NATIONAL WATER RESEARCH INSTITUTE

NWRI GRADUATE FELLOW SEMI-ANNUAL PROGRESS REPORT

Date:	November 6, 2019
Project Title:	Bacterial Opportunistic Pathogens in Drinking Water Treatment Systems and the Influence of Disinfectants
Graduate Fellow:	Katherine Dowdell
Advisor:	Professor Lutgarde Raskin
Institution:	University of Michigan

Background and Introduction

Opportunistic pathogens (OPs) are a growing public health concern in the United States and abroad. OPs are microorganisms that are not generally harmful to healthy adults, but may pose risks to vulnerable populations, such as the elderly or immunocompromised people. Bacterial OPs of particular concern include *Legionella pneumophila*, *Pseudomonas aeruginosa*, and species in the genus *Mycobacterium*, referred to as nontuberculous mycobacteria (NTM). NTM are commonly found in water and soil and, while more than 170 species are included in this group, only approximately 20 species are considered clinically-relevant (Falkinham, 2015; Tortoli, 2014). NTM in laboratory-scale experiments have shown resistance to disinfection and the ability to readily form biofilms (Taylor, Falkinham, Norton, & LeChevallier, 2000; Wang, Sui, Yuan, Li, & Lu, 2019). Some species have been observed to survive uptake by amoebae, and it is hypothesized this interaction with amoebae may increase NTM's virulence (Cirillo et al., 1999; Thomas & Ashbolt, 2011). One consequence of these properties is that NTM are commonly found in engineered water systems, including the plumbing and taps of homes and businesses (Donohue et al., 2015; Falkinham, 2018). Several studies have reported increasing rates of NTM pulmonary infection over the past two decades (Donohue, 2018; Shah et al., 2016). While numerous factors are likely contributing to this rise, it is hypothesized that increased exposure through drinking water is a factor in the rising rates of infection (Dowdell et al., 2019).

Hypothesis

In many drinking water treatment systems, ozone is used prior to filtration for disinfection and to improve the removal of organics. Biological filtration, which involves the colonization of traditional granular filtration media by microorganisms, is increasingly used to improve the removal of bioavailable organic carbon compounds formed during ozonation. Given the physiology of NTM and, as suggested by results of previous studies, it is possible that some NTM survive disinfection in full-scale drinking water treatment systems (Kotlarz et al., 2018). If so, it is plausible that NTM would benefit from the reduced competition in disinfected waters and colonize biological filtration media. **We hypothesize that the use of ozone prior to biological filtration processes in drinking water treatment may preferentially select for microorganisms more resistant to oxidants, such as NTM, in the filter influent and in the biological filter media.** Few full-scale studies have investigated how NTM

concentrations and viability are impacted by drinking water treatment. Even fewer studies have evaluated the impact of ozonation followed by biological filtration on NTM in filters and finished water.

Study Goals and Report Objectives

The overarching goal of this research is to evaluate how drinking water treatment practices influence the concentrations of viable NTM in drinking water. In addition to evaluating concentrations of clinically relevant NTM species and viability, this work aims to evaluate how the regulation of NTM genes associated with survival strategies may be impacted by treatment methods. We are testing our hypotheses using experiments focused on two full-scale drinking water systems: 1) a biological filtration plant that uses pre-ozonation and backwashes the filters with monochloraminated water in Michigan, and 2) a drinking water system that employs multiple biological filtration and pre-ozonation steps without finished water disinfection in Switzerland.

With the reported rise in the rate of NTM pulmonary infections and the suggested link between pulmonary infection and water, it is imperative that we assess the risk of NTM pulmonary infection corresponding to contact with drinking water. The first step in assessing the risk is measuring the concentrations of NTM in drinking water and determining which species are present and viable. This research seeks to characterize the concentrations and virulence of clinically relevant species of NTM in drinking water to provide a basis for risk analysis and evaluate how current practices could be improved to decrease the public health risk posed by bacterial OPs in drinking water.

Progress to Date

Work since the previous progress report has focused on analysis of flow cytometry data and processing and analysis of bacterial isolates collected from the Swiss drinking water treatment plant. Additionally, planning is underway for a new study evaluating concentrations of opportunistic pathogens in a Michigan drinking water treatment plant. The monochloramine backwash pilot testing has been concluded and samples are being analyzed.

Experimental Design

The objectives of the research in Michigan and Switzerland are to characterize the impact of oxidation and biofiltration on the microbial community and NTM in drinking water by analyzing samples collected from various points in full-scale drinking water treatment systems. Molecular methods including polymerase chain reaction (PCR), quantitative PCR (qPCR), digital droplet PCR (ddPCR), reverse transcription (RT)-qPCR, and DNA sequencing will be used to determine the concentrations of NTM and identify which species are present. Sequencing will also be used to determine the composition of the overall microbial communities in the samples. Flow cytometry and culture-based methods are being employed to determine cell concentrations, viability, and species present.

Both the Michigan and Swiss water treatment systems use ozonation followed by biological filtration. However, there are significant differences in the treatment trains of the systems. The Michigan treatment system treats a blend of groundwater and surface water using coagulation, flocculation, and lime softening followed by ozone-biofiltration with granular activated carbon (GAC) and monochloramine disinfection (Figure 1). In contrast, the Swiss treatment plant treats surface water using multiple ozonation and biofiltration steps, including a final slow sand filtration step. There is no final oxidation of the water and no oxidant residual is used in the distribution system of the Swiss system. Figure 2 provides a schematic of the Swiss water treatment plant.

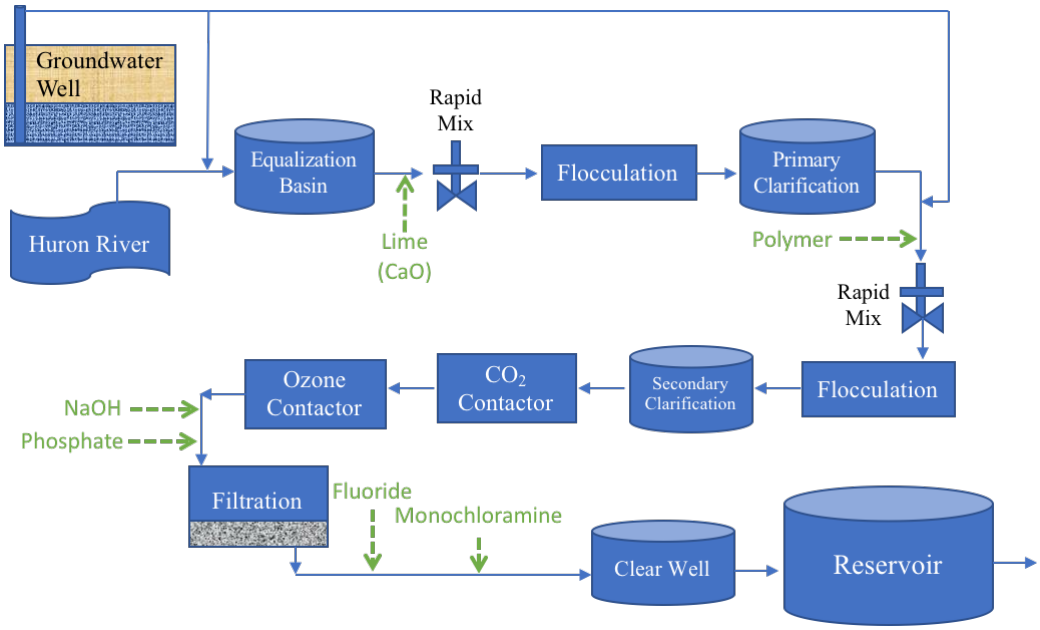

Figure 1. Schematic of the Michigan drinking water treatment plant where sampling is being conducted

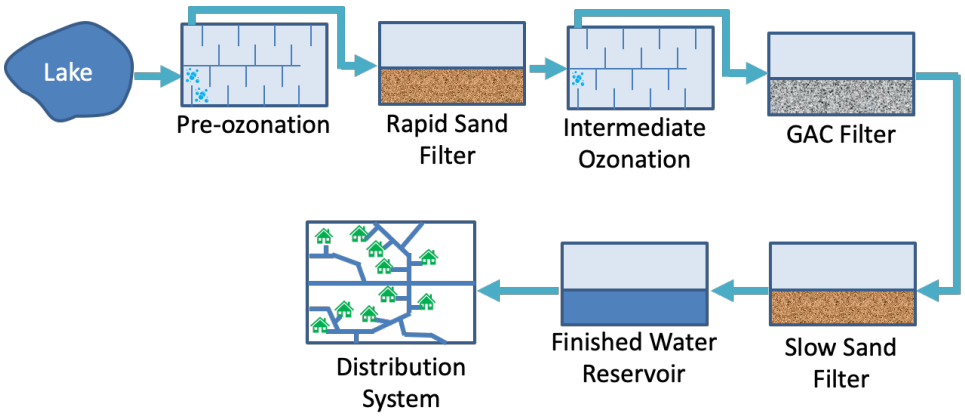

Figure 2. Schematic of the Swiss drinking water treatment plant where samples were collected in Spring 2019

Samples for DNA analyses are collected using 0.22-micron Sterivex cartridge filters (EDM Millipore, Germany) using sterile methods and are stored at -80 °C until processing. Testing is ongoing to determine the DNA extraction method that yields the highest amount of high-quality DNA with the least contamination. The extraction method selected will be used for both the Swiss and Michigan DNA samples. NTM concentrations will be determined using ddPCR with primers targeting the NTM *atpE* gene, a single copy housekeeping gene. Microbial community analysis will be conducted using primers targeting the V4 region of the bacterial 16S rRNA gene and Illumina MiSeq sequencing. Water samples from the Swiss study were analyzed using flow cytometry as described in the previous report.

In addition to DNA-based and flow cytometric methods, culture-based methods are also being used to characterize the viable bacteria present in the treatment systems. In the Swiss work, samples for culture were collected during and after each ozonation step. The residual ozone was neutralized using sodium thiosulfate. Samples were cultured onto R2A agar media plates by pipetting 1 milliliter (mL) onto the surface of the media

and spreading with a sterile spreader. The plates were incubated in the dark at 20 °C for at least one week. Isolates were cut from the agar and frozen in sterile microcentrifuge tubes. DNA from these isolates is being extracted using a previously described method (Liu et al., 2002). The near full-length bacterial 16S rRNA gene is being amplified using PCR (Takahashi, Tomita, Nishioka, Hisada, & Nishijima, 2014). After PCR, products are purified using the QIAquick PCR purification kit (QIAGEN, Germany), then sent for Sanger sequencing at the University of Michigan Advanced Genomics Core. The Sanger sequencing chromatograms, which show the raw output from sequencing, are read to determine the DNA sequence of the samples. Raw reads range from approximately 1,200 to 1,300 base pairs (bps). The chromatograms are reviewed and trimmed using the 4Peaks Software (Griekspoor & Groothuis, 2015). Trimming is required for sequencing outputs to remove primer regions and portions of the reads where the base calls are less accurate. The trimmed reads are approximately 700 to 850 bps. The trimmed forward and reverse sequences are combined using overlapping regions. The combined sequences are searched using the National Center for Biotechnology Information (NCBI) Nucleotide Basic Local Alignment Search Tool (BLAST) using the Nucleotide collection database and the megablast algorithm (Madden, 2013).

The Michigan water samples will be cultured using filter concentration and selective media to recover NTM, *Pseudomonas aeruginosa*, and amoebae. Filter concentration will be required due to anticipated low concentrations of colony forming units (CFU) per mL. Plating will be done either in duplicate or triplicate. A subset of the colonies counted as target organisms will be confirmed using Sanger sequencing. Additionally, the Legiolert and Pseudalert kits (IDEXX, USA) will be used to obtain the most probably number (MPN) of *Legionella pneumophila* and *Pseudomonas aeruginosa* in a subset of the samples.

Results and Discussion

The analysis of flow cytometry data from the Swiss samples was completed during this reporting period. Results show that ozonation significantly reduces, but does not fully eliminate, the intact cell populations. Figure 3 provides a subset of the results. The plots show the intact cell count results for the raw surface water, pre-ozonation effluent, and the intermediate ozone effluent, respectively. Each dot on the plots is considered an event, and the events within the red dotted region (gated area) are considered to be intact cells. The gating for this analysis was developed by conducting pre-testing using tap water, raw surface water, and ozonated water samples. Each plot represents the number of events in 50 microliters of sample.

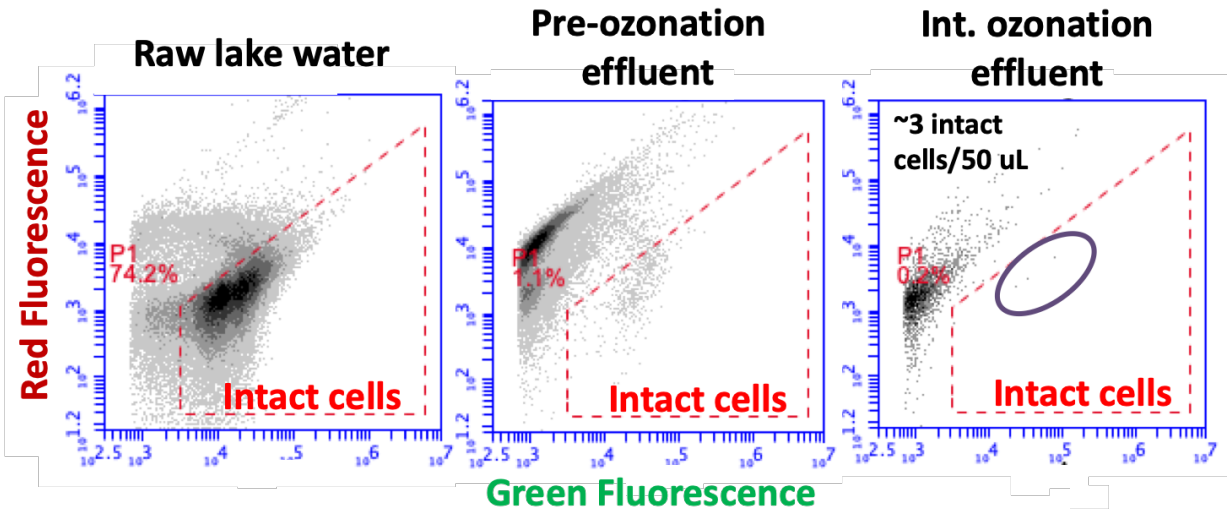

Figure 3. Results of intact cell flow cytometry of Swiss water samples. The dots falling within the red dashed area are considered intact cells.

As shown in Figure 3, ozonation substantially reduces the intact cell population, with the effluent of the intermediate ozonation step showing the lowest intact cell concentration. These plots also show, however, that a few intact cells remain after both ozonation steps.

The presence of viable cells in both ozone effluents is supported by the results of culturing. Viable bacteria were recovered from all samples, with the lowest CFU/mL observed in the intermediate ozone effluent. Work is ongoing to identify the isolates, but a subset of the results is shown in Table 1. The genera reported in Table 1 were the entries returned by BLAST with the highest percent identity.

Table 1. Results to date of sequencing of bacterial isolates from Swiss water sampling

Ozone contactor	Sample location	Bacterial isolates identified
Pre-ozone	Pre-ozone midpoint	*Flavobacterium* sp., *Acidovorax* sp.
	Pre-ozone effluent	*Achromobacter* spp, *Bacillus* sp., *Caulobacter* sp.
Intermediate ozone	Int. ozone midpoint	*Sphingomonas* sp, *Bacillus* sp.,
	Int. ozone effluent	*Bacillus* spp.

Of the isolates identified to date, it is notable that several belong to genera known to contain opportunistic pathogens. The genera listed in Table 1 that include at least one known OP are *Flavobacterium*, *Achromobacter*, *Sphingomonas*, and *Bacillus* (LiPuma, 2010; Manfredi et al., 1999; Okinaka, Pearson, & Keim, 2006; Ryan & Adley, 2010). Further analysis of isolates, combined with the results of the DNA-based work, is expected to allow for initial conclusions relating to hypotheses. Further sequencing of a subset of isolates will be used as needed to determine the species and strains.

As noted previously, testing is underway to select the DNA extraction method that will be used for collected Swiss samples and for samples from upcoming work in Michigan. The extraction kits being evaluated include MB Biomedical's FastDNA SPIN kit and QIAGEN's PowerWater kit. Kits are being evaluated based on 1) total DNA yield, 2) yield of *atpE* genes, and 3) quality of DNA.

Conclusions

The results to date from the Swiss sampling reveal that ozonation does significantly decrease the concentrations of intact cells in drinking water, but does not fully inactivate all bacteria. Several of the organisms identified thus far are from genera known to contain at least one opportunistic pathogen. Further testing is needed to confirm whether the isolates themselves belong to species known to be OPs. Analysis of the Swiss DNA samples will allow for assessment of changes to the microbial community with each treatment step, and will allow for quantification of OPs of interest, including NTM. Sampling at the Michigan treatment plant will begin this winter and continue for one year. Results of Michigan sampling will be compared to those from the Swiss water, with the goal of identifying how similarities and differences in the microbial communities and OP concentrations relate to treatment processes. This work will support efforts to better understand how our drinking water treatment processes shape the microbial community of our water and how that relates to public health.

Next Steps

Upcoming work includes the continued identification of the bacterial isolates obtained from the Swiss water samples and the characterization of Swiss water samples once a DNA extraction method is selected. Sampling of the Michigan water system will begin this month and run through Fall 2020.

References

Cirillo, J. D., Cirillo, S. L. G., Yan, L., Bermudez, L. E., Falkow, S., & Tompkins, L. S. (1999). Intracellular growth in Acanthamoeba castellanii affects monocyte entry mechanisms and enhances virulence of Legionella pneumophila. *Infection and Immunity*, *67*(9), 4427–4434. https://doi.org/0019-9567/99

Donohue, M. J. (2018). Increasing nontuberculous mycobacteria reporting rates and species diversity identified in clinical laboratory reports. *BMC Infectious Diseases*, *18*(1), 163. https://doi.org/10.1186/s12879-018-3043-7

Donohue, M. J., Mistry, J. H., Donohue, J. M., O'Connell, K., King, D., Byran, J., … Pfaller, S. (2015). Increased frequency of nontuberculous mycobacteria detection at potable water taps within the United States. *Environmental Science and Technology*, *49*(10), 6127–6133. https://doi.org/10.1021/acs.est.5b00496

Dowdell, K., Haig, S.-J., Caverly, L. J., Shen, Yun, LiPuma, J. J., & Raskin, L. (2019). Nontuberculous mycobacteria in drinking water systems – the challenges of characterization and risk mitigation. *Current Opinion in Biotechnology*, *57*, 127–136.

Falkinham, J. O. (2015). Environmental sources of nontuberculous mycobacteria. *Clinics in Chest Medicine*, *36*(1), 35–41. https://doi.org/10.1016/j.ccm.2014.10.003

Falkinham, J. O. (2018). *Mycobacterium avium* complex: Adherence as a way of life. *AIMS Microbiology*, *4*(3), 428–438. https://doi.org/10.3934/microbiol.2018.3.428

Griekspoor, A., & Groothuis, T. (2015). 4Peaks (Version v 1.8). Retrieved from www.nucleobytes.com

Kotlarz, N., Rockey, N., Olson, T. M., Haig, S.-J., Sanford, L., LiPuma, J. J., & Raskin, L. (2018). Biofilms in full-scale drinking water ozone contactors contribute viable bacteria to ozonated water. *Environmental Science & Technology*, *52*(5), 2618–2628. https://doi.org/10.1021/acs.est.7b04212

LiPuma, J. J. (2010). The Changing Microbial Epidemiology in Cystic Fibrosis. *Clinical Microbiology Reviews*, *23*(2), 299–323. https://doi.org/10.1128/CMR.00068-09

Liu, L., Coenye, T., Burns, J. L., Whitby, P. W., Stull, T. L., & LiPuma, J. J. (2002). Ribosomal DNA-Directed PCR for Identification of Achromobacter (Alcaligenes) xylosoxidans Recovered from Sputum Samples from Cystic Fibrosis Patients. *Journal of Clinical Microbiology*, *40*(4), 1210–1213. https://doi.org/10.1128/JCM.40.4.1210-1213.2002

Madden, T. (2013). The BLAST Sequence Analysis Tool. In *The NCBI Handbook, 2nd edition [Internet]*. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK153387/

Manfredi, R., Nanetti, A., Ferri, M., Mastroianni, A., Coronado, O. V., & Chiodo, F. (1999). Fiavobacteriurrt spp. Organisms as opportunistic bacterial pathogens during advanced HIV disease. *Journal of Infection*, *39*(2), 146–152. https://doi.org/10.1016/S0163-4453(99)90007-5

Okinaka, R., Pearson, T., & Keim, P. (2006). Anthrax, but Not Bacillus anthracis? *PLoS Pathogens*, *2*(11), e122. https://doi.org/10.1371/journal.ppat.0020122

Ryan, M. P., & Adley, C. C. (2010). Sphingomonas paucimobilis: A persistent Gram-negative nosocomial infectious organism. *Journal of Hospital Infection*, *75*(3), 153–157. https://doi.org/10.1016/j.jhin.2010.03.007

Shah, N. M., Davidson, J. A., Anderson, L. F., Lalor, M. K., Kim, J., Thomas, H. L., … Abubakar, I. (2016). Pulmonary *Mycobacterium avium-intracellulare* is the main driver of the rise in non-tuberculous mycobacteria incidence in England, Wales and Northern Ireland, 2007–2012. *BMC Infectious Diseases*, *16*(1), 195. https://doi.org/10.1186/s12879-016-1521-3

Takahashi, S., Tomita, J., Nishioka, K., Hisada, T., & Nishijima, M. (2014). Development of a Prokaryotic Universal Primer for Simultaneous Analysis of Bacteria and Archaea Using Next-Generation Sequencing. *PLoS ONE*, *9*(8), e105592. https://doi.org/10.1371/journal.pone.0105592

Taylor, R. H., Falkinham, J. O., Norton, C. D., & LeChevallier, M. W. (2000). Chlorine, chloramine, chlorine dioxide, and ozone susceptibility of *Mycobacterium avium*. *Applied and Environmental Microbiology*, *66*(4), 1702–1705. https://doi.org/10.1128/AEM.66.4.1702-1705.2000

Thomas, J. M., & Ashbolt, N. J. (2011). Do free-living amoebae in treated drinking water systems present an emerging health risk? *Environmental Science & Technology*, *45*(3), 860–869. https://doi.org/10.1021/es102876y

Tortoli, E. (2014). Microbiological features and clinical relevance of new species of the genus *Mycobacterium*. *Clinical Microbiology Reviews*, *27*(4), 727–752. https://doi.org/10.1128/CMR.00035-14

Wang, J., Sui, M., Yuan, B., Li, H., & Lu, H. (2019). Inactivation of two mycobacteria by free chlorine: Effectiveness, influencing factors, and mechanisms. *Science of the Total Environment*, *648*, 271–284. https://doi.org/10.1016/j.scitotenv.2018.07.451