Factors of Full-Scale Drinking Water Treatment that Contribute to Risk of Opportunistic Infectious Disease

by

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Abstract

Each year, 16,000 hospitalizations and 3,000 deaths caused by non-tuberculous mycobacteria occur in the USA. Non-tuberculous mycobacterial infections are primarily transmitted by water. This work helps to explain why mycobacteria are present in the finished water produced by drinking water treatment plants, and identifies treatment plant design and operational considerations to minimize the risk of waterborne mycobacterial infection.

It was found that viable microbial cell concentrations decreased significantly in the first ozone contact chamber of multi-chamber ozone contactors in a full-scale drinking water treatment plant. However, cell concentrations rose in subsequent chambers across the contactors. This increase resulted from detachment from biofilms on contactor surfaces and from biomass in sediments within a hydraulic dead zone. The biofilms downstream of the dead zone contained a significantly higher relative abundance of mycobacteria than biofilms in earlier chambers. Viable mycobacteria populations were detected in ozone contactor effluents and in biologically-active carbon (BAC) filters downstream. These findings present an argument for improved hydraulic efficiency in multi-chamber contactors, e.g., through decreasing chamber width, and underscore the importance of filter maintenance practices that avoid reinforcing the presence of mycobacteria.

During BAC filter backwashing with water containing monochloramine, monochloramine concentrations at the top of filter beds were measured to be similar to concentrations previously shown to be sub-lethal for the mycobacterial species *Mycobacterium avium*. *M. avium* dislodged during filter backwashing upregulated mammalian cell entry gene *mce1C* substantially. *M. avium*'s response to disinfectant exposure during backwashing raises the possibility of an adaptive response that increases its resistance to disinfection. Its upregulation of mammalian cell entry genes has implications for its role as an intracellular pathogen, and is consistent with laboratory scale findings that sub-lethal monochloramine exposure induces mycobacterial virulence factors. Where possible, utilities should limit the use of backwash water with disinfectant to reduce selecting for disinfectant-resistant bacteria.

Mycobacteria can resist residual disinfectant in distribution systems and, therefore, distribution systems represent a transmission route of mycobacteria in treated waters to consumers. Despite higher concentrations of mycobacteria reported in water disinfected with chloramine compared to chlorine, the type of disinfectant used to provide a residual disinfectant during distribution of water was not a significant predictor of infection in a case control study of patients at the University of Michigan's academic medical center Michigan Medicine. However, the use of drinking water primarily sourced from a surface water body compared to a groundwater source was found to be significantly associated with infection. This result suggests that monitoring mycobacteria in source waters used for drinking water production may be a proactive strategy that can be implemented by drinking water treatment plants.

The results presented in this dissertation suggest that specific drinking water treatment plant design and operational considerations minimize risk of waterborne infectious disease. This work should assist water system managers to improve the microbial quality of drinking water to promote health for everyone, including individuals sensitive to opportunistic bacterial infections.

Chapter 1

Introduction

Managing the microbial quality of drinking water is primarily aimed at minimizing illness caused by waterborne pathogens. The introduction of filtration and chlorination into public water treatment during the early twentieth century dramatically reduced mortality rates in major cities (Cutler et al., 2005) and classic drinking water-associated diseases such as cholera and typhoid are rare today in high-income countries. However, drinking water-associated illnesses remain a public health problem in the United States. From 2001-2012, 187 outbreaks associated with drinking water were reported to the Centers for Disease Control and Prevention (CDC) Waterborne Disease Surveillance Program. These outbreaks resulted in at least 9,991 cases and 41 deaths (Beer et al., 2015; Blackburn et al., 2004; Brunkard et al., 2011; 2013; Liang et al., 2006; Yoder et al., 2008). These figures likely represent only a fraction of the actual cases. Healthcare expenditure data showing 40,000 hospitalizations and 9.6 million US dollars in healthcare costs for infections caused by pathogens primarily transmitted by water (Collier et al., 2012), indicate that annual incidence of drinking water-associated illness cases are likely much higher than the number of cases associated with reported outbreaks. Furthermore, a substantial fraction of drinking water-associated disease is caused by opportunistic bacterial pathogens that do not have specific regulatory limits set by the US Environmental Protection Agency (EPA).

Non-tuberculous (i.e., atypical or environmental) mycobacterial species have come to the forefront as an unregulated, opportunistic pathogen group of concern. The majority of research on this group has focused on the *Mycobacterium avium* complex, which is the most frequently identified cause of non-tuberculous mycobacterial pulmonary infection. Each year 16,000 hospitalizations (Collier et al., 2012) and 3,000 deaths (Mirsaeidi et al., 2014) caused by non-tuberculous mycobacteria occur in the USA. The increasing prevalence of mycobacterial infections (Adjemian et al., 2012; Donohue et al., 2016; Marras et al., 2007; Olivier et al., 2003; Prevots et al., 2010; Roux et al., 2009) combined with abundant evidence of infections associated with exposure to contaminated drinking water (Falkinham et al., 2008; Fleming et al., 2006; Glover et al., 1994; Thomson, R. et al., 2013; Thomson, Rachel et al., 2013; Vonreyn et al., 1994), have highlighted mycobacteria as

an emerging public health challenge for drinking water systems. Research has focused on factors of distribution systems (Falkinham et al., 2001; Gomez-Smith et al., 2015; Le Dantec et al., 2002; Norton, C. D. et al., 2004; Thomson et al., 2011; Torvinen et al., 2004; Tsintzou et al., 2000; Vaerewijck et al., 2005; Wang et al., 2012; Whiley et al., 2014) and premise plumbing (Buse et al., 2014; Feazel et al., 2009; Proctor et al., 2016; Williams et al., 2013) that increase abundances of mycobacteria. The classification of *Mycobacterium avium* and other bacteria as "opportunistic premise plumbing pathogens (OPPP)" (Falkinham, 2015; Falkinham, Hilborn, et al., 2015; Falkinham, Pruden, et al., 2015) reflects that these bacteria are frequently detected in premise plumbing. However, they are also found in source waters and throughout water treatment plants (Hilborn et al., 2006; King et al., 2016; Lee et al., 2000). At seven drinking water treatment plants in the USA, mycobacteria were detected in treated water even when they were not detected in source water (King et al., 2016).

Growing evidence suggests that biologically active carbon (BAC) filtration, which has become a popular technology in drinking water treatment plants in North America (Brown et al., 2016), influences the microbial quality of treated water through the presence of viable microorganisms in filter effluents. Point-of-use filters containing granular activated carbon are well known to be colonized by mycobacteria and these species can be found in their effluents (Falkinham, 2010; Holinger et al., 2014; Rodgers et al., 1999). However, mycobacteria have not been reported as widely by bacterial 16S rRNA gene-based surveys in full-scale BAC filters (Lautenschlager et al., 2014; Pinto et al., 2012). They may be present but in low relative abundance and require sequencing of mycobacterial genes for identification. Few studies have reported on the diversity of mycobacterial species in full-scale BAC filters and their effluents. Furthermore, the impact of filter maintenance practices such as backwashing on mycobacteria in filters is poorly understood.

Based on recent surveys of biofiltration plants in North America, it is clear that GAC biofiltration is practiced using a wide range of configurations, with 63% of biofiltration plants using ozone pretreatment (Brown et al., 2016; Upadhyaya et al., 2017). Non-ideal mixing conditions in multi-chamber ozone contactors (Kim et al., 2010) can reduce disinfection efficacy. As a result, viable mycobacteria may be present in ozone contactor effluents and may colonize granular media filters following ozonation. While disinfection reduces the abundance of viable microorganisms in BAC filter effluents, it may not be sufficient to inactivate disinfectant resistant microbial populations (Batte et al., 2003; Chiao et al., 2014; Norton, Cheryl D et al., 2000). Importantly, several

mycobacteria are disinfectant resistant as free-living or intracellular microorganisms (Berry et al., 2010; Pelletier, P. et al., 1988; Pelletier, P. A. et al., 1991; Taylor et al., 2000). Furthermore, *Mycobacterium avium* cells demonstrated an "adaptive response" when populations previously exposed to sub-lethal concentrations of disinfectant (0.5-2 mg/L) exhibited increased resistance to higher disinfectant concentrations (5-10 mg/L) (Luh et al., 2008). These findings present important considerations for water treatment processes. The fate of mycobacteria through water treatment and the role of treatment processes in selecting for mycobacteria is an important area that needs more attention.

The body of research in this dissertation evaluates full-scale drinking water treatment processes, with a focus on disinfection and biofiltration processes, and their contribution to the presence of mycobacteria in treated water. The primary goal was to combine techniques from the fields of environmental engineering, environmental microbiology and molecular biology with an epidemiologic investigation of public health data, to identify water treatment design and operational factors needed for the successful control of waterborne opportunistic disease. Chapter 2 reports a study of viable microorganisms during ozonation at a full-scale drinking water treatment plant, using culture-based and culture-independent techniques, and provides evidence for factors that contribute to the presence of mycobacteria in ozone contactor effluents. Chapter 3 presents an investigation of the response of *Mycobacterium avium* in BAC filters to the drinking water disinfectant monochloramine during filter backwashing. Chapter 4 provides a review of the literature to describe relationships between cellular and genetic responses of mycobacteria to the disinfectant monochloramine, and genetic factors involved in mycobacterial infection. Chapter 5 reports a case control study of patients tested for mycobacterial infection at Michigan Medicine to test an association between mycobacterial infection and water treatment practices for municipal water supplied to patient home. Chapter 6 provides concluding remarks and public health significance.

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Chapter 2

Biofilm Detachment Drives Microbial Water Quality from Full-Scale, Multi-Chamber Ozone Contactors

2.1 Abstract

Concentrations of viable microbial cells were monitored using culture-based and culture-independent methods across multi-chamber ozone contactors in a full-scale drinking water treatment plant. Membrane-intact and culturable cell concentrations in effluent from ozone contactors were 1,200-3,750 cells/mL and 200-3,850 CFU/mL, respectively. Viable cell concentrations decreased significantly in the first ozone contact chamber, but rose, even as ozone exposure increased, in subsequent chambers. The increasing cell concentrations in water samples collected from sequential ozone contact chambers resulted from detachment from biofilms on contactor surfaces and from biomass present within lime softening sediments that had accumulated within a hydraulic dead zone. Biofilm community structures on baffle walls downstream and upstream from the hydraulic dead zone were significantly different from each other ($p < 0.05$). The biofilms downstream of the hydraulic dead zone contained a significantly ($p < 0.05$) higher relative abundance of bacterial taxa that are resistant to disinfectants and which contain members of opportunistic bacterial pathogens (*Mycobacterium* and *Legionella* populations). These results have important implications as the effluent from ozone contactors is often treated further in biologically active filters and bacteria in those effluents continuously seed filter microbial communities.

2.2 Introduction

Disinfection in full-scale water treatment systems is often less effective than predicted by lab-scale inactivation experiments. Factors that reduce disinfection efficiency in real systems include differential disinfectant resistance of different types of microbes (Chiao et al., 2014), matrix effects such as the presence of flocs (Czekalski et al., 2016), which protect microorganisms from disinfectant exposure, and oxidant demand exerted by natural organic matter (NOM) or inorganic compounds in untreated or partially treated water. Ozonation is widely applied for water treatment as ozone inactivates microbial pathogens (Hunt et al., 1999), decomposes NOM, including

disinfection byproduct precursors, removes taste-, odor- and color-causing compounds (Nerenberg et al., 2000; Saroj et al., 2005), oxidizes inorganic contaminants (Sung et al., 2015; Zhang, Y. et al., 2013), and improves clarification and filtration by inducing formation of precipitates or through other mechanisms (Jekel, 1998). Viable microbial cells have been reported to remain present after ozonation of raw water (Hammes et al., 2008) or partially treated water (Czekalski et al., 2016; Lee et al., 2000; Vital, Hammes, et al., 2012) in full-scale or pilot-scale systems, even though ozone is a strong oxidant and is a more effective disinfectant than chlorine and chloramine (Ramseier et al., 2011). Given that some opportunistic pathogens (e.g., *Mycobacterium avium*) are resistant to disinfection, including to ozonation (Taylor et al., 2000), and given the differential resistance to disinfection within a mixed microbial community (Chiao et al., 2014), understanding the potential for selection of disinfectant-resistant microorganisms during ozonation is imperative to optimize drinking water treatment and protect public health.

Reduced disinfection efficiency is relevant for drinking water treatment plants (DWTPs) that employ vertically baffled, multi-chamber ozone contactors. Non-ideal flow conditions such as short-circuiting and internal recirculation in such contactors can lead to insufficient mixing and reduced ozone exposure (Kim et al., 2009). A combination of non-ideal hydrodynamic conditions and the production of readily biodegradable substrates through oxidation of NOM may increase the potential for biofilm formation on contactor surfaces. Furthermore, biofilm extracellular polymeric substances can protect microorganisms from oxidant exposure and growth in biofilm can reduce their sensitivity to oxidative stress(Berry et al., 2009). Nevertheless, surveys of microbial communities in full-scale DWTPs (Czekalski et al., 2016; Lee et al., 2000; Pinto et al., 2012; Vital et al., 2010) so far have treated ozone contactors as “black boxes,” focusing on the planktonic microbial community in water sampled from contactor inlets and outlets only.

In this study, viable microbial cell concentrations were monitored at five locations across multi-chamber ozone contactors at a full-scale DWTP to gain insight into processes that shape the microbial quality (i.e., microbial concentrations and community composition) of ozonated water. Furthermore, the bacterial community structures in sediments and in biofilms on baffle walls in an ozone contactor were evaluated to explain how non-ideal flow conditions influence the microbial community in ozone contactor effluent.

2.3 Materials and methods

2.3.1 Drinking Water Treatment Plant

The maximum capacity of the Ann Arbor DWTP is 50 million gallons per day (MGD) (1.9×10^5 m^3/day) and the average daily water production is 15 MGD (5.7×10^4 m^3/day). Pinto et al. previously provided a description of the Ann Arbor DWTP (Pinto et al., 2012). Briefly, raw water is obtained from the Huron River (60-90%) and from groundwater wells (10-40%). The DWTP provides lime softening, coagulation, flocculation, sedimentation, ozonation, filtration, and chloramination. Before ozonation, CO_2 contactors reduce the pH of the water to approximately 7.8 (mean pH was 7.77 ± 0.36 for December 2015 through December 2016). Ozonated water is applied to biologically-active, dual media filters containing granular activated carbon and sand. Free chlorine and ammonia are added to the filter effluent to produce monochloramine at a concentration of approximately 3 mg Cl_2 /L for residual disinfection in the distribution system.

2.3.2 Ozone Contactors

The Ann Arbor DWTP has four parallel ozone contactors each containing seven chambers separated by vertical baffle walls and inlet and outlet basins (Figure 2.1). Two of the four contactors are typically operated to meet demand. Onsite ozone generators that use liquid oxygen generate a stream with approximately 7-10% ozone. Ozone is dispersed by ceramic fine bubble diffusers into chambers 2 and 3 of each contactor. Specifically, 70% of the ozone generated is dispersed into chamber 2 and 30% is dispersed into chamber 3. Water flows by gravity counter-currently to the ozone in chambers 2 and co-currently in chambers 3. A sufficient ozone dose is added to maintain 0.1 mg/L ozone residual in the effluent of chamber 2, the first chamber where ozone gas is added, in order to achieve 1-log virus inactivation credit under the United States Environmental Protection Agency (U.S. EPA) Surface Water Treatment Rule. The effluent of the seventh chamber in each contactor flows over a weir to dissipate dissolved gases, and water from the two contactors in operation is mixed in an outlet basin.

In each contactor, five sample lines extend from the contactor chambers to an ozone analyzer (Figure 2.1). Samples collected from sample line 1 represent clarified water after pH adjustment but before ozone exposure (CT = 0). Water from sample lines 2, 3, and 4 represent water after contact with ozone gas in chambers 2 and 3, and water after exposure to any residual ozone in chamber 4, respectively. Water from sample line 5 (in the outlet basin) rarely had detectable levels

of ozone. The retention time for a complete contactor is approximately 10 min. The width of chamber 3 (i.e., the distance between consecutive baffle walls) is twice the width of the other chambers to provide additional contact time.

2.3.3 Sample Collection

Water samples were collected from three contactors over the course of a year (Table 2.1). 500 mL water samples were collected into sterile plastic bottles containing sodium thiosulfate (5 × stoichiometric requirement (SR) to quench 0.5 mg/L ozone) from each sample line. Before sample collection, each sample line was flushed until meter readings of ozone concentration were stable (approximately 3 min).

2.3.4 Ozone Exposure (CT) Calculation Method

Ozone concentration and temperature in each sample line were monitored by online Ozone 499A OZ Sensors. The ozone exposure or CT (concentration × time) for each chamber was calculated as the average ozone concentration (C) in the chamber multiplied by the effective contact time (T10). For reaction chambers 4-7, where no ozone is added, a first order ozone decay model was used to determine the average ozone concentration. Residual ozone concentrations at sample line 5 (in the outlet basin) were typically below the quantification limit (0.01 mg/L). The CT calculation method is described in detail in Table 2.2.

2.3.5 Heterotrophic Plate Counts (HPC)

HPC were conducted using the pour plate method according to Standard Methods 9215 B (APHA, 2005). Samples were diluted (1:100, 1:10; 1:5, or 1:2) with sterile phosphate buffered saline (PBS, pH = 7.2 ± 0.2) solution and 1 mL diluted sample was mixed with liquefied R2A agar in triplicate and plated (n = 6 plates per sample). Colony forming units (CFUs) were counted using a Quebec Darkfield Colony Counter (Reichert, Inc., Depew, NY) after seven days of incubation at 28 °C.

2.3.6 Taxonomic Classification of Isolates

For one sampling day, 190 isolates were cut from R2A plates for phylogenetic analysis of culturable populations. A random number generator in R (base language, “sample”) was used to select the colonies that would be cut from each plate to reduce bias from picking colonies with specific size or morphology. 26, 29, 30, 47 and 58 colonies were picked from plates corresponding

to samples from lines 1-5 and dilution factors of 10, 2, 2, 2, and 10, respectively. The selected colonies (biomass with associated agar) were stored at -80 °C.

Colonies were thawed at room temperature and 50 µL of nuclease-free water was added to each tube. Successive heating and freezing of the liquid was used to release cells from agar and lyse cells. Specifically, the cell suspension was heated to 95°C for 5 min in a Mastercycler Thermocycler (Eppendorf AG, Hamburg, Germany). Then the cells were put at -80°C to freeze completely. Afterward they were thawed at room temperature, sonicated for 5 min, heated again to 95°C for 5 min, frozen completely at -80 °C and thawed again. Finally, cells were heated to 95°C for 7 min and the crude DNA extracts were quantified spectrophotometrically using absorbance at 260 nm (Nanodrop ND-1000, Nanodrop Technologies, Wilmington, DE).

A near full length fragment of the bacterial 16S rRNA gene was amplified with primers 8F and 1387R (Chiao et al., 2014). The PCR products were treated with ExoSAP-IT *Express* PCR Product Cleanup per the manufacturer's instructions (USB Corporation) and submitted for Sanger Sequencing at the University of Michigan DNA Sequencing Core (Ann Arbor, MI). Forward and reverse reads were assembled into contigs using SeqMan Pro software (DNA Star, Inc). The median sequence length was 1,270 base pairs. One of the 190 sequences had 23 ambiguous base pairs and was removed, but the remaining sequences had less than three ambiguous base pairs and no sequences had more than 14. Sequences were aligned against the SILVA database (Pruesse et al., 2007) and clustered into operational taxonomic units (OTUs) using a 97% similarity cutoff with the opticlust algorithm in mothur (version 1.39.0) (Schloss et al., 2009). Sequences were classified using a naïve Bayesian classifier (Wang, Q. et al., 2007) trained against a 16S rRNA gene training set provided by the Ribosomal Database Project (RDP) (Cole et al., 2009).

2.3.7 Flow Cytometry Analysis

EDTA pretreatment has been used in cell staining to increase cell permeability of gram-negative bacteria (Berney et al., 2007; Ramseier et al., 2011). EDTA pretreatment resulted in significantly more cells detected from sample line 1 (before ozone exposure) (data not shown); however, the effect of EDTA was not consistent with samples from lines 2-5. Inconsistent effects of EDTA pretreatment on samples has been reported by others (Nescerecka et al., 2016). As a result, EDTA pretreatment was discontinued. 1 mL aliquots of samples were stained with propidium iodide (PI, Thermo Fisher Scientific, Waltham, MA) and SYBR Green I (SGI, ThermoFisher) at final

concentrations of 2.97 μM PI and 1X SGI. After staining, samples were incubated at 37°C for 15 min to improve cell staining (Barbesti et al., 2000; Van Nevel et al., 2013). CountBright^{TM} absolute counting beads (Life Technologies Cat#C36950, Carlsbad, CA) were added to each sample per the manufacturer's instructions to determine flow cytometer process volume. All samples were stained and analyzed in duplicate and stored on ice until analysis with an LSR Fortessa cell analyzer (BD Biosciences, San Jose, CA). Events were triggered off fluorescence in the SGI OR PI channels above a threshold of 200. For each sample, forward and side-scatter as well as fluorescence in the 610/10, 530/30, and 780/60 nm wavelengths were measured for detection of PI positive, SGI positive, and bead events. At least 10,000 events were collected for each sample, and no compensation was applied to sample measurement. Data analysis was carried out using FlowJo software (FlowJo, Ashland, OR). Electronic gating of events in a density plot of SGI and PI fluorescence channels was used to determine the number of events positive for SGI and negative for PI (membrane-intact cells).

2.3.8 Organic Carbon Analysis

Each quenched sample was filtered through sterile 0.45 μm nylon syringe filters (Fisherbrand) previously washed with 40 mL MilliQ water and 10 mL of sample, and the filtrate was frozen for dissolved organic carbon (DOC) analysis with a Total Organic Carbon Analyzer TOC-V CSH (Shimadzu, Columbia, MD).

500 mL samples were collected into carbon-free amber bottles containing sodium thiosulfate and submitted to Eurofins Eaton Analytical (South Bend, IN) for assimilable organic carbon (AOC) analysis according to Standard Methods 9217B (APHA, 2005). At Eurofins Eaton Analytical, water samples were aliquoted into vials (40 mL each) and pasteurized (70-80 °C for 30 min). *Spirillum* NOX and *Pseudomonas fluorescens* P-17 strains, which have different substrate specificities, were inoculated into each vial at an initial concentration of 500 CFU/mL and then incubated at 25 °C. Their growth was determined on R2A agar plates in duplicate on days 5, 6 and 7 (n = 6 plates per sample). The average growth number of culturing flasks across the three consecutive days was converted into AOC concentration based on the growth yields of the NOX and P-17 strains for acetate (4.1×10^6 and 1.2×10^7 CFU/μg acetate-C for P17 and NOX, respectively). AOC determined by P-17 growth and AOC determined by NOX growth were added and expressed as the total AOC of the samples.

2.3.9 Contactor Shutdown Experiment

Water flow and ozone supply for one contactor were turned off, and water samples were taken before and after shutdown. Samples were collected from all chambers in the contactor before shutdown and from chamber 2 at 2 and 9.45 h after shutdown. Immediately after shutdown, water from chamber 2 was collected into a sterile glass bottle protected from light and kept at room temperature to act as a control. HPC and flow cytometry analyses were carried out on water from sample line 2 to monitor changes in viable cell concentrations following contactor shutdown.

2.3.10 Ozone Contactor Biofilm and Sludge Samples

After the shutdown experiment, the contactor was drained and ozone was allowed to degas from the concrete walls for two weeks. Then, biofilm was swabbed from specific areas (18.8 cm^2 each) on baffle walls from chambers 2-5 in duplicate (duplicate biofilm samples were swabbed next to each other at the same height (about 5 ft) from the floor) and from one diffuser in chamber 3 (n = 9 biofilm samples). Each swab was placed in sterile PBS. Swabs were vortexed for 2 min and sonicated for 2 min to release biomass. The biomass was filtered onto 0.2 µm polycarbonate filters (Millipore) and filters were stored at -80 °C. Sludge from chamber 3 was collected into a sterile Falcon tube (Corning, Corning, NY) and stored on ice for up to one hour before storage at -80 °C.

Total DNA was extracted from biofilm and sludge samples using a modified recipe of the Universal Extraction Lysis Buffer and a phenol-chloroform-isoamyl extraction protocol (Haig et al., 2017; Hill et al., 2015) to improve recovery of difficult-to-lyse bacteria. DNA was also extracted from the sludge using a Fast DNA Spin Kit (MP Biomedicals) to compare yield and diversity estimates with DNA extracted with the phenol-chloroform procedure. DNA was also extracted from reagents used during DNA extraction and from one of the sterile swabs used for sample collection. These controls were processed in parallel with the samples. PCR, sample multiplexing, and Illumina MiSeq sequencing of partial 16S rRNA genes were performed by the Microbial Systems Laboratory at the University of Michigan Medical School (Ann Arbor, Michigan). Barcoded dual-index primers specific to the V4 region of the 16S rRNA gene (Caporaso et al., 2011; Kozich et al., 2013) were used to amplify the DNA. PCR reactions included 5 µL of 4 µM forward and reverse primers, 0.15 µL of AccuPrime Taq DNA High Fidelity Polymerase (Life Technologies), 2 µL of 10x AccuPrime PCR Buffer II (Thermo Fisher Scientific), 1 µL of DNA template, bovine serum albumin (0.5 mg/mL final concentration), and

PCR-grade water. PCR conditions consisted of 2 min at 95 °C followed by 30 cycles of 95 °C for 20 s, 55 °C for 15 s, 72 °C for 5 min, followed by 72 °C for 10 min. Sequencing was done on the Illumina MiSeq platform using a MiSeq Reagent Kit V2 500 cycles according to the manufacturer's instructions with some modifications (Kozich et al., 2013).

16S rRNA gene sequences from all samples were curated using mothur (version 1.39.0) (Schloss et al., 2009). During sequence curation, sequences that were within two nucleotides of each other were merged using the pre.cluster command. After preclustering, 439 sequences in the control samples (putative contaminants) were removed from the 6,429 unique sequences in the dataset using the remove.seqs command (6.8% sequences removed). Curated sequences were clustered into OTUs using a 97% similarity cutoff with the opticlust algorithm (Westcott et al., 2016). Subsampling was performed based on the sample with the lowest number of sequences, resulting in 4,387 paired-end reads per sample. Sequences were classified using a naïve Bayesian classifier (Wang, Q. et al., 2007) trained against a 16S rRNA gene training set provided by the RDP (Cole et al., 2009). An RDP consensus taxonomy was generated for each OTU.

2.3.11 Thermal Gravimetric Analysis (TGA) of Sludge

TGA analysis was performed to estimate the composition of contactor sludge. A sample of the contactor sludge was weighed, dried in an oven at 103 °C for one hour and weighed again to determine water loss. The sediment was subject to TGA in an oxygen atmosphere. Temperature was ramped up from an ambient temperature to 800 °C at a heating rate of 10 °C min^{-1} in a TA Q500 (TA Instruments, New Castle, DE). Continuous online records of weight loss and temperature were used to plot a derivative TGA curve.

2.3.12 Statistical Analyses

Statistical significance was based upon the probability of $p < 0.05$. A Wilcoxon test was used for comparison of cell concentrations. An Analysis of Molecular Variance (AMOVA) was used to test for significant separation of samples on the NMDS plot. A Pearson correlation coefficient was used for the correlation analysis with individual OTUs and shifts in samples along axes in the NMDS plot.

2.4 Results

Because the Ann Arbor DWTP did not operate the same ozone contactors throughout the sampling campaign, water samples were collected from three contactors on different days (Table 2.1). Plant operators change ozone feed gas concentrations and influent flow rates through the contactors daily and this contributed to the variability in ozone CT profiles across the sampling days (Figure 2.1). Therefore, viable cell concentrations are plotted against sample line numbers (1-5) instead of ozone CT. The average cumulative ozone CT was 0.96 ± 0.32 mg-min/L across eight sampling days (Figure 2.1).

2.4.1 Cell Concentrations Across Ozone Contactors

Culturing and culture-independent flow cytometry were used to monitor microbial communities during ozone disinfection in multi-chamber contactors. Figure 2.2 shows viable cell concentrations measured by culturing or culture-independent flow cytometry. Viable cell concentrations were significantly and consistently reduced in chamber 2, the first chamber where ozone contact occurs, corresponding to an ozone exposure of 0.26 ± 0.06 mg-min/L (Figure 2.1). HPC based inactivation monitoring showed 1.88 ± 0.47 log reduction from 1,110 ± 420 CFU/mL before ozonation to 17 ± 19 CFU/mL in chamber 2 (Figure 2.2b). As expected, greater numbers of intact cells were measured using flow cytometry than with HPC. However, a similar log reduction of 1.86 ± 0.19 was observed from 106,000 ± 31,000 intact cells/mL before ozonation to 1,610 ± 730 intact cells/mL in chamber 2 (Figure 2.2a). The levels of culturable and membrane-intact cells increased in the third chamber, even as ozone exposure increased by 0.63 ± 0.17 mg-min/L (Figure 2.1). Specifically, in chamber 3, there was a significant ($p < 0.00001$) increase in the level of culturable cells to 750 ± 350 CFU/mL, and the level of intact cells increased to 2,700 ± 1100 cells/mL ($p = 0.05$). Intact cell concentrations increased further in chamber 4 ($p = 0.05$), but culturable cell concentrations did not increase significantly. In spite of increasing intact cell concentrations in chamber 3, and sometimes chamber 4, ozonation significantly reduced intact cell concentrations overall (1.62 log reduction, $p = 0.0001$), with concentrations ranging from 1,200 to 3,750 intact cells/mL in water from the outlet basin (sample line 5). The levels of culturable bacteria in the outlet basin varied over an order of magnitude, from 200 to 3,850 CFU/mL. In comparison with cell concentrations before ozonation (sample line 1), there was a slight reduction in culturable cells after ozonation (sample line 5) on three sampling days, but there were significant ($p < 0.0001$)

increases in CFU concentrations after ozonation on three other days in one contactor in particular (Figure 2.9). The culturability (i.e., the ratio of HPC to membrane-intact cells) of water samples before ozonation was 0.9 ± 0.4% (mean ± range) but increased 12-fold to 10.4 ± 7.8% after ozonation (Figure 2.10).

2.4.2 Microbial Growth Substrates

Dissolved organic carbon (DOC) levels did not change significantly during ozonation (Figure 2.3). The average concentration of DOC across the five sampling lines was 4,000 ± 300 µg/L (mean ± standard deviation). However, AOC levels increased significantly ($p < 0.0001$) after ozone exposure began in chamber 2. The average AOC level, based on growth of *Spirillum NOX* and *Pseudomonas fluorescens* P-17 strains combined, was 36.2 ± 0.27 µg/L (mean ± range) before ozonation and increased to 115 ± 12 µg/L (mean ± range) after ozonation. The increase in AOC was caused by an increase in growth substrates specific for the *Spirillum* NOX strain, known to grow primarily on carboxylic acids (Van der Kooij et al., 1984).

2.4.3 Cell Concentrations after Contactor Shutdown

A "no flow" experiment was conducted in the contactor to probe biofilm detachment. Under stagnation conditions, the longer contact of bulk water with contactor surfaces provides an opportunity to distinguish between the biofilm detachment processes of erosion and sloughing (Picioreanu et al., 2001; Rittmann, 1989). Cell concentrations were monitored in the stagnant water in a contactor after influent water and ozone gas supply were shut off. Concentrations in the water in chamber 2, the contact chamber where the greatest reduction in viable cell levels occurred (Figure 2.2), were monitored. Immediately after contactor shutdown, we collected water from chamber 2 to act as an experimental control and incubated this sample at room temperature in the dark. The water in the control had the same planktonic cell concentration and growth substrate level as the stagnant water in chamber 2 at the start of the experiment. However, an increase in cell concentrations in the control would be explained only by planktonic cell growth or recovery, not detachment of cells from biofilm or sediments. Residual ozone concentrations dropped to below quantification (0.01 mg/L) after 12 min in chamber 2 (Figure 2.11). The initial CFU and intact cell concentrations were 10 ± 4 CFU/mL and 1,660 ± 110 intact cells/mL, respectively. The CFU concentration in chamber 2 increased significantly 2 h after shutdown to 800 ± 210 CFU/mL, and then increased further 9.5 h after shutdown to 4,800 ± 1,300 CFU/mL (Figure 2.4a). The

concentration of intact cells increased similarly (Figure 2.4b). In contrast, the concentration of culturable cells in the control sample decreased and was below detection after 2 h and remained below detection during the 9.5 h experiment. The levels of membrane-intact cells in the control decreased slightly to 820 ± 30 intact cells/mL at 2 h, and then increased slightly at 9.5 h to 1,710 intact cells/mL (Figure 2.4b).

2.4.4 Taxonomic Classification of Isolates

The colonies on R2A plates generally became smaller and more uniform (mostly white colonies) with increasing ozone exposure (Figure 2.12). Figure 2.5 shows taxonomic classification of 16S rRNA gene sequences from 190 isolates, clustered into 19 OTUs, from one sampling day. OTUs related to *Pseudomonas*, *Blastomonas* and *Limnobacter* spp. predominated among the 13 genera cultured from water before ozone exposure (CT = 0 mg-min/L). The relative abundance of *Pseudomonas* decreased and *Blastomonas* OTUs were not detected after ozone exposure began (CT = 0.30 mg-min/L), whereas the relative abundance of *Limnobacter* increased substantially and was greater than 80% for all samples after ozone exposure began. Other bacteria cultured from water in the outlet basin (sample line 5) were represented by OTUs related to *Hydrogenophaga* or unclassified *Comamonadaceae* spp. Isolates before ozonation belonged to *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*, but isolates after ozonation began were mostly *Betaproteobacteria* and, by the end of ozonation, all cultured populations were *Betaproteobacteria*.

2.4.5 Biofilm and Sludge Samples

High-throughput sequencing of the V4 region of the 16S rRNA gene was used to investigate the bacterial community structure of biofilm samples collected from the walls in chambers 2 through 5 of one ozone contactor. An OTU classified as *Limnobacter* (OTU0003) was present in biofilm sampled from the wall in chamber 2 (1.22% relative abundance), and its relative abundance increased substantially in biofilm on the left wall of chamber 3 (28.8%). This OTU was also present in biofilm on a diffuser in chamber 3 (2.1% relative abundance). However, *Limnobacter* spp. were not detected in biofilm samples on the right wall in Chamber 3, or in biofilm samples from chambers 4 and 5.

An accumulation of sludge near the right wall in chamber 3 was observed and it was expected that this sludge would contain calcium carbonate from the lime-softening process. Indeed, TGA of the

sludge (Figure 2.13) showed significant weight loss occurred between 542 and 830 °C, corresponding to the decarbonation of calcium carbonate (Alarcon-Ruiz et al., 2005). Smaller weight losses occurring at 502 °C and between 183 and 407 °C may have corresponded to dihydroxylation of calcium hydroxide and volatilization of organic content, respectively.

DNA yields from baffle wall biofilm samples after the dead zone were significantly higher than yields from wall biofilm before the dead zone (p = 0.036) (Table 2.3). Furthermore, non-metric multidimensional scaling of Θ_{yc} distance ordinations indicated that the biofilm community structures of samples collected before and after the dead zone clustered separately (Figure 2.6), and that the separation was statistically significant (p = 0.013). In addition, the variation in community structure across the biofilm samples before the dead zone was higher (0.211) than the variation in community structure across samples in locations after the dead zone (0.067). An OTU (OTU0001) classified as *Gp4* in *Acidobacteria* became significantly more abundant in biofilm samples after the dead zone (p = 0.036). The relative abundance of this OTU was significantly correlated with a shift in the biofilm community structure along NMDS axis 1 ($p < 0.001$), shifting from the community structure before the dead zone toward the community structure after the dead zone. In addition, the relative abundance of OTUs classifying as *Mycobacterium* and *Legionella*, two genera that contain species of public health importance as opportunistic pathogens, increased significantly in biofilm samples after the dead zone (p = 0.036 and p = 0.036 for *Mycobacterium* and *Legionella*, respectively) (Figure 2.7). The bacterial community in the sludge that accumulated near the right baffle wall in chamber 3 contained sequences classified as *Gp4* OTU0001 (1.50% relative abundance) as well as *Mycobacterium* and *Legionella* (0.41% and 2.4% relative abundance, respectively) (Figure 2.7). The Shannon diversity indices of biofilms from baffle walls varied between 3.1 and 4.5 (mean of 4.02 ± 0.45, Table 2.4), whereas the Shannon diversity indices of biofilm from a diffuser and from the sludge in chamber 3 were 5.2 and 5.0, respectively. Shannon evenness ranged from 0.58 to 0.86 across all samples, with the sludge bacterial community being the most even.

2.5 Discussion

2.5.1 Viable cell concentrations

The observed 1.62 log reduction of membrane-intact cells in the ozone contactors is consistent with 1-2 log reductions of membrane-intact cells reported for ozonation of partially treated raw

waters in two other full-scale DWTPs (Vital, Dignum, et al., 2012). The relatively high variation in HPC levels (coefficient of variation of 0.79) in water from the outlet basin (sample line 5) combined with the order of magnitude greater culturability of the bacteria in water samples after ozonation, resulted in modest HPC reductions during ozonation in comparison with HPC reductions reported for a pilot-scale ozone contactor column (1.5 log) (Norton et al., 2000) and a baffled ozone reactor treating secondary wastewater effluent (1.4-1.6 log) (Czekalski et al., 2016). An increase in both HPC and intact cell concentrations in chamber 3 indicates that microbial growth occurred there or, through another mechanism, viable microbial cells were introduced to the water. The overall increase in HPC levels after chamber 2 was such that HPC levels exiting a contactor were sometimes higher than levels entering the contactor (Figure 2.9). Similarly, at a DWTP in the Netherlands, levels of heterotrophic bacteria isolated on conventional plate count agar increased after ozonation, although the increase was not observed when R2A agar was used (Vital, Dignum, et al., 2012).

Near 2-log reduction of HPC and intact cell concentrations after the first ozone contact chamber (chamber 2) was observed. A previous investigation of bacterial concentrations at the same plant, which was conducted just three months after ozonation was implemented, reported 2.6 log reduction of HPC determined by culturing on R2A, from 5.43×10^3 CFU/mL in water after softening and settling to 13 CFU/mL in water leaving the ozone contactors (Lee et al., 2000). These results suggest the significant reductions that were originally achieved over the full contactor length have been constrained to the first ozone contact chamber over decades of operating the contactors, in spite of the plant's annual maintenance and cleaning practices. In terms of a multibarrier approach to water treatment, the first contact chamber is still operating as a barrier to the microbial community in the partially treated raw water. Importantly, dynamics in chambers 3-7 are shaping the quantity and community composition of microorganisms in the water exiting the contactors.

2.5.2 Biofilm Detachment

The increase in viable cell concentrations after the first contact chamber cannot be attributed to planktonic cell regrowth considering the short residence time in the contactors. The doubling rates required to produce such an increase would be much shorter than the fastest doubling rates reported for microorganisms (e.g., 9.8 min for *Vibrio natriegens* (Eagon, 1962)). However, biomass

detachment from biofilm on contactor surfaces is possible. The contactors are made from poured concrete. Carbonation or leaching of calcium hydroxide over time would reduce the pH of concrete and thereby produce favorable conditions for biofilm formation. Although the components of concrete are not expected to provide substrate for microbial metabolism, AOC in the raw water, and AOC generated in chamber 2 (Figure 2.3) could provide a continuous supply of growth substrates to wall surfaces. Higher concentrations of AOC may result in higher growth rates of biofilm bacteria (Tsai, 2005) in ozone contactors. More rapidly growing biofilms have a higher detachment rate than slow-growing biofilms (Boe-Hansen et al., 2002; Picioreanu et al., 2001), providing a possible mechanism whereby biomass detachment from ozone contactor surfaces may increase planktonic cell concentrations.

The release of biomass from contactor surfaces was not flow dependent. Biomass can detach from biofilm on contactor surfaces continuously by erosion (Rittmann, 1989) or through discrete detachment events (i.e., sloughing). The causes for erosion and sloughing come from forces created by fluid moving past the biofilm (Picioreanu et al., 2001). The times when viable cell concentrations exiting a contactor were greatest did not correlate with days when the plant was operating at a higher flow rate. Had sloughing from biofilms on contactor surfaces been important, increasing cell concentrations would be expected with increased flow through a contactor. Within 2 h of shutting off water flow and ozone gas supply to one contactor, HPC and intact cell concentrations increased in chamber 2, but not in chamber 2 water stored separately, suggesting that contributions from train surfaces were behind the increasing viable cell concentrations observed during ozonation. This also suggests that planktonic cell recovery or repair of membrane integrity were not important factors, although it is possible that a slower decay of ozone in the control may have resulted in a greater ozone exposure and, subsequently, lower viable cell concentrations in the control at 2 and 9.45 h. The culture-based and culture-independent data taken together suggest that, at least within a 2 h time period, the increase in culturable and intact cells observed during ozonation was not likely a result of planktonic cell recovery, and was more likely caused by detachment from biofilms in contactors. Recent research has shed light on the importance of biofilm in determining planktonic community structure in distribution systems (Liu, S. et al., 2016) and premise plumbing. The importance of biofilm detachment in multichamber ozone contactors designed for plug-flow conditions presents a similar story to the role of biofilm

detachment driving microbial water quality in drinking water distribution systems, which are also designed for plug-flow conditions.

2.5.3 Biofilm community structure

Results from the contactor shutdown experiment, and other evidence discussed above, indicate that biofilm detachment is largely responsible for the microorganisms detected in water samples. The presence of *Limnobacter* spp. in biofilm samples from baffle walls may therefore be one reason why *Limnobacter* was isolated from water samples. This underlines the importance of understanding the factors that shape the microbial community structure of biofilm on contactor surfaces in order to control the microbial populations in ozonated water. The community structure of the biofilm on baffle walls may be shaped by (i) resistance to oxidative stress from ozone exposure, (ii) a succession of bacteria (Ling et al., 2015; Okabe et al., 2007) that are capable of colonizing concrete surfaces, (iii) a preference for AOC generated by ozonation, or (iv) a combination of these factors. A shift to smaller colony sizes after ozone contact in chamber 2 (Figure 2.12) is consistent with the notion that ozone exposure selected for disinfectant-resistant microorganisms as bacteria with lower specific growth rates often exhibit higher resistance to stress (Berney et al., 2006; Mah et al., 2001; Taylor et al., 2000), although this is not always the case (Berry et al., 2009). After ozonation there was an enrichment for *Limnobacter* spp., which have also been detected in biofilms from water distribution networks (Liu, R. et al., 2014; Wang, H. et al., 2012) and premise plumbing (Feazel et al., 2009). The differential resistance of *Limnobacter* to ozone disinfection is unclear since ozone inactivation kinetics for *Limnobacter* spp. have not been reported. However, in a recent study, *Limnobacter* increased in relative abundance significantly (to 2.1%) in surface water three days after disinfection with photocatalytic ozonation (Becerra-Castro et al., 2016). Another possible reason for the prevalence of *Limnobacter* is the selection for this genus on the concrete surfaces of the ozone contactors. *Limnobacter* spp. have the ability to grow chemolithoheterotrophically by oxidation of thiosulfate to sulfate (Spring et al., 2001). Heterotrophic and autotrophic sulfur-oxidizing bacteria are involved in microbially induced concrete corrosion (Grengg et al., 2015; Ling et al., 2015; Okabe et al., 2007; Santo Domingo et al., 2011) and microbial succession on concrete usually results in biofilm dominated by thiosulfate-oxidizing bacterial species. In the partially treated source water at the Ann Arbor plant, the primary source of sulfur is expected to be sulfate but thiosulfate may also be present. The enrichment for *Limnobacter* may have been related to a selection for *Limnobacter* on the

concrete surfaces in particular, although other canonical thiosulfate-oxidizing bacteria (*Thiobacillus* spp.) typically associated with microbially induced concrete corrosion (Grengg et al., 2015; Okabe et al., 2007; Parker, 1945; Santo Domingo et al., 2011) were not detected in biofilm samples. *Limnobacter* was expected to be abundant in all biofilm samples based on its predominance among isolates from contactor water samples (Figure 2.5), but the community structure of biofilm samples changed significantly on baffle walls past a dead zone in chamber 3 (discussed below).

2.5.4 Dead zone effects

The accumulation of sediments in chamber 3 was expected because the width of this chamber (i.e., the distance between consecutive baffle walls) is twice the width of chamber 2. As the water velocity through chamber 3 decreased by half the velocity in chamber 2, more sediments would be deposited in chamber 3. The ozone contactors were designed with one chamber of a greater width to provide for additional disinfectant contact time. However, a greater width in chamber 3 is also expected to increase internal recirculation inside the chamber and cause more areas with insufficient mixing (dead zones) (Kim et al., 2009). The accumulation of sludge observed near the right baffle wall in chamber 3 was in a location predicted to be a dead zone by modeling of flow through multichamber contactors (Kim et al., 2009).

The increase in viable cell concentrations in chamber 3 can be attributed in part to sediment accumulation there, caused by the non-ideal hydraulic conditions. Furthermore, the significantly different community structure in biofilm past the dead zone raises the possibility that microorganisms in sludge in the dead zone seeded biofilms downstream. Lime softening precipitates would be expected to have a high pH that may not be considered favorable for microbial growth, but the diversity of the total bacterial community in sludge from the dead zone was similar or greater than Shannon diversity estimates for activated sludge samples (0.98-3.53) (Boon et al., 2002; Kraigher et al., 2008), but lower than diversity estimates for biomass from full-scale biologically-active filters used for drinking water production (4.4 to 4.9 (mean, 4.7; SD 0.2)) (LaPara et al., 2015). Even though microorganisms present in the sludge may not all be viable, the potential for sludge in dead zones to harbor microorganisms that may present a public health risk needs to be evaluated further. The presence of *Mycobacterium* and *Legionella* OTUs in sludge, and an increase in their relative abundance on baffle walls past the dead zone, suggest that adverse

microbial effects of dead zones are another argument for improving hydraulic efficiency in multichamber contactors by decreasing chamber width, in spite of greater energy requirements (Zhang, J. et al., 2013) to drive the flow over and under more baffle walls. *Mycobacterium* and *Legionella* OTUs were detected in biofilm samples before the dead zone, albeit at much lower relative abundances, suggesting that these groups can resist oxidative stress in biofilms on contactor surfaces. In an earlier study performed shortly after the plant began ozonating, *Mycobacterium* spp. were not detected in raw water or after softening and settling, but *Mycobacterium* was the most common group and comprised 31% of bacterial isolates identified after ozonation (Lee et al., 2000). This suggests that disinfectant-resistant opportunistic pathogens may be enriched by ozonation. This finding could have been related to an increase in culturability after ozonation, as discussed above.

Chamber width is an important feature for plants that still have flexibility in contactor design, such as those plants converting to ozonation. More plants are expected to convert to ozonation combined with biofiltration to achieve removal of several emerging contaminants of public health concern in the water industry (Blackbeard et al., 2016; Gerrity et al., 2011; Hollender et al., 2009; Huber et al., 2005; Reungoat et al., 2012; Rosal et al., 2010). As retrofitting ozone contactors is not an option for many existing DWTPs, other options need to be considered to improve ozone disinfection efficiency. A strategy that increases ozone doses to avoid biofilm formation may be constrained by health risks posed by increasing concentrations of ozonation byproducts such as bromate (von Gunten et al., 1996) and nitrosamines (Gerrity et al., 2015). Better pretreatment before ozonation is an obvious way to reduce the amount of sediments entering ozone contactors and thereby improve ozone disinfection efficiency.

Our findings additionally suggest implications for post-ozonation processes. Ozonation is typically followed by biologically-active filtration to reduce microbial growth substrates that are produced by ozone treatment. Given their demonstrated selection for bacterial groups that resist disinfectants on ozone contactor surfaces and detach into the bulk water, ozone contactors may serve as an important seed source of these microorganisms to downstream biofilters. Biofilm control strategies in biofilters should avoid reinforcing selective pressure for disinfectant-resistant microbial populations that are seeded by ozonation to reduce levels of disinfectant-resistant, opportunistic pathogens in filters and thereby lower the likelihood of seeding the distribution networks (Pinto et al., 2012) with these microbes.

2.6 Figures and tables

Table 2.1 Overview of sampling dates, contactors sampled, mean flow rate on the day of sampling, water temperature, plenum pH, sample type collected, and methods used. Plenum pH is the pH of the water in the plenum area before entering the ozone contactors. Methods used were heterotrophic plate counts (HPC), flow cytometry (FCM), dissolved organic carbon (DOC) and assimilable organic carbon (AOC) analyses. Water flow and ozone gas supply was shut down on Oct 19, 2016, and biofilm and sludge samples were collected on November 2, 2016.

Sampling Date	Contactor Number	Mean flow rate (MGD)	Temperature (°F)	Plenum pH	Sample Type	Method
Dec. 15, 2015	3	6.8	48.7	8.4 ± 0.05	Water from lines 1-5	DOC, AOC
Apr. 22, 2016	3	6.5	56.8	7.6 ± 0.06	Water from lines 1-5	HPC
May 5, 2016	3	4.8	55.4	7.6 ± 0.01	Water from lines 1-5	HPC
May 9, 2016	3	6.5	59.7	7.7 ± 0.02	Water from lines 1-5	HPC
May 13, 2016	3	6.5	59.4	7.6 ± 0.01	Water from lines 1-5	HPC
May 23, 2016	3	7.6	63.7	7.7 ± 0.04	Water from lines 1-5	AOC
Sept 24, 2016	4	9.6	71.0	8.0 ± 0.01	Water from lines 1-5	FCM
Sept 25, 2016	4	8.6	69.8	8.0 ± 0.01	Water from lines 1-5	FCM
Oct 4, 2016	4	7.7	62.8	8.0 ± 0.01	Water from lines 1-5	HPC and FCM
Oct 19, 2016	2	7.7	61.6	7.7 ± 0.004	Water from lines 1-5	HPC and FCM
Oct 19, 2016	2	NA	NA	NA	Water from line 2 after contactor shutdown	HPC and FCM
Nov 2, 2016	2	NA	NA	NA	Biofilm and sludge	16S rRNA gene sequencing

Table 2.2 Method for determining average ozone concentration in each chamber

Chamber number	Sample Line	Chamber purpose	Direction of water flow to ozone gas	Equation for average ozone concentration
1	1	Inlet	No ozone gas added	None
2	2	Ozone contact	Counter-current	$C_0/2$
3	3	Ozone contact	Co-current	$(C_0 + C_{eff})/2$
4	4	Reaction	No ozone gas added	$\frac{C_0}{kt}(1 - e^{-kt})$
5-7		Reaction	No ozone gas added	$\frac{C_0}{kt}(1 - e^{-kt})$
Outlet basin	5	Reaction (rarely)	No ozone gas added	$\frac{C_0}{kt}(1 - e^{-kt})$

Where

C_0 is chamber influent ozone concentration
C_{eff} is chamber effluent ozone concentration
k is ozone decay constant
t is contact time

A k value was calculated that would result in an effluent concentration in chamber 4 within 1% of the measured residual. Ozone was rarely detected above 0.01 mg/L at sample line 5, but when it was greater than 0.01 mg/L, an average ozone concentration was calculated to estimate ozone exposure in the outlet basin.

Table 2.3 DNA extraction yields for biofilm and sludge samples. The second sludge sample did not amplify and so it was not included in the DNA sequencing.

Sample location	Sample Type	Wet weight (mg)	DNA yield (ng/cm^2)	DNA (ng/mg wet weight)
Chamber 2_1	Biofilm	58.1	0.02	0.01
Chamber 2_2	Biofilm	36.2	1.1	0.60
Chamber 3 left	Biofilm	21.0	0.70	0.60
Chamber 3 right_1	Biofilm	12.2	53.6	82.6
Chamber 3 right_2	Biofilm	22.3	66.4	56.0
Chamber 4_1	Biofilm	19.5	57.5	55.4
Chamber 4_2	Biofilm	30.7	106.8	65.4
Chamber 5	Biofilm	20.0	23.3	21.9
Chamber 3 diffuser	Biofilm	79.4		3.8
Chamber 3_1	Sludge	1119.4		0.04
Chamber 3_2	Sludge	850.07		0.05

Table 2.4 Summary of diversity and evenness estimates for biofilm and sludge samples. Number of sequences and observed OTUs for the dataset before and after subsampling, predicted OTUs (via Chao1 estimator), and Shannon diversity index and evenness for bacterial communities in biofilm and sludge samples from one contactor, as measured by Illumina MiSeq analysis of partial 16S rRNA gene sequences.

Sample Location	Number seqs	Observed OTUs	Subsampled seqs	Observed OTUs from subsampled ± SD	Chao1 from subsampled ± SE	Shannon index from subsampled ± SD	Shannon evenness from subsampled ± SD
Chamber 2	10,499	275	4,387	233 ± 4.8	279.9 ± 15.5	3.9 ± 0.02	0.72 ± 0.003
Chamber 2	9,680	236	4,387	202.2 ± 4.7	246.1 ± 15.1	3.7 ± 0.02	0.70 ± 0.004
Chamber 3 left	11,095	249	4,387	198.8 ± 5.4	254.5 ± 18	3.1 ± 0.02	0.58 ± 0.004
Chamber 3 right	16,904	391	4,387	296.5 ± 6.8	378.6 ± 24.3	4.5 ± 0.02	0.78 ± 0.003
Chamber 3 right	21,907	451	4,387	309.1 ± 7.8	415.1 ± 29.5	4.4 ± 0.02	0.76 ± 0.004
Chamber 4	14,988	369	4,387	284.9 ± 6.3	358.5 ± 22.0	4.2 ± 0.02	0.74 ± 0.004
Chamber 4	14,583	365	4,387	281 ± 6.5	359.2 ± 22.4	4.1 ± 0.02	0.73 ± 0.004
Chamber 5	4,387	234	4,387	234	241.5 ± 0	4.3	0.78
Chamber 3 Diffuser	12,174	847	4,387	633.3 ± 10.4	874.7 ± 39.7	5.2 ± 0.02	0.80 ± 0.003
Chamber 3 Sludge (FastSpin)	10,376	590	4,387	508.3 ± 6.9	595.8 ± 19.4	5.2 ± 0.02	0.83 ± 0.003
Chamber 3 Sludge	4,671	343	4,387	340.9 ± 1.4	354.5 ± 3.5	5.0 ± 0.01	0.86 ± 0.001

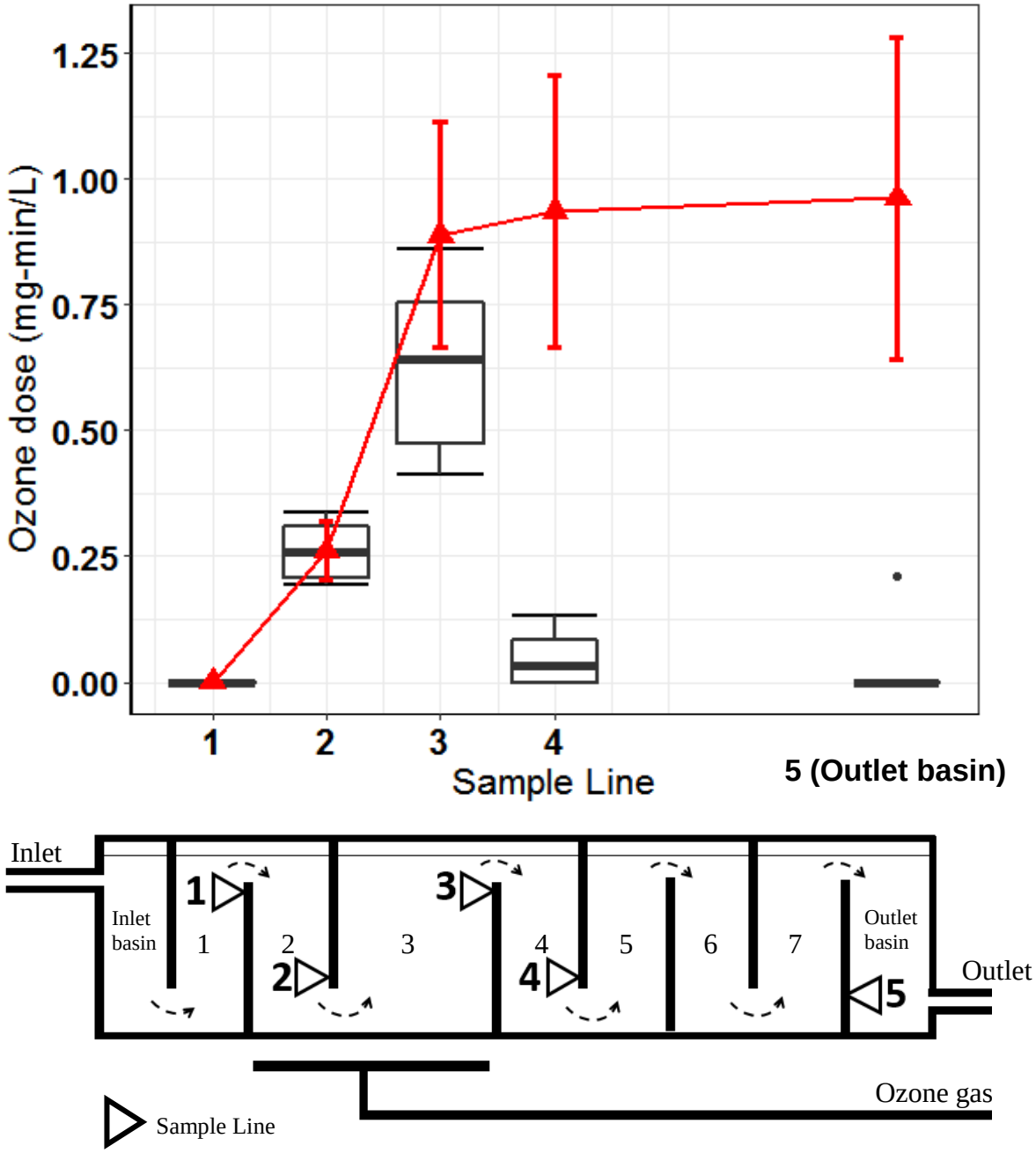


Figure 2.1 Ozone CT (average concentration × contact time) for each chamber across contactors sampled on eight days. Cumulative ozone exposure is represented by the red symbols (mean values measured on eight days; error bars represent standard deviations). Water from an inlet basin flows through seven chambers and then into an outlet basin. Ozone gas is added into chamber 2 and 3. The cumulative residence time in a contactor is approximately 10 min. The width of chamber 3 is larger than the widths of other chambers to achieve additional disinfectant contact time.

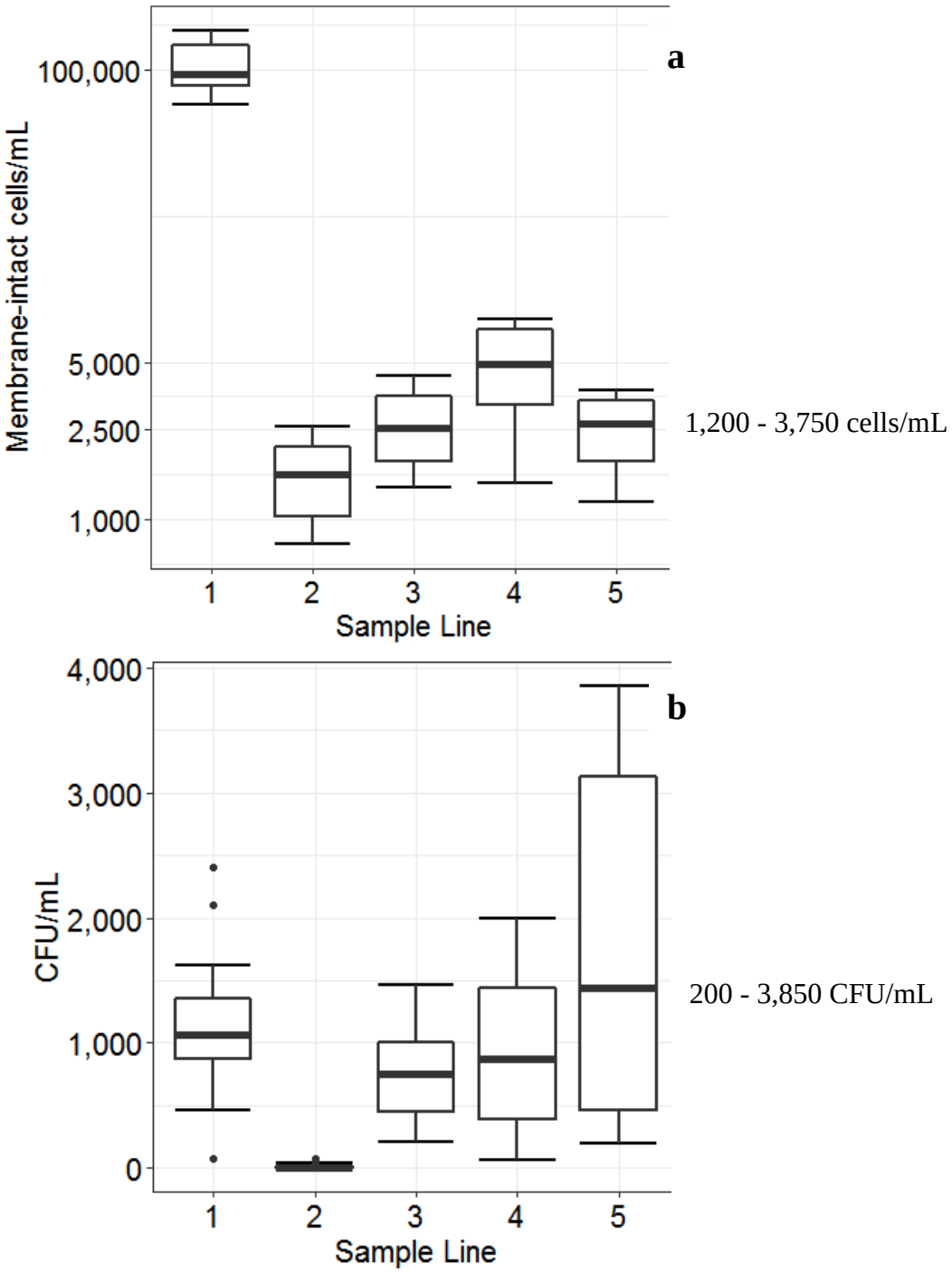


Figure 2.2 Box and whisker plots representing viable cell concentrations of lime-softened water (sample line 1) and in different chambers during ozone exposure monitored by (a) flow cytometry on four days, and (b) HPC on six days. HPC data are reported as the average of duplicate dilutions plated in triplicate (n = 6 counts per sample except for chamber 1 and chamber 4 samples collected on May 13, 2016, which had only 5 and 4 counts, respectively). Error bars represent the standard deviation of all data points. The numbers on the right side indicate the range of cell concentrations at sample line 5.

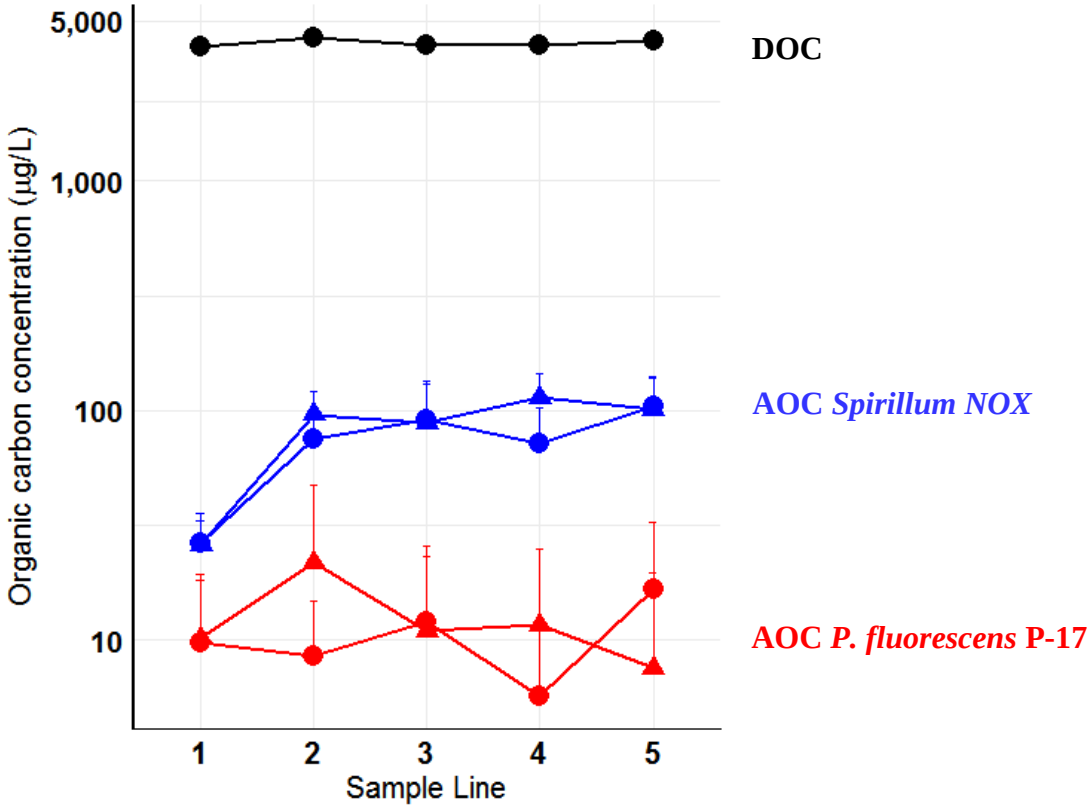


Figure 2.3 Organic carbon concentrations across an ozone contactor on two days. For dissolved organic carbon (DOC) analysis, which was measured only on one day (December 15 2015, black circles), water was filtered through 0.45 µm filters. Samples for assimilable organic carbon (AOC) analysis were collected on December 15, 2015 (circles) and May 23, 2016 (triangles), and mean AOC for *Spirillum NOX* and *Pseudomonas fluorescens* P-17 bacterial strains are reported with standard deviation.

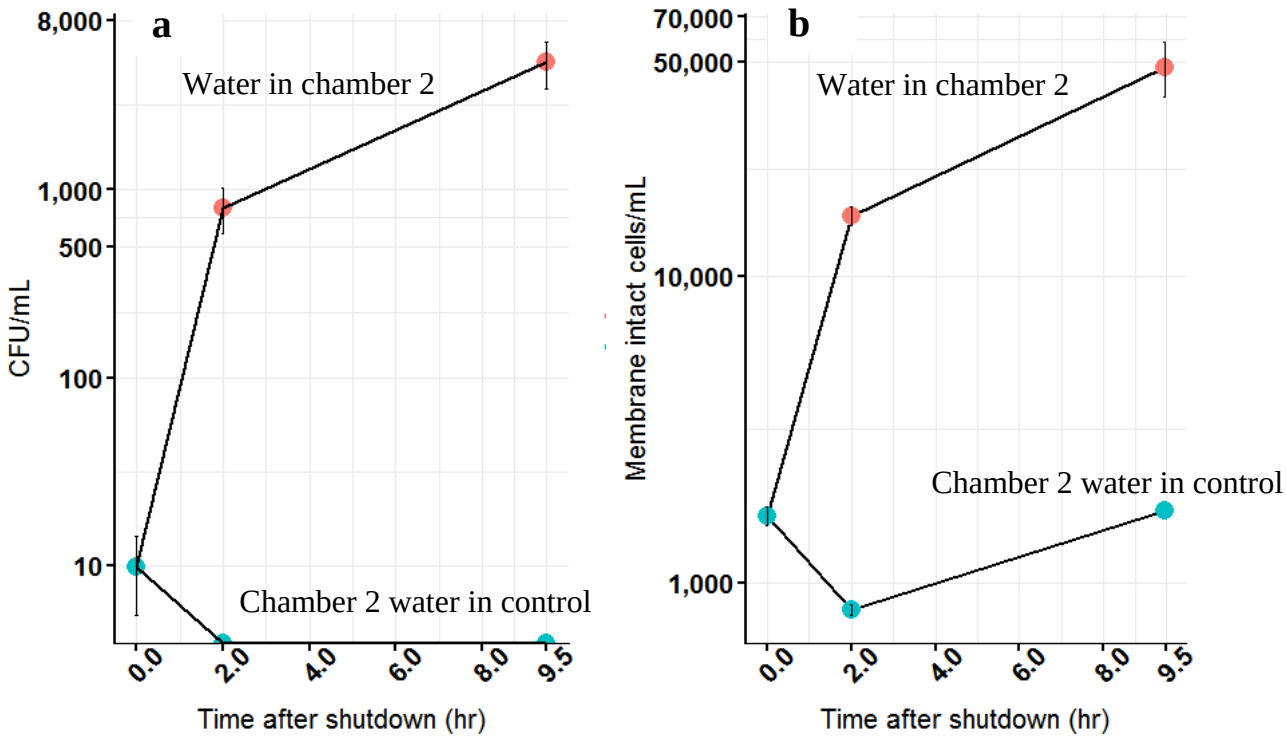


Figure 2.4 Concentrations of viable cells monitored by HPC (a) or flow cytometry (b) before and after train shutdown in water sampled from chamber 2 (pink points) and water sampled from chamber 2 stored separately as a control (blue points).

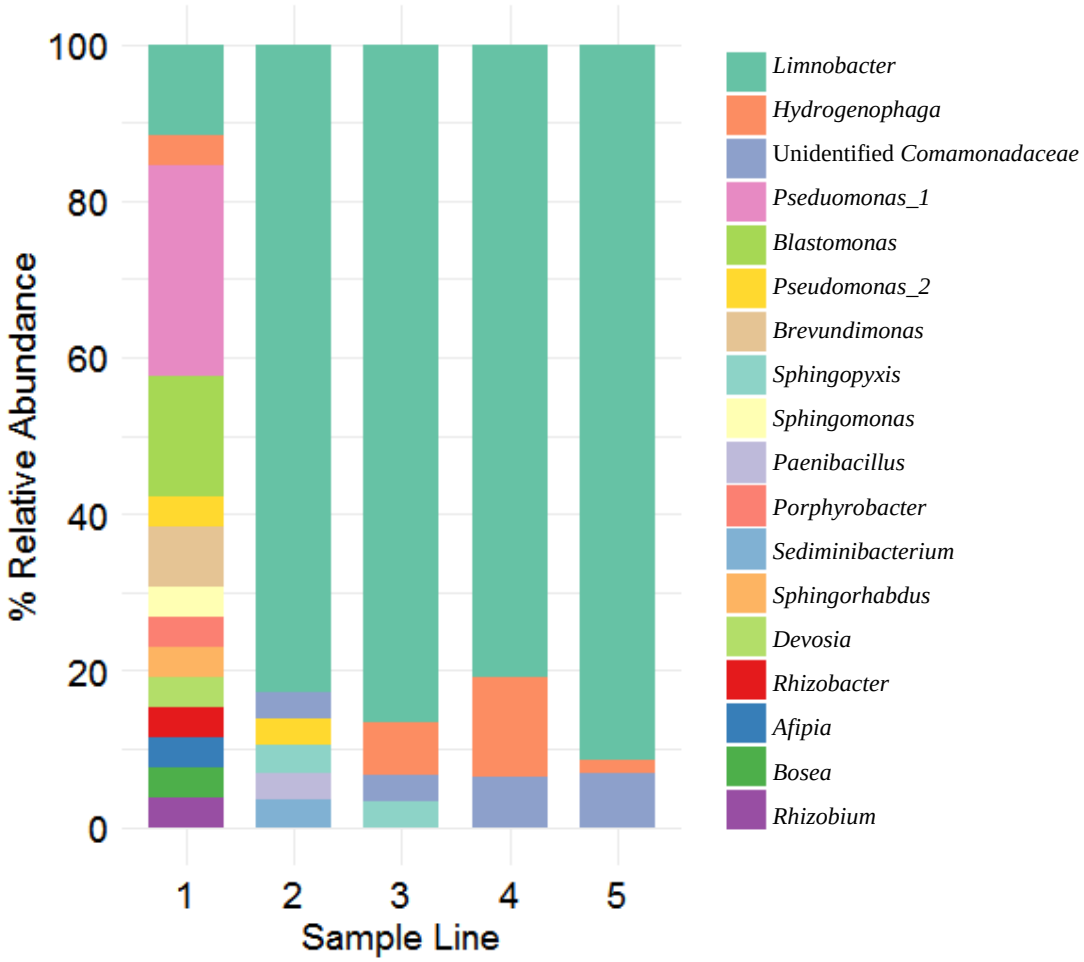


Figure 2.5 Relative abundance of cultured OTUs on November 2, 2016. The cumulative ozone exposure for samples lines 1-5 was 0.0, 0.30, 1.04, 1.12 and 1.12 mg-min/L, respectively.

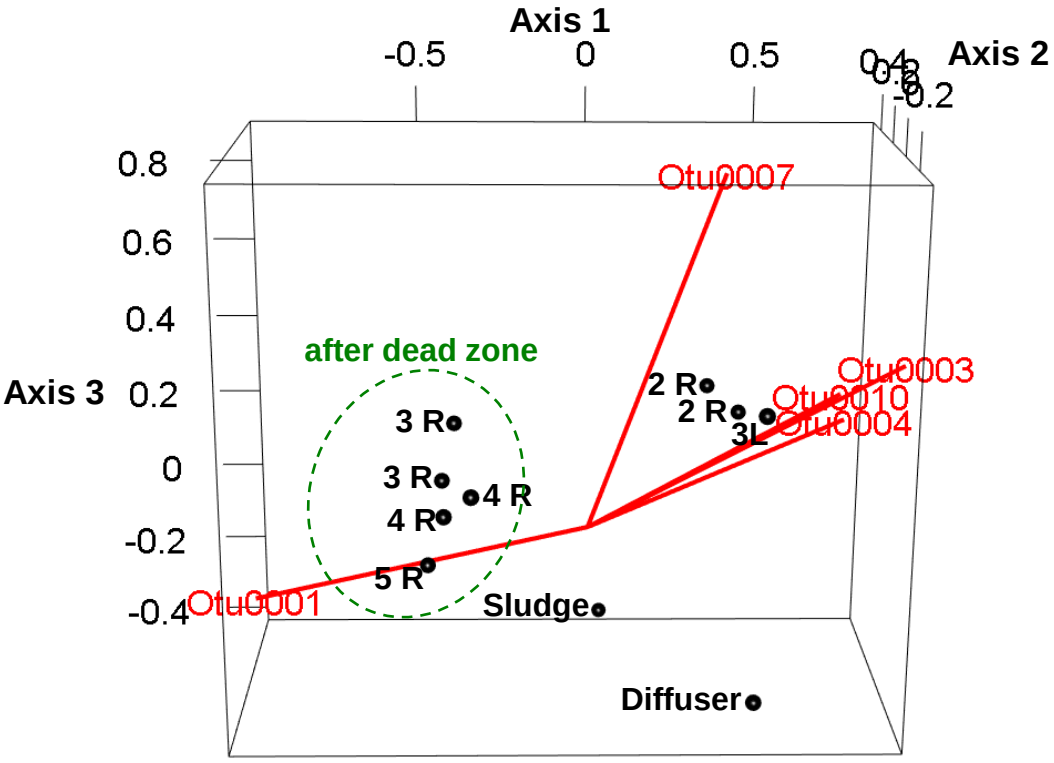


Figure 2.6 Non-metric multidimensional scaling (NMDS) plot of Θ_{yc} distance ordinations showing differences in community structure for total bacteria in biofilm and sludge samples. Biofilm samples were collected from chambers 2-5 on right or left baffle walls (indicated by R or L). Of the top ten most abundant OTUs across the samples, the OTUs with relative abundances that increased or decreased significantly ($p < 0.01$) along NMDS axis 1 (OTU0001, OTU0003, OTU0004 and OTU0010) or NMDS axis 3 (OTU0007) are shown in red. OTU0001, OTU0003, OTU0004, OTU0007 and OTU0010 were classified as *Gp4*, *Limnobacter*, *Methylophilus*, *Herminiimonas* and *Brevundimonas* populations, respectively.

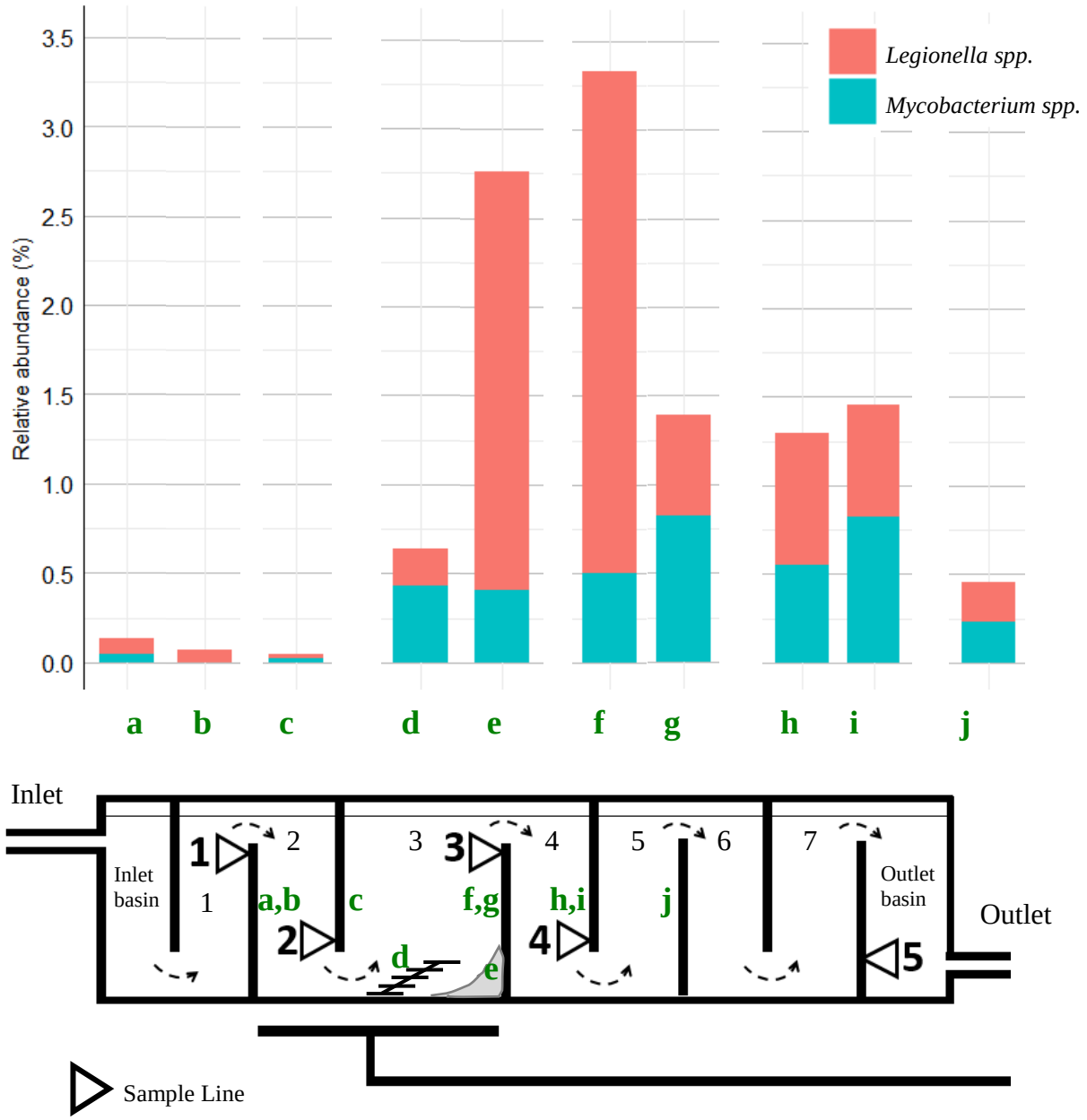


Figure 2.7 Relative abundance of 20 *Legionella* OTUs (red) and 4 *Mycobacterium* OTUs (blue) in biofilm and sludge samples.

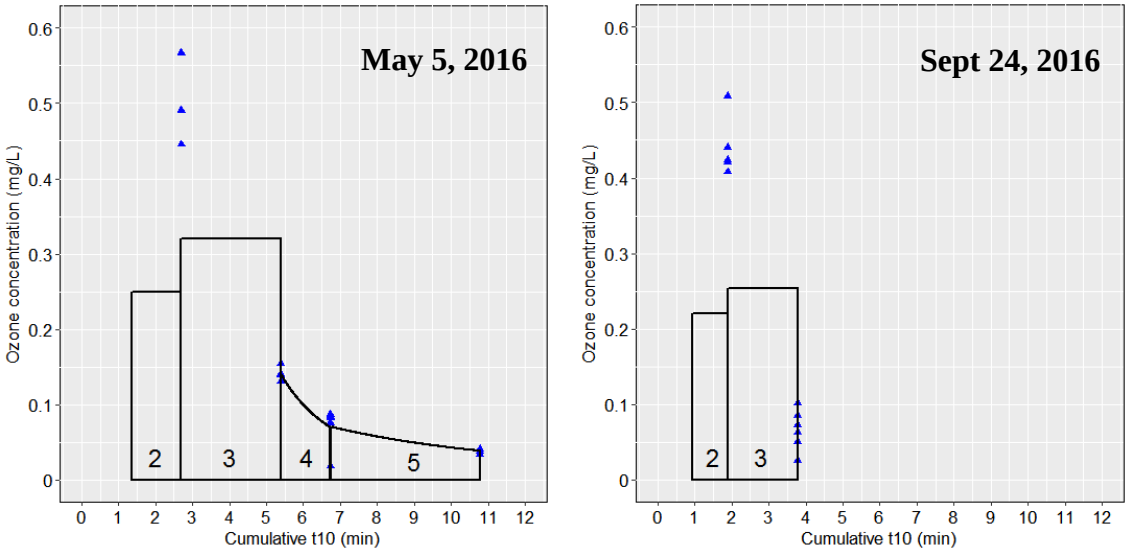


Figure 2.8 Ozone CT profile examples for two days. Average ozone concentration for each chamber determined as described above (Table S3.1). As an example, the *k* values for chamber 4 and chambers 5-7 were 1.21 min^{-1} and 0.34 min^{-1}, respectively, on May 5, 2016. The flow rate through each ozone contactor is the sum of the source waters and recycle flow divided across two contactors in service. The flow rate to the ozone building varies and is driven by consumer demand. If the mean flow rate over a sampling period was less than 6.3 million gallons per day, a baffle factor (T10/T) of 0.5 was used. If it was higher than 6.3 MGD, a baffle factor of 0.7 was used to account for short-circuiting. These factors are based on a tracer study the plant performed after construction of their ozone contactors. The area under curves of average ozone concentration plotted against T10 values for each chamber was integrated to determine ozone exposure (concentration × time) for each chamber. The blue triangles show residual ozone concentrations.

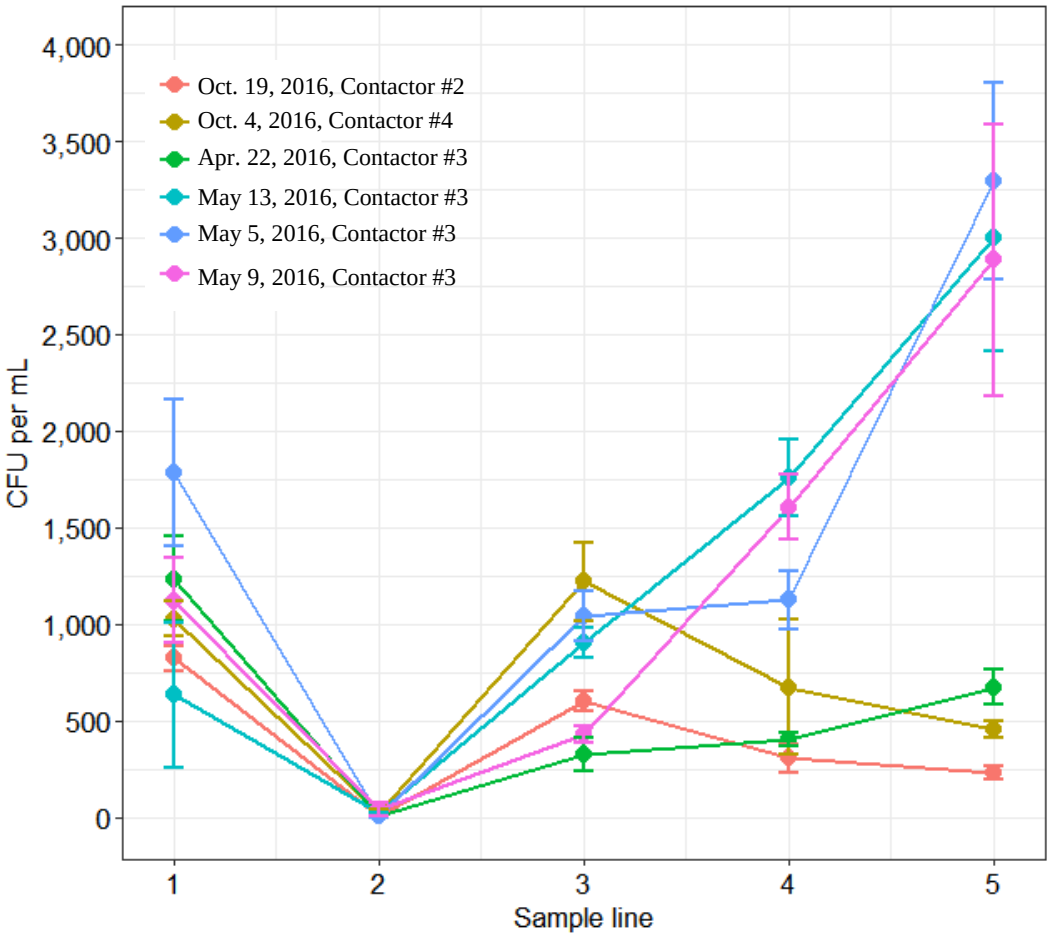


Figure 2.9 HPC concentrations for six sampling days in different chambers during ozone exposure.

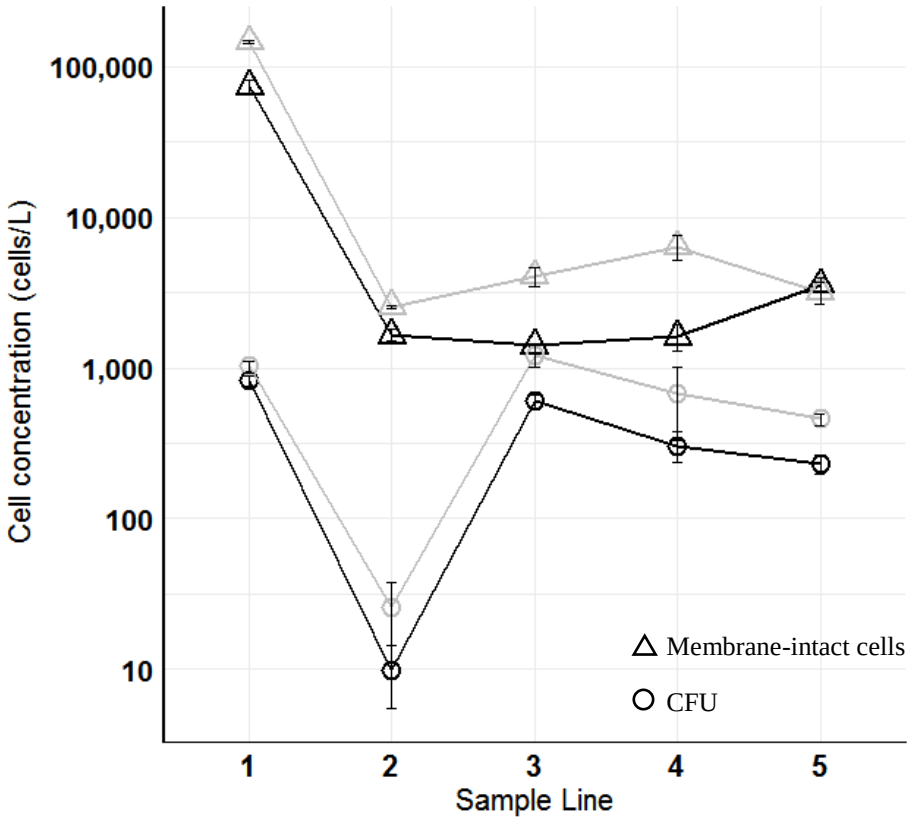


Figure 2.10 HPC and membrane intact cell concentrations on two sampling days. Culturing and flow cytometry methods were applied to water samples collected on October 4, 2016 (grey) and October 19, 2016 (black).

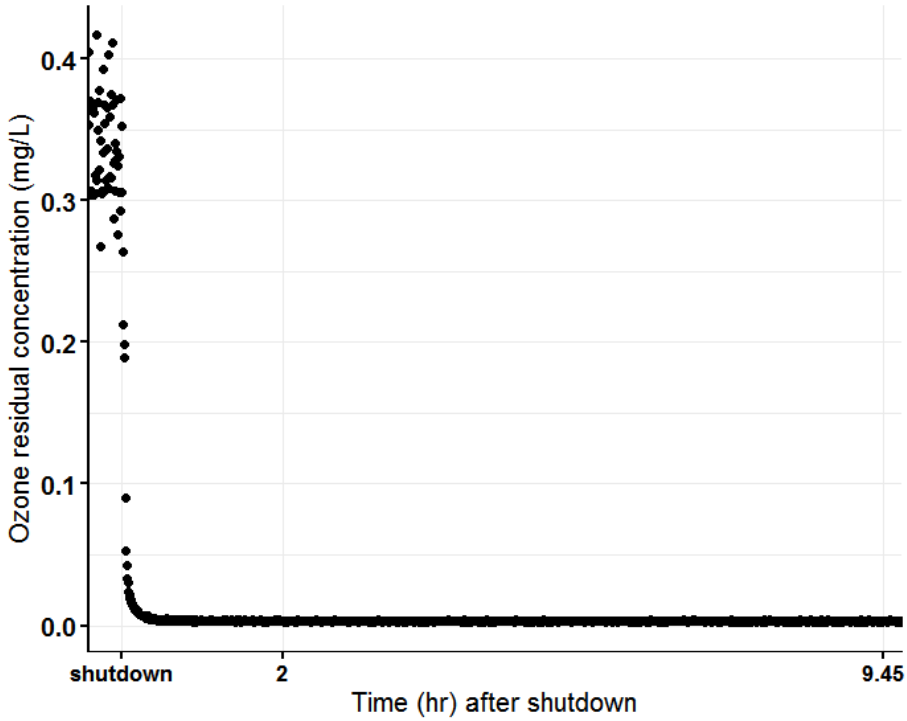
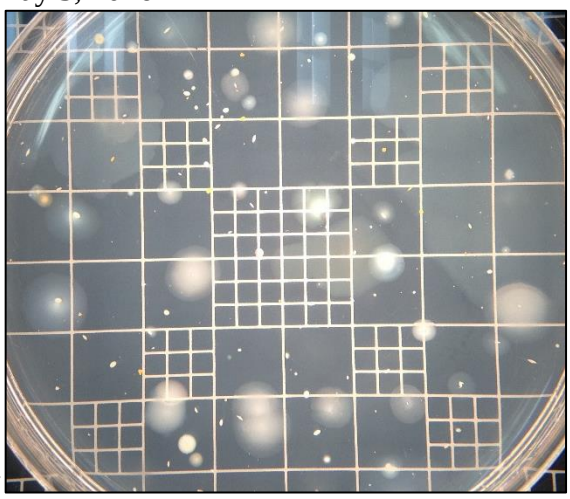
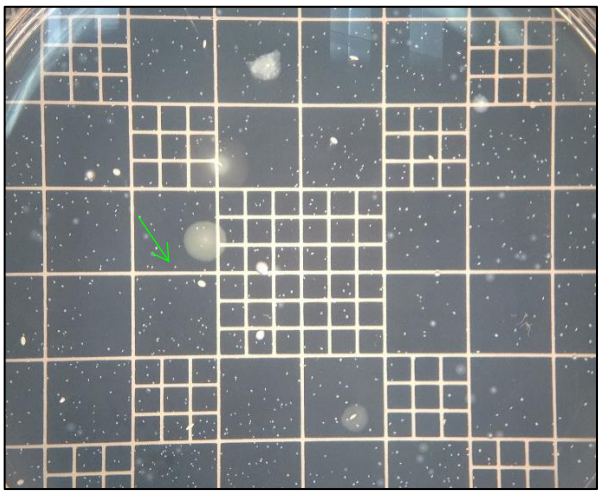


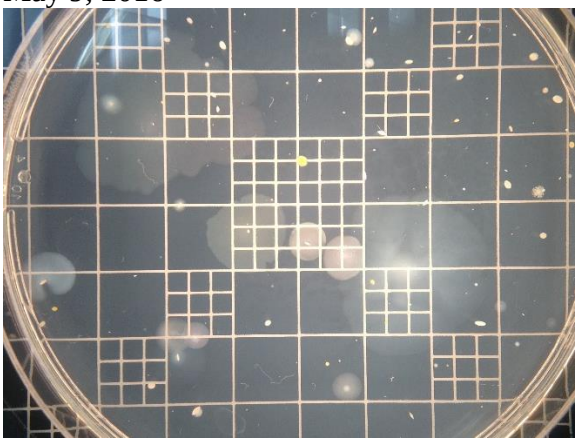
Figure 2.11 Residual ozone concentrations after contactor shutdown. HPC and flow cytometry methods were applied to water samples collected from stagnant chamber 2 at 2 and 9.45 h after shutdown.

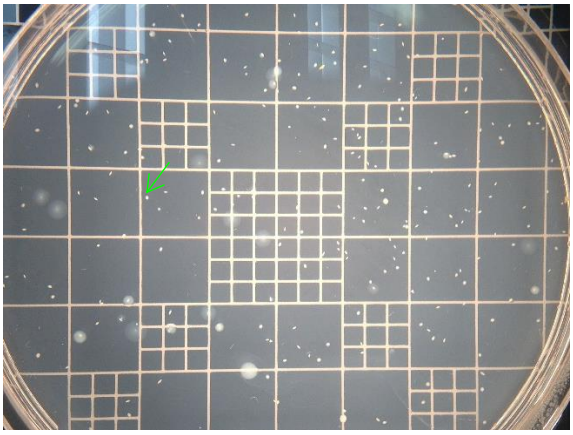
May 5, 2016



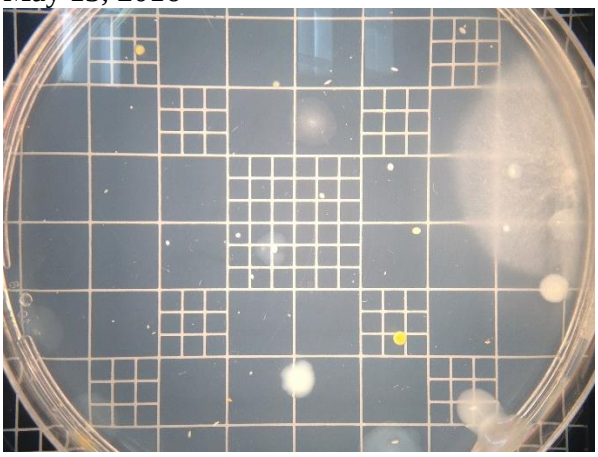


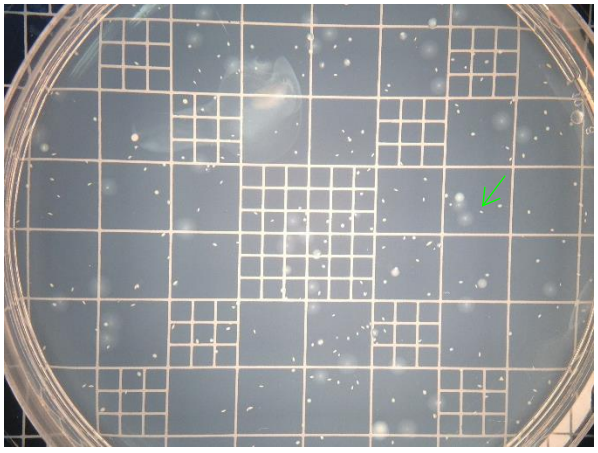
May 9, 2016



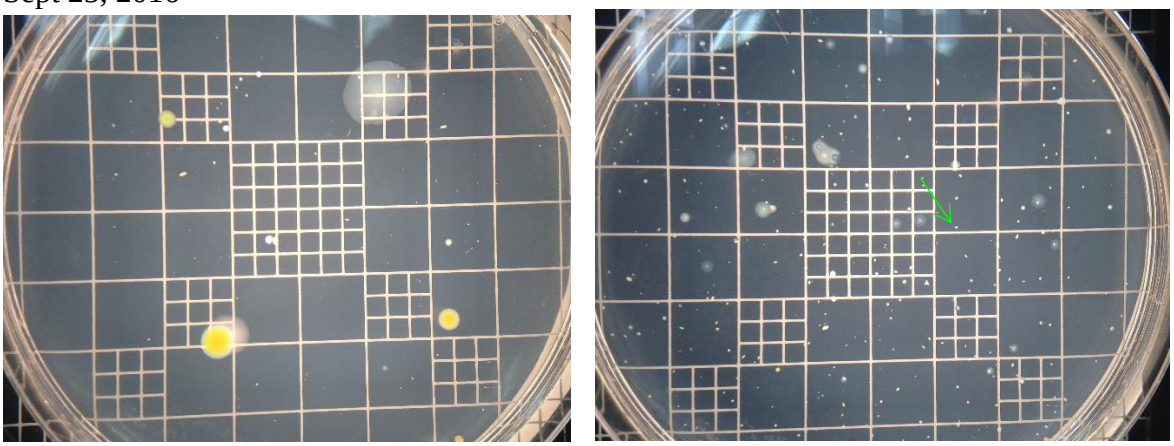


May 13, 2016

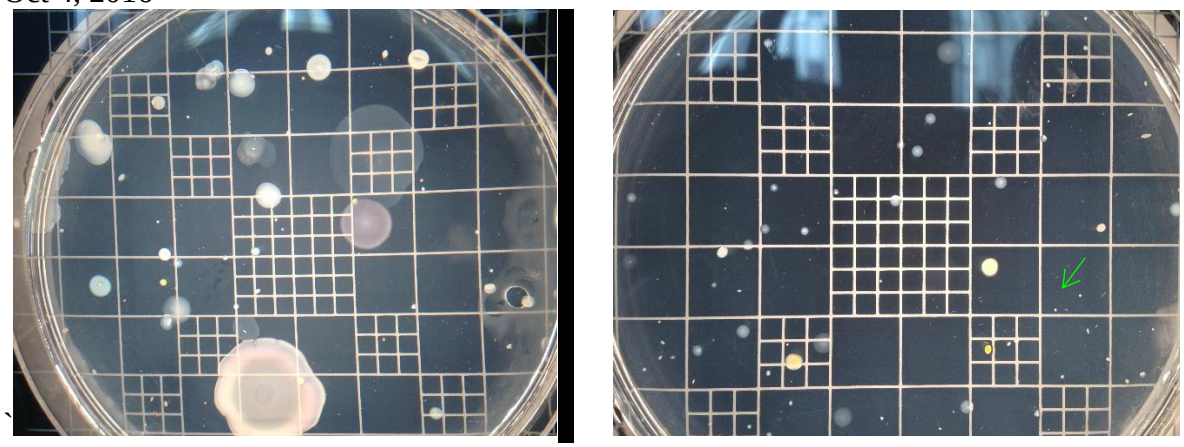




Sept 25, 2016



Oct 4, 2016



Oct 19, 2016

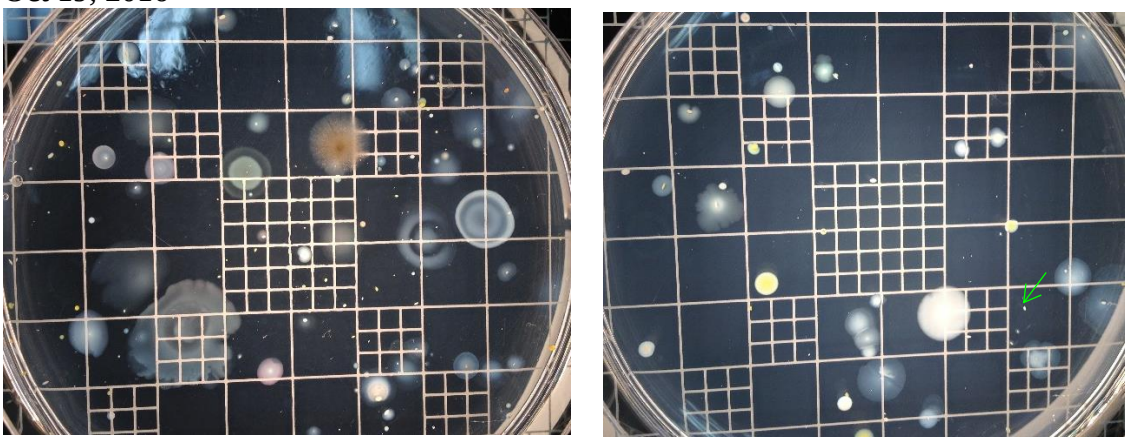


Figure 2.12 Colony morphology of R2A isolates before (left) and after (right) ozonation. Water was collected from sample lines 1 and 5, corresponding to inlet and outlet basins of an ozone contactor. Green arrows point to smaller white colonies, which became more prevalent after ozonation.

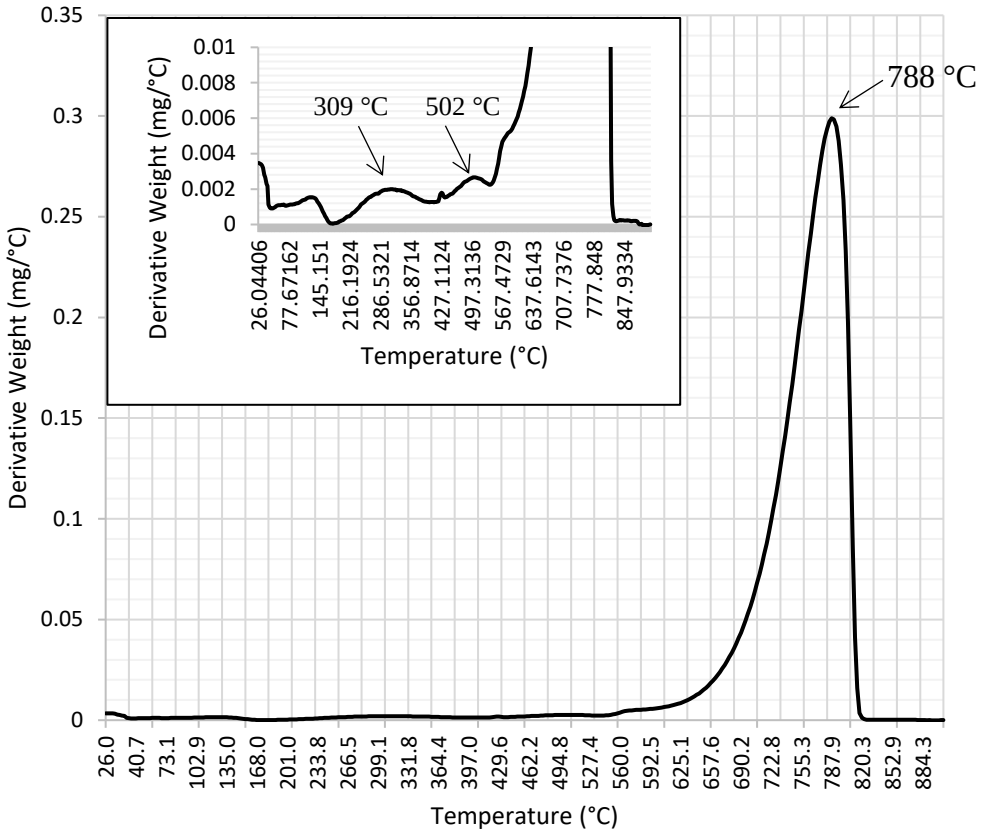


Figure 2.13 Derivative thermal gravimetric analysis (TGA) of sludge collected from an ozone contactor

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Chapter 3

Mycobacterium avium Upregulate Intracellular Parasitism Genes in Response to the Drinking Water Disinfectant Monochloramine

Note: *Mycobacterium avium* 104 and monochloramine challenge experiments were performed by David M. Berry during his PhD research at University of Michigan. Methods and results for Tables 3.1 and 3.2 and Figures 3.2 have been reprinted here with his permission.

3.1 Abstract

Mycobacterium avium is an opportunistic human pathogen capable of causing lung infections in individuals with underlying lung disease and disseminated infection in immune-compromised individuals. *M. avium* infections have been linked to exposure to drinking water. Its persistence in drinking water systems has been attributed to higher CTs (concentration × time) required for inactivation of *M. avium*, but it's response to sub-lethal disinfectant exposures has not been studied. Global transcriptional profiling of *M. avium* cells exposed to a sub-lethal concentration of monochloramine revealed that *M. avium* cells upregulate virulence factors associated with intracellular parasitism. To verify these results with an environmental microbial community, relative expression of *M. avium*'s mammalian cell entry genes in biomass from a biological activated carbon (BAC) filter was monitored during backwashing with water containing monochloramine. During BAC filter backwashing, mammalian cell entry gene *mce1C* relative expression (normalized by mycobacterial *atpE* gene level) increased substantially over 10 min, indicating that *M. avium* in the filter responded to monochloramine exposure during backwashing. *M. avium*'s response to monochloramine raises the possibility of an adaptive response that increases its resistance to disinfection and persistence in distribution systems.

3.2 Introduction

Mycobacterium avium, a member of the non-tuberculous mycobacteria (NTM) group, is an opportunistic human pathogen found in soil, surface waters and in treated drinking water (Falkinham, 2015; King et al., 2016). *M. avium* has been implicated or is suspected to be a causative agent in a variety of clinical diseases including hypersensitivity pneumonitis, cervical lymphadenitis, skin and soft tissue infections, pulmonary disease and disseminated infection.

While *M. avium* infections are of particular concern for immunocompromised individuals such as AIDS patients (Glover et al., 1994; Vonreyn et al., 1994) and individuals with predisposing conditions (LiPuma, 2010), they also occur in individuals without recognized immune dysfunction (Field et al., 2004). *M. avium* can be transmitted to humans directly from the environment through inhalation or ingestion pathways. The ingestion pathway occurs primarily through the use and consumption of contaminated drinking water, while inhalation can occur from exposure to aerosols generated from a variety of sources, including from metalworking fluids in industry, indoor swimming pools, hot tubs, showers, and water-damaged buildings (Falkinham et al., 2008; Park et al., 2016; Rickman et al., 2002; Thomson et al., 2013).

Conventional drinking water treatment has limited efficacy in removing mycobacteria such as *M. avium*. *M. avium* is resistant to inactivation with a broad array of water disinfectants, including free chlorine, chloramines, chlorine dioxide, ozone, and UV (Luh et al., 2008; Shin et al., 2008; Taylor et al., 2000; Vicuña-Reyes et al., 2008). In addition, *M. avium* can resist phagocytosis and replicate inside amoebae also present in water systems (Berry et al., 2010; Berry et al., 2006; Delafont et al., 2013; Delafont et al., 2014). Intracellular replication affords *M. avium* better protection from disinfection (Berry et al., 2010) and it may increase *M. avium* pathogenicity as the interactions with amoebae closely resemble the pathogenesis of *M. avium* infections in human macrophage cells (Danelishvili et al., 2007; Salah et al., 2009; Tenant et al., 2006). At seven of out of 25 drinking water treatment plants sampled across the United States (King et al., 2016), mycobacteria were detected in finished waters even when not detected in source waters, suggesting that water treatment may select for mycobacteria.

In this study, we characterized the response of *M. avium* to a sub-lethal dose of the commonly used drinking water disinfectant monochloramine. While the resistance of *M. avium* to disinfection is well studied (Luh et al., 2008; Shin et al., 2008; Taylor et al., 2000; Vicuña-Reyes et al., 2008), its response mechanisms are not. In lab-scale experiments, monochloramine exposure induced virulence genes associated with intracellular parasitism. To verify these results with an environmental microbial community, we monitored expression of *M. avium*'s mammalian cell entry genes in biomass from a biological activated carbon (BAC) filter during backwashing with water containing monochloramine.

3.3 Materials and methods

3.3.1 *M. avium* 104 and monochloramine challenge

Mycobacterium avium 104, an isolate from an AIDS patient(Bermudez et al., 1997), was provided by Gerard Cangelosi (Seattle Biomedical Research Institute, Seattle, Washington). *M. avium* was cultured on Middlebrook 7H9 broth supplemented with 10% albumin-dextrosecatalase (ADC) enrichment and 0.2% glycerol (Sigma-Aldrich, St. Louis, MO) and cells were harvested at an OD600 of 0.4-0.5 for all experiments.

Monochloramine was prepared and quantified as described previously (Berry et al., 2009). *M. avium* was exposed to a dose of 0.5 mg/L (as Cl2) monochloramine for several time durations at a temperature of 20 °C. All exposure conditions tested were previously determined to be sub-inhibitory (Luh et al., 2008). Monochloramine was quenched by the addition of 0.12% sodium thiosulfate (Sigma-Aldrich, St. Louis, MO).

3.3.2 Full-scale filter description and backwashing procedure

The Ann Arbor drinking water treatment plant provides lime softening, coagulation, flocculation, sedimentation, ozonation, filtration, and chloramination. The plant has 26 dual media filters that consist of 16-20 inches of GAC (Filtrasorb 300, Calgon Carbon, effective size 0.8 – 1.0 mm) on 6 inches of sand. The filter beds are supported by gravel, garnet or Leopold integral media support (IMS) caps at the bottom. Filter area is approximately 460 ft^2. At any given time, 7-10 filters are operated at 1.9-2.1 MGD flow through each filter resulting in an empty bed contact time (EBCT) of 3-6 min. Every five years, the GAC in four filters is replaced. Filter bed ages can reach up to 25,000 h of total run time before the GAC media is replaced.

Filters are backwashed after 72-96 h of operation with finished water containing 3 mg/L monochloramine as Cl_2, and a pH of 9.3. Before backwashing, the influent and effluent flow through a filter is shut off. The water in the filter is drained to the troughs (approximately 2 ft above the filter bed). The first step of backwashing is a surface wash to dislodge solids from the top of the bed. Then, backwash supply is started at 5 MGD to fluidize the media. The backwash supply flowrate is increased to 10 MGD and sometimes to 12 MGD for the remainder of the backwashing. The surface scrubbers and backwash flow are stopped once turbidity at the top of the filter bed is approximately 10 NTU, which typically takes 10-15 min. Filter bed expansion

during backwashing is approximately 40%. The backwash waste is stored in an equalization basin and then recycled to the head of the plant by combining it with source water.

3.3.3 Environmental sample collection

For a sampling event in August 2015, grab samples were collected from the top of a filter bed during backwashing at one minute intervals. The grab samples were divided for chemical and biological analyses. Water samples (approximately 3 L) were also collected from the surface water, reservoir, and filter influent and filter effluent before backwashing. Biomass samples were divided for RNA and DNA extractions separately (discussed in Molecular Methods) and filtered onto 0.2 µm polycarbonate filters (Millipore). The filters were stored in nuclease-free microcentrifuge tubes and then snap frozen on dry ice. All biomass samples were collected, filtered and stored on dry ice within 1 h. We later began using a sample line and peristaltic pump for continuous sample collection during backwashing (Figure 3.1). A sample line was dropped down into the top of a filter bed before backwashing and the line was flushed before sample collection. Once surface wash was started, a peristaltic pump began collecting water at approximately 6 mL/sec into plastic bottles (approximately 280 mL of sample per bottle). No chlorine demand was observed from the sampling line or from storage in the plastic bottles for up to 1 h (data not shown). The delay through the sampling line, from the point of sample collection until sample loading into a bottle, was 21.5 ± 10 s and this was subtracted from the sample times. In general, 12-19 samples were collected during each backwashing event.

3.3.4 Turbidity, total chlorine and fluoride measurements

Turbidity at the top of each filter bed was measured continuously by online Hach meters (Model SC200) at the water treatment plant. The maximum value that can be recorded by the meter is 2000 NTU. Signals are sent from the meters to Supervisory Control and Data Acquisition (SCADA) software. Turbidity data for each backwashing event was extracted manually.

Effluent and influent monochloramine and fluoride concentrations were measured during backwashing. Fluoride concentrations were measured as a conservative tracer. Effluent concentrations were measured at the top of filter beds. Influent concentrations were measured in water from the reservoir. Monochloramine concentrations were determined using the N, N, diethyl-p-phenylenediamine (DPD) titrimetric method according to Standard Methods 4500-Cl G(APHA, 2005) and are reported in units of mg/L as Cl_2. Fluoride concentrations were determined

using an Orion Star A214 pH/ISE Benchtop Meter (Thermo Scientific) according to Standard Methods 4500-F^- C(APHA, 2005). Normalized concentrations of monochloramine and fluoride were calculated as

$$\frac{C_e - C_{min}}{C_0 - C_{min}} \quad (1)$$

where

C_e is the effluent concentration

C_{min} is the minimum measured concentration

C_0 is the influent concentration

3.3.5 Molecular methods for lab-scale experiments

RNA was extracted using a low-pH, hot-phenol chloroform extraction method as follows. Low pH, hot-phenol:chloroform (65 ºC, pH 4.5, with IAA 125:24:1) (Ambion, Austin, TX) was added to mycobacterial cells suspended in PBS in RNase-free polypropylene tubes containing 0.5 g of 0.1 mm diameter zirconia/silica beads (BioSpec Products, Inc., Bartlesville, OK) and incubated for 5 min at 65 ºC with periodic mixing. Cells were homogenized for 2 min (Mini-Beadbeater-96, BioSpec Products, Inc., Bartlesville, OK) and incubated for 5 min at 65 ºC with periodic mixing. The tubes were centrifuged at 16,000 x *g* for 20 min at 4 ºC and the aqueous phase was transferred to a 2 ml phase-lock-gel tube (PLG Heavy, 5 PRIME Inc., Gaithersburg, MD). An equal part of the phenol:chloroform mixture was added, mixed, and centrifuged at 14,000 x *g* for 5 min at RT. The aqueous phase was transferred to another phase lock gel tube and an equal part of a chloroform:isoamyl alcohol (24:1) mixture was added, mixed, and centrifuged at 14,000 x *g* for 5 min at RT. The aqueous phase was transferred to a new tube and nucleic acids were precipitated by addition of an equal part isopropanol and a 2 h incubation at -20 ºC. Nucleic

acids were pelleted (16,000 x *g* for 30 min at 4 ºC), the supernatant was decanted, and nucleic acids were re-suspended in 90 µl RNase-free water. DNA was digested using 8 U DNase (TURBO DNA-*free* Kit, Ambion, Austin, TX) and a 30 min incubation at 37 ºC. Following DNase inactivation, RNA solution was transferred to a fresh tube and purity was spectrophotometrically determined using 260 nm/280 nm absorbance ratios (Nanodrop ND-1000, Nanodrop

Technologies, Wilmington, DE) and using a PCR control to ensure no residual DNA. RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

An expression array for *Mycobacterium avium* 104 was designed by Roche Nimblegen, Inc. using the completed genome in the NCBI database (NC_008595). The array design featured 19 different 60-mer oligonucleotide probes targeting each gene, with an overall coverage of 5,103 out of 5,120 coding sequence (CDS) regions in the genome. Four replicates of each probe were spotted on each array. cDNA was labeled with Cy-3 and hybridized to microarrays using a Nimblegen Hybridization system according to the Nimblegen protocol and scanned with a GenePix 4000B scanner. Fluorescence intensity data was extracted using NimbleScan software (Nimblegen).

Microarray data were processed using SNOMAD tools and executed in R (Colantuoni et al., 2002). Data were normalized using global mean normalization and local mean normalization to account for spatial heterogeneity on the microarray slide. Probe-level signals were combined to give a log-transformed fold-change followed by a local mean normalization to account for signal intensity bias. Corrected fold-changes for each probe were combined at the gene target level to yield fold-change, standard deviation, and p-value for each gene. Statistical significance of p-values was corrected for multiple comparisons by implementing a false discovery rate (FDR) correction (Benjamini et al., 1995). Genes were considered differentially expressed if they had an absolute fold-change of ≥ 2 and were significant at the FDR = 0.01 level.

Four upregulated genes from the microarray were selected for verification with qRT-PCR. These genes were selected because they were the most highly induced virulence-associated genes in the microarray data for which primers could be successfully designed. For qRT-PCR analyses, cDNA was synthesized from purified RNA using the Verso 2-Step qRT-PCR kit (Thermo Fisher Scientific Inc., Waltham, MA) according to the manufacturer's instructions. For microarray analyses, 10 μg RNA was reverse transcribed to double stranded cDNA using 1 μl random hexamer primers using the Superscript Double-Stranded cDNA Synthesis Kit (Invitrogen, Carlesbad, CA) according to the manufacturer's instructions. Purified cDNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and was shipped on dry ice to Roche Nimblegen (Madison, WI) for labeling and microarray hybridizations.

SYBR Green based qRT-PCR was performed using 10 ng cDNA template and 200 nM specific primers (Table 3.1) and the Verso 2-Step QRT-PCR kit (Thermo Fisher Scientific Inc., Waltham,

MA) according to the manufacturer's instructions. The thermocycler program comprised an initial enzyme activation step of 95°C for 15 min followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s. Reactions were performed in technical duplicate and biological triplicate with a Mastercycler ep *realplex2* (Eppendorf AG, Hamburg, Germany). The threshold cycle values were normalized to levels of *rplL* (ribosomal protein L7/L12, 50S rRNA) and *rpsE* (30S rRNA protein 5) in each sample and then expression fold-changes between treated and control cells were calculated using the ΔΔ*CT* method (Nolan et al., 2006). PCR primers were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and checked for specificity for the target gene in the *M. avium* genome using the BLAST algorithm (NCBI).

3.3.6 Molecular methods for environmental samples

A phenol-chloroform procedure described previously (Haig et al., 2017) was used for DNA or RNA extractions. Phenol-chloroform at pH 8.0 or pH 4.5 was used for DNA and RNA extractions, respectively. DNA and RNA yields were determined using a Qubit Fluorometer (Life Technologies, USA). Contaminating DNA in RNA extracts was removed using a DNA-*free*™ DNA Removal Kit (Life Technologies) by diluting extracts 2x and adding 2 µL of rDNase I enzyme (1 µL was added for the first 30 min incubation at 37 °C and then µL was added for another 30 min incubation).

Quantitative PCR (qPCR) assays targeting the ATP synthase subunit C (atpE) gene in *Mycobacterium* species and the V3 region of the 16S rRNA gene in Bacteria were used to quantify total mycobacteria and Bacteria, respectively (Table 3.1). PCR products of the atpE gene for *Mycobacterium abscessus* ATCC 19977 and PCR products of the 16S rRNA gene for *Pseudomonas aeruginosa* were used for qPCR standards. For quantitative reverse transcriptase-PCR (qRT-PCR) analyses, cDNA was synthesized from purified RNA using SuperScript® VILO cDNA synthesis kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). qRT-PCR for mammalian cell entry gene *mce1C* in mycobacteria was performed. *mce1C* products from *M. avium* were used for qRT-PCR standards.

The Mastercycler Realplex Ep (Eppendorf, Hamburg, Germany) was used to perform qPCR and RT-qPCR with triplicate wells for each sample. Each 10 µl reaction contained 5 µL of Fast EvaGreen qPCR Master Mix (Biotium, Hayward, CA, USA), 0.5 µl of each primer (0.5 µM; IDT, Coralville, IA, USA), 1.2 µL Ultrapure Bovine Serum Albumin (BSA) (0.6 mg/mL final conc.)

(Life Technologies, Carlsbad, CA), 1.8 µL water and 1 µL of template. qPCR conditions consisted of 5 min at 95 °C followed by 40 cycles of 95 °C for 3 s, 61.8 °C for 30 s, 60 °C for 30 s and then melt curve analysis. For all qPCR assays, there was a linear relationship between the log of the DNA copy number in standards and the calculated threshold cycle value across the specified concentration range ($R^2 > 0.99$).

A clone library was created for partial mycobacterial *rpoB* genes from surface water, BAC, filter effluent and filter backwash samples using the TOPO TA kit (Invitrogen, Carlsbad, CA) following manufacturer's instruction. Clones were screened using amplified ribosomal DNA restriction analysis with the restriction enzyme HAEIII (Promega, UK). Representative clones of the different restriction analysis patterns were submitted for Sanger Sequencing at the University of Michigan DNA Sequencing Core (Ann Arbor, MI). Forward and reverse reads were assembled into contigs using SeqMan Pro software (DNA Star, Inc) and chimeric sequences were checked for using Bellerophon (Huber et al., 2004).

3.4 Results and discussion

3.4.1 Genetic response of *M. avium* 104 to monochloramine

Global transcriptional profiling of *M. avium* exposed to 0.5 mg/L (as Cl_2) monochloramine for 10 min was determined using microarray technology in order to identify monochloramine-responsive genes. The microarray study identified 34 genes with at least two-fold differential expression (all upregulated). Several of the upregulated genes, or homologues in other mycobacteria, have been associated with oxidative stress response in other studies (listed in Table 3.2). Table 3.2 demonstrates that many of the upregulated oxidative stress-responsive genes are virulence-associated genes or have homologues in other mycobacteria that are virulence-associated. Additionally, several upregulated genes that are not implicated in oxidative stress response are also virulence-associated. The microarray results were consistent with the observation of virulence factor induction using qRT-PCR (Figure 3.2). In another microarray study, exposure of *Staphylococcus aureus* to free chlorine induced virulence genes (Chang et al., 2007).

The results in the present study add weight to the possibility that disinfection processes may induce virulence responses in bacterial pathogens. More specifically, many of the upregulated virulence genes have been associated with the ability to enter and replicate in host cells. For example, *mce1C* is part of the mammalian cell entry operon *mce1* which is expressed by *Mycobacterium*

tuberculosis upon phagocytosis by macrophages (Graham et al., 1999). In addition, elongation factor Tu was shown to be exposed on the surface of *M. avium* subsp. *hominissuis* during early infection of macrophages, and elongation factor Tu was induced by *M. avium* subsp. *hominissuis*, *M. avium* strain 905, and *M. bovis* during macrophage infection (Brunori et al., 2004; McNamara et al., 2012; Monahan et al., 2001). Recent research demonstrated that elongation factor Tu can bind fibronectin, raising the possibility that this factor is involved in fibronectin-mediated attachment of *M. avium* subsp. *paratuberculosis* to host cells (Viale et al., 2014). Furthermore, several PE/PPE proteins were found to be involved in mycobacterial growth in macrophages (Li et al., 2005; Sampson, 2011).

3.4.2 Monochloramine exposure during BAC filter backwashing

Concentrations of mycobacteria in BAC filter effluent were substantially higher than in filter influent, based on qPCR targeting the mycobacterial *atpE* gene (Figure 3.3). Furthermore, *Mycobacterium avium* dominated the mycobacterial community in filter effluent although it was not detected in BAC (Figure 3.4). The utility routinely performs filter backwashing with finished water containing 3 mg/L (as Cl_2) monochloramine. Turbidity levels at the top of filter beds before backwashing were 11 ± 7 NTU and the levels increased two orders of magnitude to 2000 NTU or greater within a few minutes from the start of backwashing (Figure 3.5). Turbidity levels dropped from the peak more gradually, from 2000 NTU to 9 ± 4 NTU by the end of backwashing. The turbidity profiles suggest a rapid dislodgement of particulates initially, followed by a more gradual dislodgement until backwashing was stopped. A similar profile was observed with mycobacterial concentrations in filter backwash (Figure 3.6).

Monochloramine concentrations were reduced after flow through BAC filter beds in all filters (Figure 3.7). Effluent monochloramine concentrations usually reached a plateau prior to the end of backwashing and the final concentration averaged 1.7 ± 0.59 mg/L across the filters. These levels are higher than concentrations previously shown to be sub-lethal for *M. avium* in laboratory inactivation experiments (i.e., 0.5 mg/L as Cl_2) (Luh et al., 2008) but *M. avium* grown in drinking water is more resistant to inactivation than *M. avium* cultured on medium (Taylor et al., 2000) so the higher concentrations we observed may still be sub-inhibitory for some populations. The persistence of monochloramine at measurable levels was expected, as it is less reactive than other disinfectants such as chlorine. In addition, the high velocity, advective flows used for backwashing

will limit the reaction of monochloramine with BAC surfaces. Fluoride concentrations in filter backwash generally approached reservoir concentrations (Figure 3.8) so the observed reduction in monochloramine concentrations was not caused by poor hydraulics.

qRT-PCR for the *M. avium mce1C* gene showed that *mce1C* relative expression (normalized by *atpE* gene level) increased in filter backwash effluent after 4 min and was 15-fold higher over 8 min of backwashing than levels before backwashing (Figure 3.9). Either the upregulation observed reflects a stress response of mycobacteria in the reservoir water used for backwashing, or it reflects the stress response of filter biofilm bacteria exposed to monochloramine during backwashing. Since *mce1C* expression was not detected in reservoir water (data not shown), the data supports a mechanism where short exposure to disinfectant during backwashing induces a stress response from mycobacteria attached to filter media.

3.4.3 Public health significance

M. avium's response to monochloramine during backwashing raises the possibility of an adaptive response that may increase its resistance to disinfection and persistence in drinking water networks. The upregulation of intracellular parasitism genes suggests that disinfection resistance may involve replicating in amoebae, as intracellular *M. avium* are better protected from harsh environments. An important question that remains to be answered is whether *M. avium*'s response to monochloramine increases its pathogenicity. Where possible, water treatment plants should limit the use of backwash water with disinfectants to reduce selection for disinfectant-resistant bacteria in their filters. Studying *M. avium*'s response to monochloramine from batch experiments to a full-scale drinking water filter opens new perspectives for water treatment science and ways to advance public health.

3.5 Figures and tables

Table 3.1 Primers used in this study

Gene	Primer	Oligonucleotide Sequence (5'-3')	Reference
EF-Tu	MAV_0417 forward MAV_0417 reverse	GATCACGGCAAATCGACTC AGCCCAAATCGATGGTCAG	This study
rpfA	MAV_0996 forward MAV_0996 reverse	GGCGAATGGGATCAGGTAG GTGTTGATGCCCCAGTTG	This study
oxyR	MAV_2838 forward MAV_2838 reverse	GGATGGCACTGGGTGACTAC CCGTAGGTGTTGAGGGACAG	(Geier et al., 2008)
ahpC	MAV_2839 forward MAV_2839 reverse	AGCACGAGGACCTCAAGAAC GTGACCGAGACGAACTGGAT	(Geier et al., 2008)
ahpD	MAV_2840 forward MAV_2840 reverse	GTACGCCAAGGATCTCAAGC GTACTTGCCGTCCAAGAAGC	(Geier et al., 2008)
MAV_4349 (PPE family)	MAV_4349 forward MAV_4349 reverse	GTTGGGTTCGGTTCGAAAG GTTGGGGTGACTTGCTTTTC	This study
rpsE	MAV_4448 forward MAV_4448 reverse	GATGGCGACAAGAGCAACTAC CTTGGAGACTCGGTTGATGG	This study
rplL	MAV_4507 forward MAV_4507 reverse	CAAAGATGTCCACCGACGAC AGCAGGGTCATCTCCTTGAAC	This study
mce1C	MAV_5013 forward MAV_5013 reverse	GATCAAGACCGACACCATCC AGAACGCGTCGTAAATCTGG	This study
atpE	atpE forward atpE reverse	CGGYGCCGGTATCGGYGA CGAAGACGAACARSGCCAT	(Radomski et al., 2013)
rpoB	rpoB forward rpoB reverse	GATGAGGTGCTGGCAGA AYTTGATGGTCARCAGYTCC	(Haig et al., 2017; Macheras et al., 2011)

Table 3.2 *M. avium* genes upregulated after 10 min exposure to 0.5 mg/L (as Cl_2) monochloramine, detected with microarray and/or qRT-PCR. Evidence that the gene or a homologous protein in another *Mycobacterium* strain is involved in oxidative stress or virulence is also indicated. If no gene annotation was available in NCBI, the annotation of a homologous protein was substituted when possible. N.S. indicates non-significant differential gene expression.

Locus	Microarray fold-chang e	qRT-PCR fold-change	Oxidative Stress	Virulence	Annotation	References
MAV_0019	2.2 ± 1.2				Serine/threonine protein kinases Drp72	
MAV_0053	2.0 ± 1.1				Hypothetical protein	
MAV_0054	2.0 ± 1.1			60% similar to Mtc28 (MtubT9_010100002141) of *M. tuberculosis* T92	Mtc28 (proline rich 28 kDa antigen) (*M. tuberculosis* T92)	(Manca et al., 1997)
MAV_0177	2.7 ± 1.4				Copper resistance protein CopC	
MAV_0417	2.2 ± 1.3	2.8 ± 0.2	30% similar to EF-Tu (RV0685) of *M. tuberculosis* H37Rv	30% similar to EF-Tu (Rv0685) of M. tuberculosis H37Rv 30% similar to EF-Tu (Mb0704) of *M. bovis* AF2122/97	Elongation factor Tu GTP binding domain-containing protein	(Manganelli et al., 2001; Monahan et al., 2001)
MAV_0510	2.5 ± 1.4			69% similar to Rv3657c of M. tuberculosis H37Rv	Flp pilus assembly protein TadC (54% similar to *Gordonia bronchialis* DSM 43247)	(Tomich et al., 2007)

MAV_0511	2.0 ± 1.1				Hypothetical protein	(Tomich et al., 2007)
MAV_0512	2.0 ± 1.2				TadE family protein (57% similar to *Geobacter bemidjiensis* Bem)	(Tomich et al., 2007)
MAV_0578	2.2 ± 1.1				Transcriptional regulator, LuxR family protein	(Patel et al., 2006)
MAV_0995	2.0 ± 1.1				Molybdopterin converting factor, subunit 2	
MAV_0996	2.2 ± 1.1	2.7 ± 0.6	71% similar to RpfA (Rv0867c) of *M. tuberculosis* H37Rv		Resuscitation-promoting factor RpfA	(Kana et al., 2008; Kendall et al., 2004)
MAV_1257	2.1 ± 1.2				Hypothetical protein	
MAV_1346	2.1 ± 1.1				PE family protein	
MAV_1722	2.0 ± 1.1				Resuscitation-promoting factor RpfE (*M. tuberculosis* H37Rv)	(Fisher et al., 2002; Kana et al., 2008)
MAV_2328	2.5 ± 1.5				S-adenosyl-methyltransferase MraW	

MAV_2329	3.5 ± 1.3				Hypothetical protein	
MAV_2429	2.0 ± 1.2				PPE family protein	
MAV_2838	N.S.	15.6 ± 0.5	75% similar to OxyR (AF034861) of *M. marinum* ATCC 15069 *M. avium* 104		Hydrogen peroxide-inducible genes activator, OxyR (*M. marinum* ATCC 15069)	(Geier et al., 2008; Pagán-Ramos et al., 2006)
MAV_2839	N.S.	237.2 ± 0.6	90% similar to AhpC (Rv2428) of M. tuberculosis H37Rv *M. avium* 104		Alkylhydroperoxide reductase, AhpC	(Fisher et al., 2002; Fontán et al., 2008; Geier et al., 2008; Hillas et al., 2000; Rohde et al., 2007)
MAV_2840	N.S.	8.5 ± 0.2	74% similar to AhpD (Rv2429) of *M. tuberculosis* H37Rv *M. avium* 104	74% similar to AhpD (Rv2429) of M. tuberculosis H37Rv	Alkylhydroperoxidase, AhpD	(Fisher et al., 2002; Fontán et al., 2008; Geier et al., 2008; Hillas et al., 2000; Rohde et al., 2007)
MAV_2956	2.1 ± 1.2				ATP-dependent RNA helicase	

MAV_3189	2.0 ± 1.2				Nicotinate-nucleotide pyrophosphorylase, NadC	
MAV_3280	2.1 ± 1.2				Hypothetical protein	
MAV_3281	3.1 ± 1.1				Hypothetical protein	
MAV_3282	2.0 ± 1.2			34% similar to PhoR (Rv0758) of *M. tuberculosis* H37Rv	Two-component regulator – sensor kinase (74% similar to HisKA (MMAR_2299) of *M. marinum* M)	(Asensio et al., 2006; Gonzalo-Asensio et al., 2008)
MAV_3640	2.6 ± 1.1				Antibiotic biosynthetis monooxygenase domain-containing protein	
MAV_3979	2.0 ± 1.1				Hypothetical protein	
MAV_4087	2.0 ± 1.2				Transferase	
MAV_4088	2.3 ± 1.1			47% similar to GmhA (MT0122) of *M. tuberculosis* CDC1551	Phosphoheptose isomerase, GmhA	(Rohde et al., 2007)
MAV_4349	2.9 ± 1.3	2.2 ± 0.4		98% similar to PPE36 of *M. avium* subsp. *avium*	PPE family protein	(Mackenzie et al., 2009)
MAV_4381	2.0 ± 1.1				Oxidoreductase	

MAV_4464	2.3 ± 1.1		94% similar to rplP (Rv0708) *M. tuberculosis* H37Rv	94% similar to RplP (Rv0708) *M. tuberculosis* H37Rv	50S ribosomal protein L16	(Manganelli et al., 2001)
MAV_4774	2.1 ± 1.1				Hypothetical protein	
MAV_4792	2.0 ± 1.1				Hypothetical protein	
MAV_4872	2.0 ± 1.3				PPE family protein	
MAV_4986	2.1 ± 1.1				ErfK/YbiS/YcfS/YnhG family protein	
MAV_5013	2.2 ± 1.2	6.8 ± 0.4	80% similar to Mce1C (Rv0171) of *M. tuberculosis* H37Rv	80% similar to Mce1C (Rv0171) of *M. tuberculosis* H37Rv	Mammalian cell entry protein Mce1C	(Gioffré et al., 2005)

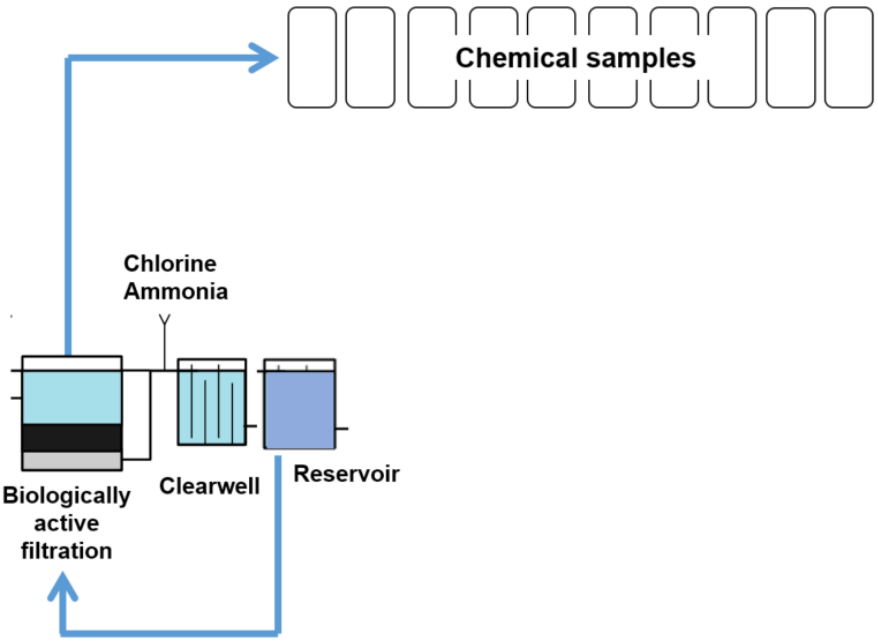

Figure 3.1 Sampling diagram for environmental samples

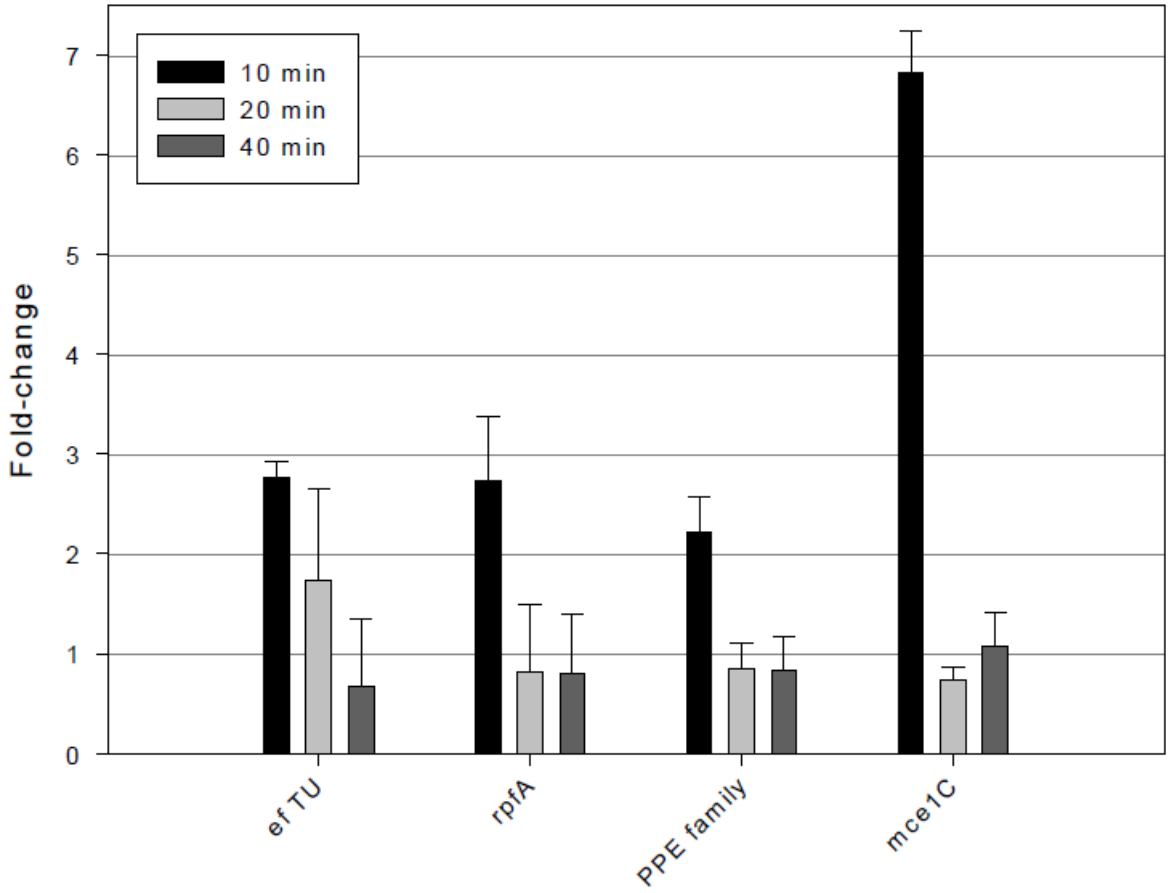

Figure 3.2 Expression levels of monochloramine-sensitive virulence-associated genes in *M. avium* 104 at different exposure times to 0.5 mg/L monochloramine as Cl_2. Error bars indicate standard deviations of replicate measurements calculated using the ΔΔCT.

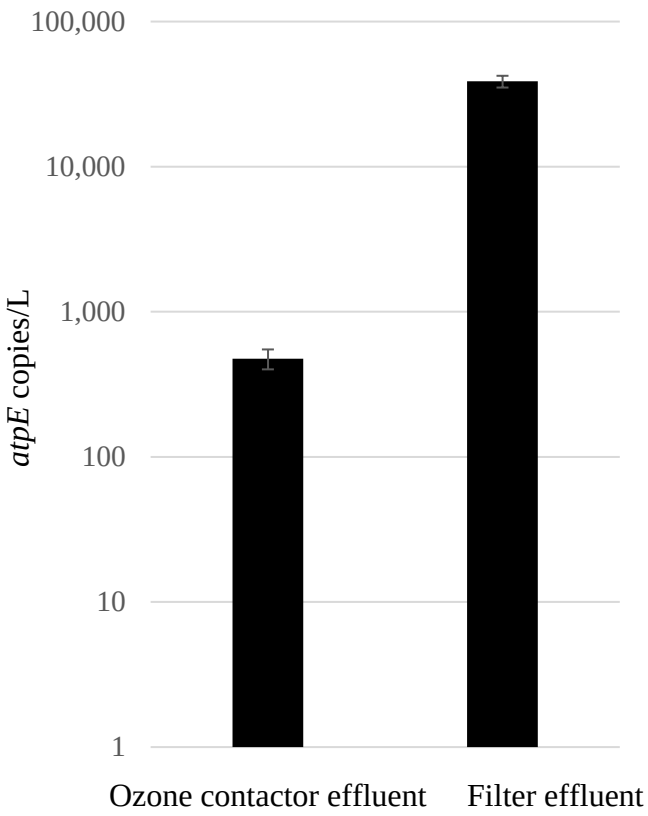

Figure 3.3 Mycobacterial concentrations in one influent and one effluent water sample from a full-scale BAC filter. Error bars represent standard deviation of triplicate qPCR reactions.

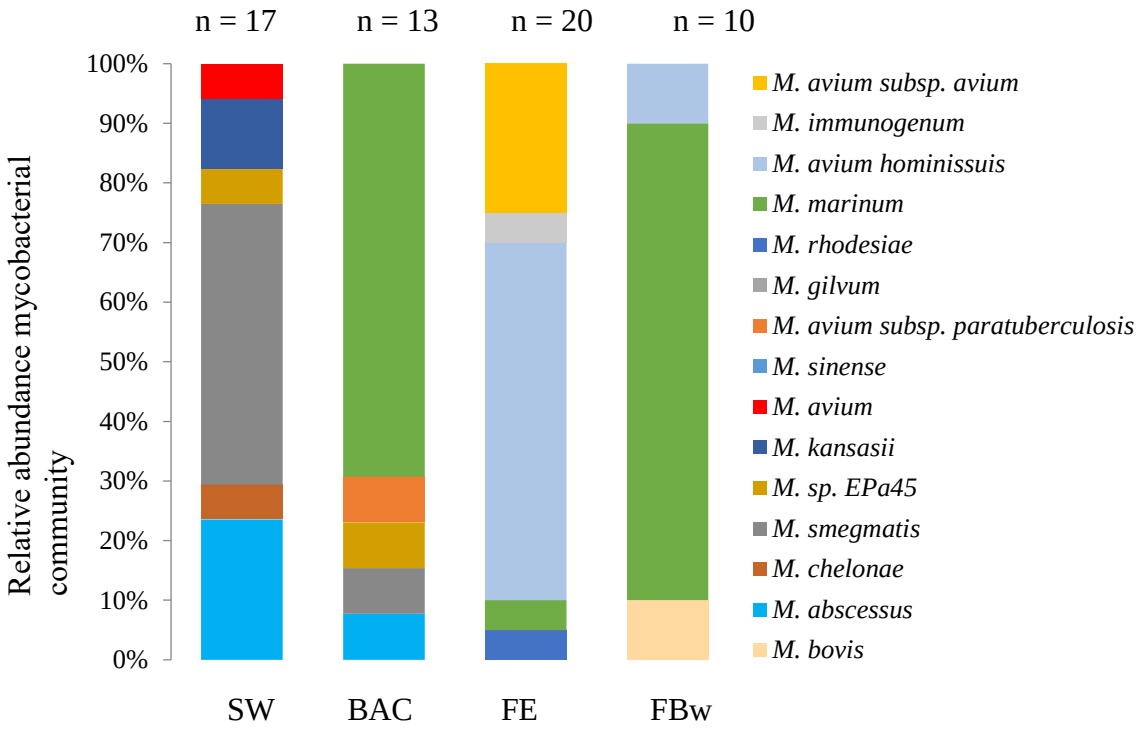

Figure 3.4 Relative abundances of *Mycobacterium* species, based on clone library analysis of *rpoB* genes, in a sample of surface water (SW), BAC, filter effluent (FE) or filter backwash water (FBw). Number of clones is indicated above each bar.

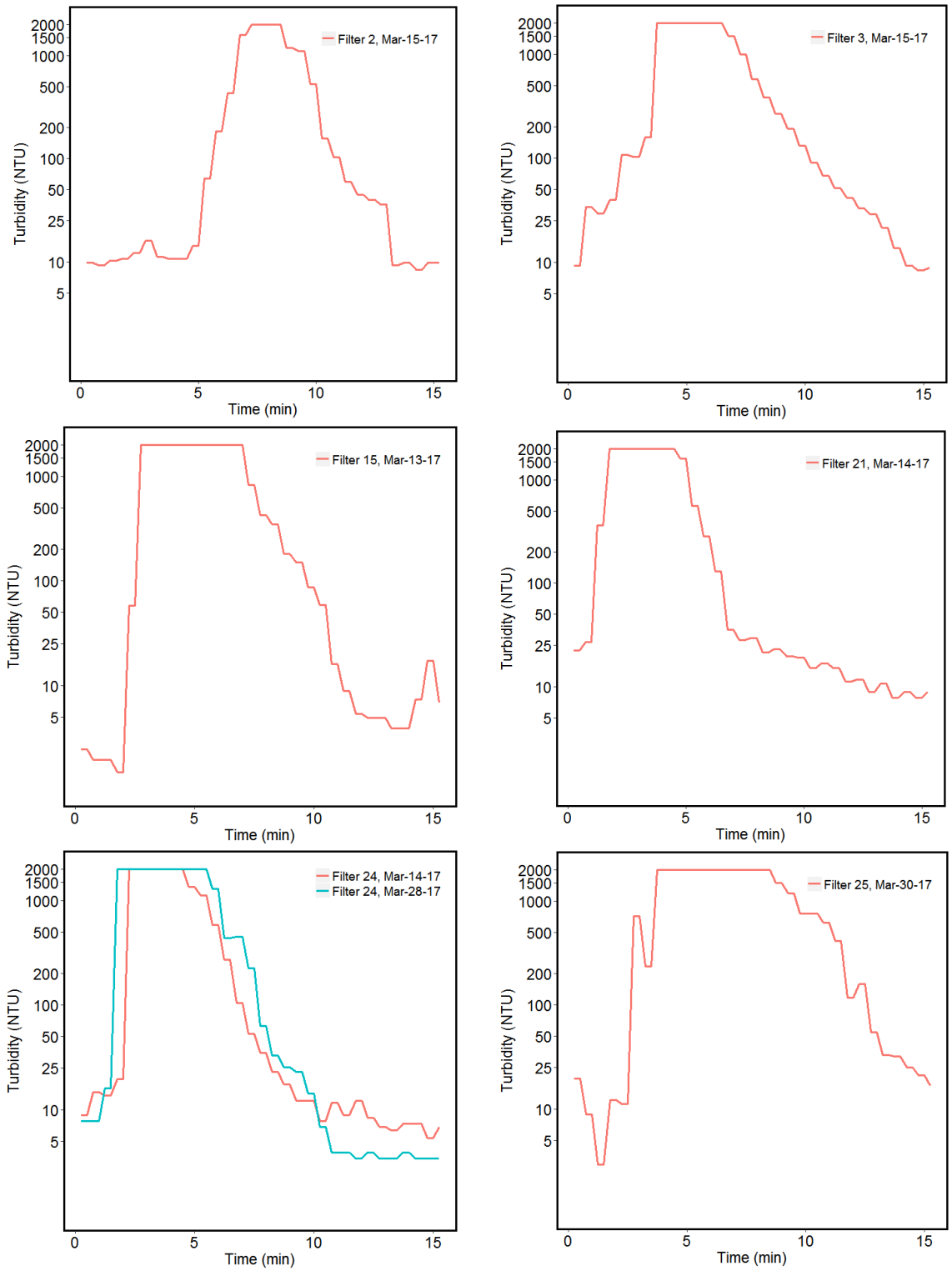

Figure 3.5 Turbidity levels at the top of full-scale filter beds during backwashing

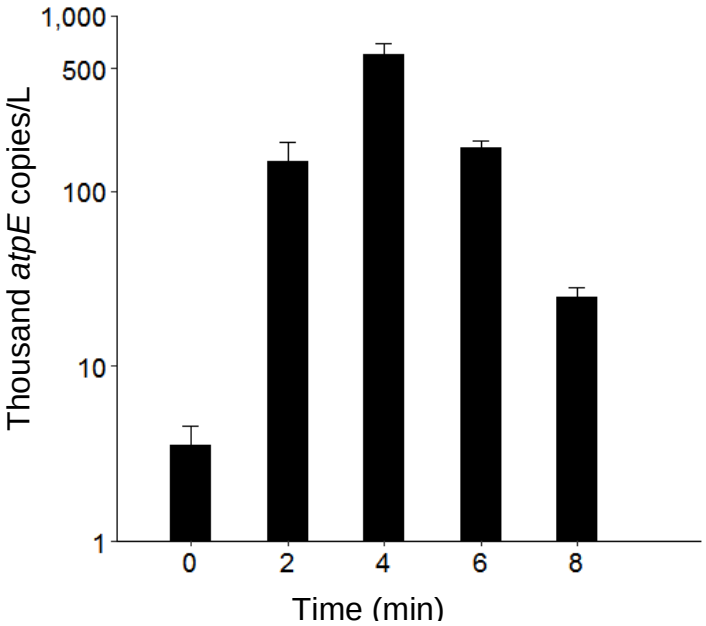

Figure 3.6 Mycobacterial concentrations measured by qPCR for the *atpE* gene in water samples (n = 5) collected from the top of a full-scale filter bed during backwashing. Error bars represent standard deviation of triplicate qPCR reactions.

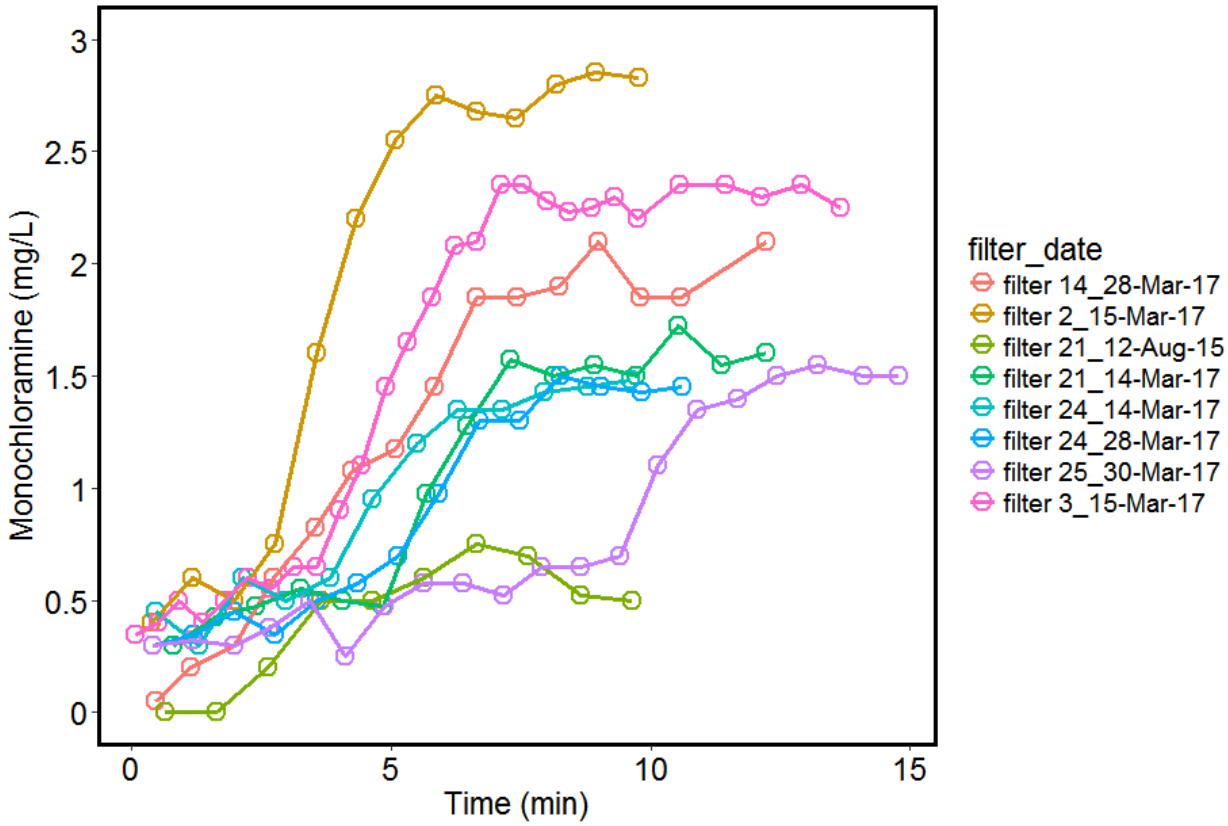

Figure 3.7 Effluent monochloramine concentrations as Cl_2 during backwashing of full-scale BAC filters with finished water containing approximately 3 mg/L monochloramine as Cl_2 and 0.7 mg/L fluoride.

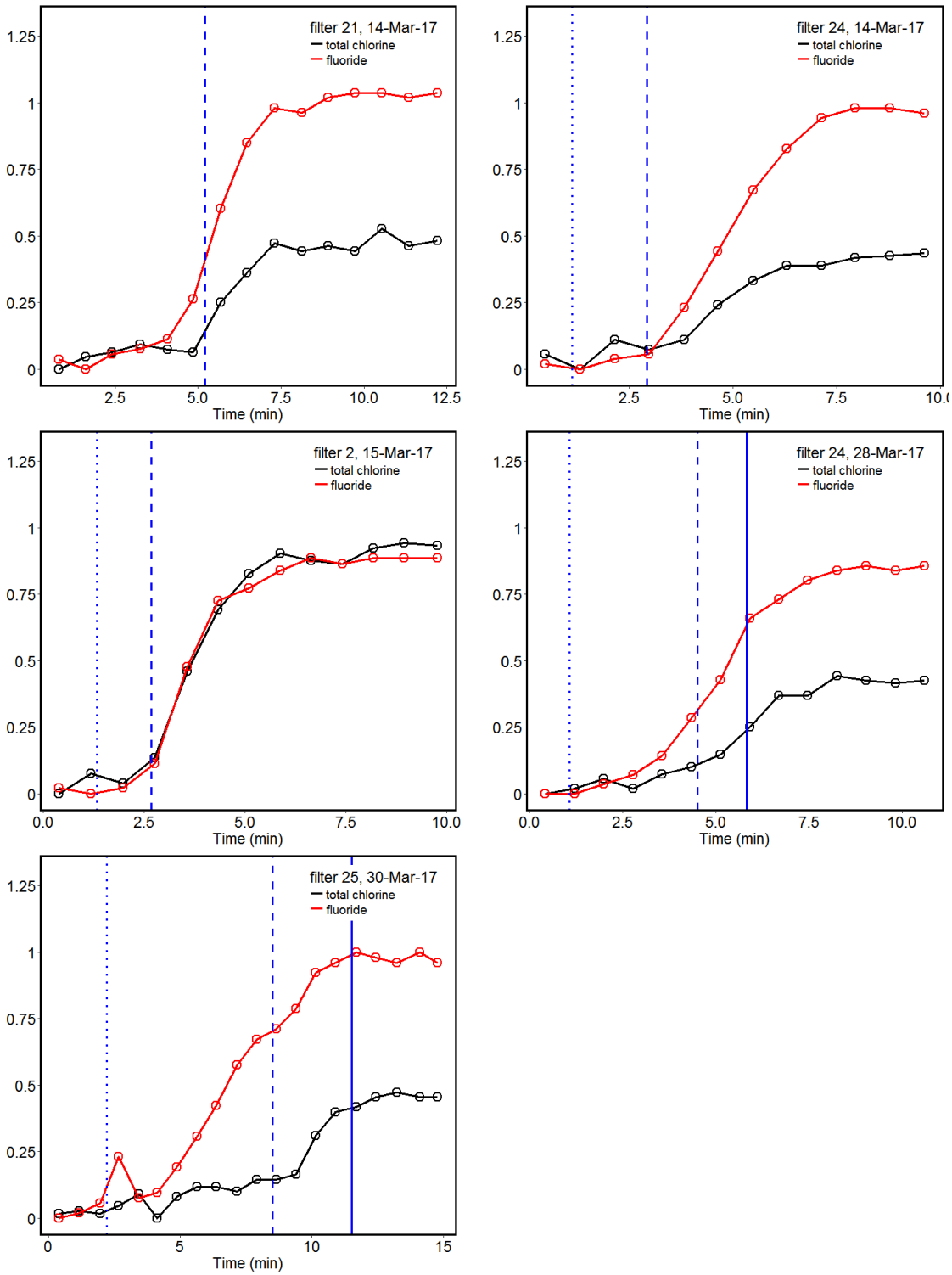

Figure 3.8 Effluent normalized monochloramine as Cl_2 (black) and fluoride (red) concentrations during backwashing of full-scale filters with finished water containing approximately 3 mg/L monochloramine and 0.7 mg/L fluoride. Backwash flowrates were increased from 5 MGD (dotted blue line) to 10 MGD (dashed blue line) and sometimes to 12 MGD (solid blue line).

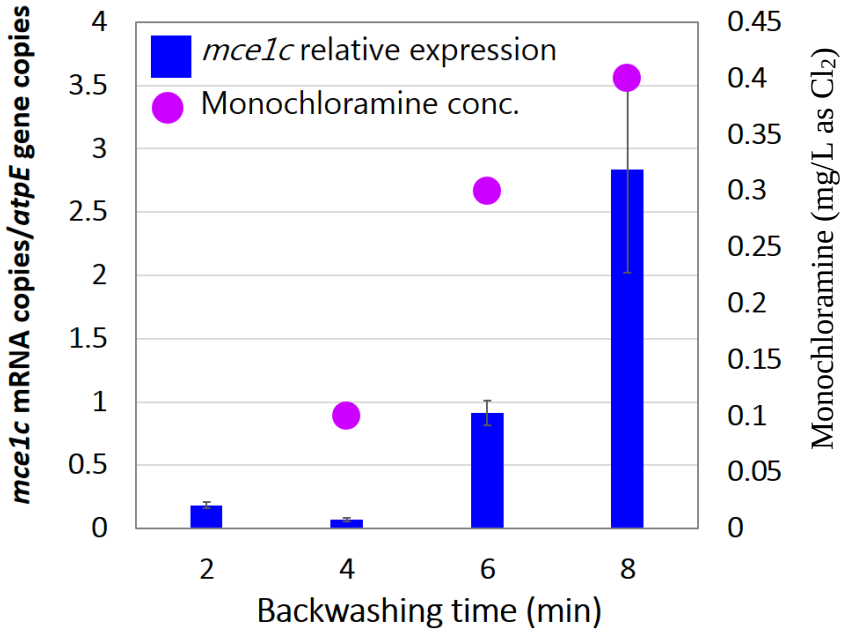

Figure 3.9 Relative expression of *Mycobacterium mce1C* gene and monochloramine concentration as Cl_2 during BAC filter backwashing

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Chapter 4

Sub-lethal Monochloramine Exposure Induces Virulence Factors in Non-tuberculous Mycobacteria

4.1 Abstract

Non-tuberculous mycobacteria (NTM) are abundant, stable members of microbial communities in drinking water systems worldwide. Application of chemical disinfectants during drinking water treatment selects for disinfectant-resistant NTM. Furthermore, NTM strains causing human infections have been genetically matched to strains recovered from drinking water, suggesting that drinking water is a source of mycobacterial infections. Several recent studies have reported that disinfection with chloramine, as compared with chlorine, is associated with a higher abundance of NTM in drinking water. However, the question of whether sub-lethal exposure to chloramine impacts NTM virulence has not received much attention. In this review, we summarize the current knowledge in this emerging area. Sub-lethal exposure to chloramine has direct effects on NTM, including increasing cell envelope permeability, which allows chloramine to enter the cytoplasm and likely selects for NTM populations that have the ability to repair oxidative damage or avoid damage by oxidants. NTM respond to chloramine by upregulating genes involved in intracellular parasitism, suggesting that chloramine exposure promotes NTM entry and replication inside host cells. Chloramine exposure also induces genes involved in biofilm formation, and thus may result in increased NTM persistence in building plumbing, facilitating human exposure to NTM. Knowledge of the sub-lethal effects of disinfectants on microorganisms, particularly the effects of chloramine on NTM, is critical to devise strategies to reduce opportunistic infections from drinking water exposure.

4.2 Introduction

Non-tuberculous mycobacteria (NTM) are common in soil and water. Unlike the obligate intracellular *Mycobacterium* species (i.e., *Mycobacterium tuberculosis*, *Mycobacterium leprae* and *Mycobacterium bovis*), NTM can replicate intracellularly as well as outside of host cells. Indeed, NTM are abundant, stable members of microbial communities in drinking water systems

worldwide (Bahram et al., 2012; Donohue et al., 2015; Holinger et al., 2014; Le Dantec et al., 2002; September et al., 2004) and drinking water is a well-documented source of NTM infection for susceptible individuals (du Moulin et al., 1988; Fleming et al., 2006; Fujita et al., 2014; Kline et al., 2004; Marshall et al., 2011; Thomson et al., 2013; Vonreyn et al., 1994; Wallace et al., 2013). While most centralized water treatment plants use chemical disinfectants (e.g., chlorine or chloramine) to inactivate pathogenic microorganisms and prevent their regrowth in distribution system pipes ("Long Term 2 Enhanced Surface Water Treatment Rule US EPA 2006a,"), maintaining a residual disinfectant concentration does not completely eliminate microorganisms in drinking water (Chiao et al., 2014; Pinto et al., 2012). In fact, several recent studies have demonstrated that disinfection with chloramine, as compared with chlorine, is associated with a higher abundance of NTM in drinking water (Baron et al., 2014; Donohue et al., 2015; Norton et al., 2004; Pryor et al., 2004; Whiley et al., 2014). In addition to encountering chloramines in drinking water, NTM are exposed to chloramines through the process of human infection itself, as chlorinated, nitrogenous compounds are generated during the immune system response to invading microorganisms (Test et al., 1984; Thomas, E. L. et al., 1991). Further, chloramine has been proposed as a promising topical antimicrobial (Gottardi et al., 2013).

Given the diversity of environments in which NTM may encounter chloramines, and considering that the incidence of NTM infections is increasing in the US and other countries (Adjemian et al., 2012; Bar-On et al., 2015; Qvist et al., 2015), it is critical to understand how NTM respond to sub-lethal concentrations of chloramine. In comparison with studies that investigated how environmental exposure to low levels of antibiotics impacts bacterial antibiotic resistance, sub-lethal effects of drinking water disinfectants on opportunistic, waterborne pathogens typically receive little attention in reviews of drinking water disinfection (Ngwenya et al., 2013; Shaibu-Imodagbe, 2013). Questions such as “Does exposure to chloramine in drinking water impact the ability of NTM to cause disease?” cannot yet be answered. A thorough analysis of the literature will generate hypotheses and guide future research in this emerging area.

In this review, we discuss cellular effects and physiological adaptations of NTM species to chloramine exposure, focusing on effects and pathways that may select for virulent NTM. Finally, we provide suggestions for future research investigating waterborne, opportunistic pathogens in disinfected drinking water.

4.3 Chloramine Disinfection

4.3.1 Chloramine Use in Drinking Water

Chloramines form when ammonia reacts with aqueous chlorine. Chloramines include monochloramine (NH_2Cl), dichloramine ($NHCl_2$) and trichloramine (NCl_3). Monochloramine, the preferred form of chloramine, is preferentially formed when the pH is above 7.5 and total chlorine to ammonia (as N) ratio by weight is 5:1 or less (Kirmeyer et al., 2004). Monochloramine is the second most commonly used disinfectant in US drinking water treatment systems after free chlorine. For example, nine of the top 20 most populated cities in the US, representing more than 33 million individuals, are serviced by utilities that use chloramine disinfection (Table 4.1). In European countries, chloramine disinfection is less prevalent, but chloramine has been used by treatment plants in the United Kingdom (Response to Request for Information 2971: Chloramination of UK Tap Water [https://www.whatdotheyknow.com/request/19663/response/51080/attach/2/final%20reply.pdf], October 2009), Finland (Lipponen et al., 2002; Torvinen et al., 2004), Spain and Sweden (Lenntech Website [http://www.lenntech.com/processes/disinfection/regulation-eu/eu-water-disinfection-regulation.htm] accessed 7/27/2015).

Chlorine disinfection of water containing natural organic matter or inorganic compounds such as bromide or iodide results in the formation of hundreds of disinfection byproducts, some of which lead to increased risk of cancer (Bull et al., 1990; Melnick et al., 1994) and birth defects (Hwang, B.-F. et al., 2008; Nieuwenhuijsen et al., 2008). Drinking water concentrations of certain disinfection byproducts, namely trihalomethanes, haloacetic acids, bromate and chlorite, are regulated in the US and European Union. These regulations, among other issues including taste and odor preferences for chloramine, have prompted many treatment plants to use chloramines, which produce fewer of the currently regulated disinfection byproducts than chlorine. However, chloramine can produce other disinfection byproducts such as iodinated species (e.g., iodoacetic acid and iodoform) (Richardson et al., 2008; Wei et al., 2013) and nitrogeneous species (e.g., haloacetonitriles) that are substantially more cytotoxic and genotoxic than brominated and chlorinated disinfection byproducts (Attene-Ramos et al., 2010; Krasner et al., 2006; Muellner et al., 2007; Plewa et al., 2010; Plewa et al., 2008), yet are not currently regulated by the US Environmental Protection Agency (EPA)'s Disinfectants and Disinfection Byproduct Rules (*Stage*

1 Disinfectants and Disinfection Byproducts Rule, 1998; *Stage 2 Disinfectants and Disinfection Byproducts Rule*, 2005). Furthermore, changes in water quality caused by converting from chlorination to chloramination can have unintended consequences. For example, lead can be released from plumbing materials (Edwards, M. et al., 2004), resulting in elevated lead blood levels in drinking water consumers (Edwards, Marc, 2014; Miranda et al., 2007). In addition, if the formation of chloramine is not carefully controlled, residual ammonia in drinking water can result in nitrification in distribution systems which, in turn, can reduce drinking water pH and exacerbate corrosion problems (Zhang et al., 2009).

Because of the negative effects associated with chloramine, some consumers have petitioned against the use of chloramine (e.g., the not-for-profit "Citizens Concerned about Chloramine" group in the San Francisco Bay Area, California, and "Tulsans Against Chloramine" in Tulsa, Oklahoma). Nevertheless, increasing regulations for disinfection byproducts formed primarily by chlorine disinfection will continue to incentivize drinking water utilities to convert from chlorine to chloramine disinfection (DISINFECTION; Seidel et al., 2005). In 2007, thirty percent of 241 utilities in the US reported using chloramine disinfection, and eight percent were considering or planning a switch to chloramine (DISINFECTION). The data in Table 4.1 suggest that the percentage of utilities that use chloramine disinfection in the US today is much larger, although no comprehensive database of disinfectant use is available to support this assertion. To date, the conversation about the health effects of chloramine has largely focused on respiratory and skin effects caused by human exposure to chloramine or chloramine-associated disinfection byproducts. There has been far less investigation into the unintended effects of chloramination on the microbial community in drinking water, particularly how chloramination impacts the abundance and virulence of opportunistic pathogens. This represents an important area for research especially considering that several recent studies have reported that disinfection with chloramine, as compared with chlorine, is associated with a higher abundance of NTM in drinking water (Donohue et al., 2015; Gomez-Alvarez et al., 2012; Pryor et al., 2004; Whiley et al., 2014).

4.4 Mechanisms of Bacterial Inactivation by Chloramine

Disinfectants inactivate microorganisms by damaging essential cellular functions. Compared to free chlorine, monochloramine is a weaker oxidant that reacts more slowly with biomolecules. In this section, we summarize the recent literature describing how bacteria are inactivated by

monochloramine to provide background for the subsequent discussion of effects of chloramine exposure on NTM.

4.4.1 Cell membrane integrity

While relatively low concentrations of chlorine cause rapid leakage of intracellular material from gram-positive and gram-negative bacteria (Venkobachar et al., 1977; Virto et al., 2005), cell envelope damage by monochloramine was limited for *Escherichia coli* K (Jacangelo et al., 1991). Compared with chlorine, monochloramine is effectively inert toward fatty acids and polysaccharides in lipids and peptidoglycan (Dodd, 2012). Likewise, exposure to monochloramine at a concentration × time (CT) of 240 mg as Cl_2-min/L caused only nonlethal cell membrane damage to *Pseudomonas aeruginosa* PAO1 cells, although some peptidoglycan was degraded (Xue et al., 2014). Even though chloramine exposure decreased the culturability of *Legionella pneumophila*, nonculturable cells had intact membranes and exhibited esterase activity (Alleron, Laëtitia et al., 2008). For bacterial cells in drinking water treated with different disinfectants, Ct values required to compromise cell membrane integrity were higher for monochloramine than chlorine, as measured by flow cytometry using a combination of SYBR Green I and propidium iodide staining (Ramseier et al., 2011). For mycobacteria, the thick, lipid-rich outer layer of their cell envelope is considered an efficient permeability barrier to antimicrobials (Medjahed et al., 2010). However, exposure of *Mycobacterium avium* 104 cells to 0.5 mg/L monochloramine (as Cl_2) permeabilized their cell membranes within five min, as determined by measuring the uptake of propidium iodide by cells, suggesting that even the complex cell wall of mycobacteria does not present a significant barrier to chloramine entry (Berry, 2009).

4.4.2 Sulfhydryl groups

In reactions with amino acids, monochloramine reacts fastest with sulfur-containing amino acids (methionine, cysteine, and cystine) and tryptophan (Boyle, 1963; Davies et al., 1999; Jacangelo et al., 1985; Scully et al., 1991). It is theorized that chloramine reactions with sulfhydryl groups (R-SH), also called thiols, inhibit proteins and subsequently inactivate cells because sulfhydryl groups are essential to numerous enzymes' active sites (Jacangelo et al., 1991). At pH values relevant for drinking water disinfection, the chlorine in monochloramine can transfer to amine ($R\text{-}NH_2$) groups (Snyder et al., 1982), and the resulting organic chloramines (R-NHCl) as well as monochloramine

can oxidize two sulfhydryl groups to form disulfides (R-S-S-R) or produce sulfur compounds with higher oxidation states (e.g., elemental sulfur) (Boyle, 1963; Jacangelo et al., 1985; Jacangelo et al., 1987, 1991).

The addition of reducing agents to culture media can improve recovery of chlorine or chloramine-stressed cells (Watters et al., 1989), suggesting that inactivation by these disinfectants is reversible. The extent to which sulfhydryls are oxidized by monochloramine to form disulfides is thought to be important for reversing monochloramine-induced damage. For example, disulfides were reduced to sulfhydryls in the presence of reducing agents (62), yet elemental sulfur was not observed to be reduced back to sulfhydryls (Boyle, 1963). It is plausible that microorganisms damaged by monochloramine disinfection can recover within reducing environments of drinking water distribution systems. The prevalence of sulfate-reducing microorganisms found in biofilms located underneath corrosion tubercles in a chloraminated distribution system is consistent with the presence of anaerobic niches in these environments (Gomez-Smith et al., 2015).

The notion that monochloramine inactivation is reversible is also consistent with results reported by Hwang and colleagues (Hwang, C. et al., 2012). They sampled drinking water from a distribution system that periodically transitions from chloramine to chlorine disinfection. Comparison of chlorinated and chloraminated samples showed that exposure to chloramine at different times over a two year period resulted in microbial community structures that were quite similar to each other, whereas exposure to free chlorine resulted in more variable community structures (Hwang, C. et al., 2012). These results suggest that chloramine-induced effects are more reversible, and damage induced by chloramine exerts a deterministic, selective pressure on populations that survive chloramine disinfection.

Chloramine inactivation of *M. avium* increases with decreasing pH (Luh et al., 2008). This pH dependence is consistent with a sulfhydryl-dependent model of inactivation, as chloramine reactivity with protein sulfhydryl groups also increases with increasing acidity (or decreasing pKa) of sulfhydryl groups (Peskin et al., 2006). Additionally, monochloramine inactivation of *E. coli*, *M. avium* and *Mycobacterium chelonae* increases with increasing initial monochloramine concentrations (Jacangelo et al., 1991; Luh et al., 2008; Pelletier, P. et al., 1988; Pelletier, P. A. et al., 1991). This may be due to protein backbone fragmentation caused by formation of organic chloramines at lysine residues, leading to increased access of chloramines to less accessible

sulfhydryl groups (Hawkins et al., 1998; Luh et al., 2008). Jacangelo and colleagues (1991) observed that inactivation of *E. coli* B by monochloramine corresponds to an inhibition of substrate dehydrogenation (Jacangelo et al., 1991). Additionally, when proteins in crude extracts were first denatured by treatment with sodium laurel sulfate, greater oxidation of sulfhydryls was observed than in intact bacteria exposed to chloramine, suggesting that some sulfhydryl groups in intact proteins are protected from oxidation by chloramine (Jacangelo et al., 1987). Recently, Xue and colleagues (2014) reported that, compared with polysaccharide-based biofilms produced by *P. aeruginosa* PAO1, penetration of *Pseudomonas putida*'s protein-based biofilms required higher Ct values with monochloramine (Xue et al., 2014). One caveat worth mentioning is that protein-based biofilms contained more void spaces, which would result in higher mass transport into the biofilm and easier access to sulfhydryl groups (Xue et al., 2014). Extracellular polymeric substance (EPS) surrounding cells may react with monochloramine and protect against cell membrane damage. However, EPS is probably more protective against chlorine than chloramine given that chlorine is quickly consumed during reactions with biofilms (Chen et al., 1996; De Beer et al., 1994), whereas monochloramine reacts more slowly with EPS constituents (Chen et al., 1996; Lechevallier et al., 1990). Yet, the type of EPS appears to matter more with monochloramine as protein-based biofilms would consume more monochloramine than polysaccharide-based biofilms.

4.5 Impacts of Sub-lethal Concentrations of Chloramine

A recent review of the literature concluded that bacteria respond to reactive chlorine species, including chloramines, through mechanisms for resisting oxidative stress, protein unfolding and sulfur starvation (Gray, M. J. et al., 2013). However, little is known about how NTM in particular respond to chloramine in drinking water, or what effect chloramine exposure has on NTM virulence. Nevertheless, to summarize the current knowledge in this emerging field, we have collected as much relevant material from the literature as possible. We present this information below in five sub-sections (and provide a visual summary in Figure 4.1). Because few studies have examined the effects of chloramine on mycobacteria specifically, we also discuss the effects of chloramine on other bacteria where that information is instructive, and occasionally refer to studies of different oxidants. Within each of the five subsections below, we present hypotheses on how chloramine exposure impacts the persistence and virulence of NTM in drinking water.

4.5.1 Cell Envelope Permeability

The bacterial cell envelope is a critical barrier to antimicrobials encountered in the environment. The cell envelope is composed of either one (gram-positive) or two (gram-negative) lipid membranes and a layer of peptidoglycan of variable thickness. Exposure to antimicrobials can change the lipid or protein composition of cell envelope components, which, in turn, can affect the permeability of the cell and modulate the amount of antimicrobial that enters the cytoplasm. As described above, monochloramine does not severely damage cell membranes, as bacteria retain their membrane integrity even after inactivation by monochloramine. A key step in this resistance may be the upregulation of genes involved in repairing the outer membrane after chloramine-induced stress. Indeed, when exposed to 1 mg/L monochloramine (as Cl_2), *E. coli* K12 MG1655 cells upregulated genes involved in lipid A and lipopolysaccharide (LPS) biosynthesis (Holder et al., 2013). In a separate study, *E. coli* K12 MG1655 cells exposed to 1 mg/L monochloramine (as Cl_2) upregulated *ybgF* nearly two fold (Berry, D. et al., 2010). YbgF coordinates peptidoglycan synthesis and outer membrane constriction during *E. coli* cell division (Gray, A. N. et al., 2015). While *Mycobacterium* species do not produce LPS, their cell walls contain lipoarabinomannan and mycolic acids conjugated to arabinogalactan. These large glycolipids form a thick outer barrier which contributes to their impermeability to antimicrobials (Lambert, 2002).

As described above, sub-lethal concentrations of monochloramine rapidly permeabilized mycobacterial cell membranes (Berry, 2009). Likewise, *M. bovis* exposed to free chlorine repressed cell wall synthesis enzymes (*fab*G4, *fad*E24) and mycolic acid fatty acid synthetases (*des*A1, *des*A2) (Jang et al., 2009a), suggesting increased permeability. Therefore, the question is raised of whether NTM respond to chloramine by upregulating pathways to maintain membrane integrity. Expression levels of genes encoding three proline-proline glutamate (PPE) proteins were significantly upregulated by *M. avium* strain 104 after 10 min exposure to 0.5 mg/L of monochloramine (as Cl_2) (Berry, 2009). PPE proteins are a large and polymorphic family of mycobacterial proteins, and many PPE proteins are located in and/or on the mycobacterial cell envelope (Fishbein et al., 2015). Several PPE proteins modulate envelope composition or integrity in response to local cues. For example, *M. tuberculosis* mutants with insertions in genes encoding particular PPE proteins exhibited increased resistance to the hydrophilic antibiotic ampicillin (Danilchanka et al., 2008). Understanding how PPE proteins alter the mycobacterial envelope in

response to monochloramine, and whether increased permeability induced by monochloramine impacts virulence, warrants more detailed analysis.

Genes encoding the nonspecific DNA binding protein Dps, which protects against chromosomal single-stranded breaks under conditions of oxidative stress (Gomez-Alvarez et al., 2012), were observed to be more abundant in microbial biomass from chloraminated vs. chlorinated drinking water (Martinez et al., 1997). These data are consistent with the model that chloramine, as compared with chlorine, enters the cytoplasm of cells more effectively. Therefore, if chloramine exposure induces permeability of NTM in drinking water, it likely selects for populations that efficiently eliminate reactive oxygen or reactive chlorine species by intracellular antioxidants.

4.5.2 Cellular Antioxidants

Most bacteria produce low molecular weight, non-protein thiols that maintain the reducing potential of the cytoplasm against oxidation by reactive chlorine species and other oxidants by one of two mechanisms. First, thiols can scavenge oxidants to limit the oxidation of cellular components (Chesney et al., 1996; Gray, M. J. et al., 2013). Second, small thiol species form reversible disulfides with protein-associated thiols (i.e, "S-thiolation"), protecting thiol-containing residues from further oxidation (Chi et al., 2014). As described above, chloramine reactivity with protein thiols is thought to be an important mechanism by which chloramine inactivates bacterial cells. Indeed, sub-lethal monochloramine exposure rapidly depleted intracellular thiols in *M. avium* strain 104 (Berry, 2009). Thus, exposure to chloramine likely selects for NTM populations that effectively eliminate intracellular reactive oxygen and reactive chlorine species. There is some evidence for this assertion with chlorine: metagenomic analyses of raw and treated water from a drinking water treatment plant in China showed that chlorination was associated with higher abundances of genes encoding enzymes in the glutathione biosynthesis pathway (e.g., glutathione synthase) (Chao et al., 2013). While glutathione is the dominant thiol in bacteria, most *Actinobacteria,* including mycobacteria, contain *mycothiol* instead of glutathione (Johnson et al., 2009; Newton et al., 1996). Compared to wild-type cells, mycothiol-deficient *Mycobacterium smegmatis* and *M. tuberculosis* mutants exhibited greater susceptibility to oxidants (Buchmeier et al., 2006; Newton et al., 1999; Rawat et al., 2002). Although none of the genes known to be involved in mycothiol biosynthesis were significantly upregulated by *M. avium* strain 104 in response to monochloramine, the cells upregulated oxidoreductase genes as well as genes related

to alkylhydroperoxide reductase (*ahpC*) and alkylhydroperoxidase (*ahpD*) after 10 min exposure to 0.5 mg/L monochloramine (as Cl_2) (Berry, 2009). Reductases and peroxidases serve complementary roles to thiols by reducing oxidants and protecting cells from damage. After sub-lethal treatment with diamide (5 mM), a thiol-specific damaging agent that has been used to study the thiol-disulfide stress response in a number of bacteria (126-128), *M. bovis* BCG cells increased their levels of AhpC protein approximately five fold and *ahpC* mRNA nearly three-fold (Dosanjh et al., 2005). Other genes induced either by *M. tuberculosis* and *M. bovis* exposed to diamide (128, 129) or by *E. coli* exposed to 1.0 mg/L monochloramine (as Cl_2) (Berry, D. et al., 2010) are the thioredoxin reductase genes *trxC, trxB* and *trxB2* and thioredoxin, an antioxidant protein that reduces other proteins by cysteine thiol-disulfide exchange. Likewise, starved *E. coli* K12 AT980 cells exposed to 1 mg/L monochloramine (as Cl_2) at 20°C upregulated a glutathione peroxidase gene (*btuE*) 8.5-fold (Du et al., 2015)..

If chloramine exposure selects for NTM populations that efficiently eliminate reactive oxygen and chlorine species, this selection would not only generate NTM strains with increased resistance to oxidative stress and increased fitness in the environment, but may also result in more virulent NTM. Production of reactive chlorine species is a mechanism used by phagocytic immune cells to eliminate invading pathogens. For example, after a microbe is phagocytosed, NADPH oxidase in phagocytic cells generates superoxide and hydrogen peroxide, which myeloperoxidase enzymes subsequently convert to free chlorine. In addition, chlorinated, nitrogenous compounds are generated during the immune system response to invading microorganisms (Test et al., 1984; Thomas, E. L. et al., 1991). A recent paper described a mobile Integrative Conjugative Element of *Legionellae* that confers resistance to oxidative stresses generated by free chlorine or the macrophage NADPH oxidase (Flynn et al., 2014). Strains carrying this element replicated in macrophages 10-fold more efficiently than did those that lacked the element (Flynn et al., 2014), demonstrating that resistance to oxidative stress acquired in the environment results in more virulent strains. This raises the possibility that chloramine disinfection (and possibly other water treatment processes) inadvertently increases the burden of opportunistic NTM pathogens, and the risk of human infection.

Intracellular antioxidants can also act as signaling molecules to activate virulence gene expression in intracellular pathogens. For example, glutathione non-covalently binds and activates PrfA, the master virulence regulator in *Listeria monocytogenes*, which coordinates the pathogen's

intracellular survival strategies (Reniere et al., 2015). This interesting observation raises the question of whether sub-lethal chloramine exposure induces master virulence regulators in NTM. Mycobacteria encode a PrfA homologue (based on alignment using the Basic Local Alignment Search Tool by the National Center for Biotechnology Information [http://blast.ncbi.nlm.nih.gov/Blast.cgi]), but this factor has not been linked to NTM virulence, and it is unclear whether expression of this gene, or other regulatory genes, is activated by mycothiol under conditions of oxidative stress. However, in *M. tuberculosis*, the thiol-reactive chemical diamide induced the alternative sigma factor *sigH*, as well as 39 other genes whose expression depended on *sigH* (Ghosh et al., 2013). Several of the genes included in the *sigH* regulon are mammalian cell entry genes (*mceA1*, *mceC*, *mce5A*, *mceB2* and *mceD*), which promote entry and replication inside host cells. Indeed, *M. tuberculosis* mutants lacking the alternative sigma factor *sigH* were more susceptible than wild-type cells to diamide-induced stress (Ghosh et al., 2013; Manganelli et al., 2002). Likewise, *M. avium* subsp. *paratuberculosis sigH* mutants survived poorly inside of activated bovine macrophages (Ghosh et al., 2013). In the next section, we investigate how chloramine exposure may impact intracellular parasitism by NTM.

4.5.3 Intracellular Parasitism

Free-living amoebae are unicellular, eukaryotic microorganisms that persist in soil and water as either dormant, stress-resistant cysts, or as trophozoites that actively feed on bacteria by phagocytosis. Certain *Mycobacterium* species survive phagocytosis and digestion by amoebae in part by preventing phagosome-lysosome fusion, thus enabling mycobacteria to exploit amoebae as hosts for replication (Cirillo et al., 1997). NTM are frequently associated with amoebae in drinking water systems (Ovrutsky et al., 2013; Thomas, J. M. et al., 2011; Thomas, V. et al., 2007) and amoebae recovered from disinfected drinking water have been shown to harbor NTM (Delafont et al., 2013; Delafont et al., 2014). In grazing experiments with *M. avium* subsp. *hominissuis* 104 and *Acanthamoeba castellanii* Neff, the proportion of infected amoebae increased linearly with the relative abundance of *M. avium* in a bacterial community (Berry, David et al., 2010). Therefore, the higher relative abundance of NTM in chloraminated drinking water systems, combined with the stability of chloramine and its ability to reduce overall bacterial levels (e.g., (Lechevallier et al., 1990)), raises the possibility that chloramine disinfection promotes intracellular parasitism of amoebae by NTM.

Understanding the contribution of intracellular parasitism to the overall occurrence of NTM in drinking water is clearly important because intracellular NTM are more difficult to inactivate by disinfection than free-living NTM (Berry, David et al., 2010). An additional reason to study intracellular parasitism of amoebae by NTM is the notion that interactions between NTM and protozoan hosts in the environment may contribute to the evolution of cellular processes that equip mycobacteria to infect and survive inside macrophages and epithelial cells (Salah et al., 2009). For example, *M. avium* genes involved in protein translation and energy metabolism upregulated during infection of *A. castellanii* are also upregulated during macrophage infection (Danelishvili et al., 2007; Tenant et al., 2006), suggesting that mycobacteria rely on similar mechanisms to colonize both cell types, as has been demonstrated for other intracellular pathogens such as *L. pneumophila* (Gao et al., 1997). Importantly, replication within amoebae enhanced the ability of *M. avium* to colonize the intestine and replicate in the liver and spleen in the beige mouse model of infection (Cirillo et al., 1997). Because intracellular replication has the potential to generate NTM populations that pose increased risk to human health, it is important to determine the impact of monochloramine exposure on the ability of NTM to enter and replicate inside amoebae in drinking water systems.

Using metagenomics analyses, Gomez-Alvarez and colleagues (2012) identified DNA sequences classified as *Mycobacterium* mammalian cell entry (*mce*) genes in chloraminated but not in chlorinated drinking water (Gomez-Alvarez et al., 2012). The ability of *mce* genes to promote mycobacterial intracellular replication was demonstrated when these *M. tuberculosis* genes were shown to be sufficient to confer to multiple *E. coli* strains the ability to invade mammalian cells (Arruda et al., 1993). Homologues of the four *mce* operons identified in *M. tuberculosis* (*mce1, mce2, mce3,* and *mce4*) are encoded by multiple *Mycobacterium* species, including *M. avium, M. bovis,* and *M. smegmatis* (Haile et al., 2002).. Moreover, when exposed to 0.5 mg/L monochloramine (as Cl_2) for 15 min, *M. avium* 104 cells upregulated *mce1C*, which is part of the *mce1* operon expressed by *M. tuberculosis* upon phagocytosis by macrophages (Berry, 2009; Graham et al., 1999).

In addition to the *mce* loci, other genes associated with intracellular parasitism are induced after chloramine exposure. For example, *M. avium* strain 104 cells were observed to upregulate a PPE family protein two-fold (Berry, 2009) and several PE/PPE proteins were found to be involved in mycobacterial growth in macrophages (Li et al., 2005; Sampson, 2011). Furthermore, *M. avium*

strain 104 induced elongation factor Tu 2.2-fold after chloramine exposure (Berry, 2009). Elongation factor Tu was shown to be exposed on the surface of *M. avium* subsp. *hominissuis* during early infection of macrophages, and elongation factor Tu was induced by *M. avium* subsp. *hominissuis*, *M. avium* strain 905, and *M. bovis* during macrophage infection (Brunori et al., 2004; McNamara et al., 2012; Monahan et al., 2001). Recent research demonstrated that elongation factor Tu can bind fibronectin, raising the possibility that this factor is involved in fibronectin-mediated attachment of *M. avium* subsp. *paratuberculosis* to host cells (Viale et al., 2014).

Finally, in the context of elucidating the role of intracellular parasitism in NTM occurrence in chloraminated drinking water, it is interesting to note that chloramine exposure has been observed to induce genes required for intracellular replication of other waterborne, opportunistic bacterial species. For example, monochloramine exposure induced the viable but nonculturable (VBNC) state in *L. pneumophila* Lens HL 0350 5056 (Alleron, Laëtitia et al., 2008), and this VBNC state was characterized by the accumulation of macrophage infectivity potentiator protein Mip (Alleron, Laetitia et al., 2013) required for intracellular replication (Cianciotto et al., 1992). Interestingly, *L. pneumophila* VBNC cells can be resuscitated through co-incubation with *Acanthamoeba* cells (García et al., 2007; Hwang, M. G. et al., 2006; Steinert et al., 1997).

4.5.4 Biofilm Formation

A biofilm is a collection of microorganisms attached to and growing on a biological or non-biological surface, surrounded by an extracellular polymeric substance (EPS) matrix (Hall-Stoodley et al., 2004). Numerous surfaces in drinking water distribution systems and building plumbing favor the formation of biofilms containing NTM (Feazel et al., 2009; Mullis et al., 2013; September et al., 2004). Using a metagenome-based approach, genes associated with *Mycobacterium*-related EPS biosynthesis were detected in chloraminated but not in chlorinated drinking water (Gomez-Alvarez et al., 2012). Biofilm formation by NTM in drinking water systems is concerning because it complicates efforts to inactivate NTM by applying disinfectants (Berry et al., 2009; Steed et al., 2006), and it enables NTM to persist in building plumbing and thus facilitates human exposure to NTM. Therefore, it is important to investigate whether NTM adapt to chloramine exposure by forming biofilms.

Mycobacteria attach to hydrophobic surfaces through interactions of the exposed fatty acid tails of their glycopeptidolipids (Recht et al., 2000; Vaerewijck et al., 2005). No studies have investigated

whether the hydrophobicity of mycobacterial cells is altered by exposure to chloramine. However, *E. coli* K12 strain $J62_{RP4}$ cells treated with 2.0 mg/L free chlorine for 15 min aggregated and exhibited greater cell surface hydrophobicity, as measured by a salt aggregation test (Arana et al., 1999). Furthermore, increasing chlorine concentration from 0.16 to 1.0 mg/L promoted significantly faster attachment of drinking water microbes to glass coupons (0.144 h^{-1} versus 0.065 h^{-1}) (Liu et al., 2014), possibly because of increased cell surface hydrophobicity or other factors. In pilot-scale drinking water distribution systems, as chlorine concentration increased from no chlorine to 0.7 mg/L, the fraction of culturable bacteria present in biofilms also increased from 19% to 63% (Srinivasan et al., 2008).

If indeed mycobacterial cells exposed to disinfectant become more hydrophobic, this surface modification could facilitate aggregation and/or faster attachment to surfaces and may contribute to the increased NTM colonization of showerhead surfaces in households supplied with disinfected water compared with those supplied with well water without disinfectants (described above, (Feazel et al., 2009)). In addition, some bacterial species are equipped with cellular appendages, such as fimbriae or pili, which act as tools to attach to surfaces (Kirov et al., 2004; Vatanyoopaisarn et al., 2000). Brief exposure of *M. avium* 104 cells to 0.5 mg/L monochloramine (as Cl_2) induced two *tad* genes (*tadC* and *tadE*) (Berry, 2009), which encode machinery required for the assembly of adhesive fimbrial low-molecular-weight protein pili (Tomich et al., 2007). Similarly, in response to 1 mg/L monochloramine (as Cl_2), *E. coli* MG1655 cells upregulated genes involved in attachment (fimbriae, pili, and curli genes), while repressing genes that mediate motility (flagella) (Holder et al., 2013). In a separate study, planktonic *E. coli* cells exposed to monochloramine down-regulated eight genes associated with motility (Berry, D. et al., 2010).

Besides increasing its resistance to disinfection, as was demonstrated with *Acinetobacter* EB22 exposed to monochloramine, aggregation of NTM may also facilitate biofilm growth. Boles and Singh (2008) reported that biofilm growth of *P. aeruginosa* produced extensive genetic variation, resulting from mutagenic repair of double stranded breaks caused by stress from oxidants generated in the biofilm (Boles et al., 2008). Therefore, NTM growth in biofilms may increase the abundance of diverse NTM strains. Furthermore, compared with planktonic conditions, the increased cell-to-cell contact within biofilms may facilitate conjugal DNA transfer between NTM strains (Nguyen et al., 2010).

4.6 Future Work and Recommendations

Several recent studies discussed above established that NTM proliferate in chloraminated drinking water systems. Therefore, understanding how exposure to sub-lethal concentrations of chloramine impacts NTM virulence is essential to devising strategies to reduce mycobacterial infections through drinking water exposure. Below we discuss areas we found to be particularly lacking in the current literature and make recommendations for future research investigating sub-lethal effects of drinking water disinfectant on NTM.

Determining whether the abundance of NTM is higher in chloraminated systems than in chlorinated systems is difficult to do by comparing results across studies for a number of reasons. Specifically, the molecular and culture-based methods used have different sensitivities and specificities, and the reporting units often are not directly comparable (e.g., qPCR targeting mycobacteria in biofilms are reported as gene copies/cm^2 or gene copies/g biomass). While it is not possible to resolve all of these methodological concerns, reporting more detailed information would be helpful. For example, most studies of microbial communities in drinking water provide limited information about the disinfection process (e.g., disinfectant type, concentration of disinfectant used, and time of exposure to disinfectant) and report few chemical and physical data on drinking water and drinking water system characteristics. More thorough reporting of relevant properties would greatly facilitate interdisciplinary collaboration and enable better comparisons across studies.

In addition to the need for standardized and detailed reporting to enable comparison across studies, the scarcity of metadata has resulted in missed opportunities to understand whether lab-scale studies are representative of drinking water conditions. For example, the limited availability of nutrients in drinking water is expected to impact NTM's sub-lethal response to monochloramine, similar to how adaptation to starvation increased *E. coli* resistance to chlorine (Du et al., 2015). Therefore, studies investigating the oxidative stress response in NTM would benefit from including assessment of the effect of nutrient availabity in their experimental model.

The higher abundance of NTM in chloraminated drinking water is possibly affected by chloramine-associated disinfection byproducts. This relationship has not been investigated in detail, probably because many of the byproducts formed by chloramine treatment are not regulated and, therefore, not monitored. However, propane-grown cells of *Mycobacterium vaccae* JOB-5

were reported to co-metabolically degrade 150 ng/L of *n*-nitrosodimethylamine (NDMA), a chloramine-associated byproduct, to below 20 ng/L (Sharp, J. O. et al., 2010), and other microbial populations can degrade NDMA (Fournier et al., 2009; Sharp, J. et al., 2005). More lab-scale and field monitoring campaigns are needed to understand whether the diverse metabolic capabilities of *Mycobacterium* species contribute to the prevalence of these microbes in chloraminated drinking water systems. Reporting disinfection byproduct concentrations as additional metadata should be encouraged.

The ability of intracellular pathogens to acquire iron from host cells is critical to pathogenesis (Sritharan, 2000). Mycobacteria acquire iron by synthesizing and secreting siderophores such as extracellular exochelin and mycobactin (De Voss et al., 2000). Treatment of *M. bovis* BCG with hydrogen peroxide induced genes encoding ferric uptake regulation protein FurA, and *mbtC* and *mbtB* which encode mycobactin, an iron-chelating compound (Jang et al., 2009b). Considering that chloramine disinfection can increase dissolution of metals into drinking water, future research should investigate how exposure to sub-lethal concentrations of chloramine in distribution systems, and during overnight stagnation in building plumbing, impacts siderophore production and other iron acquisition strategies of NTM.

4.7 Conclusions

The study of sub-lethal effects of disinfectants on waterborne, opportunistic pathogens is an emerging field. NTM encounter chloramines in a variety of environments, including in an increasing number of drinking water systems worldwide. Several studies of full-scale and simulated drinking water systems have reported that chloramine, as compared with chlorine, is associated with a higher abundance of NTM. However, our understanding of inactivation and associated effects on NTM remains limited. For example, results of lab-scale inactivation studies of NTM with monochloramine are often not consistent with data reported on the survival of NTM species in chloraminated full-scale drinking water systems. Chloramine exposure increases the permeability of the cell envelope of EM, allowing chloramine to enter the cytoplasm. Intracellular chloramine likely selects for NTM populations that efficiently repair oxidative damage or eliminate reactive chlorine species. Chloramine also induces genes involved in intracellular parasitism, suggesting that chloramine disinfection promotes NTM entry and replication inside host cells. Thus, chloramine treatment may enrich for more virulent NTM strains. Furthermore,

chloramine exposure induces genes involved in biofilm formation, suggesting that chloramine disinfection increases NTM persistence in building plumbing and facilitates human exposure to NTM. Moreover, NTM growth in biofilms may result in greater microdiversity and facilitate interactions with species present in chloraminated systems, including nitrifying and denitrifying microorganisms. More research is needed to improve our understanding of how disinfectants alter the abundance and virulence of opportunistic, waterborne pathogens. Design of successful strategies will require an interdisciplinary effort by researchers from several fields, including environmental engineering, microbiology, microbial ecology, and epidemiology.

4.8 Tables and figures

Table 4.1 Community water systems of the top 20 most populated US cities (listed in order of most populated) based on US Census Bureau as of July 1, 2014. Name, population served and source water information obtained from US EPA Safe Drinking Water Information System online [http://www.epa.gov/enviro/facts/sdwis/search.html]. This data set does not include systems that service less than 10,000 people. Disinfectant information was obtained from utility websites and/or recent water quality reports accessed on July 27, 2015. Systems that use chloramine disinfection are shaded grey.

City, State	Name	Population Served	Source Water	Disinfectant	Reference
New York, NY	New York City System	8,271,000	Surface	Chlorine	New York City 2014 Drinking Water Supply and Quality Report [http://www.nyc.gov/html/dep/pdf/wsstate14.pdf]
Los Angeles, CA	Los Angeles City Dept. of Water and Power	3,894,439	Surface	Chloramine	Los Angeles County Waterworks Districts [http://dpw.lacounty.gov/wwd/web/YourWater/WaterQuality.aspx]
Chicago, IL	Chicago Chicago Heights Chicago Ridge IL-American Chicago Suburban North Chicago	2,695,598 30,392 14,305 12,522 19,877	Surface Surface Surface Surface Surface	Chlorine	City of Chicago [http://www.cityofchicago.org/city/en/depts/water/supp_info/education/water_treatment.html]
Houston, TX	City of Houston	2,201,027	Surface	Chloramine	City of Houston Drinking Water Operation [http://www.publicworks.houstontx.gov/utilities/drinkingwater.html]
Philadelphia, PA	Philadelphia Water Department	1,600,000	Surface	Chloramine	City of Philadelphia Water [http://www.phila.gov/water/wu/drinkingwater/treatment/Pages/default.aspx]
Phoenix, AZ	City of Phoenix	1,500,000	Surface	Chlorine	City of Phoenix [https://www.phoenix.gov/waterservices/waterquality]
San Antonio, TX	San Antonio Water System	1,596,714	Surface	Chlorine	San Antonio Water System [http://www.saws.org/Your_Water/WaterQuality/water_quality_concerns/faqs.cfm]
San Diego, CA	City of San Diego	1,326,200	Surface	Chloramine	City of San Diego From Source to Tap [http://www.sandiego.gov/water/quality/watersources/treatmentprocess/index.shtml]

Dallas, TX	Dallas Water Utility	1,253,000	Surface	Chloramine	City of Dallas Water Quality Information [http://dallascityhall.com/departments/waterutilities/Pages/water_quality_information.aspx]
San Jose, CA	San Jose Water Company	998,000	Surface	Chloramine	San Jose Water Surface Disinfection [http://www.sanjoseca.gov/index.aspx?NID=1582]
Austin, TX	City of Austin Water & Wastewater	903,570	Surface	Chloramine	Austin Water Frequently Asked Questions [http://www.austintexas.gov/department/frequently-asked-questions-about-h2o]
Jacksonville, FL	JEA Major Grid	703,750	Groundwater	Chlorine	2014 Water Quality Report [https://jacksonvillenc.gov/DocumentCenter/View/1131]
San Francisco, CA	SFPUC City Distribution Division	837,442	Surface	Chloramine	San Francisco Water Power Sewer [http://sfwater.org/index.aspx?page=357]
Indianapolis, IN	Citizens Water Indianapolis	876,728	Surface	Chloramine	2014 Drinking Water Report [http://www.citizensenergygroup.com/Documents/Reports/Water-Quality-2014]
Columbus, OH	Columbus Public Water System	1,152,993	Surface	Chlorine	City of Columbus Water Treatment Process [http://columbus.gov/Templates/Detail.aspx?id=16049]
Fort Worth, TX	City of Forth Worth	748,450	Surface	Chlorine	Fort Worth Water Treatment Plants [http://fortworthtexas.gov/water/info/default.aspx?id=6058]
Charlotte, NC	Charlotte Water	818,005	Surface	Chlorine	Charlotte Water [http://charmeck.org/city/charlotte/Utilities/PublicationsandEducation/Pages/howwatertreated.aspx]
Detroit, MI	City of Detroit	713,777	Surface	Chlorine	Detroit Water and Sewerage [http://www.dwsd.org/pages_n/water101.html]
El Paso, TX	El Paso Water Utilities Public Service B	631,442	Surface	Chlorine	2014 El Paso Water Utilities Drinking Water Report [http://www.epwu.org/water/pdf/dwr_2014.pdf]
Seattle, WA	Seattle Public Utilities	836,000	Surface	Chlorine	2014 Drinking Water Quality Report [http://www.seattle.gov/util/cs/groups/public/@spu/@water/documents/webcontent/1_039275.pdf]

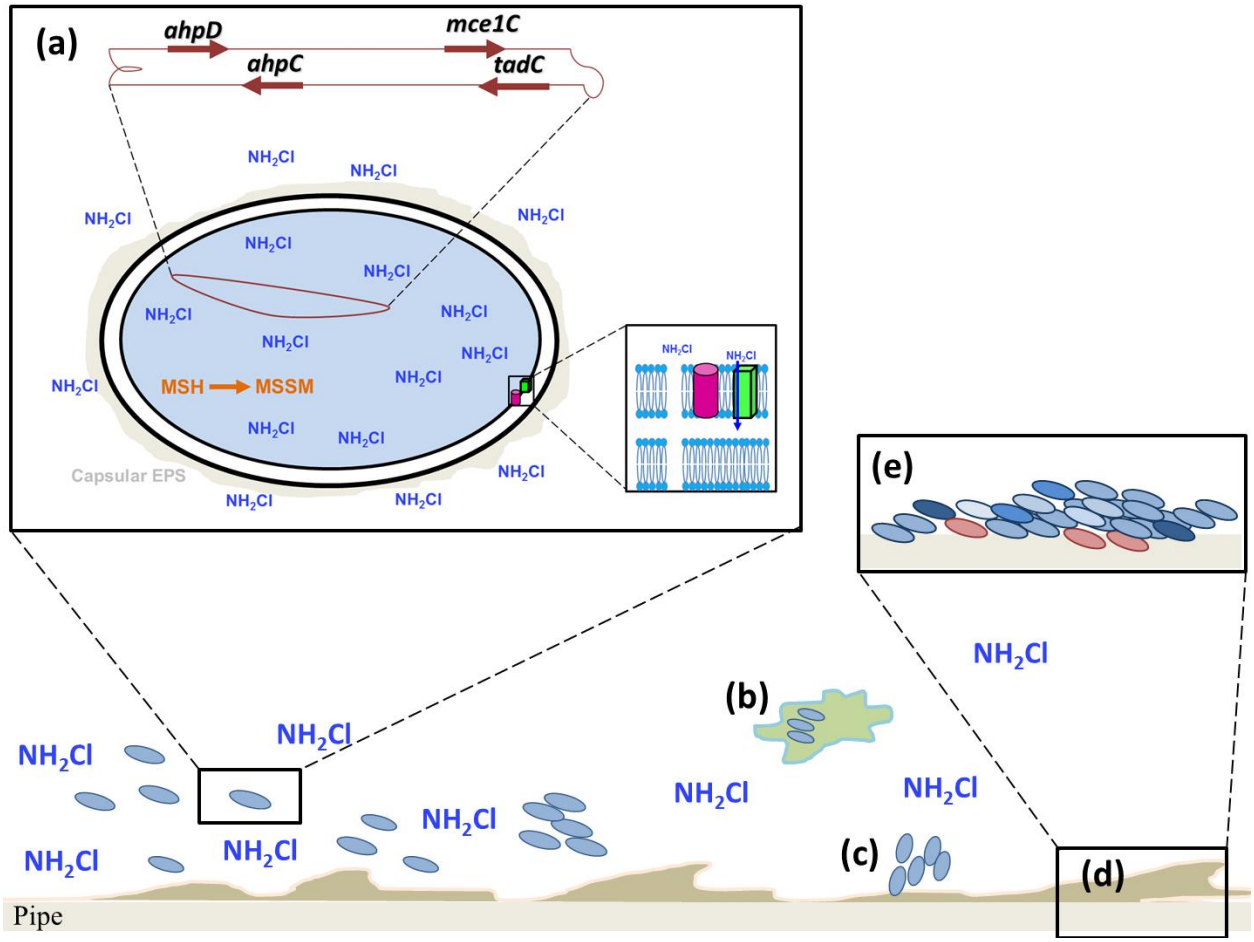

Figure 4.1 Proposed model of mycobacteria response to sub-lethal monochloramine levels (a) Monochloramine permeabilizes the cell envelope, enters the cytoplasm and, reacts with intracellular mycothiol (MSH) to form disulfides (MSSM represents mycothiol disulfide). NTM respond to monochloramine by upregulating reductase and peroxidase genes (*ahpD*, *ahpC*) and several other factors which are important for entry and survival inside host cells (*mce1C*) and biofilm formation (*tadC*). Therefore, chloramine exposure may (b) induce NTM to enter protozoan cells in drinking water systems and (c-d) aggregate and form biofilms. Furthermore, (e) NTM growth in biofilms may facilitate NTM microdiversity (depicted by the different shades of blue) and interspecies interactions with other microorganisms found in chloraminated systems, including nitrifying/denitrifying microorganisms (red cells).

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Chapter 5

Is Chloramine Disinfection of Municipal Drinking Water a Risk Factor for Non-tuberculous Mycobacterial Infection?

5.1 Abstract

Mycobacteria are frequently recovered from water, including municipally treated drinking water. The levels of mycobacteria have been reported to be higher in drinking water disinfected with chloramine than in drinking water disinfected with chlorine, suggesting that chloramine disinfection of drinking water has the potential to increase human exposure to mycobacteria and the risk of mycobacterial infection. A case-control study of patients tested for mycobacterial infection at the University of Michigan medical center (Michigan Medicine) was performed and logistic regression was used to identify water treatment practices significantly associated with mycobacterial infection. Disinfectant type (chloramine or chlorine) was not a significant predictor of mycobacterial infection, contrary to expectations. However, use of a surface water body compared to a groundwater source was a significant predictor of mycobacterial infection.

5.2 Introduction

Non-tuberculous mycobacteria (NTM) cause opportunistic infections in individuals sensitive to bacterial infection. NTM are responsible for several types of infections including pulmonary, extra-pulmonary, skin and soft tissue infection. From 1997 to 2007, the annual prevalence of pulmonary NTM disease among US adults aged 65 years or older increased 8.2% per year, from 20 to 47 cases/100,000 persons (Adjemian et al., 2012). From 2008 to 2013 in five US states, the number of patients who had positive specimens for NTM rose from 8.7 to 13.9 per 100,000 persons (Donohue et al., 2016). The observed increase in NTM disease likely reflects increased awareness of and testing for NTM combined with true increases in prevalence caused by an aging population and longer lifespan of individuals with predisposing conditions (e.g., cystic fibrosis or HIV). In some cases, however, aging and other risk factors cannot completely account for the increasing prevalence of infection (Al-Houqani et al., 2012), and therefore increased exposure to NTM through environmental sources is suspected.

NTM are primarily transmitted by water (Collier et al., 2012). Proximity to surface water bodies was associated with NTM infection in children with cystic fibrosis (Bouso et al., 2017). Mycobacteria are present in natural water bodies used as sources for drinking water production (King et al., 2016). They have also been detected in treated drinking water samples from across the USA (Donohue et al., 2015; King et al., 2016). Reasons for their presence in treated waters include their protection from disinfection through biofilm formation on disinfection contactor surfaces (Chapter 2), their growth in biologically-active carbon filters (Chapter 3) as well as a range of previously studied mechanisms (Chapter 4). NTM were detected in treated waters from nine out of 25 drinking water treatment plants (DWTPs) sampled across the USA (King et al., 2016). Distribution systems therefore represent an important transmission route of mycobacteria from natural waters to household taps.

Public water systems that use surface water or groundwater under the influence of surface water are required to disinfect water supplies before distribution to customers. A residual disinfectant in distribution systems is provided to limit microbial regrowth. Chlorine is the most common disinfectant used for residual disinfection. However, regulations on byproducts formed by chlorine have prompted many utilities to convert to chloramine, a disinfectant produced through the reaction of chlorine and ammonia. Chloramine is a weaker oxidant than chlorine and laboratory-scale studies have shown that many bacteria, including mycobacteria, require longer exposure to chloramine than chlorine for inactivation (Taylor et al., 2000).

Several studies of full-scale and simulated drinking water systems have determined that water disinfected with chloramine has higher abundances of NTM. For example, significantly more NTM were cultured from chloraminated cold water samples, as compared with chlorinated samples, from 68 taps in 25 states across the USA (Donohue et al., 2015). Higher concentrations of *Mycobacterium avium* complex, a subgroup of NTM that causes a variety of human diseases, were detected in a chloraminated drinking water distribution system compared with a chlorinated distribution system, based on qPCR targeting internal transcribed spacer regions (Whiley et al., 2014). In another study, Norton and colleagues (2004) investigated the impact of nutrient levels, pipe material and disinfectant type on *M. avium* concentrations in water and biofilm samples from a pilot-scale distribution system. Using nested-PCR targeting mycobacterial 16S rRNA genes, they found that *M. avium* levels on copper surfaces were significantly greater when water was disinfected with chloramine compared with chlorine (Norton et al., 2004). Furthermore, a

comparison of metagenomes of drinking water from distribution system simulators showed that chloraminated drinking water had higher levels of NTM genes (19.7%) than chlorinated drinking water (1.9%) (Gomez-Alvarez et al., 2012).

The clearest evidence that chloramine exposure increases the absolute or relative abundance of NTM has come from studies of full-scale systems that monitored bacterial communities in drinking water before and after converting from chlorine to chloramine disinfection. In one public water system in the US, NTM abundance increased in distribution system biofilm and water samples, based on denaturing gradient gel electrophoresis analysis, following a change from chlorine to chloramine disinfection (Pryor et al., 2004). The same study reported that, after the switch to chloramination, the number of NTM positive biofilm and water samples increased 9.4% and 36.5%, respectively, in samples collected from hot water heaters and showerheads. Similarly, Baron and colleagues (2014) reported a significant increase in the relative abundance of NTM in hot water samples from a tertiary care hospital in the USA after installation of an on-site monochloramine system (Baron et al., 2014). The relative abundance of NTM increased from less than 0.4% during chlorination to greater than 1.2% after 6 months of chloramine use, based on Illumina sequencing of 16S rRNA genes. However, a culture-based analysis of drinking water from the same system did not show significant changes in the abundance of NTM (Duda et al., 2014). While some studies did not report a significant difference in NTM abundance between chlorinated and chloraminated drinking water (Bautista-de los Santos et al., 2016; Holinger et al., 2014), or after installation of on-site monochloramine injection in a hospital drinking water system (Hicks et al., 2009), the numerous aforementioned examples of higher NTM levels in chloraminated compared to chlorinated tap water raise the possibility that municipal disinfection of drinking water with chloramine increases human exposure to NTM and thereby increases risk of infection.

To investigate a potential relationship between municipal drinking water treatment practices and NTM infection, we collected information on public water systems in Michigan and designed a case-control study among patients tested for NTM at the University of Michigan academic medical center (Michigan Medicine) from 2000-2015.

5.3 Materials and methods

5.3.1 NTM Test Data

Under approval from the University of Michigan institutional review board, we obtained records of all NTM smears and cultures performed at Michigan Medicine from January 2000 through September 2015 (i.e., the study period). Data obtained for each tested patient also included demographic information such as address, age and sex at the time of culture collection, and clinical test information such as anatomical site of isolation, diagnosis codes and result text. Raw data were filtered to exclude cultures not performed, cultures associated with institutional and post office box zip codes, and duplicate records caused by test ordering practices. Each record was categorized separately as smear and culture positive, negative or unknown based on string matching to the reported result text. For the purposes of further analysis, all records categorized as culture positive and records with unknown cultures and positive smear were categorized as positive. Species identification was performed on positive records using string matching. Unidentifiable positive records associated with a patient with an identified positive record within 400 days were presumptively identified.

Risk factors for NTM infection were taken from the International Classification of Diseases, Ninth and Tenth Revisions, Clinical Modification (ICD-9-CM and ICD-10-CM) codes on the list of immunocompromising conditions in the Prevention Quality Indicators Technical Specifications published by the Agency for Health Research and Quality (*Agency for Healthcare and Research Quality*. *Prevention quality indicators technical specifications*, 2010; Collier et al., 2012) and ICD-9-CM and ICD-10-CM codes for structural lung diseases associated with NTM infection (i.e., Alpha-1 antitrypsin, COPD, cystic fibrosis, non-CF bronchiectasis, pneumoconiosis and pulmonary alveolar proteinosis) (Chan et al., 2013; Lake et al., 2016; Mirsaeidi et al., 2013). A culture was considered to be from a patient at risk for NTM infection if the culture was collected prior to or up to two years following a year the patient received a diagnosis for a risk factor.

Positive cultures came from multiple anatomical locations which were categorized as “Lung”, “Blood”, “Skin and musculoskeletal”, “Gastrointestinal”, “Genitourinary”, “Sinus”, “Lymphatic”, “Central nervous system”, “Other” or “Missing/unknown.” Pulmonary isolations of *M. gordonae* are frequently regarded as a contaminant (Freeman et al., 2012; Morimoto et al., 2015; Smith et al., 2016). To avoid false positive results, single positive cultures of *M. gordonae* from a lung or

unknown site were removed from the dataset (335 cultures or 13.6% of positive results were removed).

5.3.2 Municipal Water Disinfection Practices

A list of public water systems in Michigan and each system's source water and residual disinfectant type was obtained through two Freedom of Information Act Requests (EPA-HQ-2015-001745 and EPA-HQ-2015-009061) to the United States Environmental Protection Agency (US EPA) Office of Ground Water and Drinking Water. The data came from US EPA's Safe Drinking Water Information System and included public water system ID, name, primary source (surface water or groundwater), facility ID and address and treatment process. Treatment process specified one of three disinfection types: (1) gaseous chlorination for post-disinfection, (2) gaseous chlorination for pre-disinfection, or (3) chloramines. Water treatment plants servicing correctional facilities (two plants), mobile and resort communities (five plants), or golf courses (two plants) were not included in the study. We inferred each system's service area based on system address and name (e.g., the water system for the city of Ann Arbor is named "City of Ann Arbor") and we assumed all patient addresses inside city limits receive municipally treated water. Cities that do not have their own water treatment system and purchase water service from elsewhere were not represented in the US EPA data. The Great Lakes Water Authority is a regional water and sewer authority that services nearly 40 percent of water customers in Michigan. It operates multiple water treatment plants that use chlorine for residual disinfection. All Great Lakes Water Authority customer cities were included in the study. Information on disinfection practices of public water systems servicing 24 cities that were not represented in the US EPA data and are not Great Lakes Water Authority Customers, were obtained by reviewing annual water quality reports for each city.

Addresses for culture results were passed to Google Maps API for geocoding through R (Ihaka et al., 1996). Shapefiles for cities in Michigan were accessed from the State of Michigan's ArcGIS Online Open Data Portal. Since the probability a person receives municipally treated water is higher for persons living in cities, only test results with addresses located inside Michigan city limits were candidates for a "water cohort."

5.3.3 Propensity Score Matching

A propensity score for chloramine disinfection was estimated for each patient in the water cohort by logistic regression of patient age, sex and risk factors at the time of the first NTM positive result

(for NTM positive patients) or a randomly selected negative result (for negative patients). Patients having chloramine disinfection were matched 1:2 using nearest neighbor matching of propensity scores with patients having chlorine disinfection. Propensity score matching was performed with the MatchIt package (Ho et al., 2006) in R.

5.3.4 Logistic Regression Modeling

Logistic regression modeling was used to identify factors significantly associated with NTM infection. First, a mixed effect logistic regression model was used to assess the significance of patient age, sex and risk factors for NTM infection in predicting the probability of a positive test result. In this model, patient and sample year were modeled as random effects to account for patients with multiple tests and changes in lab or clinical practices at Michigan Medicine over the study period, respectively. Next, a logistic regression model was fit to a water cohort nested within patients tested for NTM at Michigan Medicine to estimate the significance of disinfectant type in predicting NTM infection. Model covariates included patient characteristics as well as characteristics of each patient's city of residence. For example, patient age, sex, and risk factors for NTM infection, year of sample collection and distance from patient address to Michigan Medicine were included in the model. Furthermore, water disinfectant (i.e., chlorine or chloramine), source water (i.e., surface water or groundwater) median household income, and percent individuals over 65 years old for the city corresponding to the patient's home address were included in the model.

US 2010 Census Demographic data (total population, age, and sex) were collected from the US Census Bureau. Median household income data for each city were supplied by U.S. Census Bureau's American Fact Finder. Lastly, the model was run with a dataset of patients having water disinfected with chloramine matched to patients with chlorine. A p value less than 0.05 was considered statistically significant. Adjusted odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. All statistical analyses were conducted using R.

5.4 Results

5.4.1 NTM cases at Michigan Medicine, 2000-2015

61,786 specimens from 29,942 patients were submitted for NTM testing at Michigan Medicine during the study period. After putative *M. gordonae* contaminants were removed, there were

61,045 test results remaining (2,135 positive and 58,910 negative). Although the study period spans fifteen years, the maximum number of years separating specimen collections was less than or equal to three for a majority (88.1%) of the 11,620 patients who were tested for NTM multiple times during the study period (Table 5.1).

Each year during the study period, 387,020 ± 34,300 patients (mean ± standard deviation) were billed for services at Michigan Medicine. There were 2,135 NTM positive results from 1,244 patients over the fifteen year study period (Figure 5.1). Annual prevalence of NTM infection among patients ranged from 1.48 per 10,000 (2001) to 2.66 per 10,000 (2009). While most (71.4%) positive patients had only one positive result during the study period, 192 patients (15.4%) had two positives, 77 patients (6.1%) had three positives and 87 individuals (7.0%) had more than three (Table 5.2). The age across NTM positive patients at year of first positive result was 53.2 ± 20.4 (mean ± standard deviation) and the median age was 57 (Table 5.3). 33% of NTM positive patients (410 patients) were 65 years old or older, which is substantially larger than the fraction of the Michigan population comprised by individuals with age greater than or equal to 65 years old (Table 5.4), based on US Census Data. NTM were isolated from multiple sources but most were pulmonary isolates (Table 5.5). Species identification was available for 2,342 (94.8%) isolates. *Mycobacterium avium* isolations predominated (1,070 cultures, 50.1%), followed by *M. chelonae* (327 cultures, 15.3%), and *M. gordonae* (161 cultures, 7.5%).

65.9% of NTM positive patients (i.e., 1,408 patients) had diagnoses suggesting they were at risk for infection. Their most common risk factors, in decreasing order of frequency, were related to lung transplantation (ICD-9 codes 996.84 and V42.6), cystic fibrosis (ICD-9 codes 277.00, 277.02 and 277.03), bronchiectasis (ICD-9 codes 494.0 and 494.1), human immunodeficiency virus (ICD-9 code 042), chronic airway obstruction (ICD-9 code 496), emphysema (ICD-9 code 492.8), complications of transplanted bone marrow (ICD-9 code 996.85), and severe protein-calorie malnutrition (ICD-9 code 262).

In a mixed effects logistic regression model fit to the 61,045 test results, an increase in age ($p = 0.027$) and having a risk factor for NTM infection ($p = 0.039$) were significantly associated with an NTM positive specimen (Table 5.6).

5.4.2 Water Cohort

The majority (58,976 or 96.1%) of test addresses at date of specimen collection were located in the state of Michigan and 25,619 tests addresses were located within Michigan cities. Of these tests, 23,463 (91.6%) had addresses located in the 140 cities for which we acquired information on municipal water treatment practices (Table 5.7). Water systems for the cities of Ann Arbor, Lansing and East Lansing reported using chloramine for residual disinfection and these cities converted to chloramine in 1985, 1990 and 2000, respectively. The remaining 137 cities are serviced by water systems that use chlorine for residual disinfection. Furthermore, 102 cities are serviced by water systems that use a surface water source (e.g., lakes or rivers) as their primary source for water production. There were 38 cities serviced by water systems that use groundwater from wells as their primary source. The "water cohort" was comprised of 11,321 patients, including 542 NTM positive patients and 10,779 patients with negative results only. Of the 11,321 patients, 2,213 and 9,109 had addresses from cities using chloramine and chlorine for residual disinfection of the municipal water supply, respectively. Propensity score matching yielded 4,426 control patients having chlorinated water matched with 2,213 patients with chloraminated water. In logistic regression modeling with the matched patients, disinfectant type (chloramine) was not significantly associated with NTM infection ($p = 0.93$) (Table 5.7). However, the use of a surface water body as the primary water source was significantly associated with infection ($p = 0.04$) (Table 5.7).

5.5 Discussion and conclusions

Epidemiologic data can be used to evaluate engineering decisions but gaps in data on NTM infection (i.e., gaps caused by the limited reporting of NTM disease cases) and municipal water treatment practices need to be addressed for continued evaluation of water treatment practices as risk factors for NTM infection.

To test whether municipal water practices are associated with NTM infection, we acquired all NTM records from patients at Michigan Medicine for a fifteen-year period. The finding that disinfectant type of home water was not a significant predictor of mycobacterial infection suggests that an increasing use of chloramine for drinking water disinfection does not significantly increase the burden of mycobacterial disease. However, the use of a surface water source compared to a

groundwater source was significantly associated with infection and this finding needs to be verified in future studies.

Other findings from our study are in agreement with previous reports of NTM epidemiology. For the Michigan Medicine cohort, having a risk factor for NTM disease (i.e., an immunocompromising condition or structural lung disease) and older age were significantly associated with an NTM positive specimen. Similarly, significant increases in prevalence of pulmonary NTM isolations were observed with increasing age of residents of three North Carolina counties (Smith et al., 2016). Sex was not a significant predictor of NTM infection in the present study and NTM isolation prevalence was similar between genders in the North Carolina study. In contrast, the probability of NTM isolation was significantly higher in females with low body mass index in a cohort of University of Illinois Medical Center patients (Mirsaeidi et al., 2013).

Limitations of our study include that only NTM tests performed at Michigan Medicine were considered. Since other labs in Michigan besides Michigan Medicine perform NTM testing, an estimation of NTM prevalence was not possible nor was it the goal of the study. Additionally, NTM infection was defined as having a positive culture over the study period. Information on patients' histories of NTM infection prior to 2000 was not available. Therefore, some infected patients may have become infected before the study period (and, importantly, before their city converted to chloramine for residual disinfection of the drinking water supply).

An assumption was made that all residences located within a city's limits are serviced by the city's community water system. This assumption is expected to be accurate for larger cities (we estimate less than 5% misclassification for Ann Arbor, Lansing, and Grand Rapids based on information from the Michigan Department of Environmental Quality) but may not be accurate for smaller cities. The municipal water service coverage in Ann Arbor is approximately 97%. Matching culture addresses to a utility billing spreadsheet would connect a residence with municipal water service directly, but billing spreadsheets are often not resolved enough for this purpose. For example, each entry in a billing spreadsheet represents a water meter, not an individual residence, so when multiple residences in an apartment building are connected to the same meter, only that meter is listed on the billing spreadsheet, not the individual residences.

Of the 140 cities included in our study, only three cities–Ann Arbor, East Lansing and Lansing–reported using chloramine for water disinfection. A utility's choice between chlorine and

chloramine for disinfection is primarily driven by source water quality. Surface waters with higher levels of natural organic matter (e.g., rivers) have greater potential to form disinfection byproducts regulated by US EPA. The majority of cities in Michigan rely on great lakes water as their drinking water source. The low byproduct formation potential for great lakes water combined with the added cost and inconvenience of using chloramine explains the low frequency of chloramine use for water disinfection in Michigan. However, many more public water systems in states such as Texas and Florida reported using chloramine. Besides the relatively few chloraminated cities in the study, the location of those cities presented a potential bias. As a large academic medical center, Michigan Medicine may attract patients with underlying risk factors for NTM infection to live in cities nearby the center. Therefore, distance between each individual's address and Michigan Medicine was included as a fixed effect in logistic regression modeling. Additionally, there may be other city characteristics besides the ones we modeled that describe the variability in NTM infections across the water cohort. A longitudinal study that follows NTM infection in the same city before and after a change in disinfectant type would be one approach to address this concern.

5.7 Tables and figures

Table 5.1 Maximum number of years separating tests from 11,620 patients tested multiple times over the study period

Maximum years	Number of Multi-tested Patients	Percent of Multi-tested Patients
0	7,627	65.6
1	1,525	13.1
2	639	5.5
3	453	3.9
4	328	2.8
5	261	2.2
6	197	1.7
7	152	1.3
8	122	1.1
9	86	0.7
10	69	0.6
>10	161	1.4

Table 5.2 Number of NTM isolations for 1,244 NTM positive patients at Michigan Medicine during the study period

Number of Patients	Number of NTM Isolations	Percent Culture Positive Patients	Percent Patients Tested for NTM
888	1	71.4	2.97
192	2	15.4	0.64
77	3	6.1	0.26
28	4	2.3	0.09
25	5	2.0	0.08
8	6	0.6	0.03
26	>6	2.1	0.09

Table 5.3 Age, sex and immune status at year of first positive NTM result for 1,244 NTM positive patients

Group	Number of Patients
Sex	
Male	631
Female	613
Age	
≥90	7
80-89	72
70-79	198
60-69	267
50-59	246
40-49	168
30-39	103
20-29	78
10-19	60
<10	45
Immune status	
Compromised	685
Not	559

Table 5.4 Table 5-4 Demographics at first NTM isolation compared with Michigan demographics from 2010 US Census Bureau Data

Group	Number of Positive Patients	Percent Positive Patients (%)	Michigan US Census 2010 Data (%)
Sex			
Male	631	50.7	
Female	613	49.3	50.9
Age			
≥65	410	33.0	13.8
< 18	98	7.9	23.7
< 5	30	2.4	6.0

Table 5.5 Number (percent) of 2,135 NTM isolates by source category

Source Category	**# (%)**
Lung	1,360 (63.7)
Blood	56 (2.6)
Skin and musculoskeletal	35 (1.6)
Gastrointestinal	34 (1.6)
Genitourinary	26 (1.2)
Sinus	7 (0.3)
Lymphatic	6 (0.3)
Central Nervous System	2 (0.1)
Other	191 (8.9)
Missing/unknown	418 (19.6)

Table 5.6 Mixed effects logistic regression of 61,045 NTM test results (2,135 positive and 58,910 negative) from 29,796 patients at Michigan Medicine for 2000-2015

Variables	***p*-Value**	**OR (95% CI)**
Age	0.027	1.01 (1.00-1.02)
Sex (male)	0.145	0.77 (0.53-1.10)
Immune status (predisposed)	0.039	1.39 (1.02-1.90)

Table 5.7 Primary source (groundwater or surface water) and disinfectant type for 140 Michigan cities included in the study. Ann Arbor, East Lansing and Lansing (bold) reported using chloramine for disinfection.

	City	Primary Source	Disinfectant		City	Source	Disinfectant
1	Adrian	SW	Chlorine	71	Lapeer	SW	Chlorine
2	Allen Park	SW	Chlorine	72	Lathrup Village	SW	Chlorine
3	Alma	GW	Chlorine	73	Lincoln Park	SW	Chlorine
4	Alpena	SW	Chlorine	74	Linden	GW	Chlorine
5	**Ann Arbor**	**SW**	**Chloramine**	75	Livonia	SW	Chlorine
6	Auburn Hills	SW	Chlorine	76	Lowell	GW	Chlorine
7	Battle Creek	GW	Chlorine	77	Madison Heights	SW	Chlorine
8	Bay City	SW	Chlorine	78	Manistique	SW	Chlorine
9	Belding	GW	Chlorine	79	Marshall	GW	Chlorine
10	Belleville	SW	Chlorine	80	Melvindale	SW	Chlorine
11	Benton Harbor	SW	Chlorine	81	Memphis	SW	Chlorine
12	Berkley	SW	Chlorine	82	Menominee	SW	Chlorine
13	Birmingham	SW	Chlorine	83	Midland	SW	Chlorine
14	Bloomfield Hills	SW	Chlorine	84	Milan	GW	Chlorine
15	Brighton	GW	Chlorine	85	Monroe	SW	Chlorine
16	Buchanan	GW	Chlorine	86	Morenci	GW	Chlorine
17	Burton	SW	Chlorine	87	Mt Clemens	SW	Chlorine
18	Cadillac	GW	Chlorine	88	Mt Pleasant	GW	Chlorine
19	Carson City	GW	Chlorine	89	Muskegon	SW	Chlorine
20	Center Line	SW	Chlorine	90	New Baltimore	SW	Chlorine
21	Charlevoix	SW	Chlorine	91	New Buffalo	SW	Chlorine
22	Charlotte	GW	Chlorine	92	Niles	GW	Chlorine
23	Cheboygan	GW	Chlorine	93	Northville	SW	Chlorine
24	Chelsea	GW	Chlorine	94	Novi	SW	Chlorine
25	Clare	GW	Chlorine	95	Oak Park	SW	Chlorine
26	Clawson	SW	Chlorine	96	Orchard Lake Village	SW	Chlorine
27	Coldwater	GW	Chlorine	97	Owosso	GW	Chlorine
28	Croswell	GW	Chlorine	98	Petoskey	GW	Chlorine
29	Dearborn	SW	Chlorine	99	Pleasant Ridge	SW	Chlorine
30	Dearborn Heights	SW	Chlorine	100	Plymouth	SW	Chlorine
31	Detroit	SW	Chlorine	101	Pontiac	SW	Chlorine
32	Dexter	GW	Chlorine	102	Port Huron	SW	Chlorine
33	East Grand Rapids	SW	Chlorine	103	Portage	GW	Chlorine
34	**East Lansing**	**GW**	**Chloramine**	104	Richmond	SW	Chlorine

35	Eastpointe	SW	Chlorine	105	River Rouge	SW	Chlorine
36	Ecorse	SW	Chlorine	106	Riverview	SW	Chlorine
37	Escanaba	SW	Chlorine	107	Rochester	GW	Chlorine
38	Essexville	SW	Chlorine	108	Rochester Hills	SW	Chlorine
39	Farmington	SW	Chlorine	109	Rockwood	SW	Chlorine
40	Farmington Hills	SW	Chlorine	110	Romulus	SW	Chlorine
41	Fenton	GW	Chlorine	111	Roseville	SW	Chlorine
42	Ferndale	SW	Chlorine	112	Royal Oak	SW	Chlorine
43	Flat Rock	SW	Chlorine	113	Saginaw	SW	Chlorine
44	Flint	SW	Chlorine	114	Saline	GS	Chlorine
45	Fraser	SW	Chlorine	115	Sault Ste Marie	SW	Chlorine
46	Garden City	SW	Chlorine	116	South Lyon	SW	Chlorine
47	Gibraltar	SW	Chlorine	117	Southfield	SW	Chlorine
48	Gladstone	SW	Chlorine	118	Southgate	SW	Chlorine
49	Grand Blanc	GW	Chlorine	119	St Clair Shores	SW	Chlorine
50	Grand Ledge	GW	Chlorine	120	St Ignace	SW	Chlorine
51	Grand Rapids	SW	Chlorine	121	St Joseph	SW	Chlorine
52	Grosse Pointe	SW	Chlorine	122	Sterling Heights	SW	Chlorine
53	Grosse Pointe Farms	SW	Chlorine	123	Sturgis	GW	Chlorine
54	Grosse Pointe Park	SW	Chlorine	124	Sylvan Lake	SW	Chlorine
55	Grosse Pointe Woods	SW	Chlorine	125	Taylor	SW	Chlorine
56	Hamtramck	SW	Chlorine	126	Tecumseh	GW	Chlorine
57	Harbor Beach	SW	Chlorine	127	The Village of Grosse Pointe Shores A Michigan City	SW	Chlorine
58	Harper Woods	SW	Chlorine	128	Trenton	SW	Chlorine
59	Hastings	GW	Chlorine	129	Troy	SW	Chlorine
60	Hazel Park	SW	Chlorine	130	Utica	SW	Chlorine
61	Highland Park	SW	Chlorine	131	Village of Clarkston	SW	Chlorine
62	Hillsdale	GW	Chlorine	132	Walled Lake	SW	Chlorine
63	Howell	GW	Chlorine	133	Warren	SW	Chlorine
64	Huntington Woods	SW	Chlorine	134	Wayne	SW	Chlorine
65	Inkster	SW	Chlorine	135	Westland	SW	Chlorine
66	Jackson	GW	Chlorine	136	Wixom	SW	Chlorine
67	Kalamazoo	GW	Chlorine	137	Woodhaven	SW	Chlorine
68	Keego Harbor	SW	Chlorine	138	Wyandotte	SW	Chlorine
69	Lake Angelus	SW	Chlorine	139	Wyoming	SW	Chlorine
70	**Lansing**	**GW**	**Chloramine**	140	Ypsilanti	SW	Chlorine

Table 5.8 Logistic regression analysis results

Variables	**Variable Type**	***p*-Value**	**OR (95% CI)**
Age	Patient	0.002	1.01 (1.00-1.01)
Sex (male)	Patient	0.262	0.88 (0.71-1.10)
Risk factors (compromised)	Patient	< 0.0001	3.34 (2.67-4.17)
Sample year	Patient	0.168	0.98 (0.96-1.01)
Travel distance to Michigan Medicine	Patient	0.132	0.88 (0.75-1.04)
Total population	City	0.448	1.06 (0.91-1.25)
Median household income	City	0.998	0.99 (0.86-1.16)
Percent of population over 65 years old	City	0.675	1.04 (0.88-1.22)
Percent of population black	City	0.324	0.91 (0.74-1.10)
Disinfectant type (chloramine)	City	0.927	0.98 (0.69-1.40)
Water source (surface water)	City	0.040	1.48 (1.02-2.16)

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Chapter 6
Conclusions and Public Health Significance

This research was undertaken to contribute to our understanding of the presence of opportunistic bacterial pathogens, especially non-tuberculous mycobacteria, in treated drinking water. Previous research on opportunistic bacteria has focused on intervention strategies in distribution systems and premise plumbing, but the results presented in this dissertation suggest that specific drinking water treatment plant design and operational considerations can improve microbial water quality and minimize risk of waterborne infectious disease.

First, this research highlights the importance of biofilm formation and non-idealities of full-scale, multi-chamber contactors used for disinfection, in influencing microbial water quality as a whole (Chapter 2). Ozone is considered a gold standard for water treatment as laboratory scale experiments have reported rapid inactivation of bacteria by ozone (Ramseier et al., 2011). In full-scale, multi-chamber ozone contactors, however, it was found that microbial detachment from biofilms on contactor surfaces, and from biomass within sediments in a hydraulic dead zone, were responsible for the presence of viable microorganisms, including mycobacteria, in contactor effluents. Previous research has shown that bacterial growth in biofilm can induce bacterial resistance to disinfectant (Berry et al., 2009). It is therefore advisable to minimize biofilm formation in disinfection contactors.

Health risks posed by disinfectant and disinfection byproducts limit the concentration of disinfectant that can be used, making a strategy of increasing CT (concentration × time) to avoid biofilm formation on contactor surfaces unrealistic. Instead, research is needed to investigate contactor materials and utility maintenance practices that minimize biofilm formation. Additionally, the observed increase in microbial concentrations following dead zones is an argument for improving particle removal before disinfection to avoid sediments entering contactors, and improving hydraulic efficiency in multi-chamber contactors by decreasing distance between baffle walls (i.e., chamber width) in contactors.

Approximately 15,000 viable mycobacterial cells per liter, representing four mycobacterial 16S rRNA gene-based OTUs, were detected in full-scale ozone contactor effluents (Appendix). Ozonated water was treated further in biologically-active carbon (BAC) filters. Some viable bacterial populations that were detected in ozone contactor effluents, including one mycobacterial OTU, were also detected in the BAC filters, highlighting the interconnectedness of the drinking water microbiome. The finding of only one mycobacterial OTU from the ozone contactors in filter BAC may represent competitive exclusion of other mycobacterial OTUs in the filter microbial community. Factors selecting for one type of mycobacteria over another deserve more attention and more research is needed on interactions between pathogenic and non-pathogenic mycobacterial species in drinking water systems. High-throughput microbiology tools with the ability to differentiate mycobacterial species are needed since partial 16S rRNA gene sequencing does not provide the resolution required to identify species for most bacteria. Sequencing of mycobacterial *rpoB* genes showed diverse mycobacterial species were present in BAC of a full-scale filter and *Mycobacterium avium* was the dominant species in filter effluent. This work underscores the importance of filter maintenance practices that avoid reinforcing the presence of mycobacteria. For example, filter backwashing performed with finished water containing disinfectant presents an opportunity for selective effects by disinfectant exposure.

Building on previous reports that pure cultures of bacteria respond to disinfectant exposure by upregulating virulence factors (reviewed in Chapter 4), this research investigated the response of *M. avium* to disinfectant exposure during backwashing of full-scale BAC filters. The observed upregulation of *M. avium*'s mammalian cell entry gene *mce1C* in response to monochloramine, and during filter backwashing with water containing monochloramine, raises the possibility of an adaptive response that promotes disinfection resistance. Disinfectant resistant mycobacteria would explain the recovery of mycobacteria from the distribution system even when sufficiently high residual disinfectant is measured (Haig et al., 2017). The upregulation of intracellular parasitism genes in particular has implications for *M. avium*'s interactions with amoebae and for its role as an intracellular pathogen. For example, its persistence in drinking water systems may involve replicating in amoebae, as intracellular *M. avium* are better protected from harsh environments (Berry et al., 2010). It is unclear whether *M. avium*'s response to monochloramine increases its virulence or, in other words, reduces its infectious dose. While sub-lethal monochloramine exposure has been shown to induce mycobacterial virulence factors (reviewed in Chapter 4), the

impact of sub-lethal disinfectant exposure on bacterial virulence in animal trials appears not to have been studie but is important for evaluating the impact of drinking water disinfection on human health.

Utilities should explore limiting the use of backwash water with disinfectants to reduce selection for disinfectant-resistant bacteria. For example, research is needed to test the impact of dechlorinating backwash supply on filter microbial communities, focusing on whether this strategy reduces mycobacterial levels while achieving the same or better filtration performance. This research could result in strategies for utilities to reduce the levels of disinfectant-resistant, opportunistic pathogens in filters and thereby lower the likelihood of introducing these microbes into distribution networks (Pinto et al., 2012).

Even though higher mycobacterial levels have been reported in tap water disinfected with chloramine compared to chlorine, disinfectant used for residual disinfection of water supplied to patient home was not a significant predictor of mycobacterial infection in a case control study of patients at the University of Michigan's academic medical center Michigan Medicine (Chapter 5). This result suggests that an increasing use of chloramine for drinking water disinfection does not significantly increase the burden of mycobacterial disease. However, the use of a surface water source compared to a groundwater source was significantly associated with infection. This impact of a surface water source on mycobacterial infection will need to be confirmed by other studies but it points to the importance of source water quality and the greater potential for microbial contaminants in surface waters. This is an interesting finding in light of water scarcity pressures forcing water purveyors to consider alternative water sources such as reclaimed water.

The use of experimental methods in this dissertation to monitor for mycobacteria through water treatment has pointed to specific dynamics that explain why mycobacteria are present in finished water from treatment plants. Furthermore, epidemiologic data was used to evaluate potential associations between mycobacterial infection and residual disinfectant type (chlorine or chloramine) or source water type (surface water or groundwater). Gaps in data caused by the limited reportability of mycobacterial infection (Buser et al., 2015; Winthrop et al., 2017) and the lack of a centralized, curated database describing water treatment practices and finished water quality characteristics for all public water treatment plants (Chini et al., 2016) need to be addressed for better evaluation of water treatment practices as risk factors for opportunistic infection.

This research provides useful information for the better management of microbial water quality and the production of drinking water that is safe for everyone, including immune compromised individuals. While the research in this dissertation was largely focused on mycobacteria, several of the observations made are relevant for other waterborne bacteria. Decisions for modifications to water treatment practices should consider the drinking water microbiome as a whole and aim to improve microbial water quality, as opposed to making treatment process changes that reduce abundances of a single type of bacteria. In other words, findings from this dissertation should be integrated with previous research on other waterborne bacteria for improving microbial water quality. If changes in water treatment plant design and operational considerations can improve microbial water quality and, for example, reduce waterborne infections at least by half, 20,000 hospitalizations at a cost of $485 million per year (Collier et al., 2012) would be prevented.

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Appendix

Viable Bacteria in Full-scale Ozone Contactor Effluents Are Also Present in Downstream Biologically Active Carbon Filters

A.1 Introduction

Biologically active carbon (BAC) filtration is becoming a popular technology in drinking water treatment plants in North America. Based on recent surveys of biofiltration plants in North America, 63% of plants practice biofiltration with ozone pretreatment (Brown et al., 2016). Non-ideal mixing conditions in multi-chamber ozone contactors can reduce the effectiveness of ozone (Kim et al., 2010). As a result, microbial populations that are relatively resistant to disinfection may survive ozone treatment. Fluorescence activated cell sorting (FACS) combined with bacterial 16S rRNA gene sequencing was used to identify viable bacteria present in the effluent from an ozone contactor at a full-scale drinking water treatment plant (DWTP). Furthermore, total bacteria in BAC filters following ozonation at the DWTP were characterized by 16S rRNA gene sequencing to identify viable bacterial populations in filter communities that may have been seeded by ozonation.

A.2 Materials and methods

A.2.1 Sample collection

The Ann Arbor DWTP provides lime softening, coagulation, flocculation, sedimentation, ozonation, filtration, and chloramination. Ozonated water is applied to biologically-active, dual media filters containing granular activated carbon and sand. The DWTP has four parallel ozone contactors each containing seven chambers separately by vertical baffle walls, and inlet and outlet basins. In each contactor, five sample lines extend from the contactor chambers to an ozone analyzer. Water samples were collected from sample lines 1, 3, 4 and 5, corresponding with clarified water before ozone exposure in chamber 1, water during contact with ozone in chamber 3, water after exposure to residual ozone in chamber 4 and water from an outlet basin

Water samples (500 mL) were collected into sterile plastic bottles containing sodium thiosulfate (5 × stoichiometric requirement (SR) to quench 0.5 mg/L ozone) from each sample line sequentially. Before sample collection, each sample line was flushed until meter readings of ozone concentration were stable (approximately 3 min).

BAC cores were collected from five BAC filters before backwashing in December 2016. A BAC core was collected from one filter (Filter 25) before rinsing. BAC from top, middle and bottom fractions of each core were collected into sterile falcon tubes and snap frozen on dry ice.

A.2.2 Fluorescence activated cell sorting

Samples were stained with propidium iodide (PI, ThermoFisher) and SYBR Green I (SGI, ThermoFisher) at final concentrations of 2.97 μM PI and 1X SGI. After staining, samples were incubated at 37°C for 15 min to improve cell staining.(Barbesti et al., 2000; Van Nevel et al., 2013) A FACSAria II cell sorter (BD Biosciences, San Jose, CA) at the University of Michigan Flow Cytometry Core was used for cell sorting.

The trigger parameter defines a threshold for an event. For the first sorting dataset (August 2016), events were triggered off of forward scatter. For the second sorting dataset (November 2016), events were triggered off fluorescence in the SGI OR PI channels above a threshold of 200. For each sample, forward and side-scatter as well as fluorescence in the 610/10 and 530/30 wavelengths were measured for detection of PI positive and SGI positive events. No compensation was applied to sample measurement. Electronic gating of events in a density plot of SGI and PI fluorescence channels (Figure 1) was used to identify events for sorting (i.e., events positive for SGI and negative for PI).

A.2.3 DNA extraction and 16S rRNA gene sequencing

DNA was extracted from sorted cells using a modified recipe of the Universal Extraction Lysis Buffer and a phenol-chloroform-isoamyl extraction protocol (Haig et al., 2017; Hill et al., 2015). DNA was precipitated with polyethylene glycol (PEG) 6000 to improve DNA recovery from the relatively few cells in the sorted samples. In a comparison of precipitation methods using a DNA standard, higher DNA yields were observed using PEG 6000 than precipitation by ammonium acetate and isopropanol (data not shown). DNA was extracted from BAC using a FastDNA Spin

Kit (MP Biomedicals) (Santa Ana, California, USA). Higher DNA yields were observed for BAC samples using this kit than with our phenol chloroform method (data not shown).

We were concerned that contaminating DNA derived from sheath fluid in the cell sorter or from reagents used during nucleic acid extraction or PCR could confound our analysis. Therefore, DNA was also extracted from samples of sheath fluid, reagents used during DNA extraction, and PCR blanks. These control samples were processed in parallel with the samples.

PCR, sample multiplexing and Illumina MiSeq sequencing of partial 16S rRNA genes were performed by the Microbial Systems Laboratory at the University of Michigan Medical School (Ann Arbor, Michigan). Barcoded dual-index primers specific to the V4 region of the 16S rRNA gene(Caporaso et al., 2011; Kozich et al., 2013) were used to amplify the DNA. The sorted cell extracts were amplified using a touch down PCR procedure. PCR reactions included 5 µL of 4 µM forward and reverse primers, 0.15 µL of AccuPrime Taq DNA High Fidelity Polymerase (Life Technologies), 2 µL of 10x AccuPrime PCR Buffer II (Thermo Fisher Scientific), 3 µL of DNA template and PCR-grade water. After an initial denaturation at 95 °C for 2 min, 20 cycles of touch down PCR were performed (95 °C for 20 s, 60 °C for 15 s, with a temperature decrease of 0.5 °C per cycle, and 72 °C for 5 min), followed by 20 cycles of 95 °C for 20 s, 55 °C for 15 s, 72 °C for 5 min, and a final extension step at 72 °C for 10 min. PCR reactions for the BAC extracts had the same components except that only 1 µL of template was used and bovine serum albumin was added (0.5 mg/mL final concentration). PCR conditions consisted of 2 min at 95 °C followed by 30 cycles of 95 °C for 20 s, 55 °C for 15 s, 72 °C for 5 min, followed by 72 °C for 10 min. Sequencing was done on the Illumina MiSeq platform using a MiSeq Reagent Kit V2 500 cycles according to the manufacturer's instructions with some modifications.(Kozich et al., 2013)

16S rRNA gene sequences from all samples were curated using mothur (version 1.39.5) (Schloss et al., 2009). During sequence curation, sequences that were within two nucleotides of each other were merged using the pre.cluster command. After pre-clustering, 2289 sequences in the control samples (putative contaminants) were removed from the 21767 unique sequences in the dataset using the remove.seqs command (10.5% sequences removed). Curated sequences were clustered into operational taxonomic units (OTUs) using a 97% similarity cutoff with the opticlust algorithm (Westcott et al., 2016). Sequences were classified using a naïve Bayesian classifier (Wang et al.,

2007) trained against a 16S rRNA gene training set provided by the Ribosomal Database Project (RDP) (Cole et al., 2009). An RDP consensus taxonomy was generated for each OTU.

For a comparison of membrane-intact OTUs in ozone contactor effluents and OTUs in BAC filters, sequences from top, middle and bottom BAC core factions from each filter was combined into a composite filter sample. Sequences from ozone contactor chambers 3, 4 and 5 were combined into a composite sample representing ozone contactor effluent.

A.3 Tables and figures

Table A.1 (a) Top ten overlapping OTUs across membrane-intact fractions after ozonation (sequences from ozone contactor chambers 3, 4 and 5 combined) and total bacteria from BAC (sequences from all filter samples combined) (b) two overlapping OTUs from groups with known opportunistic pathogenic species

OTU	Taxonomy	Intact after ozonation relative abundance (%)	Filter relative abundance (%)
Otu0008	*Bradyrhizobiaceae*	10.4	14.5
Otu0010	*Hyphomicrobiaceae*	0.33	14.0
Otu0023	*Nitrospira*	0.09	8.49
Otu0024	*Brevundimonas*	1.69	7.91
Otu0026	*Betaproteobacteria*	0.07	6.40
Otu0033	*Reyranella*	3.40	5.64
Otu0037	*Burkholderiales*	0.84	1.53
Otu0040	*Betaproteobacteria*	0.55	5.39
Otu0048	*Alphaproteobacteria*	0.68	4.57
Otu0057	*Nitrosomonas*	2.81	4.20
…			
Otu0545	*Mycobacterium*	0.56	0.02
Otu0537	*Stenotrophomonas*	0.04	0.06

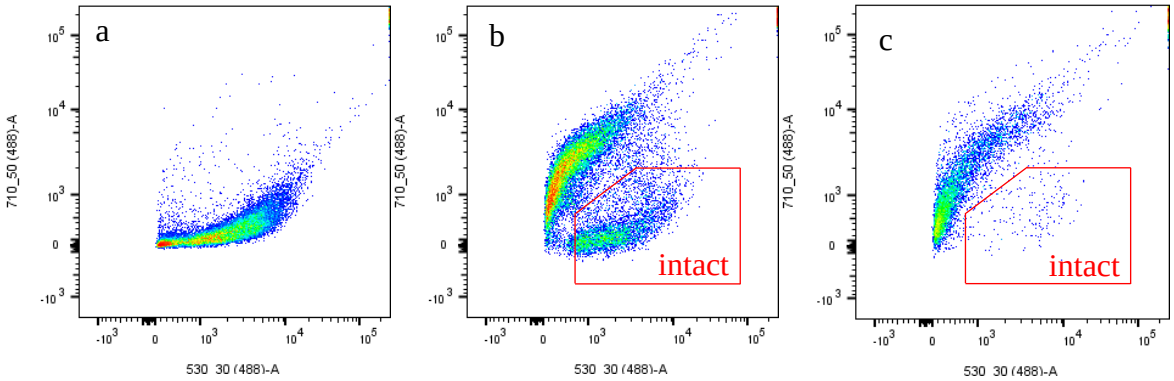

Figure A-0.1 Example dot plots and gating used for fluorescence activated cell sorting. Water from chamber 1 (before ozonation) was stained with (a) SYBR Green I or (b) a combination of SGI and propidium iodide (PI). Significantly fewer SGI positive, PI negative events (i.e., membrane-intact cells) were detected in water from chamber 5 (after ozonation) (c) stained with SGI and PI.

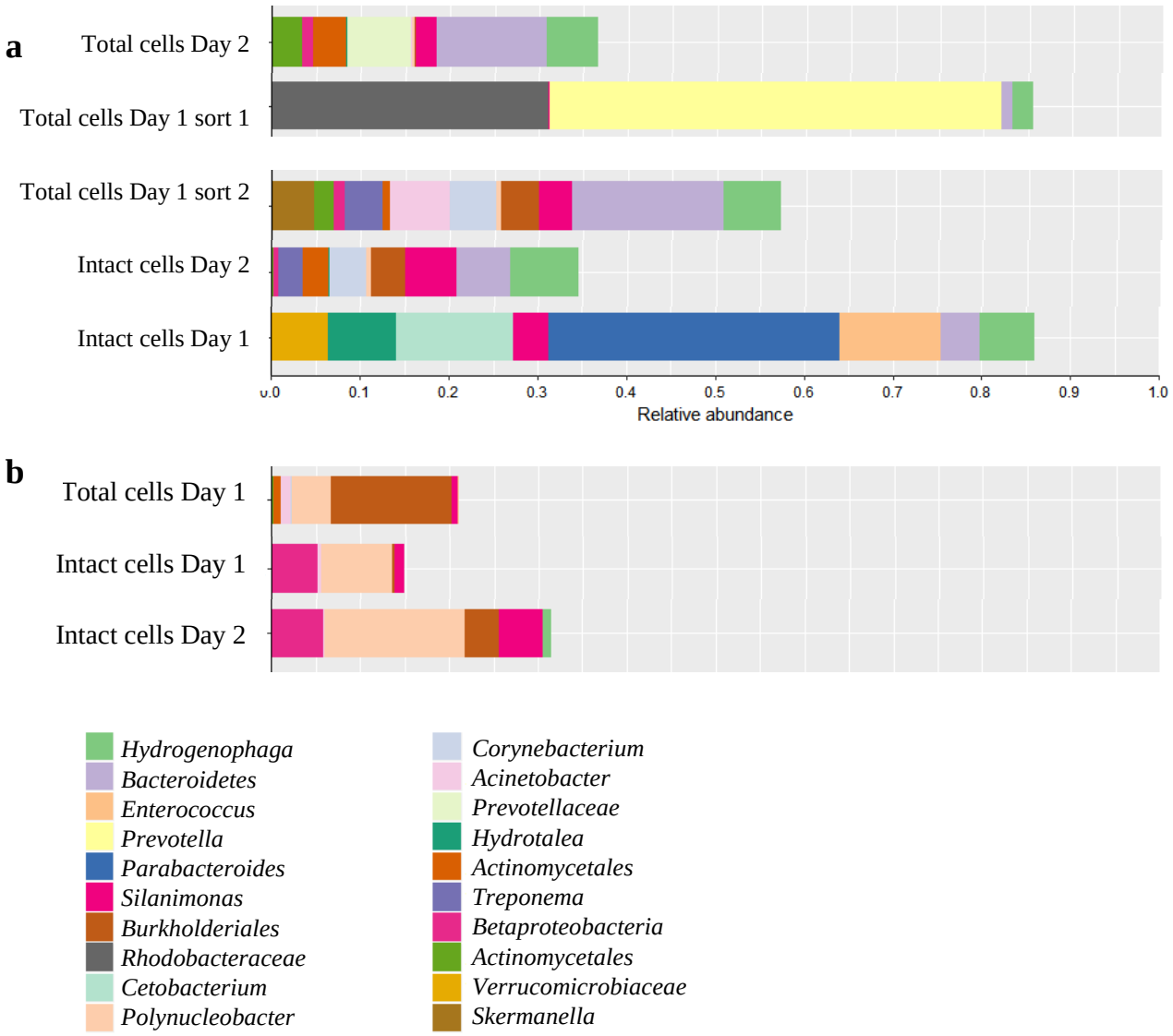

Figure A-0.2 20 most abundant OTUs from total and membrane-intact fractions from chamber 1 (before ozonation) for samples collected in (a) August 2016 and (b) November 2016. The flow cytometry trigger was set to forward scatter for the August 2016 samples but it was changed to fluorescence for the November 2016 to improve detection of smaller cells.

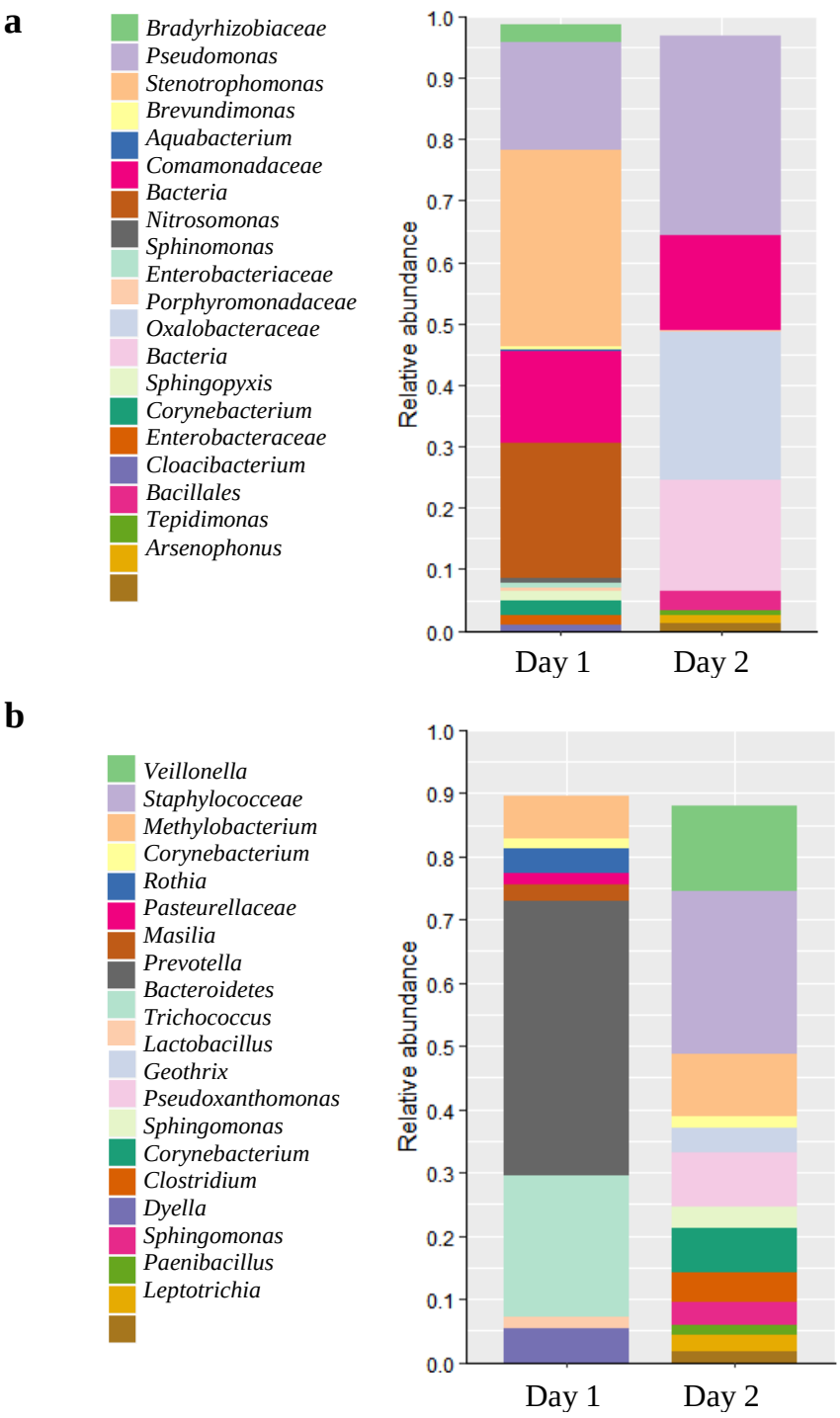

Figure A-0.3 20 most abundant OTUs in membrane-intact fraction of water samples from chamber 5 (after ozonation)

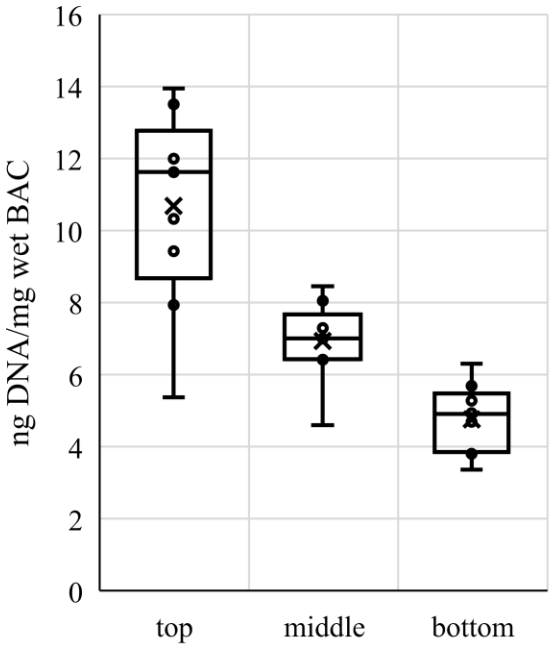

Figure A-0.4 DNA extraction yields from BAC samples from top, middle and bottom fractions of all filters sampled

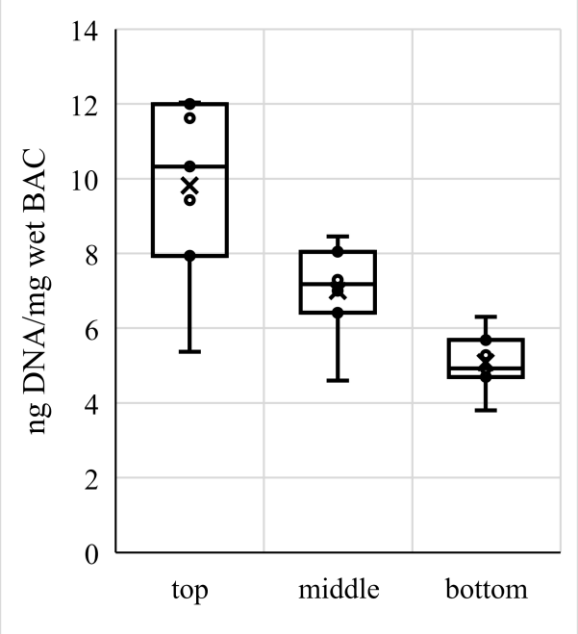

Figure A-0.5 Figure A 1 DNA extraction yields from BAC samples from top, middle and bottom fractions of filters sampled before backwashing

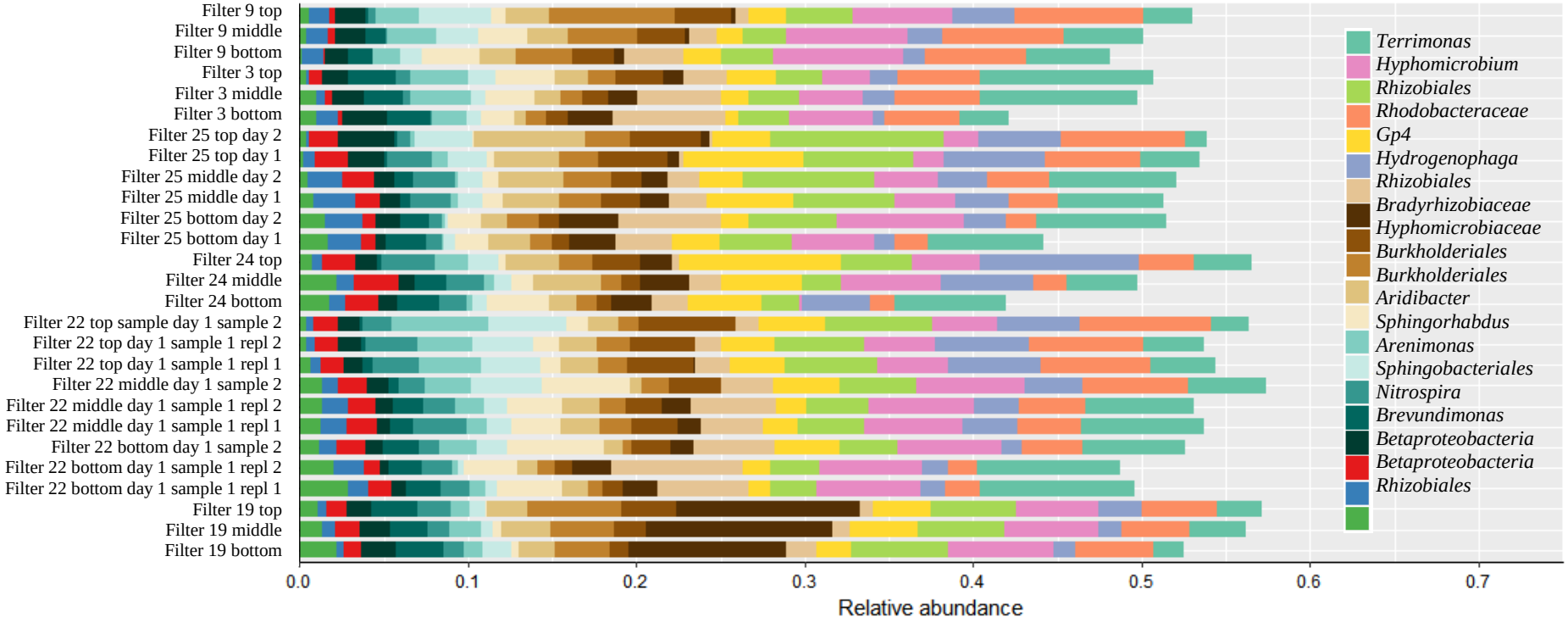

Figure A-0.6 Relative abundance of 20 most abundant OTUs across top, middle and bottom fractions from full-scale BAC filters sampled before backwashing (filters 9, 3, 24, 22, 19) or after backwashing with greater than 24 h stagnation (filter 25). Filters 22 and 25 were each sampled on two days. Replicate sampling and extractions (repl) were sequenced for samples from filter 22.

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