	111.1	9	999.9	Impactor A	3000	49.995	0.060006001	0	48	25	21	14	3	0	orange, yellow, white
49	1-Apr	RKC Quad	111.1	9	999.9	Impactor B	3000	49.995	0	0	48	25	21	14	0	0	0
50	1-Apr	RKC Quad	111.1	9	999.9	Impactor C	3000	49.995	0	0	48	25	21	14	0	0	0
51	1-Apr	RKC Quad	111.1	9	999.9	Impactor D	3000	49.995	0.020002	0	48	25	21	14	1	0	white
52	1-Apr	RKC Quad	20	50	1000	LilC	5000	30	0.333333333	0	48	25	21	14	28	0	yellow, orange, white
53	1-Apr	RKC Quad	20	50	1000	LilC	5000	30	0.966666667	0	48	25	21	14	29	0	yellow, orange, white

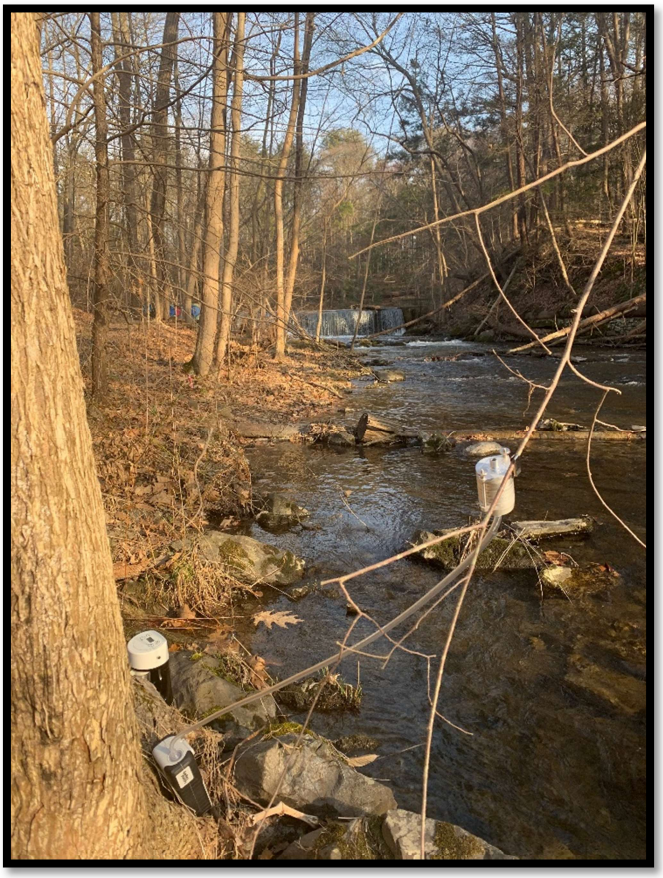
Additional images of sampling events:

RKC forest sampling site

RKC Quad sampling site

Cooling tower atop library

Rooftop of library taken before a sampling event.



Wastewater Plant Outflow