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Bathing in Bacteria: Mycobacteria Relative Abundance on Showerhead Biofilms

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BATHING IN BACTERIA:

MYCOBACTERIA RELATIVE ABUNDANCE ON SHOWERHEAD BIOFILMS

BY

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Abstract

*Mycobacterium* is a genus of Actinobacteria that contains many human pathogens, causing diseases such as tuberculosis, leprosy, and nontuberculous mycobacteria (NTM) lung disease. Diagnosed cases of NTM lung disease, are increasing rapidly across the United States. Recent studies have indicated that this respiratory infection is acquired through the inhalation of aerosolized mycobacteria derived from the biofilms found on the inside of household showerheads.

To assess the factors that influence mycobacteria abundance on the interior biofilms of showerheads we analyzed bacterial and protistan communities from showerhead samples gathered across the United States. Further, we investigated water chemistry, specifically total chlorination concentration and pH, and geographical variables to better understand the factors influencing the relative abundance of mycobacteria.

After analyzing 191 samples, we found that *Mycobacterium* was the most common genus in showerhead biofilms, with a mean relative abundance of 19.2%. We found that mycobacteria were more abundant in biofilms with water sourced from municipal water than from well-water. This could be due to the higher chlorine content of municipal water, as mycobacteria are known to have mechanisms to resist chlorine. This is corroborated by the fact that mycobacteria were more abundant in samples with higher total chlorine concentrations. Mycobacterial abundances were not influenced by the pH of the water, the geographical location of the sample, or the relative abundance of free-living amoebas (FLA).

Our results indicate that showerhead biofilms from chlorinated, municipal water supplies are more likely to contain mycobacteria. This indicates that individuals using these
showers may be at a higher risk of developing NTM lung disease and that the addition of chlorine to household water may be linked to the increasing incidence of this disease.
Acknowledgements

I would like to sincerely thank all of the members of the Fierer lab who gave me the opportunity to be involved in the Showerhead Microbiome Project. Especially Dr. Noah Fierer for advising me every step of the way and challenging me to think deeper about the microbial world around us. To Angela Oliverio for helping me understand the wonderful world of protists and statistical analysis, and to Matt Gebert whose analytical direction was vital. I would also like to give a special thanks to Dr. Christopher Lowry for opening my eyes to the potential benefits of mycobacteria and for providing numerous suggestions along the way. I would also like to thank Dr. Eric Stade and Dr. David Sherwood for agreeing to be part of my committee. And finally to Riley Brady for his editorial mastery. None of this would have been possible without all of you.
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1. Introduction

As our understanding of the microbial world has expanded through technological advances and scientific investigation, we have begun to uncover the rich relationships microbes share with society. Many human-associated microbial communities have been explored, such as the those found in our housing (Luongo et al., 2016), on our bodies (Huttenhower et al., 2012), and even with domestic pets (Huttenhower et al., 2012). Recently there has been an increasing interest in investigating other microbial communities, especially those associated with human health. One such community of interest is the biofilms found on the inside of showerheads.

Previous studies have shown that *Mycobacterium*, a genus containing opportunistic pathogens, is very common on our showerheads (Thomson et al., 2013). Mycobacteria have been found to be relatively abundant in showerheads and members of this group are present in more than 50% of showerheads in the United States (Bautista-de los Santos et al., 2016; Falkinham, 2016). It has been suggested that this household association could have a large impact on human health, as some species of mycobacteria have been found to have immunoregulatory effects that could possibly prevent psychiatric disorders, while other species cause nontuberculous mycobacteria (NTM) lung disease (Reber et al., 2016; Matthews and Jenks, 2013; Thomson et al., 2013).
NTM lung disease is a respiratory infection that is most often contracted by inhaling virulent mycobacteria into the lungs, causing chronic lung ailment if left untreated (Parrish et al., 2008). Although mycobacteria are found in most tap water, they are 100 times more abundant in showerhead biofilms (Feazel et al., 2009). This is because they are adapted to survive in low-nutrient moist areas (Feazel et al., 2009). Due to the thick hydrophobic coating of their cell walls, mycobacteria can be resistant to many disinfectants and detergents (Falkinham et al., 2001). Further, some mycobacteria species have genes for antibiotic resistance (Telenti and Iseman, 2000). Because of this, an antibiotic treatment of over one year is often required to cure NTM lung disease (Falkinham, 2016).

The prevalence of NTM lung disease in the United States has been rapidly increasing at a rate of 10% per year over the past 20 years (Falkinham, 2016). Currently there are over 90,000 diagnosed cases of NTM lung disease per year in the United States. However, this number is likely underestimated, as many people do not seek treatment for the infection (Falkinham, 2016).

The link between clinical isolates of NTMs from patients and the NTMs on their showerheads is becoming increasingly apparent, highlighting the importance of aerosolized showerhead NTMs in disease transmission (Thomson et al., 2013). 95% of NTM lung disease patients that were studied had mycobacteria present in their household water (Thomson et al., 2013). Further, some studies have shown that clinical isolates from a given patient exactly match the strain present in their showerhead (Falkinham et al., 2008). *Mycobacterium avium* complex (MAC) accounts for 50-80% of NTM lung disease infections in the United States, making it the most common cause of NTM lung disease (Hoefsloot et al., 2013; Parrish et al., 2008) MACs have been isolated from showerheads and drinking water and are thought to live inside free-living amoebas (FLA) and to be
resistant to chlorination (Hruska and Kaevska, 2012).

The prevalence of NTM lung disease varies across the United States. Hot spots have been identified in regions such as Hawaii, Louisiana, southern California, Florida, Wisconsin, and the New York metropolitan area (Adjemian et al., 2012; Abe et al., 2016). It is not yet understood why these regions are hot spots for NTM lung disease, but it has been suggested that chemical properties of the water associated with these showerheads support NTM growth (Hruska and Kaevska, 2012). In this study, we target four main hypotheses to better understand why NTM lung disease is more prevalent in certain areas, and why its incidence is increasing nationwide.

First, we hypothesized that mycobacteria abundance would increase with decreasing latitude, as many reported hot spots for NTM lung disease are in warmer regions of the U.S. Second, we hypothesized that mycobacteria would be more abundant in showerhead samples with higher chlorine levels. More specifically, we expect them to be more abundant in municipal water than well-water showerheads, as municipal water is generally treated with disinfectants such as chlorine. Mycobacteria are disinfectant-resistant, and 10-100 times more resistant to chlorine than \textit{E. coli}. Thus, they survive high-chlorine conditions, while other bacteria once present in the water are killed (Falkinham et al., 2001; Pryor et al., 2004; Bautista-de los Santos et al., 2016).

Third, we hypothesized that mycobacteria would be more abundant in showerheads that have a higher water pH. Previous studies have shown that mycobacteria are more abundant in aquatic systems with a pH greater than 7.7, as they appear to grow better under more basic conditions (Castillo-Rodal et al., 2012; Hruska and Kaevska, 2012).
Lastly, we hypothesized that mycobacteria would be more abundant in samples that have a greater abundance of free-living amoeba (FLA). Previous studies have proposed that mycobacteria have a parasitic or endosymbiotic relationship with FLA (Ovrutsky et al., 2013; Parrish et al., 2008). It is likely that pathogenic mycobacteria are able to live inside FLA without being digested. This adaptation might allow mycobacteria to survive inside the alveoli in human lungs, in turn causing NTM lung disease (Al-Quadan et al., 2012).

To test these hypotheses, a citizen-science project was initiated to obtain 191 samples of showerheads across the United States. In this study, we analyzed prokaryotic and eukaryotic communities found on biofilms inside these showerheads along with the chemistry of the source water. In doing so, we hope to determine how household water chemistry, house location, or FLA abundance may influence the relative abundance of mycobacteria. This information could then be used to identify households or regions that are at higher risk for NTM lung disease.
2. Methods

We assessed microbial communities found on the inner biofilms of 191 showerheads from citizen scientists across the United States. These citizens voluntarily followed our specific protocol for sampling the inside of their showerhead and for taking measurements of their shower water chemistry, and completed a detailed questionnaire about their showering habits (see Appendix). The participants were also invited to leave their contact information so they could be sent data from their own showerhead in order to encourage future participation in this study.

We looked at the genus *Mycobacterium*, and did not discriminate between pathogenic and non-pathogenic strains, as the sequenced gene region only provided resolution down to the genus level. As there are many different species of mycobacteria, some of which are always pathogenic, opportunistically pathogenic, or benign, it is difficult to identify specific bacterial sequences as certainly pathogenic. Some strains of mycobacteria also differ in their pathogenicity based on their geographical region (Hoefsloot et al., 2013). Due to this, we looked at trends across the *Mycobacterium* genus, which could give us indications of areas to explore further with specific *Mycobacterium* species.
2.1 Citizen Scientist Sampling Protocol

Each participant was advised to select the shower in his or her household that was used most frequently, that was wall-mounted, and that had a removable head so that the biofilm on the interior of the showerhead could be easily accessed for swabbing. They were then instructed to sample the inside of the showerhead using a sterile double swab while wearing nitrile gloves, sampling the inner face of the showerhead, close to the water holes. They would then put the swabs immediately back in the sterile container to be sent to us. They used 5-in-1 water chemistry testing strips that included five color pads, one color pad for each property: pH, total alkalinity, total hardness, free chlorine and total chlorine. They were instructed to turn on the shower to a comfortable temperature for showering and place the entire test strip under the flow of water for one second. They would then wait 30 seconds and compare the pH, total alkalinity and total hardness color pads to the Aquacheck Test Strip Color Guide (Elkhart, IN) and record the corresponding values. Then they would place the strip under the flow of water for ten seconds to assess total chlorine and free chlorine. Lastly, they used a second test strip for nitrate and nitrite, and a third strip for total iron concentrations following a similar process. The participants then returned an envelope to us containing their showerhead swab, data they collected for the water chemistry variables, and the completed questionnaire including their geographical coordinates (see Appendix).

2.2 Sample Processing

Samples were stored at -20°C while waiting to be processed so that the communities remained representative of the original biofilms. Once enough samples had been returned to fill two 96-well plates, we began the DNA extraction process. We extracted the DNA from each sample using the PowerSoil htp-96 well Isolation Kit (MoBio, Carls-
bad, CA). Extraction blanks of sterile swabs were also included to control for possible processing contamination. In order to assess the prokaryotic and eukaryotic community diversity, the DNA was amplified in triplicate using PCR with two separate primer sets. The first primer set was barcoded 515f/806r that targeted the V4 region of the 16S rRNA gene for bacterial diversity analysis and identification of *Mycobacterium* at the genus level (Caporaso et al., 2012). The second primer set targeted the gene encoding 18S SSU rRNA, to use for eukaryotic diversity analysis (Amaral-Zettler et al., 2009). Amplification was confirmed using gel electrophoresis and the amplicon concentrations were cleaned and normalized using SequalPrep Normalization Kit (Thermo Fisher Scientific, Waltham, MA) before pooling 5uL of each sample into a test tube. Sequencing was performed on the MiSeq (Illumina, San Diego, CA) at the University of Colorado Boulder Next Generation Sequencing Facility using a 500 cycle V2 reagent kit (2x250bp chemistry)(Caporaso et al., 2012).

### 2.3 Prokaryote Data Analysis

We obtained 16S rRNA gene sequences and first demultiplexed them, trimmed the reverse read to 150 base pairs, and then merged the forward and reverse reads. Singletons and replicates were removed using USEARCH pipeline (Edgar, 2013). OTUs were assigned to sequences within 97% similarity, and then assigned taxonomic affiliations using Greengenes (McDonald et al., 2012). All further statistical analyses were performed in R programming language.

In order to normalize reads per sample, we rarefied the bacterial sequences to 8000 reads. This excluded the blanks and no-template controls from our data, which indicated minimal contamination. After rarefication, 171 samples remained.
The relative abundances of different bacterial genera were calculated to determine the overall composition of the showerhead communities. Spearman's correlations were then run on the genus level of bacteria to see if any bacterial genera correlated with the relative abundance of mycobacteria.

13 independent variables were analyzed: pH, source of showerhead water (municipal water or well-water), total chlorine, free chlorine, nitrate, nitrite, total iron, hardness, total alkalinity, longitude, latitude, interior showerhead finish (brass, bronze, copper, nickel, no finish), and whether the showerhead had a filter. To assess any relationships between the independent variables and mycobacteria abundance, Spearman's correlations were run on continuous independent variables and Kruskal-Wallis on categorical variables. A \( p \)-value less than 0.05 was considered as statistically significant.

2.4 Eukaryote Data Analysis

We obtained 18S rRNA gene sequences and demultiplexed them, trimmed them and removed singletons using the USEARCH pipeline (Edgar, 2013). We then clustered sequences of 97% similarity into OUTs and assigned these to taxonomy based on Protist Ribosomal Reference (PR2) database (Guillou et al., 2012). For this study, we specifically focused on the protists found on showerhead biofilms and their potential relation to mycobacteria. Because of this, we removed all other Metazoa (animals), Embryophicia (plants), Bacteria, and Fungi sequences from the analyses. In order to normalize the sequences per sample, we rarefied to 500 sequences, which resulted in 116 samples.

We calculated the relative abundances of the most dominant protist orders and examined the dominant phylotypes. To assess any relationship between mycobacteria and FLA abundance, we compared the relative abundance of the Amoebozoa phylum
and specific FLA genera to the relative abundance of mycobacteria using Spearman's correlations.
3. Results

Figure 3.1 and Table 3.1 display the ten most abundant bacterial genera on showerhead biofilms. *Mycobacterium* was the dominant genus at a median relative abundance of 6.7% (mean of 19.2%). Other abundant genera were *Sphingomonas* and *Sphingobium*, at median relative abundances of 4.4% (mean of 16.8%) and 0.04% (mean of 9.9%) respectively.

Figure 3.2 illustrates the geographical distribution of samples across the United States with a color gradient highlighting the relative abundance of mycobacteria in each sample. The samples received from our citizen scientists are biased towards the east coast and suggest hotspots of mycobacteria occurrence in southern California and New England. However, we did not find a clear relationship between mycobacteria abundance and latitude ($\rho = -0.02; p = 0.8$).

We compared the relative abundances of mycobacteria to each of the independent variables that were gathered. Mycobacteria were found to be significantly more abundant in municipal water ($n=152$) than in well-water ($n=15$) sources, with a $p$-value of 0.003 using the Kruskal-Wallis test (Figure 3.3). In line with this, mycobacteria were found to be more abundant in samples with higher total chlorine concentrations ($p < 0.10$) using the Spearman’s test. In order to investigate the impacts of total chlorine further, we broke the variable into three groups: high (>0.5ppm, $n=35$), medium (0.0-0.5ppm,
n=70), and low chlorine (no detectable chlorine, n=62). Figure 3.4 displays the abundance of mycobacteria for each of these groupings, illustrating the differences between low and high chlorine concentrations.

We also split pH into high (>6.9, n=21), medium (6.21-6.9, n=56), and low (<6.2, n=88) groups. We found that within the pH range sampled, mycobacteria abundance was not affected by differences in pH ($p = 0.982$). None of the other independent variables were correlated with relative abundance of mycobacteria. A full summary of these results can be found in Table 3.2 and Table 3.4.

Figure 3.5 and Table 3.3 display the ten most abundant orders of protists. The most abundant protistan order was Echinamoebida with a median relative abundance of 42% (mean 48%). Within Echinamoebida, the dominant families were Vermamoebidae, Vexilliferidae and Acanthamoebida, which are all families of FLA. We did not find a correlation between the phylum Amoebozoa and mycobacteria, nor any other taxonomic group down to the phylotype level within Amoebozoa.

Table 3.1: Minimum, maximum, median, mean and standard deviation of the relative abundances of the top 10 bacterial genera found in showerhead samples (n=171).

<table>
<thead>
<tr>
<th>Identity</th>
<th>Min</th>
<th>Max</th>
<th>Median</th>
<th>Mean</th>
<th>STD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycobacterium</td>
<td>0</td>
<td>0.986</td>
<td>0.067</td>
<td>0.168</td>
<td>0.170</td>
</tr>
<tr>
<td>Sphingomonas</td>
<td>0</td>
<td>0.795</td>
<td>0.044</td>
<td>0.099</td>
<td>0.106</td>
</tr>
<tr>
<td>Sphingobium</td>
<td>0</td>
<td>0.882</td>
<td>0.007</td>
<td>0.092</td>
<td>0.063</td>
</tr>
<tr>
<td>Blastomonas</td>
<td>0</td>
<td>0.900</td>
<td>0.003</td>
<td>0.081</td>
<td>0.062</td>
</tr>
<tr>
<td>Bradyrhizobium</td>
<td>0</td>
<td>0.978</td>
<td>0.023</td>
<td>0.055</td>
<td>0.233</td>
</tr>
<tr>
<td>Sphingopyxis</td>
<td>0</td>
<td>0.679</td>
<td>0.004</td>
<td>0.044</td>
<td>0.074</td>
</tr>
<tr>
<td>Novosphingobium</td>
<td>0</td>
<td>0.386</td>
<td>0.002</td>
<td>0.035</td>
<td>0.083</td>
</tr>
<tr>
<td>Delfia</td>
<td>0</td>
<td>0.355</td>
<td>0.000</td>
<td>0.023</td>
<td>0.167</td>
</tr>
<tr>
<td>Methylobacterium</td>
<td>0</td>
<td>0.517</td>
<td>0.000</td>
<td>0.021</td>
<td>0.144</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>0</td>
<td>0.956</td>
<td>0.000</td>
<td>0.016</td>
<td>0.094</td>
</tr>
</tbody>
</table>
Table 3.2: Means, ranges, and correlation with mycobacteria relative abundance p-values for 9 continuous variables related to water properties. Spearman's statistical test were run on each variable individually.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean</th>
<th>Range</th>
<th>Correlation with mycobacteria relative abundance (rho)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>6.525</td>
<td>5.5 - 8.4</td>
<td>-0.052</td>
<td>0.504</td>
</tr>
<tr>
<td>Total Chlorine</td>
<td>0.636ppm</td>
<td>0 - 10ppm</td>
<td>0.128</td>
<td>0.0958</td>
</tr>
<tr>
<td>Total Iron</td>
<td>0.154ppm</td>
<td>0 - 1.5ppm</td>
<td>-0.0486</td>
<td>0.529</td>
</tr>
<tr>
<td>Nitrate</td>
<td>1.35ppm</td>
<td>0 - 15ppm</td>
<td>0.0198</td>
<td>0.799</td>
</tr>
<tr>
<td>Nitrite</td>
<td>0.093ppm</td>
<td>0 -2ppm</td>
<td>0.0448</td>
<td>0.565</td>
</tr>
<tr>
<td>Total Hardness</td>
<td>144.7ppm</td>
<td>25 - 425ppm</td>
<td>-0.0155</td>
<td>0.842</td>
</tr>
<tr>
<td>Total Alkalinity</td>
<td>120.1ppm</td>
<td>0 - 240ppm</td>
<td>0.0611</td>
<td>0.426</td>
</tr>
<tr>
<td>Longitude</td>
<td>85.86W</td>
<td>154.9W - 64.9W</td>
<td>0.0475</td>
<td>0.538</td>
</tr>
<tr>
<td>Latitude</td>
<td>37.83N</td>
<td>18.15N - 47.16N</td>
<td>-0.0167</td>
<td>0.829</td>
</tr>
</tbody>
</table>

Table 3.3: Minimum, maximum, median, mean and standard deviation of the relative abundances of the top 10 Protistan orders found in showerhead samples (n=116).

<table>
<thead>
<tr>
<th>Identity</th>
<th>Min</th>
<th>Max</th>
<th>Median</th>
<th>Mean</th>
<th>STD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Echinamoebida</td>
<td>0</td>
<td>1.000</td>
<td>0.417</td>
<td>0.480</td>
<td>0.441</td>
</tr>
<tr>
<td>Centramoebida</td>
<td>0</td>
<td>0.959</td>
<td>0.000</td>
<td>0.034</td>
<td>0.159</td>
</tr>
<tr>
<td>Chrysophyceae-Synurophyceae_X</td>
<td>0</td>
<td>0.999</td>
<td>0.000</td>
<td>0.040</td>
<td>0.160</td>
</tr>
<tr>
<td>Colpodida</td>
<td>0</td>
<td>0.957</td>
<td>0.000</td>
<td>0.039</td>
<td>0.176</td>
</tr>
<tr>
<td>Dactylopodida</td>
<td>0</td>
<td>1.000</td>
<td>0.000</td>
<td>0.065</td>
<td>0.200</td>
</tr>
<tr>
<td>Dinophyceae_X</td>
<td>0</td>
<td>0.861</td>
<td>0.000</td>
<td>0.026</td>
<td>0.140</td>
</tr>
<tr>
<td>Euglenida</td>
<td>0</td>
<td>0.991</td>
<td>0.000</td>
<td>0.027</td>
<td>0.127</td>
</tr>
<tr>
<td>Kinetoplastida</td>
<td>0</td>
<td>0.642</td>
<td>0.000</td>
<td>0.024</td>
<td>0.098</td>
</tr>
<tr>
<td>Lobosa_XX</td>
<td>0</td>
<td>0.994</td>
<td>0.000</td>
<td>0.035</td>
<td>0.163</td>
</tr>
<tr>
<td>Vannellida</td>
<td>0</td>
<td>0.999</td>
<td>0.000</td>
<td>0.026</td>
<td>0.128</td>
</tr>
</tbody>
</table>
Table 3.4: The number of samples that fell into each categorical variable tested and the correlation with mycobacteria abundance p-values. Before running ANOVA analysis on each variable individually, “unknowns” were removed. * Denotes significant ($p < 0.05$) using a Kruskal-Wallis test.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Number of Samples</th>
<th>Mean Relative Abundance of Mycobacteria</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source of Water</td>
<td></td>
<td></td>
<td>0.00307*</td>
</tr>
<tr>
<td>Well</td>
<td>15</td>
<td>0.0481</td>
<td></td>
</tr>
<tr>
<td>Municipal</td>
<td>152</td>
<td>0.184</td>
<td></td>
</tr>
<tr>
<td>Does the shower have a filter?</td>
<td></td>
<td></td>
<td>0.946</td>
</tr>
<tr>
<td>Yes</td>
<td>46</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>82</td>
<td>0.175</td>
<td></td>
</tr>
<tr>
<td>Interior Showerhead Finish</td>
<td></td>
<td></td>
<td>0.168</td>
</tr>
<tr>
<td>Brass</td>
<td>10</td>
<td>0.125</td>
<td></td>
</tr>
<tr>
<td>Bronze</td>
<td>31</td>
<td>0.178</td>
<td></td>
</tr>
<tr>
<td>Copper</td>
<td>3</td>
<td>0.0052</td>
<td></td>
</tr>
<tr>
<td>Nickel</td>
<td>11</td>
<td>0.354</td>
<td></td>
</tr>
<tr>
<td>No Finish</td>
<td>40</td>
<td>0.156</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.1: Relative abundances of the ten most common bacterial genera found across all showerhead samples. Each dot denotes the relative abundance for a given sample. The black horizontal line for each box and whiskers plot represents the median of the 171 samples.
Figure 3.2: Geographical distribution of showerhead sample sites (n=191). Relative abundance of mycobacteria is indicated by the color bar.
Figure 3.3: Differences in the relative abundance of mycobacteria in showerhead samples between municipal water (n=152) and well-water (n=15) sources.
Figure 3.4: Differences in the relative abundance of mycobacteria in showerhead samples between high (>0.5ppm, n=35), medium (0.0-0.5ppm, n=70), and low chlorine (no detectable chlorine, n=62).
Figure 3.5: Relative abundances of the ten most common Protistan orders found across all showerhead samples. Each dot denotes the relative abundance for a given sample. The horizontal line for each box and whiskers plot denotes the median of the 116 samples.
4. Discussion

4.1 Bacterial Community Analysis

The ten most common bacterial genera found in our showerhead samples (Figure 3.1) are the same major genera typically recovered in samples of household water (Hruska and Kaevska, 2012). However, the difference is that the environment in the showerhead appears to select for bacteria that are known to form extensive biofilms, to live in low nutrient concentrations, to resist disinfectants, and to pass through filters applied to most water systems. For these reasons, it is not surprising that mycobacteria are the most abundant bacteria in showerheads. Their extremely thick outer membrane appears to be impermeable and resistant to many disinfectants, providing a significant selective advantage over other bacteria (Hruska and Kaevska, 2012). Mycobacteria make extracellular polymeric substances (EPS), which are biopolymers that contain polysaccharides (Vu et al., 2009). These are produced to create the structure of the biofilm matrix, further explaining mycobacteria’s dominance in biofilms.

4.2 Geographic Distribution

There is no clear spatial dependence of mycobacteria abundance across the United States (Figure 3.2). This may be because our range in latitudes was only 18N to 47N, so we may not have had enough lower latitude samples to see any pattern. However,
mycobacteria abundances in locations that are known to be hot spots of NTM lung disease, such as Florida and southern California, do seem to be higher than at other locations (Adjemian et al., 2012). In order to further see if there are any clear hot spots, or if latitude influences mycobacteria abundance, more samples that are dispersed across the entire United States are needed. With more samples, we could also identify regions that have low abundances of mycobacteria and use this information to infer what factors lead to this preventative mechanism in these regions.

It would be interesting to investigate the clinical link between showerhead mycobacteria and NTM lung disease by examining the showerhead biofilms of high NTM lung disease incidence regions. In order to achieve this, we would need to obtain more samples from high-incidence areas and request all physicians to report the local incidence of NTM lung disease so we could better identify these regions.

**4.3 Source of Water**

As we predicted, mycobacteria were more abundant in municipal water than well-water ($p$-value = 0.003), likely due to the fact that the municipal water systems sampled had higher concentrations of chlorine ($p$-value = 0.03; Figure 3.3). The hypothesis was based on previous observations that indicate that municipal water is often treated with disinfectants such as chlorine, which kills many other bacteria, but leaves mycobacteria unscathed due to their resistant hydrophobic nature (Falkinham et al., 2001).

Only 15% of all homes in the United States source their water from wells, while the remaining homes use municipal water (Gomez-Alvarez et al., 2012). There are many differences in the chemistry, quality, and potential health risks of both sources of water. Firstly, the Environmental Protection Agency controls and monitors only municipal
water and has no jurisdiction over well-water (Gomez-Alvarez et al., 2012). Municipal water is often treated with chemicals including chlorine in order to eliminate pathogens and other contaminants (Gomez-Alvarez et al., 2012). Further, our study indicates that this treatment of water selects for Mycobacteria, likely including NTM that cause lung disease.

Conversely, well-water quality is at the discretion of the individual landowner and likely does not contain chlorine. However, agricultural chemicals can contaminate well-water, as it is sourced from local aquifers (Bautista-de los Santos et al., 2016). From our results, we predict that individuals who source their water from wells may be at lower risk of contracting NTM lung disease as mycobacteria are likely found in lower abundance in their showerheads. Although we only had 15 showerhead biofilm samples sourced from well-water and 155 samples from municipal water, the well-water samples still had significantly lower mycobacterial abundances.

4.4 Total Chlorine

We also found that mycobacteria abundance increased as total chlorine concentration in the shower water increased (Figure 3.4). Total chlorine is the combination of the free chlorine in the water, the residual chlorine dissolved as a gas, and the combined chlorine, the chlorine found in organic ammines, or ammonia, which are sometimes added to water. Most of the households we sampled had zero free chlorine as chlorine reacts rapidly in water, so is rarely “free," but most had some total chlorine present. Because of this result, chlorine addition to household water poses an additional threat on human health. It is likely that slow-growing, pathogenic strains of mycobacteria would be selected for on these biofilms, meaning that they would be aerosolized when the occupants shower and could potentially cause NTM lung disease if inhaled (Gomez-
In recent years, some municipal water treatment facilities have been changing their chlorine disinfectant from free-chlorine to monochloramine in order to prevent the buildup of disinfectant byproducts (Gomez-Alvarez et al., 2012). However, it has been suggested that the addition of monochloramine actually selects for mycobacteria (Gomez-Alvarez et al., 2012; Donohue et al., 2015). The gene expression of EPS-related genes was increased in mycobacteria in a high chloramine solution, showing their ability to withstand chloramine disinfectants via the EPS mechanism (Gomez-Alvarez et al., 2012).

The same properties that make mycobacterium pathogenic likely aid their survival against disinfectants, meaning that pathogenic mycobacteria may be further selected for on the inside of chlorinated water showerheads. Also, slower growing strains of mycobacteria such as *M. tuberculosis* and *M. avium*, both known pathogens, have been shown to be more resistant to chlorine than faster growing mycobacteria strains (Falkinham et al., 2001). It is therefore likely that they mycobacteria on showerhead biofilms in waters with higher chlorine concentrations are more likely to be pathogenic. We would suggest municipal water treatment facilities consider the effects of adding chloramine to their water systems as it may increase the number of NTM lung disease infections. A further interesting study would be to culture household water in media with different chlorine concentrations to investigate the direct effect of chlorine on mycobacteria abundance.

### 4.5 pH

Contrary to previous studies, we did not see a relationship between mycobacteria abundance and the pH of the water. It had been proposed that mycobacteria are more abun-
dant in water with pH greater than 7.7, but we did not observe this relationship (Castillo-Rodal et al., 2012). Even when the samples were split into three discrete pH groups, we still could not see a difference in the mean relative mycobacteria abundance. This would suggest that pH does not have a significant effect on mycobacteria abundance. Possibly the range of our pH values (6.0-8.4) was not large enough to show mycobacterium's dominance in more basic waters.

4.6 Associations Between Mycobacteria and Protists

Our finding of Echinamoebida being the most dominant protistal order (Figure 3.5) is consistent with recent water system literature (Valster et al., 2009). Within Echinamoebida, the dominant families of Vermamoebidae, Vexilliferidae and Acanthamoebida are also shown to be dominant FLA in household water (Valster et al., 2009). Other abundant orders were Dactylopodidia, a FLA, and Chrysophyceae-synurophyceae, a golden algae. Both of these orders have been reported in drinking water systems (Valster et al., 2009).

It has been proposed that mycobacteria may have a parasitic or endosymbiotic relationship with FLA, similar to the relationship between Legionella and Vermamoeba vermiformis (Parrish et al., 2008; Ovrutsky et al., 2013). The Legionella strains that have this parasitic relationship tend to be more pathogenic, as they encapsulate themselves in Legionella-containing vacuoles (LCVs) in order to be taken up by the FLA and not destroyed (Amaro et al., 2015). They can use this same mechanism in the human lungs within alveolar macrophages to resist degradation from lysosomes (Amaro et al., 2015). Therefore, it is thought that pathogenic mycobacteria such as NTM may use similar mechanisms to cause NTM lung disease (Ovrutsky et al., 2013).
Although we hypothesized a relationship between mycobacteria abundance and FLA, we did not find any statistical relationship between mycobacteria and the phylum Amoebozoa or any phylotype of FLA. We looked at specific genera within Amoebozoa such as *Vermamoeba vermiformis*, *Echinamoeba*, and *Acanthamoeba* and again did not find any correlation with mycobacteria. This may be because we did not have a specific species of FLA known to associate with mycobacteria to target, as there is not sufficient research in this area yet. Also, it is possible we did not see any relationship because we did not have absolute abundance data. If we were to use qPCR, we could obtain absolute abundance data and we might see that samples with a greater volume of mycobacteria would also have a greater volume of FLA. Mycobacteria also may not grow in FLA at all, or they might grow in diverse protists and thus not be selective about which ones they form endosymbiotic or parasitic relationships with.

If we obtained mycobacteria and FLA in separate cultures, we could add different concentrations of FLA to mycobacteria and monitor the response to determine whether FLA adds an advantage to the mycobacteria associated with them. We could also investigate the genes expressed during this endosymbiotic relationship and compare them to the pathogenicity genes expressed when living in human cells.

### 4.7 Potential Beneficial Health Effects of Mycobacteria

As we have previously discussed, selecting for mycobacteria with the addition of chlorine to municipal water sources may be detrimental to human health. But, there is also research that suggests that exposure to certain species of live and heat-killed mycobacteria have health benefits for rats, such as treating and preventing diseases like anxiety and stress-induced colitis (Reber et al., 2016; Matthews and Jenks, 2013) Due to their immunoregulatory effects, exposure to mycobacteria causes the immune system to re-
duce inflammation induced by proinflammatory cytokines which are linked to psychiatric disorders (Rook and Lowry, 2009). Therefore, it is possible that inhaling aerosolized mycobacteria while showering could have beneficial effects for humans and reduce their likelihood of developing psychiatric disorders. In order to assess this claim, further research is needed to see if individuals with psychiatric disorders have a lower relative abundance of mycobacteria on their showerheads. These potential beneficial effects of mycobacteria also need to be taken into consideration when developing future policies regarding water treatment and distribution.
5. Conclusion

Overall, we found that *Mycobacterium* is the dominant bacterial genera on the biofilms found inside of showerheads in the United States. Mycobacteria are more abundant in showerheads that experience higher concentrations of chlorine, possibly due to their chlorine-resistant cellular mechanisms. As a result, mycobacteria are more abundant in municipal water samples than in well-water samples, because municipal water is treated with chlorine and other disinfectants to remove pathogens. Our analyses also showed that none of the other water chemistry variables measured affected the abundance of mycobacteria to a significant degree, including pH, which had been previously reported to affect mycobacteria density. While our data show that Amoebozoa are extremely prominent on showerhead surfaces, we were unable to demonstrate an endosymbiotic relationship with FLA.

These findings lead us to believe that individuals whose household water supply is municipal water, treated with chlorine, may be at greater risk of developing NTM lung disease. This conclusion is subject to the fact that we used relative—rather than absolute—abundance. We would suggest that individuals with high chlorine content in their water try to reduce using standard activated charcoal filtration and to be aware of the potential spread of infection through aerosolized particles. In addition, municipal and state government agencies should be aware of the potential increase in NTM lung disease due
to the addition of chlorine to household water and that switching to chloramine may be worse for their citizens’ health.
Bibliography


A. Appendix

The following pages are the showerhead sample protocol and questionnaire, which were sent to the citizen scientists who registered for this project.
We are continuously exposed to a broad diversity of microbes as we go about our daily lives. In particular, every time we take a shower we are breathing in those microbes growing in our showerhead that can become dislodged and aerosolized, potentially getting into our lungs. Although most of these microbes are harmless and some may even benefit our immune systems, a small minority can potentially cause respiratory diseases in susceptible individuals.

Showering can be an important mode of disease transmission, including the transmission of Legionella (the bacterium that causes Legionnaire’s disease) and exposures to a group of bacteria that cause nontuberculous mycobacterial respiratory infections. Although these diseases are relatively rare in the U.S., and healthy individuals are unlikely to be susceptible to these bacterial pathogens, they are considered a growing problem. Currently we do not know whether those bacterial pathogens that can persist in our showerheads are more common in some regions of the U.S. than in others and what characteristics of our household water supply cause some showerheads to harbor more of these potential pathogens than others. More generally, we currently have a limited understanding of what microorganisms live in our showerheads and the factors that influence their distributions.

We thank you for participating in this project as your involvement is critical to its success. Please follow these instructions carefully to sample the microbes from your showerhead, measure the chemistry of your shower water, and complete the associated questionnaire. If you are interested in receiving the results from your home and getting updates on the progress of this study, please include your email address on the Questionnaire and Data Sheet so we can contact you.

What your sampling kit contains:

1. One (1) pair of blue nitrile gloves (Latex-Free)
2. One (1) double swab in transport tube (with a unique identifying barcode attached)
3. One (1) 50 mL conical tube (water sample collection tube for IRON TEST ONLY)
4. Three (3) tests strips: 5-in-1, Nitrate/Nitrite, Total Iron
5. One (1) silver packet of Iron Reducing Powder
6. One (1) return envelope (pre-addressed, pre-paid) (6in x 9in)
7. One (1) Questionnaire and Data Sheet (with a unique identifying barcode attached)
8. One (1) Showerhead Sampling Protocol

Estimated time for completion: 20 minutes

1. Remove the entire contents of the padded mailing envelope. Place everything in a dry area, away from the water source you will be testing.
2. Remove the three (3) testing strips and silver-colored packet from the conical tube with the orange lid and place in a dry area. We will test each component one at a time. 

3. Please read all of the directions for each section before starting. Some tests require multiple steps!

Find a shower in your home that is used most frequently. This will be the shower that you will use for the entire test. To ensure that testing is as standardized as possible, and the best possible sample is obtained, please choose a standard showerhead (wall mounted that will allow you to access the interior of the showerhead for proper swabbing), and use the same showerhead for all the tests. When swabbing, try to swab as close to the inside interface of the showerhead as possible.

SHOWERHEAD BIOFILM TESTING

1. You will be sampling the INSIDE of the showerhead.
2. The sampling kit contains one (1) pair of blue nitrile gloves. The one (1) pair will be for sampling the biofilm and for sampling the shower water.
3. Put on the blue nitrile gloves and carefully remove the showerhead – Removal of showerhead will vary depending on type of showerhead.
4. Pick up the dual tip swab and carefully remove the collection tube. DO NOT TOUCH THE TIP OF THE SWABS. TOUCHING THE SWAB TO ANY SURFACE OTHER THAN THE INTERIOR OF THE SHOWERHEAD WILL RESULT IN CONTAMINATION OF THE SAMPLE! ONLY TOUCH THE BLACK HANDLE WHEN USING THE SWAB! IMMEDIATELY PLACE SWAB BACK INTO STERILE COLLECTION TUBE AFTER SHOWERHEAD HAS BEEN SWABBED.

5. Swab the inside of the showerhead thoroughly – moving repeatedly back and forth 4-5 times over the INSIDE SURFACE of the showerhead. You want to collect the biofilm (the slimy gunk) that accumulates on the inside of the showerhead. Try to sample as close as you can to the inside interface of the showerhead.
6. After you have sampled, **IMMEDIATELY** place the swab **CAREFULLY** back into the collection tube. **DO NOT SET THE SWAB DOWN. DO NOT TOUCH THE TIP OF THE SWABS.**

7. Screw the showerhead back on to your shower.
8. Place the collection tube off to the side. This will be placed in the return envelope to be sent to the University of Colorado - Boulder.

**SHOWER WATER TESTING**

Test #1: ‘5-in-1’ Test for pH, Total Alkalinity, Total Hardness, Free Chlorine, and Total Chlorine

1. Find the test strip for the ‘5-in-1’ test. It is the strip that contains five (5) individual color pads. Keep the other strips in a dry area until it is time for their specific test.
2. Turn on the shower to a temperature between 65°F and 90°F (a comfortable temperature for showering.)
3. Keep the blue nitrile gloves on and pick up ‘5-in-1’ testing strip near the base of the strip. **Keep the gloves on through the entire testing process.**
4. Pass the entire strip under the shower water stream for **1 second**, and remove. **DO NOT** shake excess water from the test strip. Hold the strip horizontal for **30 seconds**.

5. Compare the bottom three (3) color pads (pH, Total Alkalinity, and Total Hardness) to the colors on the Aquachek Test Strip Color Guide. **DO NOT THROW STRIP OUT YET!**

6. Record the color data on the data recording sheet included. If color appears to fall between two values, estimate the value. **Notice the guide diagram on the left side of the color guide. It explains the location on the test strip of each test! The TOP of each row is labeled with which test it is for!**

7. Pass the entire strip under the shower water stream **again** and hold for **10 seconds**. 
Ensure that all 5 pads are coming in contact with the shower water!

8. Now, compare the total chlorine and free chlorine color pads to the colors on the Test Strip Color Guide below. **Notice the guide diagram on the left side of the color guide. It explains the location on the test strip of each test!** The TOP of each color row is labeled with which test it is for.
9. Record the color data on the data recording sheet included. If color appears to fall between two values, estimate the value.
10. You are now done with the ‘5-in-1’ test strip, and it can be thrown away.

Test #2: ‘Nitrite/Nitrate’ Test for Nitrite and Nitrate

For this test, you will need to record data at two different time points from the same test strip - after 30 seconds and one after a total of 60 seconds have passed. Keep the strip at a horizontal level for the entire process. Do not throw test strip away until you have recorded information for both the 30 second and 60 second time points!

1. Find the test strip for the ‘Nitrite/Nitrate’ test. It is the strip that contains two (2) individual color pads. Keep the last remaining strip (‘Total Iron’) in a dry area until it is time for its testing.

2. Pick up ‘Nitrite/Nitrate’ testing strip and pass gently under the shower water stream for 1 second, and remove. Do not shake excess water from test strip
3. Hold test strip horizontal, pad side up, for 30 seconds. Compare the Nitrite test pad (BOTTOM PAD - CLOSEST TO HANDLE) to the Test Strip Color Guide below.

![Nitrite Test Strip Color Guide]

HANDLE

4. Record the nitrite color data on the data recording sheet included. If color appears to fall between two values, estimate the value.

5. After 60 seconds have passed starting from the time the strip was exposed to water, compare the Nitrate test pad (TOP PAD CLOSEST TO THE FAR END OF THE STRIP) to the Test Strip Color Guide above.

6. Record the nitrate color data on the data recording sheet included. If the color appears to fall between two values, estimate the value.

7. Test strip can now be thrown away.

Test #3: ‘Total Iron’ Test for Total Iron

![Total Iron Test Strip]

1. Find the test strip for the ‘Total Iron’ test. It should be the only remaining test strip, and should contain only one (1) individual color pad. You will also need the 50 mL conical tube (water sample collection tube) with the orange cap for water collection.
2. Make sure there is nothing remaining in the water sample collection tube. All the test strips and the Iron Reducing Powder silver packet should have been removed.

3. Uncap the collection tube, place the cap off to the side, and fill the collection tube up the 30 milliliter line (the line with the 30 next to it), approximately half way full. *Once the tube is filled to the 30 mL mark, the shower can be turned off. This will be the last of the water testing.*

4. Open the silver packet of Iron Reducing Powder and add powder contents to the 30 milliliters of water in the sample collection tube.

5. RECAP the collection tube with the orange cap and shake rapidly for 5 seconds. Remove the orange cap again and place it off to the side.
6. Dip the ‘Total Iron’ Test strip into water sample collection tube and rapidly move the color test pad back and forth underwater for **15 seconds**. Remove and shake excess water from the test strip.

7. Immediately compare the **single** test pad to Aquachek Test Strip Color Guide below. Record the color data on the data recording sheet included. If the color appears to fall between two values, estimate the value.

![Aquachek Test Strip Color Guide](image)

8. Test strip and water sample collection tube can be thrown away after the data are recorded.

**RETURNING YOUR KIT:**

Ensure that all your data has been recorded legibly and correctly, and you have filled out the questionnaire **completely and accurately** to the best of your ability. You have been provided with a postage paid return envelope. Place the **Questionnaire and Data Sheet, and the swab** into the return envelope and mail to the University of Colorado - Boulder. Everything else can either be disposed of in the trash or recycled. Seal the envelope with the adhesive strip, and put a piece of tape over the enclosure to ensure that the swab is not lost in transit. **Please try to place the packet in the mail as soon as possible, after sampling.**

Thank you for your participation! Again, if you are interested in obtaining the results from your individual showerhead, and from the showerhead collection, please provide your email at the top of the **Questionnaire and Data Sheet.**

Preaddressed, prepaid return envelope to the University of Colorado should include:

- Completed **Questionnaire and Data Sheet** (Make sure it is completely filled out)
- Showerhead sample swab

**THANK YOU!**
QUESTIONNAIRE AND DATA SHEET

ADDRESS (include street, city, state, zip code): ________________________________
__________________________________________________________________________
COUNTRY: ______________________________

EMAIL CONTACT: ________________________________

DATE: ________________________________

RECORD WATER CHEMISTRY DATA HERE:

1-in-5 Testing Kit Results:

pH: ________________________________

Total Alkalinity: ____________________ ppm

Total Hardness: ____________________ grains per gallon

Free Chlorine: ____________________ ppm

Total Chlorine: ____________________ ppm

Nitrite/Nitrate Results:

Nitrite: ____________________ ppm

Nitrate: ____________________ ppm

Total Iron Results:

Total Iron: __________ ppm (mg/L)

Circle the response that BEST answers the question:

1. What is the source of your water:
   a. Municipal water system  b. Well-water system  c. Unknown/Other

2. When was the showerhead installed:
   a. Within the last month  b. Within the last 6 months  c. More than 6 months ago  d. Do not know

***TURN SHEET OVER! THERE ARE MORE QUESTIONS ON THE BACK***
3. How frequently is your shower used (by anyone in the household):
   a. 1-4 times/week   b. 5-9 times/week   c. 10-13 times/week   d. 14+ times/week

4. How frequently do you clean the inside of your showerhead:
   a. Once a week   b. Once a month   c. Once every 6 months   d. Once a year   e. Never

5. If you clean the inside of your showerhead, what do you clean it with:

6. What material is your showerhead made of:
   a. Plastic   b. Metal   c. Solid brass   d. Combination of plastic and metal

7. What type of finish does your showerhead have INSIDE the showerhead or shower unit (if any):

8. What system best describes your showerhead:
   a. Fixed/permanent showerhead   b. Fixed Showerhead with additional hand-held unit

9. Does your showerhead have any sort of filter inside it:
   a. Yes   b. No   c. Do not know

10. How would you best describe your showerhead spray pattern:
    a. Wide coverage that feels like drenching rain   b. Drenching forced spray ideal for rinsing shampoo
    c. Targeted pattern producing a sharp, pointed spray   d. Fine misting of water (low flow showerhead)
    e. Full flood like a sink faucet   f. Multiple interchangeable spray pattern settings

11. How is your water heated?
    a. Hot water heater   b. Hot water generated by an electric tankless water heater

12. How old is your hot water heater?
    a. <1 year   b. 1-5 years   c. 6-10 years   d. >10 years   e. Do not know

13. If you have a hot water heater, how frequently is it flushed to clean?
    a. Yearly   b. Every few years   c. Never   d. Do not know

** MAKE SURE TO INCLUDE THIS SHEET IN THE RETURN ENVELOPE! **