Impact of Pipe Material on Microbial Communities in Biofilm Samples from Full-scale Drinking Water Distribution Systems

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IMPACT OF PIPE MATERIAL ON MICROBIAL COMMUNITIES IN BIOFILM SAMPLES FROM FULL-SCALE DRINKING WATER DISTRIBUTION SYSTEMS

by

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ABSTRACT
IMPACT OF PIPE MATERIAL ON MICROBIAL COMMUNITIES IN BIOFILM SAMPLES FROM FULL-SCALE DRINKING WATER DISTRIBUTION SYSTEMS

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The drinking water distribution system (DWDS) provides a habitat particularly supportive to biofilm-forming bacteria that can attach to pipe walls. These biofilms comprise the majority of bacteria in the DWDS, yet they have been understudied with respect to the pipe material on which they form. Biofilms can accelerate disinfectant decay, promote corrosion of pipes, and harbor opportunistic pathogens (OPs). The choice of pipe material could have a long-lasting effect on the characteristics of the biofilms formed in DWDSs. In this study, the impact of pipe material on biofilms residing in full-scale drinking water pipes was investigated by obtaining biofilm samples from three utilities (Milwaukee, Waukesha, and Oak Creek) and four pipe materials (cast iron, ductile iron, copper, and lead). Quantification of biomass (ddPCR targeting the 16S rRNA gene) and characterization of the microbial communities (Illumina sequencing) provided insights into how pipe materials shape biofilm microbial communities. Within a single utility, cast iron and ductile iron pipes supported higher biomass densities than copper and lead pipes. Pipe material shaped the biofilm communities within a single utility and explained 12% of microbial community dissimilarity within the dataset; pipe sample type, which is inherently a function of pipe material because tubercles only formed in iron pipes, explained 30% of microbial community dissimilarity. Iron-associated genera dominated iron pipes (especially for cast iron), and various heavy-metal resistant bacteria were identified in each pipe material. This is the second study to characterize the microbial community within full-scale lead pipes. Utility was a stronger driver of microbial community dissimilarity (21%) than pipe material, but the interactions between utility and pipe materials should be better understood. The dominance of *Mycobacterium* specifically in ductile iron pipes of the chloraminated utility suggests that the interaction of this pipe material and disinfect ant may select for the OP-harboring genus. Further, corrosion-accelerating genera in cast iron pipes were not ubiquitous in all utilities, suggesting utilities could control microbial corrosion. Lastly, detection rates of putative OPs were similar by pipe material and utility, with high detections of *Burkholderia cepacia* and *Ralstonia pickettii*, which are considered OPs of emerging concern.

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DEDICATION

San Marie V. Thomson

I would like to dedicate this thesis to my family. My parents Greg and Heidi Thomson have blessed me with their unwavering belief in me. My sister Antonia Thomson has role modeled to me equal dedication to achievement as well as to lightheartedness. Lastly, I offer a deep appreciation for passed loved ones who have supported me in spirit.

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1 INTRODUCTION

1.1 Motivation

Within the drinking water distribution system (DWDS), the microbial communities of environmental bacteria are shaped by several factors that research has attempted to better understand. Despite the addition of disinfectant residuals administered by utilities, microbes can persist in biofilms formed on the interior of pipes. Indeed, more than 90% of microbes in the DWDS inhabit biofilms (Niquette et al., 2000). Biofilms can reduce the efficacy of disinfectant residuals, promote microbial pipe corrosion, and may harbor opportunistic pathogens (OPs) (Huo et al., 2021; Morton et al., 2005; Norton et al., 2004; Sun et al., 2014; Tang et al., 2021). As bacteria in tap water have been found to more likely originate from biofilms than the treatment plant, the presence of OPs in biofilms should be monitored (Pullerits et al., 2020a; J. Zhang et al., 2018). Thus, characterizing the microbial community in drinking water biofilms and understanding the factors that influence its structure is important to ensure safe drinking water to consumers and to protect drinking water infrastructure.

The DWDS is a complex and dynamic ecosystem where many factors may act individually or in combination to impact the structure and abundance of the microbial community in biofilms. For one, season has a strong impact on the structure of the microbial community in biofilms, and temperature can affect the density of biomass (Kelly et al., 2014; Pullerits et al., 2020b; Tang et al., 2021). In addition, bulk water characteristics like hydraulic flow, pH, and nutrient availability can impact the abundance and structure of biofilms (Kelly et al., 2014; Percival et al., 1999). However, the aforementioned factors are beyond the control of engineering decision-making.

Therefore, to manage biofilms in DWDSs, special attention should be given to controllable factors like disinfectant and pipe material. Studies have demonstrated the individual impacts of disinfectant type and dose as well as pipe material, but the two factors may also interact, altering their effects (W. Li et al., 2020; H. Zhang et al., 2019).

Because most drinking water pipes remain part of the DWDS for years, choice of pipe material can have long-lasting effects. Certain pipe materials may be less likely to corrode, may better mitigate the presence of OPs, or may more effectively support a disinfectant residual. However, current knowledge has primarily relied on studies in laboratory settings because sampling from full-scale systems is challenging. Moreover, studies that have obtained full-scale samples have included only a limited number of pipe materials or have not focused their research on pipe material. Therefore, biofilms originating from several pipe materials in a full-scale DWDS need to be studied to assess the role of pipe material.

1.2 Objectives

The goal of this study was to understand the impact of pipe material on the biomass density and the microbial community of biofilms inhabiting full-scale DWDS pipes. To do this, a sampling campaign of full-scale drinking water pipes from three utilities was conducted. The utilities employed variable combinations of source water, disinfectant residual, and corrosion inhibitor. Ultimately, these utilities provided biofilm samples from four pipe materials: cast iron, ductile iron, copper, and lead. The specific research objectives were as follows:

1. Quantify the biomass density of biofilms and characterize the microbial communities inhabiting each pipe material type within the same utility

2. Compare the microbial communities occurring in different utilities based on their formation on the same pipe material
3. Investigate the influence of season, pipe age, pipe diameter, and sample type in shaping the microbial community
4. Identify opportunistic pathogens within the DWDS, focusing on differences among utilities and pipe materials

1.3 Approach

A sampling campaign of 12 pipes from Milwaukee, Wisconsin representing four pipe materials (cast iron, ductile iron, copper, and lead) was conducted to investigate the impact of pipe material within the same utility. In addition, 5 pipes from Oak Creek and 4 pipes from Waukesha were sampled to see how similar or different the microbial community might be on a pipe material from another utility, where the implemented disinfectant residual and corrosion inhibitors differed from Milwaukee.

The biomass density of the Milwaukee samples was quantified as the concentration of 16S rRNA gene copies via digital droplet polymerase chain reaction (ddPCR) to reveal how pipe materials affected biomass density within a utility. In addition, 16S rRNA gene Illumina Miseq Sequencing was used to characterize the microbial community of samples from all three utilities, offering insights into differences among pipe materials within the same utility as well as across utilities. GraphPad (GraphPad Software, San Diego, CA) and RStudio version 4.2.1 (RStudio: Integrated Development Environment for R., PBC, Boston, MA) provided tools for visualizing microbial community data, correlating ddPCR and sequencing data to categorical and nominal variables, and determining statistical significance.

1.4 Thesis Structure

Chapter 2 contains a literature review presenting the factors that impact the microbial community in biofilms of drinking water pipes, with an emphasis on the role of pipe material. **Chapter 3** describes the experimental design and the methods used to conduct the study. **Chapter 4** presents the study's results and discusses them in the context of previous research. **Chapter 5** summarizes the conclusions derived from the experimental results and provides recommendations for future research.

2 LITERATURE REVIEW

2.1 The Ecosystem within Drinking Water Distribution Systems (DWDSs)

2.1.1 Pipe Biofilms

Despite efforts by drinking water treatment plants to limit bacteria in drinking water, microbial life is ubiquitous in the drinking water distribution system (DWDS). The majority of bacteria in the DWDS – an estimated >90% – live in biofilms that line the interior of pipes (Flemming et al., 2002). Microbes that produce extracellular polymeric substances (EPS) have a survival advantage because EPS forms a protective matrix that brings bacteria close together, helps them cling to pipe walls, and protects them from disinfectants. While the bacterial community found in bulk water remains relatively stable as water travels through the DWDS, biofilms have been found to vary spatially in both abundance and structure (Henne et al., 2012).

Biofilms in DWDSs are important to study because they impact pipe corrosion and disinfectant residual, and they can harbor opportunistic pathogens (OPs). Although most bacteria found in the DWDS pose no threat to human health, OPs have been detected in drinking water pipes in several studies (Fu et al., 2021; Gomez-Smith et al., 2015; Huo et al., 2021; Pullerits et al., 2020a; Tang et al., 2021). These OPs can become hazardous to human health if released from the biofilm into the bulk water, where they could then travel to consumer taps. Microbes found in biofilm can enter bulk water when single cells detach during the maturation stage to seek new environments to colonize (S. Liu et al., 2016). Indeed, bacteria in tap water have been found to originate in biofilm and not treatment plant effluent (Pullerits et al., 2020a; J. Zhang et al., 2018) Further, the low-nutrient conditions and oxidative stress in a drinking water pipe can cause microbes to enter a dormant, viable-

but-non-culturable state from which they can resuscitate once relocated to a more hospitable environment (Fu et al., 2021).

In addition, biofilms play a key role in corrosion processes, which release particulate matter into drinking water. Pipe corrosion has consequences to infrastructure costs, consumer health, and the ability of municipalities to meet drinking water regulations. Corrosion threatens noncompliance with the Lead and Copper Rule and, more importantly, may endanger consumers drinking tap water containing heavy metals (United States Environmental Protection Agency, 1991). In addition, corroded pipes can lead to leakage or breakage in the system, incurring maintenance and replacement costs. Further, particulate matter in bulk water also supports the existence of particle-associated OPs, which are more difficult to inactivate with disinfectant residuals as compared to free-living OPs (Huo et al., 2021).

2.1.2 The Challenge of Studying Microbial Communities in DWDSs

Engineering decisions in drinking water infrastructure impact the safety of drinking water, drawing importance on research about the factors that influence biofilm growth in DWDSs. However, identifying the factors that shape biofilm communities can be a challenge due to the many individual factors involved as well as the combined effects of these factors. Even within the study of biofilms in DWDSs, the abundance and structure of the microbial community can vary based on the type of sample collected, such as whether biofilms resided on the smooth surface of the pipe or on/below corroded surfaces and structures (Gomez-Smith et al., 2015; Kimbell et al., 2021).

A dynamic and complex interaction of factors influences microbial growth in biofilms, providing a challenge to identifying key factors that influence microbial

community characteristics. Laboratory-scale studies have attempted to isolate factors by creating simulated drinking water systems (Aggarwal et al., 2018; Rożej et al., 2015). In a study on pipe material, water age, and disinfectant residual, all three factors were seen to drive microbial community structure, highlighting that these factors in combination provide unique ecosystems (Hong Wang et al., 2014). In addition, season and temperature had a stronger impact on microbial community dissimilarity at sampling locations more distant from the treatment plant, where disinfectant residuals had decreased with increased water age, emphasizing the interplay of spatial and temporal factors (Potgieter et al., 2018).

Several studies have identified temperature and season as particularly strong drivers of community structure (Kelly et al., 2014; Pullerits et al., 2020b; Tang et al., 2021). While season clearly shapes microbial community differences and biofilm abundances, there is no consensus on the effect of any particular season, with studies finding conflicting results (Potgieter et al., 2018; Siedlecka et al., 2021). Again, the inconclusive findings regarding temporal factors emphasize the complexity of the microbial ecology in drinking water systems.

Although many factors impact the microbial communities in DWDSs, only a few of them are within the control of human decision-making. Water temperature, age, and hydraulic conditions are difficult, if not impossible, to control within the extents of the DWDS. Thus, special attention should be given to factors that humans can influence, namely pipe materials and disinfection strategies. A review of these controllable factors is presented below.

2.2 Engineering Factors that Shape Microbial Communities in DWDSs

2.2.1 The Role of Disinfectants

Chlorine and chloramine, common disinfectants, are added to finished water to suppress the growth of biofilm and of opportunistic pathogens. The choice of disinfectant influences the abundance and type of bacteria that will survive, thereby shaping different microbial communities (Hong Wang et al., 2014). While chloramine has been known to penetrate biofilms more deeply, free chlorine more effectively inactivates bacteria on the surface of biofilms (Lee et al., 2011).

Some taxa have increased sensitivity or resistance to a disinfectant, depending on which disinfectant is used. The genus *Mycobacterium* contains several species known to cause infection in humans, and it has been found to persist in drinking water systems, in part due to its resistance to common disinfectants (Vaerewijck et al., 2005). In particular, *Mycobacterium* can resist chloramine disinfection (Aggarwal et al., 2018). Meanwhile, chloramine is more effective than chlorine in inhibiting the pathogen *Legionella pneumophila* (Huo et al., 2021).

Water age influences the microbial community because older water is exposed to lower disinfectant concentrations. In this way, water age and disinfectant concentration are inversely correlated: older water has higher abundances of 16S rRNA gene copies and OPs in biofilm (Huo et al., 2021; Hong Wang et al., 2014). In addition, the microbial community changes with reduced stress of disinfectants (Potgieter et al., 2018). Again, the choice of disinfectant will determine how strongly water age impacts the drinking water microbiome. In one study, chloramine decayed more rapidly in the distribution system due to nitrification (Hong Wang et al., 2014). However, it has also been

recognized that free chlorine can be scavenged by chemicals relating to iron pipe corrosion thus giving favor to chloramine (Morton et al., 2005),

Overall, protecting systems from OPs proves difficult due to the interactions of disinfectants with pipe materials. Disinfectants can decrease the pH of bulk water and can react with chemicals originating in pipe materials, accelerating corrosion rates (Huo et al., 2021; H. Zhang et al., 2019). As such, increasing disinfectant doses may be counterproductive. Corrosion products can enter bulk water as particulate matter or create scales on the interior of pipes, both of which can prevent effective disinfection. Particle-associated pathogens are more difficult to disinfect, so corrosion-related particulate matter can harbor OPs (Huo et al., 2021). Further, corrosion scales induced by high concentrations of chlorine dioxide promoted the formation of a corrosive layer that shielded biofilms from disinfection (H. Zhang et al., 2019). It is also noteworthy that disinfectants can stimulate the release of cells from drinking water biofilms (J. Zhang et al., 2018).

2.2.2 The Role of Pipe Materials

Pipe material influences microbes' ability to establish a biofilm. The differences in growth on different materials can be extreme. Niquette et al. reported 10 to 45 times higher biofilm density on iron surfaces than on plastic surfaces (Niquette et al., 2000). Further, pipe material shapes the taxonomical structure of the community. The primary factors contributing to differences in biofilms with respect to pipe material can be grouped into two categories. First, a pipe material's surface characteristics, specifically roughness, porosity, and surface charge, influence the ability of microbes to adhere to a surface and form the protective EPS. Second, the physiochemical properties associated

with pipe deterioration and corrosion can harm or help microbes in surviving on pipe surfaces. For this review, corrosion will be discussed in the following subsection though it evidently is related to pipe material.

Surfaces that have a higher surface roughness indices allow biofilm to grow thicker, supporting higher abundances of microbes. Indeed, it has been found that biomass concentration is similar on surfaces with similar surface roughness and porosity (Niquette et al., 2000). The indentations on rougher, more porous surfaces provide larger surface areas to which biofilm can attach and also provide spaces sheltered from hydraulic shear forces and disinfectants (Tang et al., 2021; Z. Zhu et al., 2014). As such, iron pipes have been found to have higher biomass densities than smoother pipes, like plastic, cement, copper, and steel (W. Li et al., 2020; Niquette et al., 2000; Hong Wang et al., 2014). Hence, corroded pipes also support higher bacterial growth as surface roughness increases with corrosion.

The surface charge of pipe materials could also contribute to both the abundance and structure of microbial communities in biofilms. For example, Zhu suggested that the colonization of *Bacillus* on different pipe materials could be related to the hydrophobic surface of the genus's spores, which would attach more readily to hydrophilic surfaces like polyethylene and less readily to hydrophobic surfaces like cement (Z. Zhu et al., 2014). In addition, stainless steel discourages biofilm growth, possibly due to its negative surface charge, and it has been suggested as material suited for pathogen control (Fu et al., 2021; Z. Zhu et al., 2014).

Although several studies have established the influence of pipe material on drinking water biofilms, Tang found that when controlling for similar pipe ages and

diameters, pipe material did not significantly affect the microbial community of biofilms in cast iron and ductile iron pipes (Tang et al., 2021). However, this study only compares two pipe materials, both which are iron pipes. Tang's finding therefore does not address the differences that may arise when comparing other metallic pipe materials, like copper and lead. Overall, research of drinking water biofilms in full-scale metallic pipes has been limited, providing a research gap.

2.2.3 The Role of Corrosion and Pipe Deterioration

The deterioration of pipes, both via leaching (in the case of plastic pipes) or corrosion (in the case of metallic pipes) can influence the abundance and structure of drinking water biofilms. In the case of cast iron pipes, corrosion generally supports higher growth due to increased surface roughness, protection from disinfectant exposure, and the provision of nutrients. Tubercles, structures that form on corroded iron surfaces, generally have higher biomass density as do the surfaces underneath them (Gomez-Smith et al., 2015; Kimbell et al., 2021).

Corrosion tubercles have been found to be unique in community structure compared to swabs of biofilm or from below tubercles (Kimbell et al., 2021). For one, tubercles can provide anaerobic niches in which anaerobic microbes dominate (Gomez-Smith et al., 2015). It is especially important to consider that corroded surfaces can support the survival of OPs. Tang (2021) found that in 13 cast iron and ductile iron mains, *Mycobacterium spp*. were 100% detected and *Legionella* was 92.3% detected (Tang et al., 2021). Between these two pipe materials, cast iron supported higher abundance of *Mycobacterium* – likely due to more corroded surfaces – than ductile iron pipes (Tang et al., 2021). In another study, it was concluded that cast iron pipes supported

OPs of higher pathogenicity as compared to copper and cement pipes (Z. Zhu et al., 2014). These studies encourage more research into the role of pipe material in the survival of pathogens.

The niches created by corroded material protect biofilms from disinfection and can retain growth-supporting nutrients (S. Liu et al., 2016). Corroded iron and steel surfaces were found to supply the macronutrients carbon, nitrogen, and phosphorus to support regrowth under low-disinfectant conditions (Morton et al., 2005). In addition, iron ions released from iron pipes have been found to support biofilm growth (Fu et al., 2021), though theoretically, they may lead to cell damage at high concentrations (Tang et al., 2021; Ye et al., 2020).

Moreover, corrosion can prevent effective disinfection, which impacts the microbial community structure and, more importantly, the growth of OPs. First, the biofilm itself, which is thicker on a rougher surface, can protect microbes from disinfectants in the water. Further, chemical products released during corrosion can interact with disinfectants. Several studies have found that chemicals near corroded iron surfaces can consume free chlorine residual (Morton et al., 2005; Norton et al., 2004). For example, *Mycobacterium* has usually been known to dominate in chloraminated systems due to its resistance to chloramine and ability to form biofilms (Aggarwal et al., 2018; Kimbell et al., 2021). However, the chemical reactions of iron corrosion products and chlorine caused chloramine to be a more effective disinfectant against *Mycobacterium* in one study (Norton et al., 2004). In another study, scaling on cast iron coupons was promoted at an increased dose of chlorine dioxide, which protected biofilms and increased richness (H. Zhang et al., 2019). As such, the interactions of disinfectants

with pipe materials are important in pathogen control and should be considered in the choice of disinfectant strategy and dose.

Although iron corrosion clearly supports biofilms, there is more research needed to understand how deterioration of other pipe materials impacts biofilm growth. The toxicity of copper ions (Cu^{2+}) originating in copper pipes generally inhibits biofilm growth (Lehtola et al., 2004; W. Li et al., 2020). However, in one lab-scale study, copper coupons supported higher biofilm growth as they corroded (Z. Zhu et al., 2014). In addition, new polyethylene pipes can leach phosphorus, a nutrient that can increase the growth potential of biofilms especially when it is limited in the bulk water (Lehtola et al., 2002, 2004). However, studies on the interactions between chemicals released from plastic and other materials and biofilms are not readily available, so other factors may be overlooked.

2.3 Noteworthy Genera Dominating Drinking Water Ecosystems

2.3.1 Opportunistic Pathogens

In characterizing microbial communities in drinking water pipes, special attention is rightfully given to the presence of OPs. As opposed to fecal pathogens, e.g. *Escherichia coli* (*E.coli*), OPs are inherent members of drinking water microbial communities (Hong Wang et al., 2013). It has been reported that 30% of the US population may be exposed to OPs (Falkinham III et al., 2015). Thus, a primary motivation for drinking water research is monitoring OPs and seeking out key factors that influence their presence.

The genus *Mycobacterium* contains several pathogenic species, and is known for showing resistance to disinfectants (W. Li et al., 2020; Webster et al., 2021). Thus,

Mycobacterium has frequently been found in drinking water distribution systems (Huo et al., 2021; Pullerits et al., 2020b; Tang et al., 2021; Hong Wang et al., 2014). In particular, *Mycobacterium avium* is surveyed in drinking water studies due to its ability to cause a number of clinical symptoms, including pulmonary disease, especially in immunocompromised individuals (Norton et al., 2004; Vaerewijck et al., 2005).

The genus *Legionella* contains the pathogenic species *Legionella pneumophila*, the source of the outbreak of Legionnaires' disease in Flint, Michigan, and has also been identified in drinking water pipes (Huo et al., 2021; Tang et al., 2021; Waak et al., 2018). A 10-year study between 2004 and 2014 showed a more than 2.85-fold increase in cases of Legionnaires' disease, where the most frequent exposure to *L. pneumophila* was via potable water (Garrison et al., 2016). *Legionella* and *Mycobacterium* have been shown to cohabitate drinking water biofilms (Vaerewijck et al., 2005).

The species *Pseudomonas aeruginosa* is an environmental OP that can occur on fresh produce and in drinking water, though in lower concentration in drinking water (Hardalo & Edberg, 1997; Huo et al., 2021; Rożej et al., 2015). This pathogen is harmful to individuals with predisposing conditions, and the at-risk group is well-defined (Hardalo & Edberg, 1997). As the OP is resistant to disinfection, Hardalo et al. argues that the carcinogenic byproducts formed in drinking water treated with high levels of disinfectants may be more harmful than the OP itself (Falkinham III et al., 2015; Hardalo & Edberg, 1997). Still, efforts to understand the occurrence of *P. aeruginosa* with the goal of managing infections continue to be of importance in drinking water research (Falkinham III et al., 2015; Fu et al., 2021).

2.3.2 Corrosion-Related Microbes

Key taxa have been identified in association with corrosion-related processes, specifically in the corrosion of iron pipes. While some taxa increase corrosion, others can inhibit it. It is evident that the structure of the microbial communities in iron pipes reflects the interspecies relationships involved in iron corrosion. When discussing these microbes in terms of iron corrosion, they are often grouped by their abilities to oxidize or reduce iron, sulfate, and nitrate.

Iron-oxidizing bacteria (IOB), sulfate-reducing bacteria (SRB), and sulfur-oxidizing bacteria (SOB) accelerate iron corrosion (Sun et al., 2014). IOB, like the genus *Gallionella*, have been associated with red water events where aqueous iron concentrations are high (D. Li et al., 2010). *Acidovorax* and *Leptothrix* are also commonly-cited IOB that cause corrosion (Gomez-Alvarez et al., 2012; D. Li et al., 2010; White et al., 2011; Y. Zhang et al., 2018). SRB and SOB largely inhabit inner layers of corroded iron, where conditions may be anaerobic, suggesting these microbes anaerobically corrode iron (Sun et al., 2014). IRB also may grow in anaerobic conditions, and the corrosion-related processes performed by SRB and SOB may promote the growth of IOB, and vice versa, ultimately furthering corrosion (Sun et al., 2014; Teng et al., 2008). A common and abundant SRB in drinking water biofilms is *Desulfovibrio* (Rożej et al., 2015; Siedlecka et al., 2021; Sun et al., 2014; Tang et al., 2021) while *Sulfuricella* is a commonly-cited SOB in these systems (Sun et al., 2014; Y. Zhang et al., 2018).

Meanwhile, iron-reducing bacteria (IRB) are associated with inhibition of iron corrosion and release (Sun et al., 2014). In particular, the formation of stable corrosion products like goethite, α-FeOOH, and magnetite, Fe_3O_4, by microbes in iron pipes

prevents the release of iron to bulk water (Sun et al., 2014). In drinking water studies, commonly identified IRB include *Pseudomonas*, *Bacillus*, and *Arthrobacter* (Gomez-Alvarez et al., 2012; H. Zhang et al., 2019; Z. Zhu et al., 2014).

Further the genus *Dechloromonas* has been identified as an abundant nitrate-reducing bacteria (NRB) that promotes the formation of a stable, compact corrosion layer that reduces release of corrosion particles in bulk water (W. Li et al., 2020; Haibo Wang et al., 2014). As it performs cellular respiration, *Dechloromonas* cycles Fe^{2+} and Fe^{3+}, consumes oxygen, and consequently promotes the formation of iron oxides and magnetite (Haibo Wang et al., 2014). This genus may be more dominant in chloraminated systems, as are other nitrifying taxa (Gomez-Alvarez et al., 2012; Lee et al., 2011). *Simplicispira* is another NRB that has been associated with cast iron pipes, though its specific role in iron corrosion has not been well-discussed (Tang et al., 2021).

2.3.3 Other Genera Commonly Detected in DWDSs

The genera frequently found to dominate drinking water biofilms are generally well-adapted for low-nutrient conditions and persist despite the presence of disinfectants. Due to the large range of conditions among – and within – distribution systems, numerous genera have been identified as dominating a particular drinking water biofilm community. However, a few genera stand out as frequently cited. The genus *Sphingomonas* is often dominant because it is well-suited to live in pipe biofilms as it effectively produces EPS, which helps it adhere to pipe walls and resist disinfection (Z. Zhu et al., 2014). It has been identified in both chlorinated and chloraminated conditions as well as on several pipe materials (W. Li et al., 2020; Tang et al., 2021; Z. Zhu et al., 2014). In addition, *Nitrospira* and *Nitrosomonas* are often identified as dominant genera

(Aggarwal et al., 2018; W. Li et al., 2020; Potgieter et al., 2018; Siedlecka et al., 2021; Tang et al., 2021). These are nitrifying bacteria and therefore have been found to dominate chloraminated systems, threatening to reduce disinfection efficacy (Shi et al., 2020). *Nitrosomonas* is known to oxidate ammonia while *Nitrospira* oxidizes nitrite (Dionisi et al., 2002). In addition, *Nitrospira* may be able to execute comamox, a process in which ammonia is completely oxidized to nitrate (Aggarwal et al., 2018). *Nitrospira* is believed to promote the stability of biofilms and is therefore considered a keystone genus (Tang et al., 2021).

2.4 Summary of Research Needs

Existing drinking water research has investigated how various factors shape the microbial community, but most studies have been limited in scope. Specifically, due in part to the difficulty in obtaining biofilm samples from full-scale drinking water pipes, existing research regarding the impact of pipe material on drinking water biofilm has largely been limited to lab-scale studies. Meanwhile, the few studies that have collected samples from full-scale pipes have not compared more than two pipe materials. Additionally, research has not compared similar pipe materials from different utilities or how pipe materials of lead, iron, and copper vary within a single utility. Lead pipes in particular have been excluded from studies on the role of pipe material in shaping microbial communities.

Due to the role of biofilms in pipe corrosion and control of OPs, it is important to more comprehensively investigate how pipe material impacts the microbial community of biofilms, both in abundance and in structure. To fill these research gaps, the present study had four research objectives:

Objective 1: Establish the impact of pipe material on the abundance and structure of the microbial community in drinking water biofilms within a utility.

Hypothesis: As compared to copper and lead pipes, the biomass density of biofilms will be higher in cast iron and ductile iron pipes, which have been shown in other studies to support higher biomass densities due to increased surface roughness and the possible provision of growth-promoting nutrients. The dominant taxa of microbial communities will be shaped by the pipe material, with dissimilarities in microbial communities observed based on pipe material.

Objective 2: Understand the variability in microbial community structure in biofilms growing on the same pipe material from different utilities.

Hypothesis: While the same pipe material will promote dominance of similar taxa, there will still be observable differences in the microbial communities based on utility, particularly due to differences in disinfectant residual and corrosion inhibitors used by the utilities.

Objective 3: Elucidate the roles of pipe age, pipe diameter, sample type, and season in comparison to the roles of pipe material and utility in shaping the microbial community of biofilms.

Hypothesis: Because the microbial community in DWDSs is influenced by many factors that can complexly shape the microbial community, there will be drivers of microbial community dissimilarity beyond pipe material and utility. In particular, season has been shown to alter the microbial community and thus is most likely to be another strong factor shaping the microbial community. Further, previous studies have demonstrated dissimilarities in the microbial community based on the origin of the

sample type, i.e., surface swab, tubercle chunk, under-tubercle swab, so sample type will also have an influence on the microbial community.

Objective 4: Improve the understanding of the impact of pipe material, utility, and sample type on the presence of OPs within the DWDS.

Hypothesis: As previous literature has found that disinfectant and pipe material can impact the survival of OPs, both the pipe material and the utility will influence the frequency with which OPs are detected. Because corrosion-related processes impact the disinfectant residual and community structure, OPs will be detected with higher frequency in corrosion-related samples, i.e., tubercle and under-tubercle samples.

3 METHODS

3.1 Experimental Design

3.1.1 Sampling Campaign from Full-Scale Drinking Water Distribution Systems

To investigate the relationship between pipe material and the microbial community in the biofilms of drinking water distribution systems, a sampling campaign of full-scale biofilm samples was completed between October 2019 and August 2021 in southeast Wisconsin, USA.

Samples were obtained from pipes in Milwaukee County, which includes the city of Oak Creek, and Waukesha County. Milwaukee County's DWDS is supplied by two treatment plants that sources its water from Lake Michigan. Both treatment plants use the same treatment processes. Oak Creek is a city within Milwaukee County that treats its drinking water separately from other cities within the county, though it also uses Lake Michigan as its source water. Meanwhile, Waukesha County sources its water from shallow sand aquifers and a deep sandstone aquifer and performs water treatment at three facilities, all which use the same treatment process and distribute to the same network of pipes. A summary of relevant information regarding each utility's water treatment can be found in **Table 3.1**.

Table 3.1 Description of drinking water utilities from which pipes were sampled

	Milwaukee	**Oak Creek**	**Waukesha**
Number of Treatment Plants	2	1	3
Source Water	Lake Michigan	Lake Michigan	St. Peter Sandstone Aquifer & shallow sand aquifers
Primary Disinfection	Ozone	Sodium Hypochlorite	Sodium Hypochlorite
Secondary Disinfection	Chlorine	None	None
Disinfection Residual	Chloramine	Chlorine	Chlorine
Corrosion Inhibitor	Phosphoric Acid	None	Sodium silicate
Other Added Chemicals	Fluoride, Alum	Fluoride	Fluoride

Milwaukee County was selected as the utility that could provide insight into how pipe material shapes the microbial community within a utility because it had a high likelihood of pipes available to sample from four different pipe materials (cast iron, ductile iron, copper, and lead). Three pipes were sampled for each of these materials, resulting in a total of 12 pipes. Samples collected from Milwaukee were studied to measure total biomass (quantification of concentration of 16S rRNA) and to identify the microbial communities (Illumina sequencing of 16S rRNA).

Meanwhile, samples obtained from pipes in Oak Creek and Waukesha were sequenced to provide points of comparison regarding microbial community composition (only Illumina sequencing of 16S rRNA). Waukesha provided two cast iron pipes, three ductile iron pipes, and one lead pipe. Oak Creek provided one ductile iron pipe and two copper pipes. **Figure 3.1** shows the locations of each sampling event. **Table 3.2** shows relevant information relating to each sampled pipe.

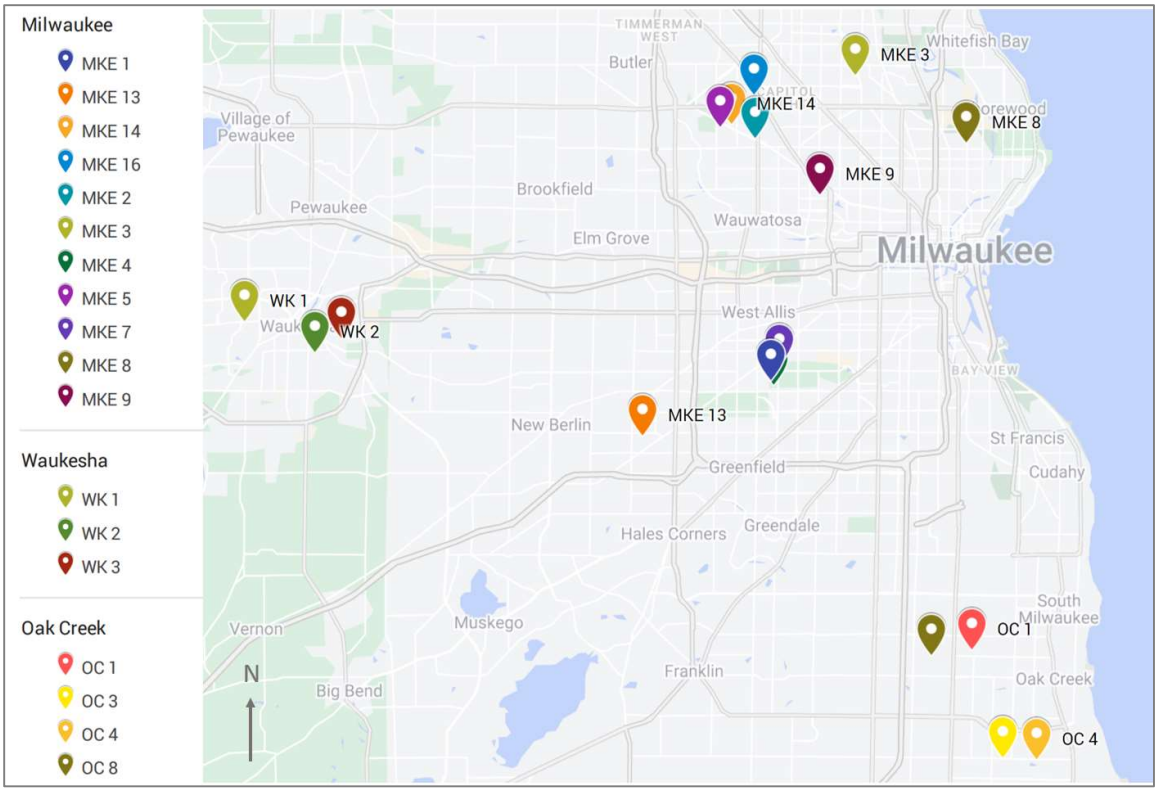

Figure 3.1 Map of Sampling Events as created with Google Maps (Google, Mountain View, CA). The map may be viewed online at the following URL: https://www.google.com/maps/d/edit?mid=1q6osD6yn85tKrwwVd-BpUMKOdV-8-jA&usp=sharing. Each sampling event provided samples from a single pipe, except for MKE 5 and WK2, which each provided samples from two pipes that were connected to one another.

Table 3.2 Description of Sampled Pipes from Milwaukee, Oak Creek, and Waukesha

Sampling Event	Material	Pipe Type	Diameter	Age	Month	Type(s) of Samples Collected
Milwaukee (MKE)						
1	Copper	Lateral	3/4"	70	October	Bio
2	Lead	Lateral	3/4"	75	October	Bio
3	Lead	Lateral	3/4"	90	October	Bio
4	Cast Iron	Main	6"	67	October	Bio, Tub, UT
5-cFe	Cast Iron	Main	12"	70	November	Bio, Tub, UT
5-dFe	Ductile Iron	Main	12"	13	November	Bio
7	Cast Iron	Main	8"	70	November	Bio, Tub, UT
8	Lead	Lateral	5/8"	120	December	Bio
9	Copper	Lateral	1"	NA	February	Bio
13	Copper	Lateral	3/4"	NA	March	Bio
14	Ductile Iron	Lateral	8"	16	August	Bio
16	Ductile Iron	Lateral	8"	21	August	Bio, Tub
Oak Creek (OC)						
1	Copper	Lateral	3/4"	55	July	Bio
4	Ductile Iron	Main	18"	34	August	Bio
5	Ductile Iron	Main	20"	39	August	Bio
6	Ductile Iron	Main	20"	39	August	Bio
7	Copper	Lateral	3/4"	NA	January	Bio
Waukesha (WK)						
1	Ductile Iron	Main	16"	51	August	Bio
2-Pb	Lead	Lateral	3/4"	106	August	Bio
2-cFe	Cast Iron	Lateral	3/4"	106	August	Bio
3	Cast Iron	Lateral	2"	73	September	Bio, Tub, UT

3.1.2 Sample Collection Methods

In coordination with Milwaukee Water Works, the Oak Creek Water and Sewer Utility, and the Waukesha Water Utility, samples were collected from full-scale mains and laterals following excavation for maintenance purposes.

Three types of biofilm samples were collected when possible: (1) biofilm swabs from the smooth surface of the pipe, (2) tubercle chunks removed from the pipe wall, and (3) under-tubercle swabs from beneath collected tubercles (Figure A-1). Although these samples all constitute pipe biofilms, this thesis will distinguish each of the three sample

types as (1) biofilm, (2) tubercle, and (3) under-tubercle samples, respectively. Samples were collected and placed into sterile tubes in the field before being transported in a cooler to the laboratory. Biofilm swabs were obtained by swabbing several locations along the inside of the pipe. Each swab contacted a surface area of approximately 1 cm^2. Biofilm swabs were obtained from all materials. Meanwhile, tubercle and under-tubercle samples were collected for only cast iron and ductile iron pipes because copper and lead pipes did not form tubercles. When present, observed tubercles were dislodged with a spatula. Each tubercle's previous location was swabbed to obtain under-tubercle biofilm samples. Following transport to the lab, each tubercle was carefully placed into a new sterile tube to obtain its wet weight. All samples were stored at -20ºC until DNA was extracted.

Table 3.3 summarizes the number of samples from each pipe that were included in the quantification of 16S rRNA via digital droplet polymerase chain reaction (ddPCR) and Illumina sequencing of 16S rRNA, as grouped by sample type.

Table 3.3 Number of Samples Quantified/Sequenced from Each Pipe

			ddPCR quantification			Illumina Sequencing		
	Sampling Event	Material	Biofilm	Tubercle	Under-Tubercle	Biofilm	Tubercle	Under-Tubercle
Milwaukee	1	Copper	6	0	0	3	0	0
	2	Lead	6	0	0	3	0	0
	3	Lead	6	0	0	3	0	0
	4	Cast Iron	6	3	3	3	3	3
	5-cFe	Cast Iron	6	3	3	3	3	3
	5-dFe	Ductile Iron	6	0	0	3	0	0
	7	Cast Iron	6	3	3	3	3	3
	8	Lead	6	0	0	3	0	0
	9	Copper	6	0	0	3	0	0
	13	Copper	6	0	0	3	0	0
	14	Ductile Iron	6	0	0	3	0	0
	16	Ductile Iron	6	2	0	3	2	0
Oak Creek	1	Copper	NA	NA	NA	3	0	0
	4	Ductile Iron	NA	NA	NA	3	0	0
	5	Ductile Iron	NA	NA	NA	1*	0	0
	6	Ductile Iron	NA	NA	NA	3	0	0
	7	Copper	NA	NA	NA	2*	0	0
Waukesha	1	Ductile Iron	NA	NA	NA	3	0	0
	2-Pb	Lead	NA	NA	NA	3	0	0
	2-cFe	Cast Iron	NA	NA	NA	3	0	0
	3	Cast Iron	NA	NA	NA	3	3	3

*sequencing was not successful for the three samples submitted

3.2 Molecular Methods

3.2.1 DNA Extractions

DNA from all samples was extracted using a FastDNA SPIN Kit (MP Biomedicals, Solon, OH) with minor modifications. The initial cell lysis was performed by three freeze-thaw cycles using liquid nitrogen, following methods established in previous studies (Kappell et al., 2018; Kimbell et al., 2021; G. W. Li et al., 2012). From this point on, the manufacturer's instructions were followed to complete the DNA extraction.

3.2.2 Quantification of 16S rRNA via ddPCR

The droplet digital quantitative polymerase chain reactions (ddPCR) was used to quantify concentrations of 16S rRNA gene copies, which have been used as an indicator of total biomass in biofilm samples (Kimbell et al., 2021). ddPCR was selected for gene quantification as studies have observed more reliable results for biofilm samples obtained from the drinking water distribution system (Kimbell et al., 2021).

Duplicates of each sample were quantified where the coefficient of variance (CoV) was ≤ 10%. In instances where the CoV was >10% between duplicate measurements of a sample, additional replicates were quantified to achieve the target CoV. In addition, negative controls were included with the ddPCR plates, and the limit of blank (LOB) was established with the equation $LoB = mean_{blank} + 1.645*(standard\ deviation)_{blank}$, based on guidelines from the Clinical and Laboratory Standards Institute (Armbruster & Pry, 2008). The LOB was established as 10.9 gene copies/µL for the raw data. To compare the LOB to the concentration of 16S rRNA quantified in biofilm swabs, the same formula that was applied to the raw data of the samples was applied to the LOB. This results in a normalized LOB of 5.74 gene copies/cm^2.

For each well of a 96-well plate, a 22-µL reaction mixture was prepared that contained 11 µL EvaGreen Supermix (Bio-Rad Laboratories Inc, Hercules, CA), 4 µL of diluted sample (1:100 dilution), 0.55 µL combined forward/reverse primers (achieving a final concentration of 250 nM for each primer), and 5.9 µL molecular grade water. The primer sequences used to quantify the 16S rRNA gene were F-(5'-CCT ACG GGA GGC AGC AG -3') and R-(5'- ATT ACC GCG GCT GCT GG -3'). Using the QX100 Droplet Generator, droplets were generated by 20 µL of the reaction mixture and 70 µL of

QX200 Droplet Generation Oil for EvaGreen. Gene amplification of the droplets within the plate was achieved by the C1000 Touch Thermal Cycler. The following conditions established gene amplification: denaturation for 10 minutes at 95˚C, annealing and extension in 39 cycles of 94˚C for 30 seconds followed by 60˚C for 60 seconds and 72˚C for 60 seconds, and lastly, signal stabilization for 5 minutes at 4˚C and 5 minutes at 90˚C. Finally, the QX200 Droplet Reader quantified the gene copies of the sample.

Analysis of the ddPCR results was done using the software QuantaSoft Analysis Pro, version 1.0.596 (Bio-Rad Laboratories Inc, Hercules, CA). Quality control was performed that excluded results with <10,000 droplets or that had ≤ 3 positive droplets (Di Cesare et al., 2018; Kimbell et al., 2021). Samples that produced such results were re-quantified.

3.2.3 MiSeq Illumina Sequencing

Sequencing of 16S rRNA genes was performed with Illumina MisSeq by MR DNA (Molecular Research LP, Shallowater, TX, www.mrdnalab.com). In addition to the DNA extracts of the samples, three extraction blanks were included in the sequencing plate as negative control samples. MR DNA provided a spreadsheet of amplicon sequence variants (ASVs) classified to genera, which were further analyzed. To distinguish between positive reads and contamination, ASVs were removed from further analysis where the average raw abundance was less than 3 times the maximum of the blanks. This reduced the number of ASVs from 762 to 194. Further, the absolute abundance was calculated to be the difference of the positive read and the maximum of the negative control.

Seven species considered to be opportunistic pathogens (OPs) were selected for further investigation. Commonly studied OPs included in this study were *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Mycobacterium fortuitum*, and *Mycobacterium avium* (Huo et al., 2021; Siedlecka et al., 2021). Based on initial findings of prevalent genera found in the samples, the pathogenic species within the genera Burkholderia and *Ralstonia* were further investigated: *Burkholderia cepacia*, *Ralstonia mannitolilytica*, and *Ralstonia pickettii*. *Ralstonia spp*. in particular is considered to be a pathogen of emerging importance (Ryan & Adley, 2014). Species counts were not adjusted based on the blanks, but detection of OPs in blanks was reported. Detection rates of these OPs were calculated as the percentage of positive detections per total number of samples in a grouping.

3.3 Statistical Analysis

Analysis of sequencing data was performed in part by the statistical program RStudio version 4.2.1 (RStudio: Integrated Development Environment for R., PBC, Boston, MA). Meanwhile, GraphPad Prism version 9.4.1 for Windows (GraphPad Software, San Diego, CA) was used to visualize and statistically analyze ddPCR results. Statistically significant results had a p-value less than 0.05.

The assumption criteria were tested for all data for which statistical tests were ran, thus allowing the appropriate test to be applied. The Shapiro-Wilk test was performed using the package ‘Rcmdr’ to test for the normality of data. To test the homogeneity of variances, Levene’s test was performed for ddPCR and alpha diversity data. When data was normal and had equal variance, the analysis of variance (ANOVA) test was performed, either using GraphPad or the ‘aov’ function in RStudio. Post-hoc ANOVA

tests were Tukey's Honest Comparison (HSD). Alternatively, when these assumptions were not met, the Kruskal-Wallis test was performed in GraphPad or with the 'kruskal.test' function in RStudio. Post-hoc Kruskal-Wallis tests used Dunn's test.

The 'phyloseq' package in RStudio was used to identify the dominant genera in subsets of the data following guidance from an introductory tutorial (LaMartina & Newton, 2019). Dominant genera were found by summing the relative abundances of each taxon within a selected data subset and sorting out those taxa with the highest sums. In addition, this package performed a principal coordinate analysis (PCoA) via the Bray-Curtis dissimilarity measurement. The 'vegan' package was used to calculate alpha diversity values, where the Shannon Index represented diversity and richness was the number of genera present. Pielou's Evenness Index was used to represent evenness of genera. Richness was reported as observed counts of unique genera. Dissimilarities in relative abundances of genera in various microbial communities were assessed using Bray-Curtis dissimilarity and the Permutational Multivariate Analysis of Variance (PERMANOVA) test via the 'adonis2' function in the package 'vegan'. An assumption of PERMANOVA is homogeneity of variance, which was assessed using the 'betadisper' function. Canonical correspondence analysis (CCA) was performed using the 'cca' and the 'envfit' functions in the 'vegan' package to determine how the microbial communities were shaped by pipe material, utility, season, pipe age, pipe diameter, and sample type.

The 'corrplot' package calculated the Spearman's rank sum correlation of the relative abundances of ASVs to provide insights into co-occurrence of taxa and correlations with pipe age and pipe diameter. The package 'ggplot2' was used to modify visual elements of heatmap plots.

4 RESULTS & DISCUSSION

4.1 Impact of Pipe Material Within a Single Utility: Milwaukee Water Works

4.1.1 Impact of Pipe Material on Biomass Density

Within Milwaukee's utility, the density of biomass (as measured by 16S rRNA gene copy number) in biofilm samples differed by pipe material, with the iron pipes having more biomass than copper and lead (Figure 4.1). Out of 111 samples, 29 samples had detections below the LOB, and all 29 of these samples were copper or lead, indicating low biomass. Another study found that iron biofilms produced the highest heterotrophic plate counts while copper biofilms produced the fewest, though this study did not include lead pipes (W. Li et al., 2020).

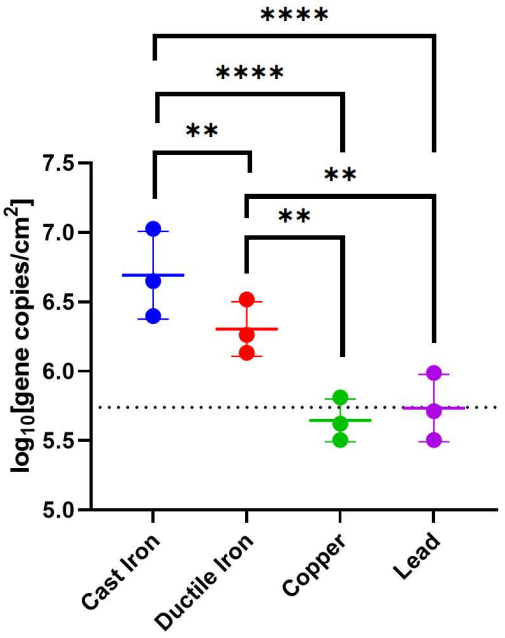

Figure 4.1 The absolute abundance of 16S rRNA gene copies in biofilm samples from Milwaukee County as measured by ddPCR. Each point represents the mean abundance of biofilm samples (n=6) from a sampled pipe. The dotted line shows the normalized LOB. Statistical significance is represented by asterisks as shown for the results of HSD following removal of data points below the LOB. Asterisks indicate level of statistical significance as follows: ($p \leq 0.0001$) = ****, $p \leq 0.001$) = ***, ($p \leq 0.01$) = **, and ($p \leq 0.05$) = *.

After removing samples that had results below the LOB, the four materials had significantly different biomass concentrations (ANOVA, $p<0.0001$). Cast iron had the highest density of biofilm growth, even as compared to ductile iron (HSD, $p=0.0051$). Cast iron has been found by other studies as the material that supports the most biofilm growth with respect to copper, steel, plastic, stainless steel, and cement materials (W. Li et al., 2020; Niquette et al., 2000; Hong Wang et al., 2014). The surfaces of the cast iron pipes were generally rougher and more corroded as compared to the swabbed surfaces of other pipe materials. As has been widely recognized, increased surface roughness promotes biofilm growth because there is a larger surface area for attachment as well as protection from disinfectants and shearing hydraulic forces (Tang et al., 2021; Z. Zhu et al., 2014). In addition, cast iron corrosion products may provide nutrients to biofilms and can react with disinfectants to reduce their efficacy (Fu et al., 2021; S. Liu et al., 2016). On the contrary, copper is known to have antimicrobial properties and to limit biomass growth (Lehtola et al., 2002; W. Li et al., 2020). Research on the biofilms of lead pipes is limited, so the present study's finding that lead yields low biomass growth is novel.

4.1.2 Impact of Pipe Material on Microbial Community Structure

Pipe material impacted the structure of microbial communities with respect to the relative abundances of genera. PCoA plots based on Bray-Curtis dissimilarities of microbial communities show clustering based on pipe material for all genera (Figure 4.2A) as well as for dominant genera (Figure 4.2B). Statistical testing using PERMANOVA affirmed the dissimilarities of microbial communities based on pipe material of all genera (PERMANOVA, $p_{adonis}=0.004$, $R^2=0.1451$, $p_{betadisp} = 0.2882$) and for the top twelve genera (PERMANOVA, $p_{adonis}=0.002$, $R^2=0.17701$, $p_{betadisp} = 0.1839$).

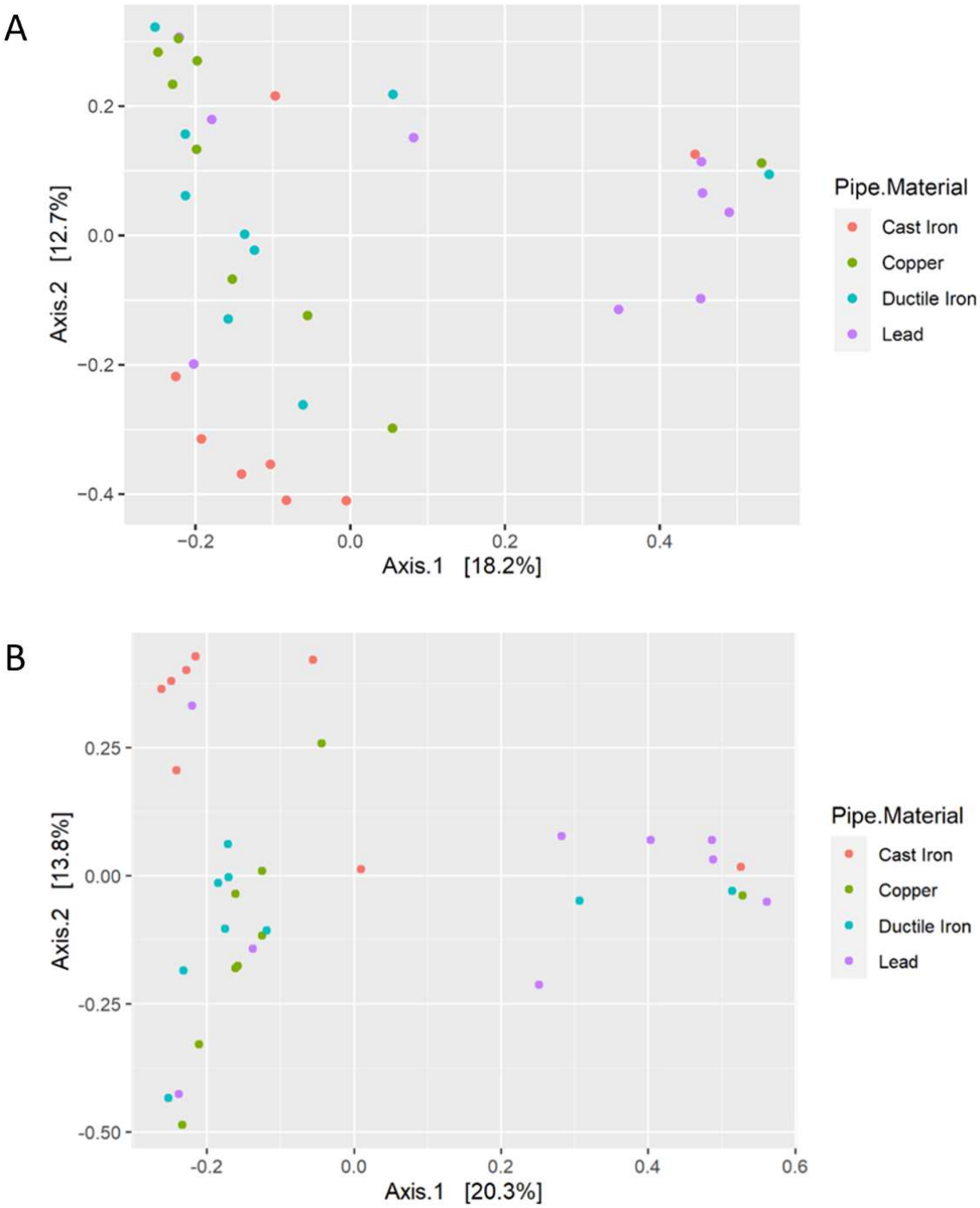

Figure 4.2 Principal Coordinate Axis (PCoA) based on Bray-Curtis dissimilarities of microbial communities in biofilm samples from Milwaukee pipes. A: Dissimilarities of all genera. B: Dissimilarities of twelve most dominant genera. Each point represents a biofilm sample, and colors distinguish the pipe material from which the sample was collected.

Pipe material did not influence the diversity or evenness of the microbial community in biofilm samples from Milwaukee pipes to a statistically significant degree, but increased richness was observed in cast iron pipes (Figure 4.3). Alpha diversity was within the range observed by other studies of full-scale, chloraminated DWDSs (Kimbell et al., 2021; Pinto et al., 2014). Likewise, evenness was similar among groups and reflected Pielou's evenness scores found in full-scale biofilms (Henne et al., 2012; Pullerits et al., 2020b). In contrast, cast iron had a richer microbial community than other pipe materials, though only to a statistically significant degree as compared to lead (Dunn's, $p=0.00251$). The increased richness in cast iron biofilms is not a reflection of the presence of rare, low-abundant OTUs because the evenness is similar in cast iron as in other pipe materials. Rather, cast iron provides an environment habitable to several taxa that co-occur in similar abundance. The interspecies relationships of iron-corroding bacteria has been documented in cast iron pipes (Sun et al., 2014; Teng et al., 2008; Y. Zhang et al., 2018). The dominant genera in cast iron pipes were distributed with similar relative abundances, affirming this study's finding based on alpha diversity that cast iron is hospitable to several co-existing taxa (Figure 4.4).

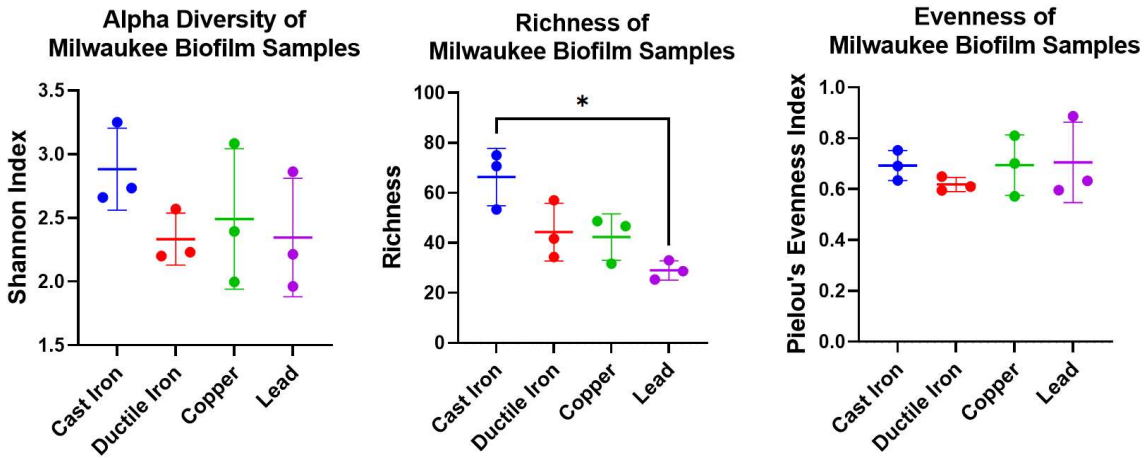

Figure 4.3 Alpha diversity measurements for biofilm samples from Milwaukee pipes. Left: Diversity as represented by the Shannon Index. Center: Richness as number of ASVs in the microbial community. Right: Evenness as represented by Pielou's Evenness Index. Asterisks indicate level of statistical significance as follows: ($p \leq 0.0001$) = ****, $p \leq 0.001$) = ***, ($p \leq 0.01$) = **, and ($p \leq 0.05$) = *.

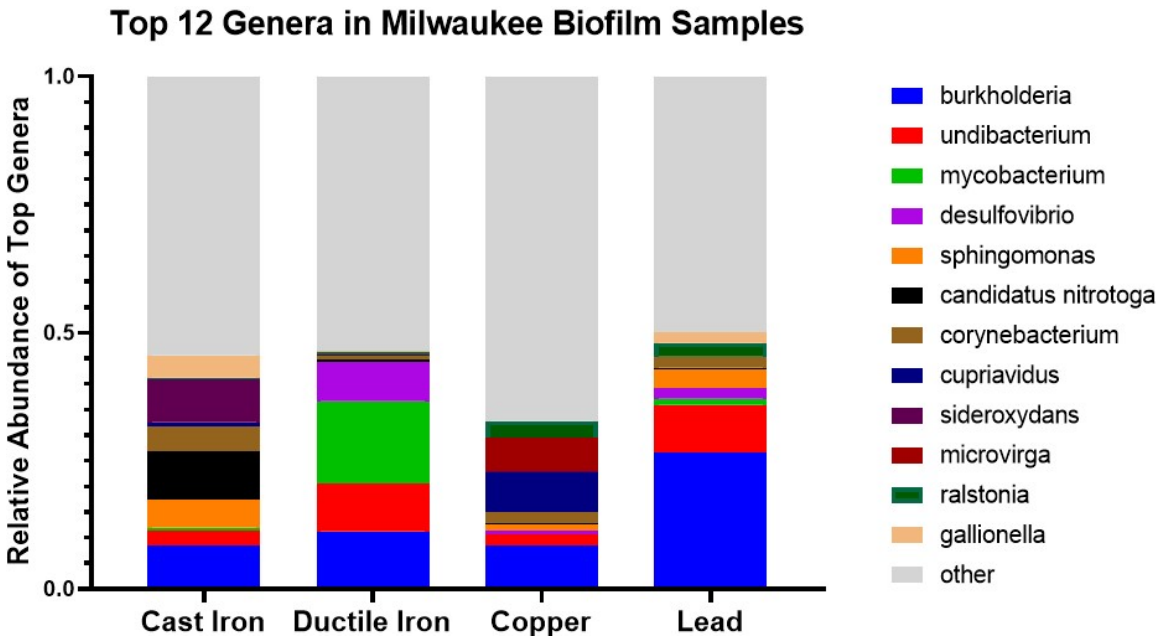

Figure 4.4 Relative abundances of the twelve dominant genera in biofilm samples of Milwaukee pipes. The relative abundance of each genus is the mean of biofilm samples as grouped by material (cast iron, n=8; ductile iron, n=9; copper, n=9; lead, n=9). Each material was represented by sampling from three pipes.

Although a few dominant genera were ubiquitous in DWDS biofilms, pipe materials created distinctive environments that allowed for the survival of unique genera

depending on pipe material. In copper pipes, *Cupriavidus* (0-47%) and *Microvirga* (0-56%) were abundant. Meanwhile, *Cupriavidus* and *Microvirga* were not detected in lead nor in ductile iron pipes (0%). In cast iron, *Cupriavidus* and *Microvirga* had a relative abundance of only 0-6% and 0-1%, respectively. Both *Cupriavidus* and *Microvirga* have been identified as having heavy metal resistance genes that protect against several heavy metals, including copper. (Monchy et al., 2007; Tapase & Kodam, 2018). In particular, *Cupriavidus* has been shown to employ several mechanisms for copper resistance (Huang et al., 2019). Whereas bacteria without these genes would succumb to cell damage when exposed to copper ions, *Microvirga* and *Cupriavidus* could colonize the copper pipes and outcompete other genera. Further, *Ralstonia* was dominant in copper pipes (0-23%), and this genus was shown to dominate when exposed to high copper concentrations (M. Zhang et al., 2018). *Ralstonia* and *Cupriavidus* are closely related and have been shown to co-occur in environments with high exposure to heavy metals (Mijnendonckx et al., 2013).

Predictably, genera associated with iron corrosion were identified in both the cast iron and ductile iron pipe biofilms. Interestingly, the cast iron and ductile iron were dominated by different corrosion-related genera. Overall, cast iron biofilms contained more dominant genera related to iron corrosion than did ductile iron. In cast iron, *Gallionella* (0-17%) and *Sideroxydans* (0-40%), two genera associated with red water events, were higher in relative abundance compared to ductile iron. Red water events occur when cast iron pipes have weak corrosion scaling, allowing iron to enter drinking water (Haibo Wang et al., 2014). Further, the identification of *Candidatus nitrotoga* in cast iron biofilms (0-29%) is uncommon yet not surprising given it thrives in the same

environments as *Nitrosomonas* and *Nitrospira*, which are commonly dominant in drinking water biofilms (Aggarwal et al., 2018; Spieck et al., 2021; Tang et al., 2021). The genus *Candidatus nitrotoga* may cycle iron when converting nitrite to nitrate, suggesting a role in corrosion of cast iron pipes (Spieck et al., 2021). Meanwhile, the dominant corrosion-related bacteria in ductile iron were only *Desulfovibrio* (0-44%) and *Undibacterium* (0-46%). In cast iron biofilms, *Desulfovibrio* was not present, and *Undibacterium* was low (0-11%). However, it should be noted that *Desulfovibrio* appeared in the tubercle and under-tubercle samples of cast iron pipes (discussed in Section 4.3.2), which is in agreement with the characterization of *Desulfovibrio* as a sulfate-reducing bacteria (SRB) that appears in anaerobic niches of cast iron pipes (Gomez-Smith et al., 2015). Corrosion-related genera are further discussed in Sections 4.2.4-4.2.5.

Notably, *Mycobacterium* (0-90%) was the most abundant genus in ductile iron pipes, which was significantly different with respect to copper pipes (HSD, $p=0.0496$). *Mycobacterium* contains several OPs and is discussed in more detail in Section 4.4.

While lead pipes have been studied with respect to physiochemical factors relating to corrosion, the microbial community in lead biofilms is understudied. Research by White et al. offers the only point of comparison for the microbial communities in this study (White et al., 2011). White et al. sequenced one full-scale lead pipe, which also included *Sphingomonas* in its dominant genera but shared no other dominant genera with Milwaukee's lead pipes. However, White et al. concluded that corrosion of the lead pipe shaped the dominant genera, which were largely associated with environments contaminated by heavy metals. Similarly, *Sphingomonas* (0-32%) and *Burkholderia* (0-

55%) can resist heavy metals, including lead (Jiang et al., 2008; White et al., 2011). *Undibacterium* (0-19%) and *Gallionella* (0-11%) are two genera usually associated with cast iron corrosion, but they were identified in the lead pipes (Kimbell et al., 2020; Sun et al., 2014). It is not clear whether the ability to oxidize iron may correspond with the survival of *Undibacterium* and *Gallionella* in lead pipes, and more research is needed. Although *Undibacterium* has shown resistance to arsenic, there is a lack of research on its resistance to other metals (M. Zhang et al., 2020). In general, lead pipes did not contain unique genera with respect to the other pipe materials, suggesting that the heavy-metal tolerant bacteria that were able to colonize lead may also have a similar ability to colonize other metals. Indeed, heavy-metal resistance genes can confer resistance to several heavy metals (Kimbell et al., 2020; Tapase & Kodam, 2018).

Burkholderia was ubiquitous in all pipe materials accounting for 0-59% in cast iron, 0-88% in ductile iron, 0-71% in copper, and 0-55% in lead. *Burkholderia* is not usually identified as a dominant taxa in full-scale drinking water studies, though it was found in the biofilm of a pilot-scale study (Z. Zhu et al., 2014). In Zhu's study of five pipe materials, four families of Burkholderiales were abundant in only cast iron and cement-lined pipes, not in copper pipes. However, this study did not classify the taxa beyond family, and it did not include ductile iron or lead pipes, meaning the dominance of *Burkholderia* in the studied materials is a novel discovery. *Burkholderia* was highest in lead pipes, which was significant with respect to cast iron (HSD, $p=0.0262$) and copper (HSD, $p=0.0187$). *Burkholderia* contains several OPs that have been identified as being of emerging global concern and is discussed more in Section 4.4.

4.1.3 Comparison of Connected Pipes

Two sampling events (MKE 5 and WK 2) provided samples from adjacent pipes of different material that were connected to one another, providing an opportunity to examine how pipe materials might impact microbial communities exposed to the same bulk water, residual disinfectant, and temperature conditions. Further, connected pipes had the same diameter. WK 2 pipes also had the same age, controlling for another factor. However, due to the small sample size, there is limited statistical power in comparing pipes and making broader inferences. Thus, the microbial communities of the connected pipes are treated as case studies and discussed solely based on relative abundances of dominant taxa.

The cast iron and ductile iron pipes from MKE 5-cFe and MKE 5-dFe shared the most dominant genus *Burkholderia*, but overall differed in relative abundances of dominant taxa (Figure 4.5). Several taxa were detected only in cast iron samples: *Nitrosomonas*, *Sphingomonas*, *Pandoraea*, and *Candidatus Nitrotoga*. Meanwhile, *Kocuria* was only detected in the ductile iron samples. The cast iron pipe had more corrosion-associated genera, reflecting findings comparing all Milwaukee cast iron and ductile pipes (Section 4.1.2).

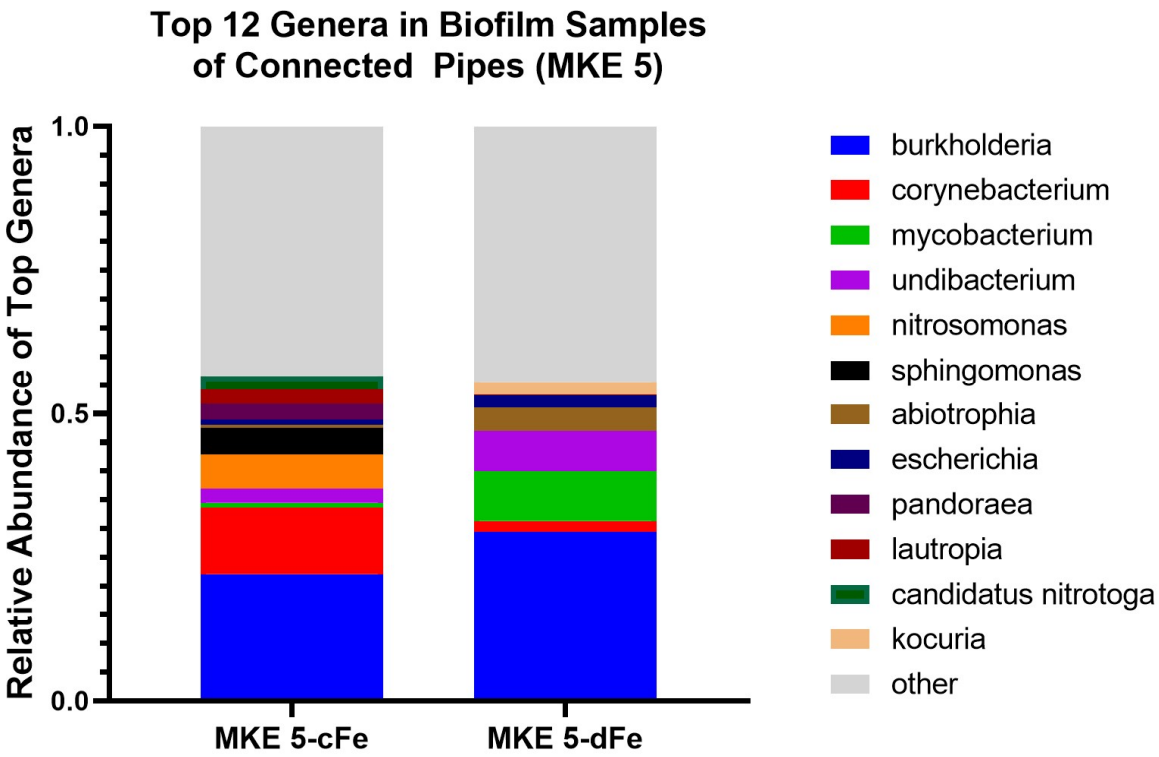

Figure 4.5 Relative abundances of the twelve dominant genera in biofilm samples of the connected cast iron and ductile iron pipes from Milwaukee sampling events MKE 5-cFe and MKE 5-dFe, respectively. The relative abundance of each genus is the mean of biofilm samples as grouped by material (n=3). These pipes presumably experienced similar characteristics of bulk water, and they had the same pipe diameter. However, the cast iron pipe was 67 years older than the ductile iron pipe.

Increased relative abundance of *Mycobacterium* in the ductile iron pipes is noteworthy due to its relevance in harboring OPs. *Mycobacterium* was found to be more abundant in the ductile iron biofilms compared to cast iron biofilms: its relative abundance ranged from only 0.1-1.3% in the cast iron pipe but from 0-26% in the ductile iron pipe. Tang et al. found that ductile iron pipes had a statistically significant higher abundance of mycobacterial gene copies as compared to a cast iron of the same diameter and similar age (Tang et al., 2021). However, there is a lack of statistical power in Tang's study as well as this study, so more research is needed to determine if ductile iron selects for *Mycobacterium* and why. The dominance of *Mycobacterium* in ductile iron pipes is

further discussed in Sections 4.2.5, where *Mycobacterium* was found to dominate ductile iron pipes from Milwaukee but not from Oak Creek and Waukesha.

Like the connected Milwaukee pipes, the comparison of the connected cast iron and lead pipes from Waukesha in the sampling events WK 2-cFe and WK 2-Pb revealed that dominant genera varied based on material (Figure 4.6). However, compared to differences observed between the Milwaukee cast iron and ductile iron pipes, differences between Waukesha's cast iron pipe and lead pipe were more pronounced. Only genera with very low relative abundances were shared by the two pipes. Further, the cast iron pipe contained several genera not found in the lead pipe: *Ralstonia*, *Cupriavidus*, *Corynebacterium*, *Methylobacterium*, *Bradyrhizobium*, and *Bacillus*. Meanwhile, the lead pipe uniquely contained *Burkholderia* and *Flavobacterium*. As previously discussed in Section 4.1.1, the genera *Cupriavidus* and *Ralstonia* exhibit heavy metal-resistance and co-occur (Mijnendonckx et al., 2013; M. Zhang et al., 2018). Further, *Ralstonia* can reduce iron and may be promote cast iron corrosion products (Sun et al., 2014; Y. Zhu et al., 2020) . On the other hand, *Burkholderia* resists lead along with other heavy metals, but it did not inhabit this lead pipe (Jiang et al., 2008; White et al., 2011).

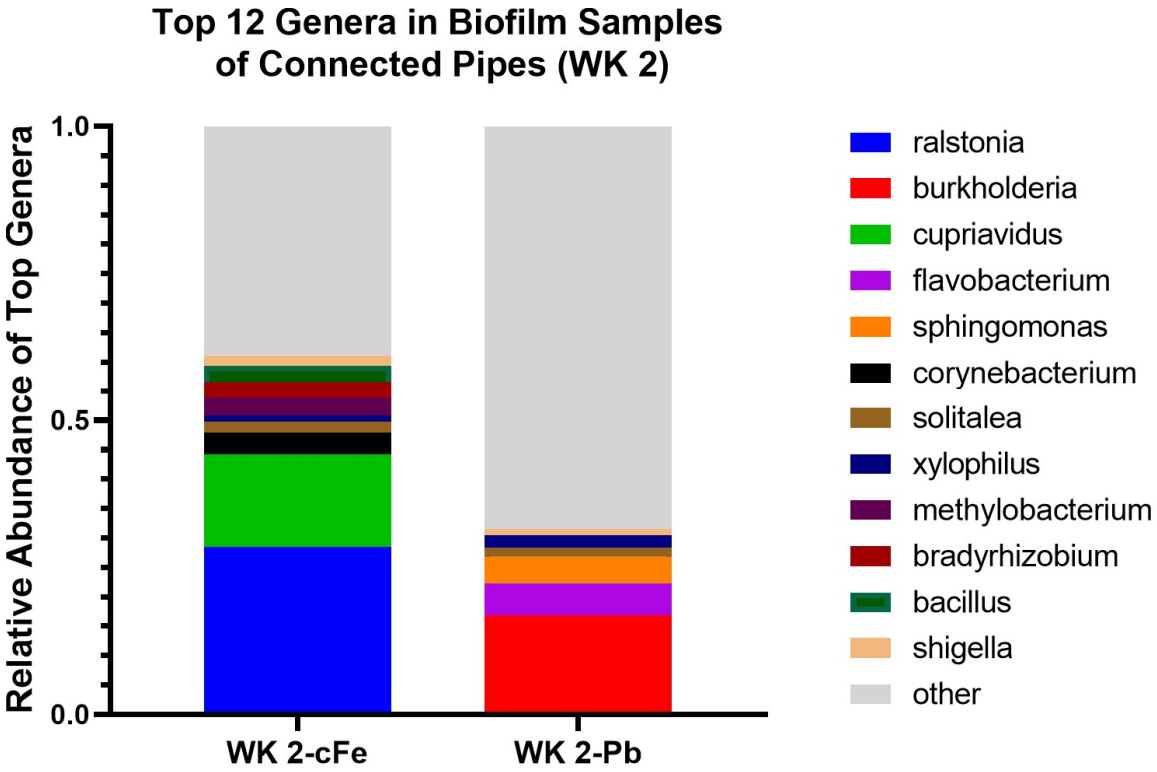

Figure 4.6 Relative abundances of the twelve dominant genera in biofilm samples of the connected cast iron and lead pipes from Waukesha sampling events WK 2-cFe and WK 2-Pb, respectively. The relative abundance of each genus is the mean of biofilm samples as grouped by material (n=3). These pipes presumably experienced similar characteristics of bulk water, and they had the same pipe diameter and pipe age.

This case study of connected pipes indicates that microbial communities that are presumably exposed to similar temperatures, bulk water quality characteristics (including pH and nutrient availability), and disinfectant residual concentrations can vary because of pipe material. For both the Milwaukee and Waukesha pipes, the dominant genera changed over a short distance. Research has shown the primacy of pipe material over proximity as well as the primacy of proximity over pipe material in shaping similar microbial communities in full-scale DWDS biofilms (Henne et al., 2012; Siedlecka et al., 2021). While the results of the present study emphasize the influence of pipe material, there is more research needed to elucidate why the dominant genera varied between adjacent materials. In the case of the connected Milwaukee pipes, corrosion-related

microbes typical to cast iron pipes were dominant in the cast iron pipe and low or undetected in the ductile iron pipe. However, the differences observed in the Waukesha pipes prompt further research, particularly in the possible role of metal resistance genes and the utilization of metal ions in redox cycling of lead pipes.

4.2 Impact of Utility in Shaping Microbial Communities on the Same Pipe Material

4.2.1 Top Genera in Each of the Three Utilities

Utility had an impact on the microbial community's composition regardless of pipe material, with Waukesha and Oak Creek exhibiting similarities in the most dominant genera (Figure 4.7). While Milwaukee treats its drinking water treatment plant effluent with a chlorine disinfectant residual, both Waukesha and Oak Creek use chloramines. Regarding corrosion inhibitors, Milwaukee uses phosphate, Waukesha uses sodium silicate, and Oak Creek does not add any corrosion inhibitor. While differences in corrosion inhibitor could explain observed differences among utilities, likewise the similar use of chloramines by Oak Creek and Waukesha could explain the observed similarities of microbial communities in these utilities.

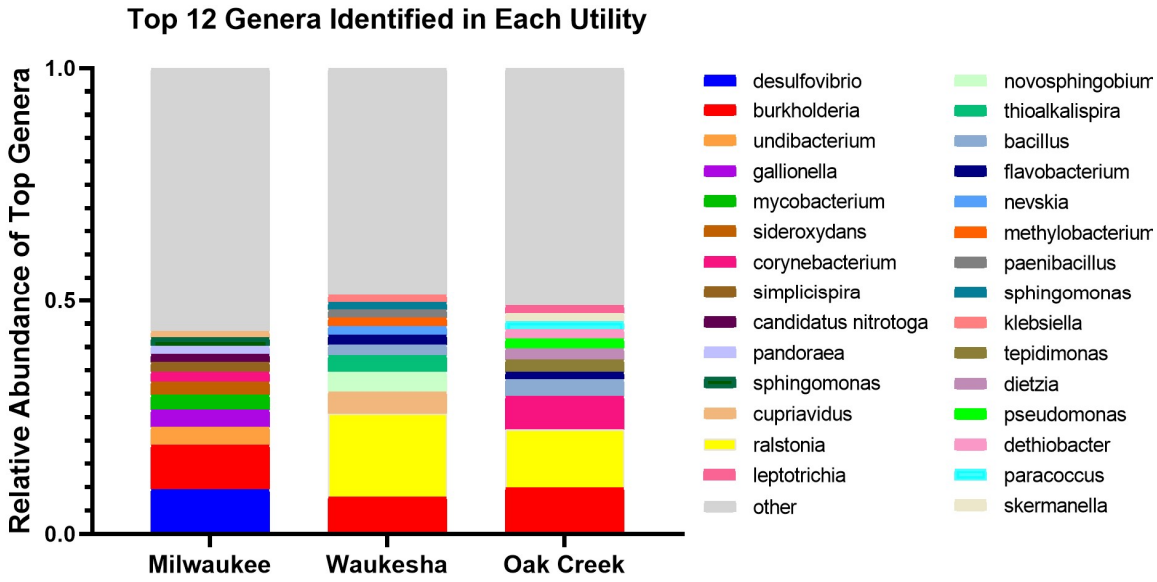

Figure 4.7 Relative abundances of the twelve dominant genera identified in each utility. For each utility, the top twelve genera were identified, and "other" was calculated as the remaining genera constituting the microbial community. The relative abundance of each genus is the mean of biofilm, tubercle, and under-tubercle samples as grouped by utility. Milwaukee included cast iron pipes (n=3), ductile iron pipes (n=3), copper pipes (n=3), and lead pipes (n=3). Waukesha included cast iron (n=2), ductile iron (n=3), and a lead pipe (n=1). Oak Creek included a lead pipe (n=1) and copper pipes (n=2).

Although *Burkholderia* was dominant in all three utilities, Oak Creek and Waukesha shared similar dominance of four genera: *Burkholderia*, *Ralstonia*, *Bacillus*, and *Flavobacterium*. Meanwhile, Milwaukee only shared two dominant genera with Oak Creek (*Burkholderia* and *Corynebacterium*) and only three dominant genera with Waukesha (*Burkholderia*, *Cupriavidus*, and *Sphingomonas*). More importantly, Waukesha and Oak Creek had similar relative abundances of their top two genera *Burkholderia* and *Ralstonia*, suggesting that similarities between these utilities promoted these genera. To illustrate the influence of utility regardless of pipe material, utilities are compared within a given pipe material in the following sections (Sections 4.2.2-4.2.5).

Further, *Mycobacterium* was included only in the dominant genera of Milwaukee. Due to its relevance in potentially containing OPs, a discussion of this finding occurs in greater depth in Section 4.2.

4.2.2 Comparison of Copper Pipes

The composition of the dominant microbial community differed between Oak Creek and Milwaukee's copper pipes (Figure 4.8). Oak Creek was dominated by four genera that accounted for a large proportion of the top genera: *Ralstonia* (0-84%), *Burkholderia* (0-43.5%), *Corynebaterium* (0-26%), and *Tepidimonas* (0-33%). The remaining eight top genera in Oak Creek had small relative abundances, with *Pseudomonas* making up 0-8% and all other genera existing below 5%. Meanwhile, Milwaukee had several dominant genera that were equally competitive. For example, similar ranges of relative abundance existed for *Lautropia* (0-25%), *Shigella* (0-18%), *Brevibacillus* (0-21%), *Corynebacterium* (0-19%), *Undibacterium* (0-19%), and *Alkanindiges* (0-15%). These differences could be shaped by the different disinfectant residuals or by the different corrosion inhibitors used in each utility. Further, the interactions of disinfectants with copper might establish differences, but there is more research needed to reveal if this is true.

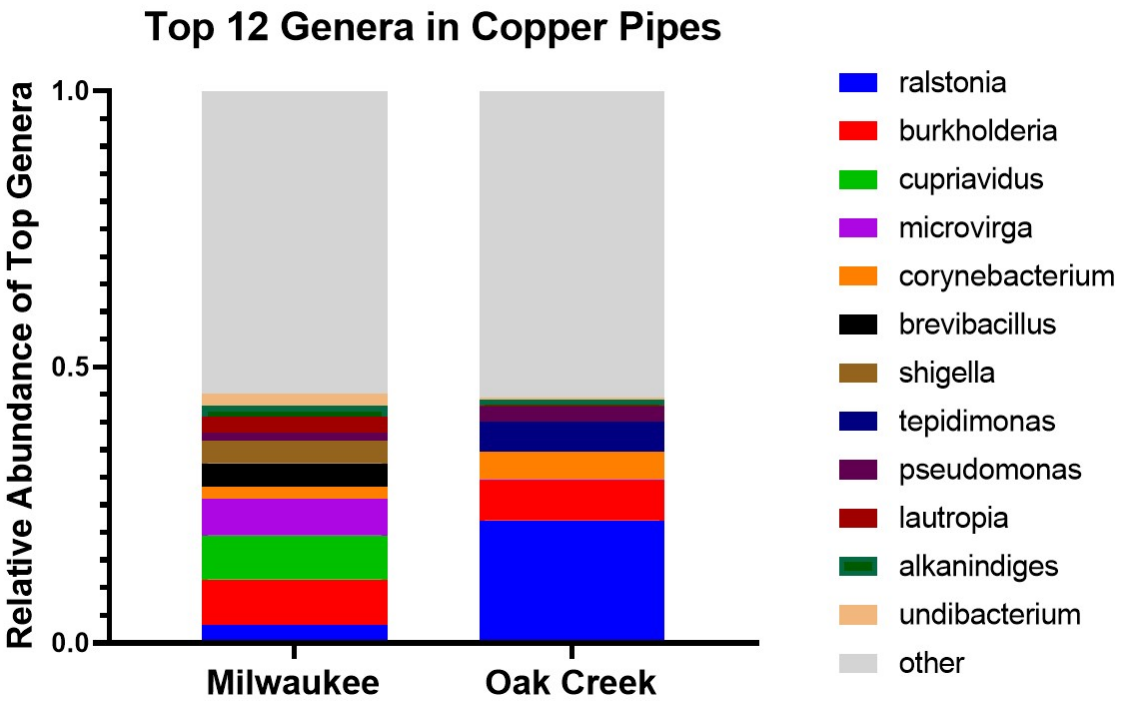

Figure 4.8 Relative abundances of the twelve dominant genera in copper biofilm samples compared for Milwaukee pipes (n=3) and Oak Creek pipes (n=2). The mean relative abundance of each genus in each utility is plotted.

The genera *Cupriavidus* and *Microvirga* were unique to Milwaukee. As discussed in Section 4.1.2, *Cupriavidus* and *Microvirga* are known to have copper resistance capabilities, so its lack of dominance in Oak Creek's copper pipes shows that its advantage in colonizing the antimicrobial surface does not alone allow it to outcompete other genera. As such, these genera may be promoted by the presence of phosphate or may resist chloramines but not chlorine.

Notably, wide ranges in relative abundances of *Ralstonia* and *Burkholderia* emphasize the point that microenvironments exist even within the same utility and the same pipe material. Despite having a lower mean relative abundance, *Ralstonia* was still dominant in Milwaukee and experienced the same range as samples from Oak Creek (0-

85% in both utilities). Likewise, *Burkholderia* had a lower average relative abundance but a larger range than Oak Creek (0-71%).

4.2.3 Comparison of Lead Pipes

Biofilms in Milwaukee's lead pipes could only be compared to samples from a singular lead pipe from Waukesha (Figure 4.9). In both utilities, lead pipes were dominated by *Burkholderia*, which contains species found to be resistant to lead (Jiang et al., 2008). In Milwaukee, *Burkholderia* ranged from 0-55%, and in Waukesha it ranged from 0-51%. In addition, *Mycobacterium* was similar in both pipes (of 0-4% in each utility). Further, *Sphingomonas* had a similar mean relative abundance, though it had a higher range in Milwaukee's pipes (0-32%). The similar dominance of *Burkholderia*, *Sphingomonas*, and *Mycobacterium* in both utilities could indicate that lead may have a stronger influence on these genera than other variable factors, but further research including samples from more lead pipes is necessary to investigate this.

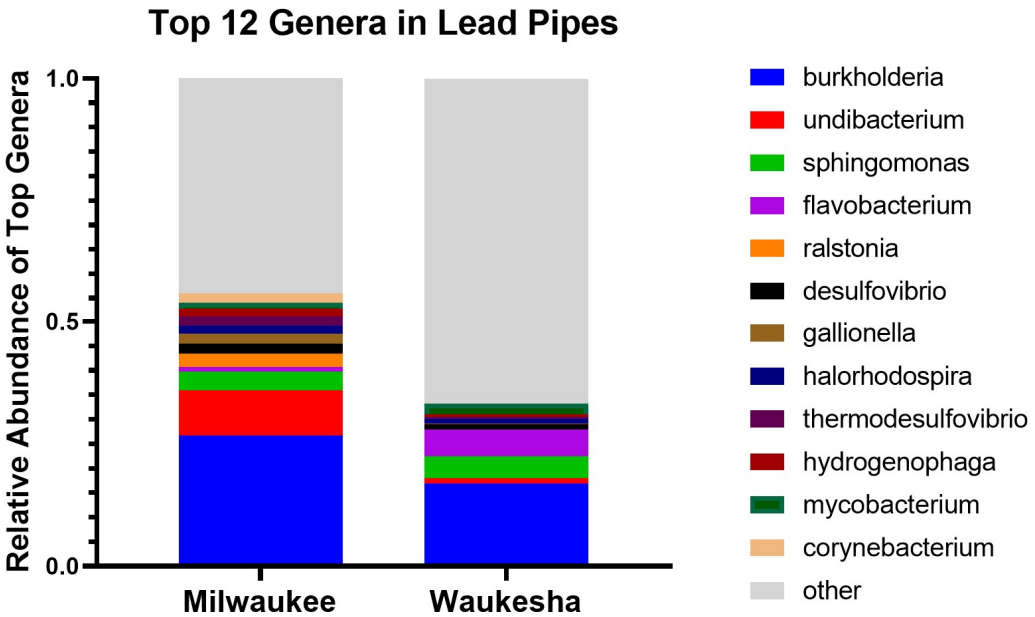

Figure 4.9 Relative abundances of the twelve dominant genera in copper biofilm samples are compared for Milwaukee pipes (n=3) and a Waukesha pipe (n=1). The mean relative abundance of each genus in each utility is plotted.

Only one lead pipe was collected from Waukesha, and the microbial community structure can be explained by many factors; Therefore, it is not appropriate to make conclusions that are too broad. For example, *Ralstonia* appeared only in Milwaukee's lead pipes, but some of Milwaukee's samples also had 0% detection of *Ralstonia*, and only three biofilm samples represent Waukesha, meaning it is not appropriate to make a broader conclusion from this finding. Likewise, *Flavobacterium* appeared as higher to Waukesha, but it had a range of 0-10% in Waukesha and 0-4% in Milwaukee, so the sample number may establish a misleading representation of *Flavobacterium*. However, overall, there is a lack of literature discussing the microbial community in lead biofilms, so these results still provide novel insights. To understand the impact of lead and utility more fully on lead pipes, further research of microbial communities in lead pipes is needed.

4.2.4 Comparison of Cast Iron Pipes

The dominant genera in cast iron biofilms differed in the Milwaukee and Waukesha utilities, with genera dominating the chloraminated Milwaukee pipes being consistent with previous studies (Figure 4.10). In a study comparing disinfectants on biofilms grown on coupons of several materials (including cast iron as well as PVC, copper, HDPE, and steel), *Sphingomonas*, *Bradyrhizobium*, and *Nitrosomonas* were also more dominant in chloraminated than chlorinated conditions (Y. Zhang et al., 2018). In another study of the same pipe materials, *Bradyrhizobium* and *Nitrosomonas* were likewise more dominant when exposed to chloramine rather than chlorine (W. Li et al., 2020). However, *Sphingomonas* dominated both chlorinated and chloraminated conditions (W. Li et al., 2020). The appearance of *Nitrosomonas* along with chloramines

is logical as the genus can oxidize ammonia (Dionisi et al., 2002). The presence of *Nitrosomonas* may be a concern as it could reduce the efficacy of the disinfectant in the Milwaukee pipes (Shi et al., 2020; Hong Wang et al., 2013). As such, observed differences could also reflect differences in overall concentrations of disinfectant residuals rather than the differences between chlorine and chloramines.

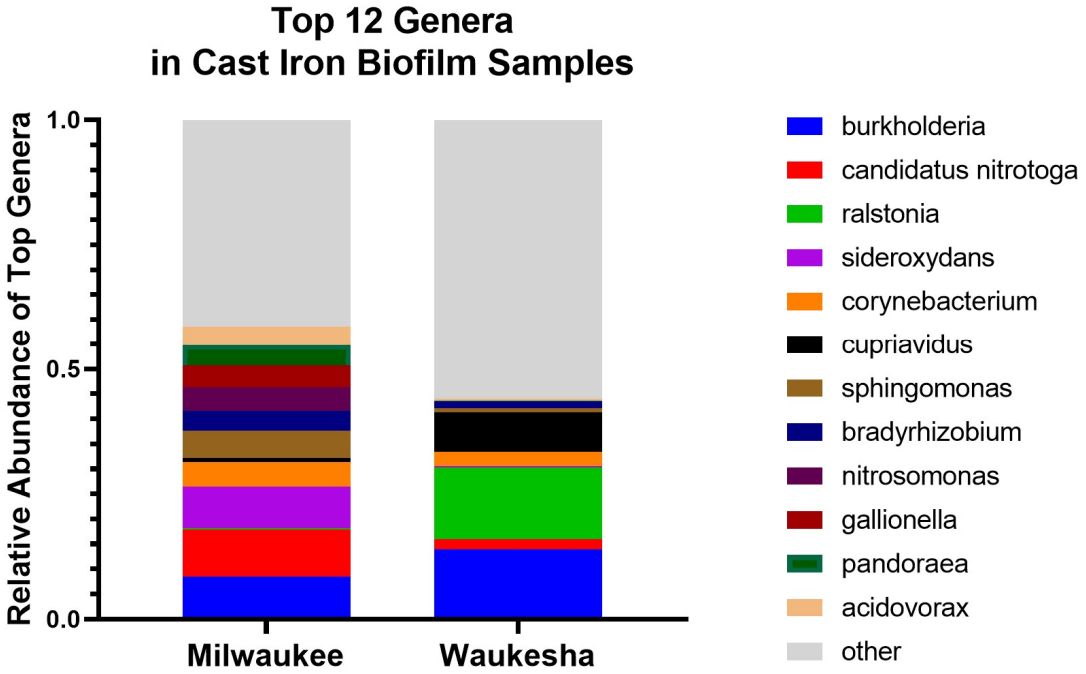

Figure 4.10 Relative abundances of the twelve dominant genera in cast iron biofilm samples are compared for Milwaukee pipes (n=3) and Waukesha pipes (n=2). Tubercle and under-tubercle samples were excluded from this comparison so that the impact of utility could be evaluated separately from sample type. The mean relative abundance of each genus in each utility is plotted.

The profile of corrosion-related bacteria in Waukesha's and Milwaukee's cast iron pipes suggests that the utilities could control microbial corrosion. Milwaukee's cast iron biofilms were dominated by the iron-oxidizing bacteria (IOB) *Sideroxydans*, *Gallionella*, and *Acidovorax*, which are known to accelerate corrosion. Further, the dominance of *Sphingomonas* and *Bradyrhizobium* indicates limited protection from

corrosion release as these genera are siderophore-producing bacteria (SPB) that produce weak, porous corrosion scales (Y. Zhu et al., 2020). Meanwhile, *Ralstonia* was much more dominant in Waukesha (0-86%) than in Milwaukee, where it was in very low abundance (0-1%). In contrast to IOB and SPB, *Ralstonia* reduces iron and is therefore an iron-reducing bacteria (IRB), a group associated with inhibited corrosion and the formation of stable corrosion products (Sun et al., 2014; Y. Zhu et al., 2020). As such, phosphate or chloramines – or a combination of these – may select for corrosion-promoting bacteria in cast iron pipes. However, recognizing the research associating the observed SPB with chloramines and noting the co-occurrence of SPB with IRB in the present study, it is plausible that chloramines could promote microbial corrosion of cast iron pipes.

Interestingly, *Cupriavidus* occurred in Waukesha (0-47%) but was low in Milwaukee (0-6%). In contrast, *Cupriavidus* was previously found to dominate in Milwaukee's copper pipes but not in Oak Creek's copper pipes (Section 4.2.2). This suggests that disinfectant and pipe material alone do not establish dominance by *Cupriavidus* but that several factors likely combine to provide it with a competitive advantage.

Unlike copper and lead pipes, cast iron pipes also had samples from tubercles and under tubercles, but this section's focus was the differences between utilities, so comparisons were made solely based on the biofilm samples. The emergence of other genera in cast iron pipes found on different types of cast iron surfaces is discussed in Section 4.3.2.

4.2.5 Comparison of Ductile Iron Pipes

Ductile iron was the only material that showed statistically significant differences in microbial community structure based on utility. Bray-Curtis dissimilarities were significant based on utility for all genera (PERMANOVA, p_{adonis}=0.040, R^2=0.1545, $p_{betadisp}$ = 0.2846) and for the top twelve genera (PERMANOVA, p_{adonis}=0.025, R^2=0.1732, $p_{betadisp}$ = 0.3151). In addition, richness differed between Milwaukee and Waukesha (HSD, p=0.0313). The differences in the microbial communities are easily observed in the top twelve genera in the ductile iron biofilms of Milwaukee, Waukesha, and Oak Creek (Figure 4.11).

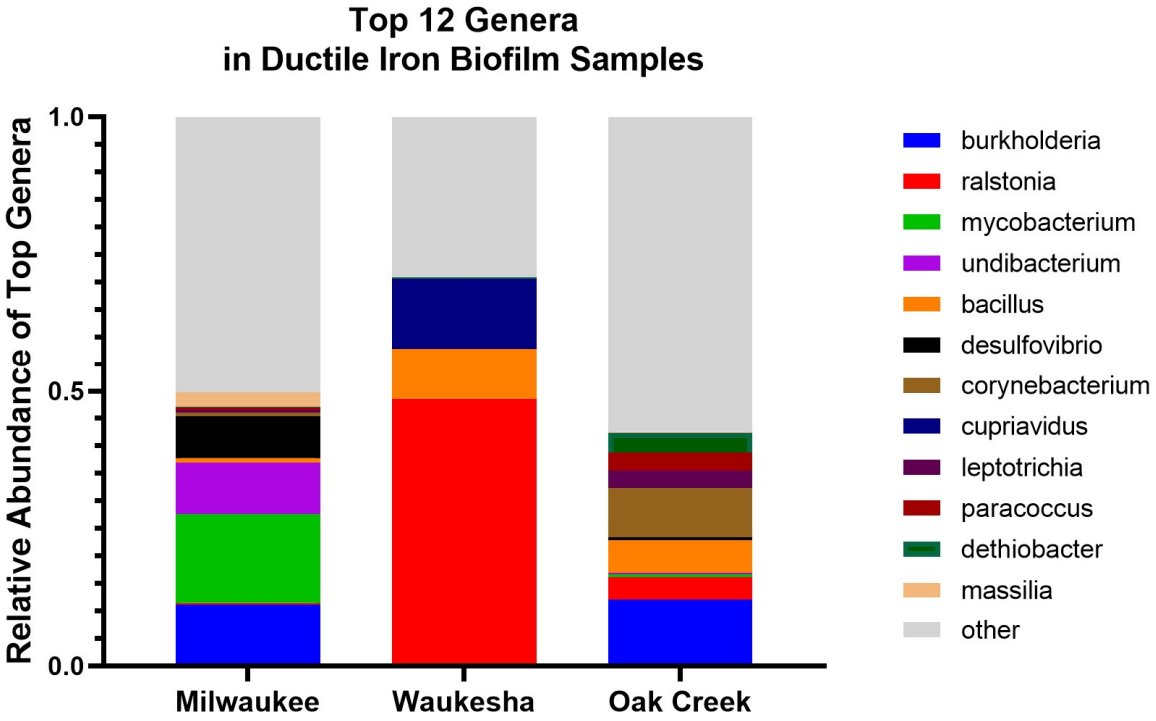

Figure 4.11 Relative abundances of the twelve dominant genera in ductile iron biofilm samples are compared for Milwaukee pipes (n=3), Waukesha pipes (n=3), and an Oak Creek pipe (n=1). The two tubercle samples from Milwaukee pipes were excluded from this comparison so that the impact of utility could be evaluated separately from sample type. The mean relative abundance of each genus in each utility is plotted.

Similar to the comparison of cast iron pipes from Milwaukee and Waukesha, Milwaukee had more corrosion-promoting bacteria while Waukesha had more corrosion-inhibiting bacteria. Like Waukesha's cast iron pipes, *Ralstonia*, which is an IRB and is associated with inhibited corrosion, dominated Waukesha's ductile iron pipes (0-97%). In addition, *Bacillus* dominated in Waukesha (0-28%), and it is an IRB that could inhibit corrosion (Sun et al., 2014). In contrast, *Undibacterium* (an IOB) and *Desulfovibrio* (an SRB), which accelerate corrosion, were both dominant in Milwaukee pipes (0-46% and 0-44%, respectively).

Several other genera were dominant in only one utility. *Massilia* only dominated in Milwaukee (0-23%), and this genus was found in the biofilm of another study of ductile iron pipes from a full-scale chloraminated system, suggesting it could have a niche in this environment (Kelly et al., 2014). *Cupriavidus* again appeared as a genus unique to Waukesha (0-38%) as it did in the comparison of cast iron pipes (Section 4.2.4). However, as previously discussed, *Cupriavidus* only appeared in Milwaukee's copper pipes and not in Oak Creek's (Section 4.3.2). Thus, further research is needed to understand which factors influence the dominance of *Cupriavidus* in the DWDS. Notably, *Mycobacterium* was dominant in Milwaukee (0-90%) but not in Waukesha (0%). Because this genus can harbor several OPs, special attention should be given to this observation, and a discussion about *Mycobacterium* in Milwaukee's ductile iron pipes is provided in Section 4.4.2.

4.3 Contribution of Other Factors Shaping the Microbial Community

4.3.1 Factors that Shape Biomass Density

In addition to pipe material, which was previously asserted to influence biomass density in Section 4.1.1, biomass density also differed based on sample type (Table 4.1). Ultimately, sample type is also a function of pipe material, since cast iron and ductile iron pipes provided corrosion tubercles and copper and lead pipes did not. Thus, it was important to determine whether sample type influenced biomass density within the same pipe material.

Table 4.1 Results of Statistical Testing Comparing Log(Abundance) of 16S rRNA

	All Samples		**Biofilm Swabs**	
	P-value	Significance	P-value	Significance
Based on Sample Type	3.23e-08	****	-	-
Based on Pipe Material	4.11e-06	****	0.000132	***

($p \leq 0.0001$) = ****, ($p \leq 0.001$) = ***, ($p \leq 0.01$) = **, and ($p \leq 0.05$) = *

Comparing samples within each pipe, under-tubercle samples had higher biomass density for all cast iron pipes, which was significant for two of the three pipes (Figure 4.12). This finding suggests that the niche below tubercles may be protected from disinfectants and hydraulic shearing forces. Further, tubercle samples had significant higher biomass densities than biofilms for MKE 4, MKE 7, and MKE 16. Although the comparison could be influenced by the different units of abundance, it is plausible that tubercles allow for more dense biofilm growth. For one, Potgieter et al. suggested that obstacles in drinking water systems may develop thicker biofilms (Potgieter et al., 2018). Since tubercles project out from the surface of a pipe, it would fit the description of an obstacle.

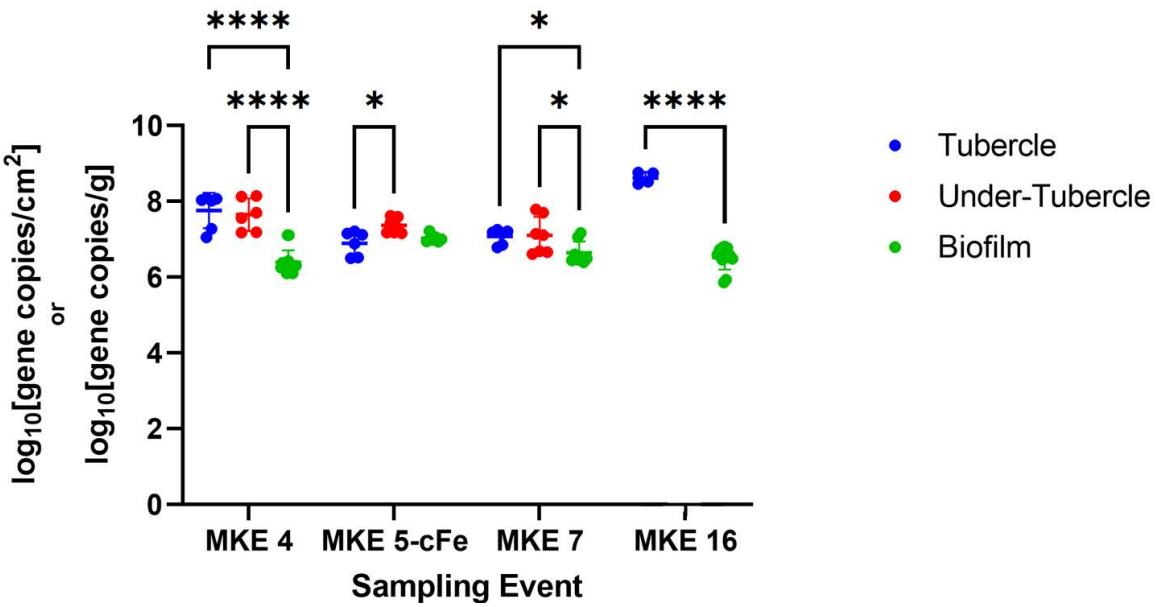

Figure 4.12 Absolute abundance of 16S rRNA for biofilm, tubercle, and under-tubercle samples. Events MKE 4, MKE 5-cFe, and MKE 7 are cast iron, and MKE 16 is ductile iron. Each point represents a sample, and color distinguishes sample types. Bars between sample points represent the mean abundance. Absolute abundance of tubercles is shown in log transformed copies/g while absolute abundance of biofilm and under-tubercle samples is shown in log transformed copies/cm^2 swabbed. Asterisks indicate level of statistical significance as follows: ($p \leq 0.0001$) = ****, $p \leq 0.001$) = ***, ($p \leq 0.01$) = **, and ($p \leq 0.05$) = *.

Pipe diameter correlated with biomass density to a significant degree, where larger pipes supported higher biomass growth, though this finding may be misleading (Table 4.2). Copper and lead pipes were all laterals while cast iron and ductile iron pipes consisted of mains and laterals. Therefore, the positive correlation of pipe diameter and biomass abundance may be reflective of differences caused by pipe materials and not by pipe diameter. This study found that iron pipes supported higher biomass growth, and other research has attributed this to differences in surface roughness, iron bioavailability, and protection from disinfectant, as previously discussed.

Table 4.2 Results of Spearman Rank Correlation for Log(Abundance) of 16S rRNA with Pipe Diameter and with Pipe Age

	All Samples			Biofilm Swabs		
	P-value	Rho	Significance	P-value	Rho	Significance
Pipe Diameter	0.00718	0.3409	**	0.000767	0.5046	***
Pipe Age	0.9686	-0.0052	-	0.37	-0.1456	-

($p \leq 0.0001$) = ****, ($p \leq 0.001$) = ***, ($p \leq 0.01$) = **, and ($p \leq 0.05$) = *

Pipe age did not have a significant impact on biofilm. To the best of our knowledge, pipes in this study had all been in the system for 13-120 years. Therefore, following initial colonization of pipe surfaces, the formation of biofilm seems to remain stable over time.

An objective of this study was to investigate the impact of season on the biomass density in the biofilms; However, samples obtained in the winter were only represented by one lead pipe and two copper pipes. As such, season would not be equitably represented by all pipes materials and could yield misleading results. However, season has been shown to impact the density of biofilms by other studies. Kelly et al., found that water temperature was higher in the summer than in the winter, and, accordingly, biofilm samples obtained in the summer produced heterotrophic plates counts 3 orders of magnitude higher than samples from the winter (Kelly et al., 2014). Higher temperatures can degrade disinfectants, and lower temperatures can decrease bacterial growth rates (S. Liu et al., 2016).

4.3.2 Factors that Shape Microbial Community Structure

Consistent with findings discussed in Sections 4.1 and 4.2, pipe material and utility influenced the microbial community by CCA (Table 4.3). Pipe material was responsible for 12% of community dissimilarity, and utility was responsible for 21% of

differences. According to these results, utility may have a stronger influence on microbial community structure, though the combined effects of utility and pipe material on microbial community are probable yet not captured by CCA.

Table 4.3 Results of Canonical Correspondence Analysis (CCA)

Factor	R^2	P-value	Significance
Pipe Age	0.0347	0.296	-
Pipe Diameter	0.0051	0.838	-
Pipe Material	0.1219	0.007	**
Utility	0.205	0.001	***
Season	0.0708	0.030	*
Sample Type	0.2998	0.001	***

($p \leq 0.0001$) = ****, ($p \leq 0.001$) = ***, ($p \leq 0.01$) = **, and ($p \leq 0.05$) = *

Sample type had the strongest impact on shaping the microbial community, explaining 30% of dissimilarity (CCA, p=0.001). Aside from a tubercle from MKE 16 that was removed and divided into two samples, the tubercle and under-tubercle samples were all obtained from cast iron pipes. Thus, microbial communities within cast iron pipes vary greatly in the microenvironments created by corrosion. Kimbell et al. identified sample type as influencing 32% of microbial community structure of a cast iron pipe from the Milwaukee's utility. For cast iron pipes from Milwaukee, clustering by sample type is observed even for samples from different pipes (Figure 4.13).

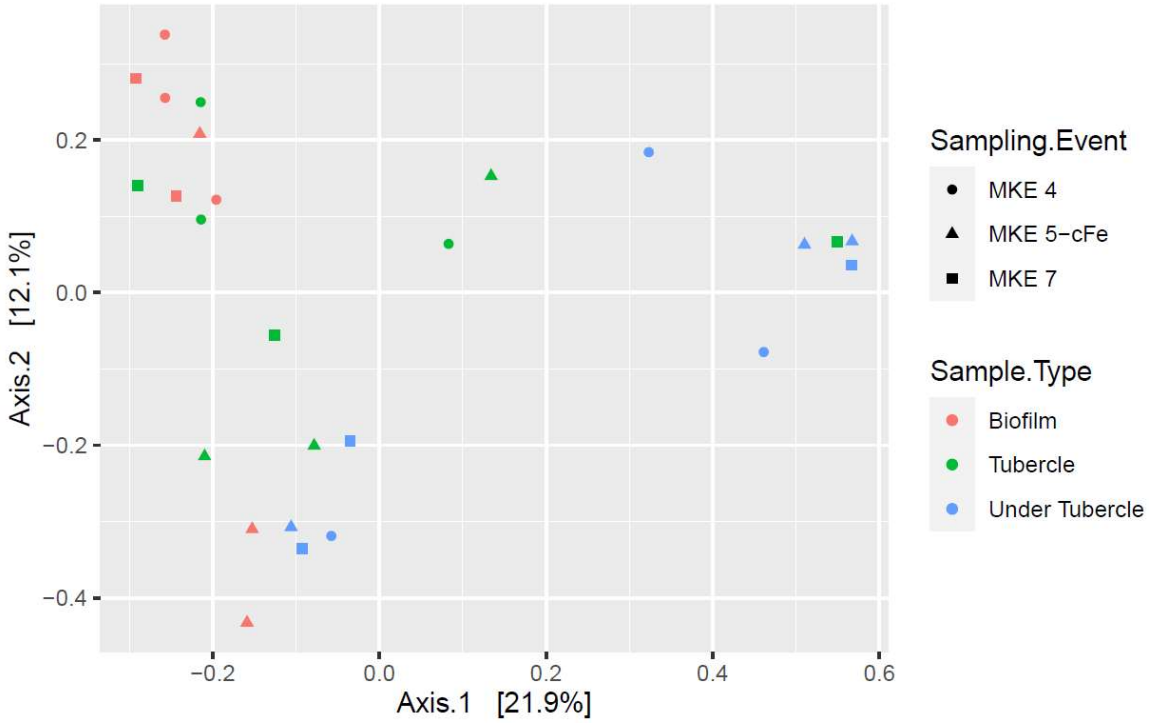

Figure 4.13 Principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarities of relative abundances of genera in biofilm, tubercle, and under-tubercle samples from Milwaukee cast iron pipes. Each point represents a sample, where colors distinguish the sample type and shapes distinguish sampling events.

The dominant genera in different sample types in cast iron pipes varied, supporting the results of the CCA (Figure 4.14). The dominant genera living on and under tubercles in Milwaukee cast iron pipes are dominated by corrosion-related bacteria. *Desulfovibrio*, an SRB, was dominant in tubercle and under-tubercle samples, where its ability to survive in anaerobic conditions allows it to outcompete other genera (Gomez-Smith et al., 2015). Another SRB *Desulfosporosinus*, which is an obligate anaerobe, dominated under-tubercle samples, affirming the claim that SRB can dominate the niche under tubercles (Hippe & Stackebrandt, 2015). The genera *Rarobacter*, *Geothrix*, and *Rhodoferax* appeared in tubercle samples. *Rarobacter* and *Rhodoferax* are facultative anaerobes, and *Rhodoferax* was identified in the loose deposits of full-scale mains

(Kämpfer, 2015; G. Liu et al., 2014). *Geothrix* is an IRB that is an obligate anaerobic, so its presence in tubercle samples demonstrates that anaerobic niches exist on tubercles, not just below them (Thrash & Coates, 2015). As previously discussed, IRB are associated with the formation of stable corrosion scales. Meanwhile, the IOB *Sideroxydans* and *Gallionella* are associated with iron release and appeared in the biofilms in contact with bulk water. Since they are not located on the "protected" areas provided by tubercles and under-tubercles, the cycling of iron by these genera may be more likely to enter the bulk water.

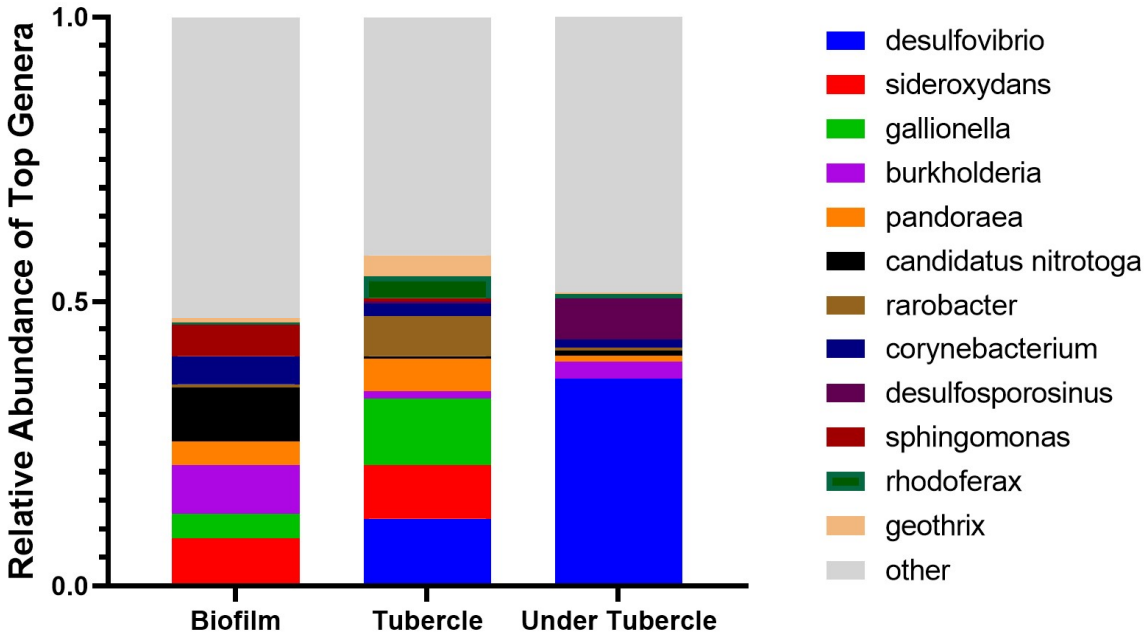

Figure 4.14 Relative abundances of the twelve dominant genera in three Milwaukee cast iron pipes for biofilm samples (n=8), tubercle (n=9), and under-tubercle samples (n=9). The mean relative abundance of each sample type is plotted.

In addition, season of sampling had significant impact on shaping the microbial communities. Season accounted for 7% of dissimilarity in the microbial community (CCA, p=0.030). Seasonal shifts in community structure have been observed, and Tang et al., asserted temperature as accounting for 11% of variation in the microbial community

(Kelly et al., 2014; Pullerits et al., 2020b; Tang et al., 2021). However, the present study was not designed to study seasonal influences, so the seasons are not equally represented in the dataset, and findings should not be overstated. Overall, there is more research needed to completely understand each season's effect because studies have found different results (Pinto et al., 2014; Potgieter et al., 2018; Siedlecka et al., 2021).

4.4 Presence of Opportunistic Pathogens

4.4.1 Detection of Opportunistic Pathogens in DWDSs

As described in Methods Section 3.2.3, seven opportunistic pathogens were chosen for further study based on their relevance in the literature and to the genera found to dominate in the present study. These species were *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Mycobacterium fortuitum*, *Mycobacterium avium*, *Burkholderia cepacia*, *Ralstonia mannitolilytica*, and *Ralstonia pickettii*. Of these, only *P. aeruginosa*, *L. pneumophila, B. cepacia*, and *R. pickettii* were identified in the dataset. Meanwhile, *M. fortuitum*, *M. avium*, and *R. mannitolilytica* were not identified at the species-level. While the depth of sequencing of this study's samples provides generally reliable results for genus data, it is less effective at sequencing to a species-level. Thus, negative detections of the OP species should not be interpreted as a true nonexistence in the samples but rather as a lack of detection based on the sequencing methods. Further, positive detections should be considered tentatively.

Just as *Burkholderia* and *Ralstonia* were overall dominant genera in the DWDS, the OPs *B. cepacia* and *R. pickettii* had high detection rates in the dataset. *B. cepacia* and *R. pickettii* had high detection rates in all three utilities, identified respectively in 97% and 88% of all samples (Figure 4.15). *B. cepacia* was ubiquitous in all utilities, with

≥95% detection in each utility. Meanwhile, *R. pickettii* was higher in Waukesha (100%) than in Milwaukee and Oak Creek (85% for each utility). *Ralstonia* is a genus that contains a few OPs considered to be of emerging concern (Ryan & Adley, 2014). Although *R. pickettii* is not considered to be highly virulent, infections can be difficult to treat due to resistance to several antibiotics (Ryan & Adley, 2014). Ryan & Adley speculated that the increase in cases of *Ralstonia spp.* infections in recent years could be due to a general rise in the at-risk, immunocompromised group. *B. cepacia* has also been found to resistant antibiotics, and it is particularly harmful to cystic fibrosis patients (Vial et al., 2011). A rise in immunocompromised patients as well as a global rise in antibiotic resistance prompts further consideration of methods to control the dominance of the genera *Burkholderia* and *Ralstonia* in the DWDS.

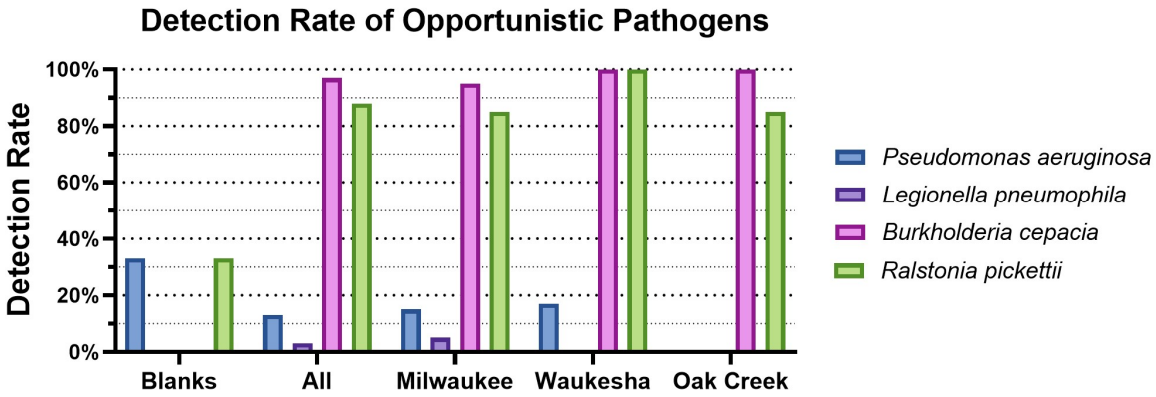

Figure 4.15 The detection rate of the opportunistic pathogens *Pseudomonas aeruginosa*, *Legionella pneumophila*, *Burkholderia cepacia*, and *Ralstonia pickettii* in the sequencing blanks (n=3), the entire sample set (n=86), Milwaukee samples (n=55), Waukesha samples (n=18), and Oak Creek samples (n=13). The detection rate is calculated as the percentage of samples with positive detection.

Although *P. aeruginosa* and *L. pneumophila* are commonly the focus of drinking water studies investigating OPs, the detections of these OPs were overall low (13% and 3% in the entire dataset, respectively). However, it is possible that these species were

present but were not reliably sequenced at this sequencing depth. On the other hand, if these results were reliable, the low detections of *L. pneumophila* and *P. aeruginosa* could indicate effective disinfection by the Milwaukee, Waukesha, and Oak Creek utilities. Overall, more rigorous sequencing technology should be used to monitor OPs in DWDSs.

Although the pathogenic species of interest in the genus *Mycobacterium* were not sequenced in the present study, *Mycobacterium* was found to dominate in the Milwaukee utility and not in Oak Creek and Waukesha (Section 4.2.1). To reiterate, *Mycobacterium* was greater in relative abundance in Milwaukee ductile iron pipes (0-90%) compared to Waukesha (0%), and Oak Creek (0-2%). Differences in disinfectant residual could account for this observed difference. Other studies have found that chloramines can promote *Mycobacterium*. In one study, switching from chlorine to chloramines increased *Mycobacterium* in the biofilms of premise plumbing (Pryor et al., 2002). Further, a lab-scale study showed that *Mycobacterium* was dominant in chloraminated reactors compared to no-disinfectant control, though the study did not include metallic pipes (Aggarwal et al., 2018). However, because findings in Section 4.3.5 revealed that the dominance of *Mycobacterium* in Milwaukee was specific to Milwaukee's ductile iron pipes and not in other pipe materials in the utility, the role of pipe material cannot be ignored. The following section (Section 4.4.2) explores the possible interaction of ductile iron and chloramines in promoting *Mycobacterium*.

4.4.2 Influence of Pipe Material in Detection of OPs

The interaction of ductile iron and chloramine specifically may promote the dominance of *Mycobacterium* based on the findings that (1) *Mycobacterium* only dominated in the chloraminated Milwaukee utility and not in the chlorinated Oak Creek

and Waukesha utilities and (2) that *Mycobacterium* only dominated in Milwaukee's ductile iron pipes and not in Milwaukee's copper, lead, and cast iron pipes. Kelly et al. similarly identified *Mycobacterium* as a dominant genus in biofilms from full-scale ductile iron pipes (n=5) in a chloraminated system (Kelly et al., 2014). In this study, OTUs of *Mycobacterium* were highest when chloramine had degraded to ammonia and free chlorine was low, which could suggest that the presence of *Mycobacterium* in Milwaukee's ductile iron pipes was the result of degraded chloramines (Kelly et al., 2014). However, the comparison of adjacent ductile iron and cast iron pipes in Milwaukee were presumably exposed to similar disinfectant residual, and *Mycobacterium* was still more dominant in the ductile iron pipe (Section 4.1.3).

As such, there should be further research on the interaction of ductile iron with chloramines to ensure an effective disinfectant residual is maintained. Further, more studies should investigate the specific impact of pipe material and disinfectant on the pathogenic species of *Mycobacterium* because the presence of the genus does not guarantee the presence of the OPs. For example, in one study, chlorine more effectively inactivated *Mycobacterium avium* compared to chlorine in iron pipes, though the study did not specify whether the material was cast or ductile iron (Norton et al., 2004). Overall, the combined effects of disinfectants and pipe material also needs to be understood to successfully target OPs.

Pipe material also impacted the detection rates of *P. aeruginosa*, *L. pneumophila*, *B. cepacia*, and *R. pickettii*, and lead specifically may offer better control of these OPs (Figure 4.16). *B. cepacia* was 100% detected in cast iron, ductile iron, and copper samples, but it was only detected at 75% in lead pipes. Further, lead had no detections of

P. aeruginosa nor *L. pneumophila*, though based on this study's sequencing depth this finding does not guarantee the OPs were not present. Although *R. pickettii* was detected at 100% in lead samples, this was only slightly higher than detections on the other materials (84-93%). Therefore, it is possible that lead pipes, when prevented from corroding with appropriate corrosion inhibitors, may be the least habitable to these three OPs. However, it should be noted that possible advantage OP control does not outweigh the potential harm done by exposure to lead itself.

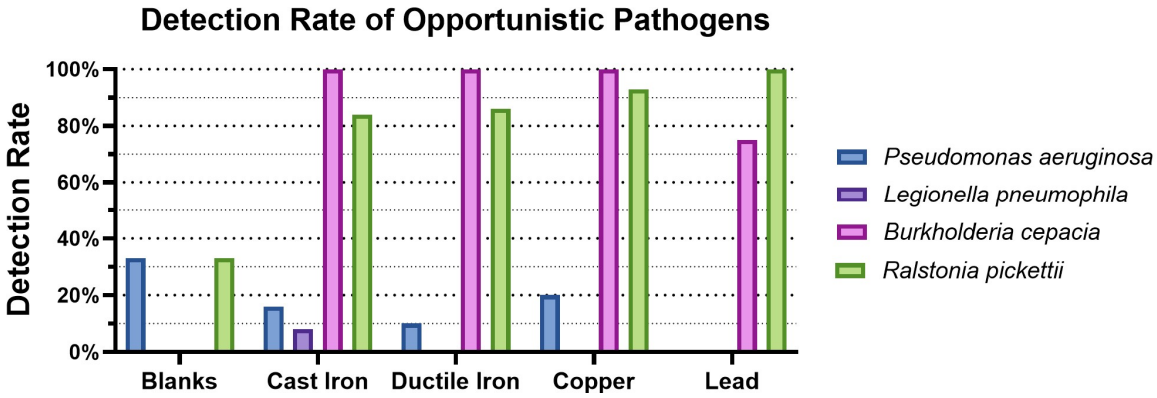

Figure 4.16 The detection rate of the opportunistic pathogens *Pseudomonas aeruginosa*, *Legionella pneumophila*, *Burkholderia cepacia*, and *Ralstonia pickettii* in the sequencing blanks (n=3), cast iron samples (n=38), ductile iron samples (n=21), copper samples (n=15), and lead samples (n=12). The detection rate is calculated as the percentage of samples with positive detection.

Ductile iron and cast iron had similar detection rates of both *R. pickettii* and *B. cepacia*. The genus *Ralstonia* is a recognized IRB, and *B. cepacia* is an SPB, so these OPs have an obvious advantage in colonizing iron pipes (Thomas, 2007). Further, other studies have proposed that corroded iron pipes can provide niches protected from disinfectant, as previously discussed. Protection from disinfectant could explain why cast iron, which presented the most visible corrosion, was the only material to detect *L.*

pneumophila (8%). Supporting this claim, *L. pneumophila* appeared highest in tubercle samples as opposed to biofilm and under-tubercle samples (14%) (Figure 4.17).

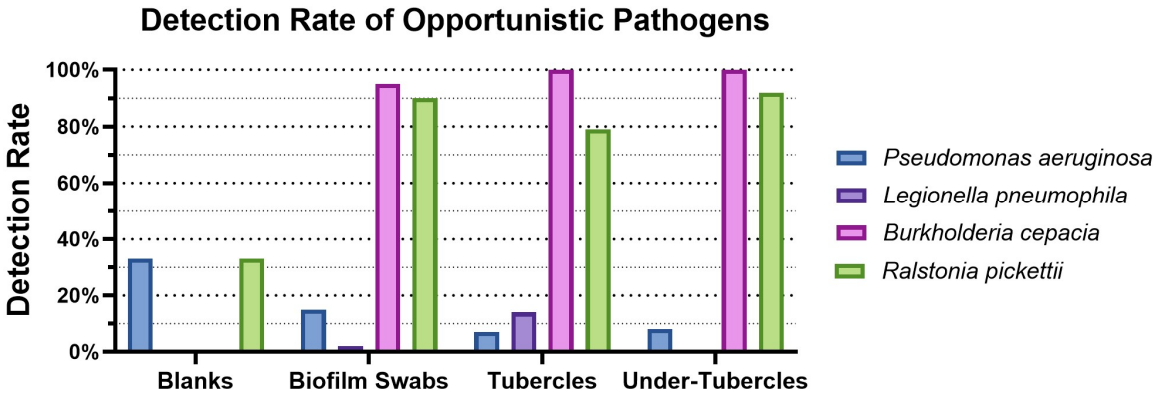

Figure 4.17 The detection rate of the opportunistic pathogens *Pseudomonas aeruginosa*, *Legionella pneumophila*, *Burkholderia cepacia*, and *Ralstonia pickettii* in the sequencing blanks (n=3), biofilm swabs (n=63), tubercle samples (n=14), and under-tubercle samples (n=12). Biofilm swabs are representative of the entire dataset while tubercle and under-tubercle samples are only from cast iron and ductile iron pipes. The detection rate is calculated as the percentage of samples with positive detection.

Although detection was overall low, *P. aeruginosa* had the highest occurrence in copper samples. Further, *P. aeruginosa* had a significant positive correlation with the copper-resistant genus *Cupriavidus*, which dominated Milwaukee's copper pipes and Waukesha' ductile iron pipes (Figure 4.18). It is also an interesting finding that *B. cepacia* detection remained high on copper pipes because, despite its metal resistant properties, Ibrahim et al. suggested the use of copper surfaces in hospitals could inhibit *B. cepacia* (Ibrahim et al., 2011).

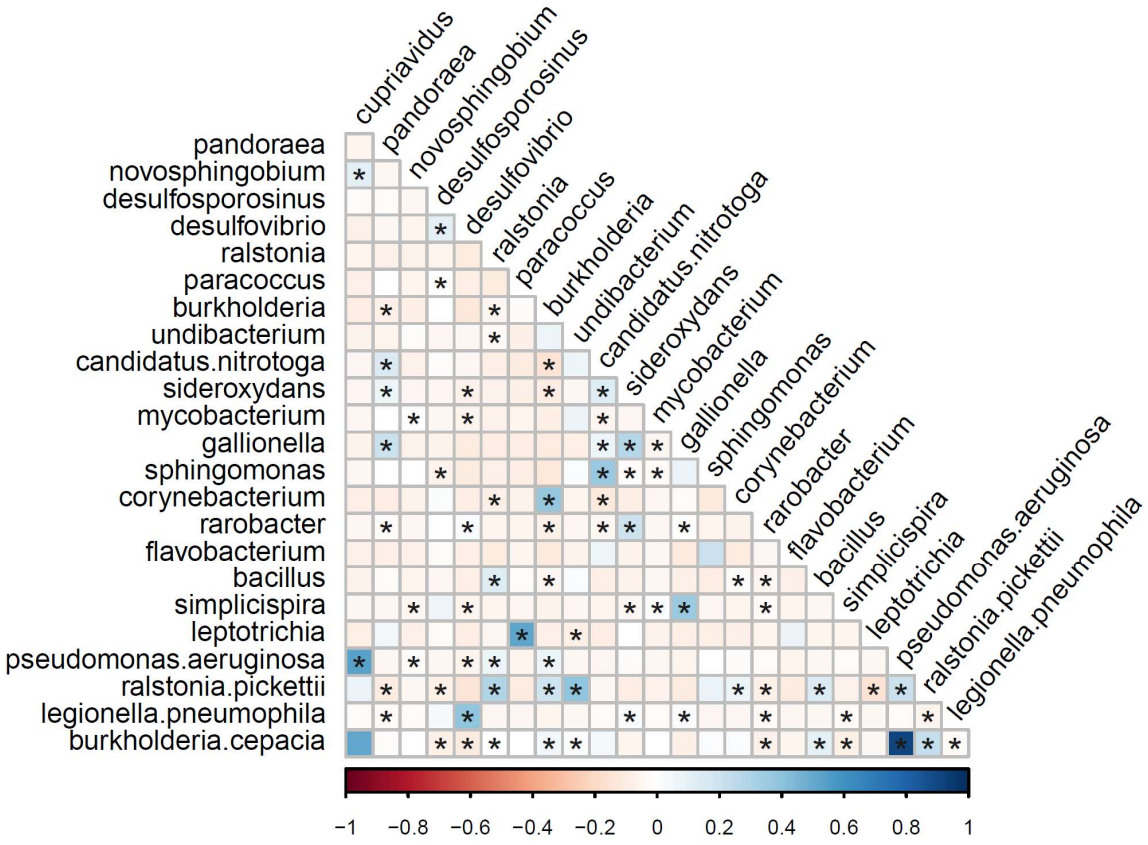

Figure 4.18 The relationships between relative abundances of the top 20 dominant genera in the dataset and the OTUs of the OPs *Pseudomonas aeruginosa*, *Legionella pneumophila*, *Burkholderia cepacia*, and *Ralstonia pickettii*. The color-scale represents rho as calculated by Spearman's rank sum correlation. Asterisks indicate statistically significant results (p-value<0.05).

The high rates of *B. cepacia* may explain the low detections of *L. pneumophila* as they may produce toxins that harm *L. pneumophila* (Hong Wang et al., 2013). Species of *Bacillus* and *Flavobacterium*, which were dominant in this study, also produce toxins harmful to *L. pneumophila* (Hong Wang et al., 2013). Although not significant, *Bacillus* and *Flavobacterium* were slightly negatively correlated with *L. pneumophila*, suggesting the possibility that these genera may have controlled *L. pneumophila* in the DWDS. However, no correlation was found between *L. pneumophila* and *B. cepacia*. Interestingly, there was a significant positive correlation between *B. cepacia* and *P.*

aeruginosa, suggesting that environments that allowed the survival of one of these OPs also supported the survival of the other OP. Wang et al. has proposed a probiotic approach to pathogen control in which genera that outcompete OPs or that produce toxins harmful to OPs are promoted, yet a greater understanding of the relationships among DWDS bacteria and influence of environmental factors must first be strengthened (Hong Wang et al., 2013). As such, the present paper provides some insights regarding co-occurring genera and OPs as well as the impact of pipe material and utility. However, follow-up research could be conducted to establish the relevance of other significant correlations among taxa in the DWDS biofilms. Such research could aim to further research of the probiotic approach of pathogen control and could likewise establish the same approach to corrosion control.

5 CONCLUSIONS

5.1 Key Findings

To address the research gap on the impact of pipe material on biofilms inhabiting DWDSs, modern molecular methods were employed to quantify and characterize the microbial community in biofilm samples from full-scale drinking water pipes. The influence of pipe material within a single utility and across the utilities was studied by analyzing biofilm samples from three utilities and four pipe materials. In addition, the influence of other factors (sample type, pipe diameter, and pipe age) were investigated, and tentative detection rates of several putative opportunistic pathogens were determined. The key findings of the present study are as follows:

1. Cast iron and ductile iron pipes promoted higher biomass densities as compared to lead and copper pipes in their biofilm samples. Cast iron supported the highest abundance with respect to all materials, and ductile iron had the second highest abundance, likely due to increased surface roughness and bioavailability of nutrients associated with corroded iron surfaces. The low biomass densities of copper pipes suggests that copper pipes may be advantageous in preventing bacterial growth in laterals. Although lead pipes also had low biomass densities, the risk of lead toxicity prohibits their use in DWDSs.

2. Regarding the microbial community composition, pipe material shaped differences in the microbial communities in a single utility. Genera associated with iron corrosion were dominant in cast iron and ductile iron pipes, and genera shown to exhibit heavy-metal resistance were identified in all pipe materials. Interestingly, *Burkholderia* has not been commonly associated with the DWDS,

but it was ubiquitous in all materials within Milwaukee samples. This is only the second study to characterize the microbial community in lead pipes.

3. While some core genera were shared by pipes of the same material across different utilities, utility also influenced the composition of the microbial communities in biofilms, emphasizing the interplay of many factors in shaping the microbial community in the DWDS. In particular ductile iron pipes in chloraminated systems may provide a niche for the genus *Mycobacterium*, which can harbor opportunistic pathogens. Further, genera that promoted corrosion of cast iron were more dominant in Milwaukee pipe samples than in Waukesha pipe samples, prompting the need for further research into how engineering decisions act in combination with environmental factors to influence microbial corrosion.
4. In addition to pipe material, the type of biofilm sample also shaped the biomass density of biofilms within a single utility. Biofilms under tubercles supported higher growth as compared to biofilm swabs, revealing that cast iron and ductile iron pipes – which already had higher biomass abundance in surface biofilm samples as compared to copper and lead – can allow for even denser biofilm growth when corrosion structures form. In order from most impactful to least impactful, the following factors shaped the microbial community to a statistically significant degree: biofilm sample type (30%), utility (21%), pipe material (12%), and season (7%). Ultimately, biofilm sample type is also a function of pipe material because cast iron and ductile iron pipes are more likely to form tubercles than copper and lead pipes. As such, the state of corrosion impacts which microbial communities are present in iron pipes. In cast iron pipes, under-tubercle

samples provided an anerobic niche that promoted dominance by bacteria associated with corrosion inhibition while bacteria associated with accelerated corrosion dominated in biofilm samples.

5. Of the putative opportunistic pathogens of interest to this study, *P. aeruginosa*, *L. pneumophila, B. cepacia*, and *R. pickettii* were identified in the dataset. The detection rates of *P. aeruginosa* and *L. pneumophila* were overall low, respectively accounting for 13% and 3% of all samples. Detection of *L. pneumophila* was limited to Milwaukee's cast iron pipes. Meanwhile, *B. cepacia* and *R. pickettii* had high detection rates, respectively accounting for 97% and 88% of all samples, and were ubiquitous in all utilities and pipe materials. The correlations of dominant genera with these opportunistic pathogens could provide insights to a probiotic approach to controlling opportunistic pathogens, as proposed by Hong Wang, et al. (2013).

To apply the findings of this study to a practical implementation, more research is still needed. Overall, the study found that pipe material influences both the abundance and the composition of microbial communities in DWDS biofilms, which emphasizes the importance of considering pipe material in engineering decisions. Further, utility and sample type had a strong impact on shaping the microbial community structure. Lastly, the opportunistic pathogens *Burkholderia cepacia* and *Ralstonia pickettii* had high detection rates in all utilities and all pipe materials, which prompts further consideration of strategies that would reduce their presence in DWDS. Recommendations for future work that builds upon the findings of the current study are presented in the following section.

5.2 Recommendations for Future Work

This is the first study to include biofilm samples from full-scale cast iron, ductile iron, lead, and copper pipes to investigate the impact of pipe material on the microbial communities of biofilms in full-scale DWDSs. Because utility and pipe material both influenced the microbial communities of biofilms, this study provides increased confidence in the potential for engineering decisions to shape a safer and more durable DWDS. For example, choosing a pipe material based on its ability to inhibit biofilm growth is possible as this study found that copper pipes had lower density biomass than cast iron and ductile iron pipes. Further, there is evidence that chloraminated ductile iron pipes could promote the presence of *Mycobacterium*, which suggests that understanding the interactions of disinfectants and pipe materials could inform selections of pipes based on the inhibition of OPs. However, this study alone cannot be used to conclude which pipe materials should be implemented for the following reasons: the study only included four pipe materials, pipe materials were not equally represented in all utilities, and selective pressures potentially provided by utilities were not completely understood.

Therefore, research occurring in other utilities and including other pipe materials could provide further insight into the influence of both pipe material and utility. For one, the present study was not able to obtain biofilm samples from plastic pipes because sampling was constrained to pipe maintenance or replacement, and excavation of plastic pipes did not occur during the sampling campaign. As such, the microbial communities of plastic pipes in the studied utilities are not known and are yet to be characterized. If biofilm samples from plastic pipes were obtained, performing molecular analyses similar to this study would allow for an interesting comparison of metallic pipes to plastic pipes.

Likewise, obtaining full-scale samples of the same pipe materials (cast iron, ductile iron, copper, and lead) but from different utilities could reveal whether engineering decisions shared by utilities promote similar dominant genera, e.g., use of the same corrosion inhibitor and disinfectant residual. In addition, the present study only quantified the abundance of biomass in the biofilms of Milwaukee to determine the role of pipe material; however, considering the strong effect of utility on the microbial community composition, additional research should investigate the abundance of biofilms in Waukesha and Oak Creek to see how utility might impact abundance.

To expand upon the limitations of this study, full-scale studies should also characterize the water quality from nearby taps or hydrants, particularly with respect to the concentration of disinfectant residuals, ammonia (which can indicate decay of chloramines), and corrosion inhibitors. The DWDS is a dynamic system and bulk water conditions can change spatially and temporally, meaning that sampling of biofilms ultimately only captures microbial community characteristics at a localized point in time. As such, having water quality data to pair with microbial community data would allow for more convincing explanations of differences/similarities in the microbial communities with respect to a utility's selective pressures. Overall, the present research provides a starting point for more informed selection of pipe materials, but more research is needed to establish which pipe materials would be best implemented by a utility.

In general, the microbial communities inhabiting full-scale DWDSs should be characterized more completely, and information should be consolidated. Due to the large quantity and complexity of variables existing within drinking water pipes, investigating the influence of a factor like pipe material in full-scale pipes becomes more effective with

greater statistical power, i.e., a larger sample size. Although full-scale drinking water studies are much less common than lab-scale studies, sequencing of full-scale biofilms has occurred (Aggarwal et al., 2018; Kelly et al., 2014; Tang et al., 2021; Zhang et al., 2018). These studies did not explicitly focus on the impact of pipe material, but the microbial community data presented in these studies (as well as that from other studies not listed here) could be included in a meta-study. A meta-study of published papers could reuse data that was previously obtained for a different objective and produce new, meaningful insights to the impact of pipe material. For this reason, researchers obtaining biofilm samples from full-scale pipes should always provide descriptions of the pipes from which sampling occurred. Unfortunately, this is not the case for many studies where the influence of pipe material has not been studied. Regardless of research objectives, full-scale studies of drinking water biofilms should include as much information as possible about each sample's origin, including pipe material, pipe diameter, and pipe age.

Further, more coordinated efforts by drinking water researchers to share their microbial community data would allow the impact of factors other than pipe material to be studied as well. This paper proposes the creation of a database specific to drinking water microbial community data for full-scale samples. Such a database would consolidate large amounts of data, including microbial community data as well as all relevant water quality, sampling, and utility data. In this way, even small-scale studies or single utilities could contribute to a larger body of research. Analyses of factors shaping microbial communities could be conducted on a larger scale, and the same data could be analyzed in several ways to address many different research objectives. For example, the present study as well as other research has found that season may drive microbial

community composition, but more research is needed to understand season's impact. As such, the inclusion of many data points from all seasons – possibly paired with temperature measurements – could address this research gap. Further, OPs could be monitored more comprehensively. At present, OPs are usually studied separately in environmental and in clinical studies. A database of OPs inhabiting DWDSs could instigate more effective communication between the environmental engineering field and the medical field, as well as other interest groups like utilities and governmental health organizations (e.g., the Center for Disease Control and Prevention).

Overall, there is a need for deeper sequencing of species-level microbial community data to identify the existence of harmful OPs in DWDSs. Many studies discuss the presence of OPs in drinking water based on the presence of genera that can contain pathogenic species, not based on the identification of the pathogenic species themselves. These studies responsibly do not overstate their findings because a genus that contains pathogen species usually also contains many non-pathogenic species. For example, if *Mycobacterium* is identified as a dominant genus in a drinking water sample, it is possible that there are no pathogens in the sample just as it is possible that *M. avium* may be harbored. As was discussed in Section 4.4, it has been suggested that an increase in clinical cases caused by emerging OPs may be due to a general rise in immunocompromised individuals who are more susceptible to infection (Ryan & Adley, 2014). This claim was made in 2014 prior to the COVID-19 pandemic, which has increased hospitalizations and infected millions of people, and may make infections by OPs even more relevant. In addition, the OPs investigated in this study have demonstrated antibiotic resistance, which has become an increasing threat to global

health. With such stakes, it is important that the DWDS inhibits growth of OPs, but utilities must first understand the potential risks present in their systems. Further, engineering decisions like choice of disinfectant residual and pipe material would be better-informed if there was more definitive research associating these factors with OP species.

Drinking water pipe biofilms can influence the presence of OPs as well as corrosion, and a deeper understanding of the co-occurrence of taxa could help to establish an innovative approach to controlling the DWDS's microbial community. There are several difficulties associated with the use of disinfectants, such as the potential to form carcinogenic and mutagenic disinfection byproducts, promotion of pipe corrosion, and decay within the system. As such, Hong Wang et al. has proposed that a probiotic approach to OP control could be used to shape a favorable microbial community in the DWDS that outcompetes OPs or is antagonistic to the survival of OPs (Wang et al., 2013). This paper cites pilot-scale studies that seeded pipes or treatment plant filters with desirable microbial communities, proposing these as potential mechanisms to inhibit OP colonization. However, a probiotic approach could also be considered as one that manipulates selective pressures like pipe material, disinfectant, or corrosion inhibitors with the goal of promoting certain genera. While current treatment strategies are generally oriented toward the removal of harmful bacteria, a probiotic approach would shift this outlook toward the promotion of bacteria beneficial to achieving engineering design goals. In this way, the probiotic approach could also be used to reduce pipe corrosion. The present study found that genera associated with accelerated iron corrosion were generally found together while genera associated with inhibited corrosion co-

occurred. A deeper understanding of the environmental factors leading to the co-occurrence of corrosion-associated bacteria could allow utilities to utilize selective pressures to encourage the growth of corrosion-inhibiting bacteria over corrosion-accelerating bacteria.

In addition, the strong influence of type of biofilm sample (biofilm swab, tubercle sample, under-tubercle swab) on both abundance and composition of the microbial communities suggests that the degree to which iron pipes have corroded may be important. Although iron pipes can remain in the DWDS for decades, the microbial community in iron pipes likely changes as corrosion progresses. While pipe age did not correlate with biomass abundance or microbial community dissimilarities in the dataset of the present study, a follow-up study should specifically investigate whether pipe age corresponds with microbial community data from cast iron and ductile iron pipes. A study that monitors the progression of corrosion and the formation of corrosion tubercles with time could provide insights to the lifespan of cast iron and ductile iron pipes with respect to a favorable microbial community. This may be important to a probiotic approach. Further, such a study could indicate whether it is more likely for iron to bulk water beyond a certain point of corrosion. Perhaps cast iron and ductile iron pipes should be replaced based on the characteristics of the microbial communities throughout the corrosion process and not just when pipes break.

Beyond full-scale studies, more lab-scale studies should be conducted to specifically understand the interactions of pipe material with disinfectant residual and corrosion inhibitors to shape biofilms. The present study highlights both pipe material and utility as having a strong impact on the structure of the microbial community in full-

scale DWDSs, but the interactions of a utility's design factors with pipe materials should be better understood. Specifically, disinfectant residuals and corrosion inhibitors may act in combination with pipe material to provide unique selective pressures, but these interactions need to be studied in a laboratory setting where each factor may be manipulated. A comprehensive study could include several combinations of pipe materials, common disinfectant residuals (chlorine, chloramine), and common corrosion inhibitors (silicate, orthophosphate) to investigate how each factor individually or in combination shapes the microbial communities of biofilms. An advantage to studying these interactions in a single study would be that the same source water could be used, controlling for potential differences in the seed microbial communities, which are influenced by temporally and spatially changing water quality characteristics. For example, the claim made in the present paper that chloraminated ductile iron pipes may support dominance of the potentially pathogenic *Mycobacterium spp.* could be further investigated. In addition, heavy-metal resistant genera were identified on the metallic pipes in this study, and Kimbell et. al, found that zinc orthophosphate could promote mechanisms that confer both heavy-metal and antibiotic resistance. As such, it is important to determine if corrosion inhibitors promote heavy-metal resistant genera on certain pipe materials.

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APPENDIX

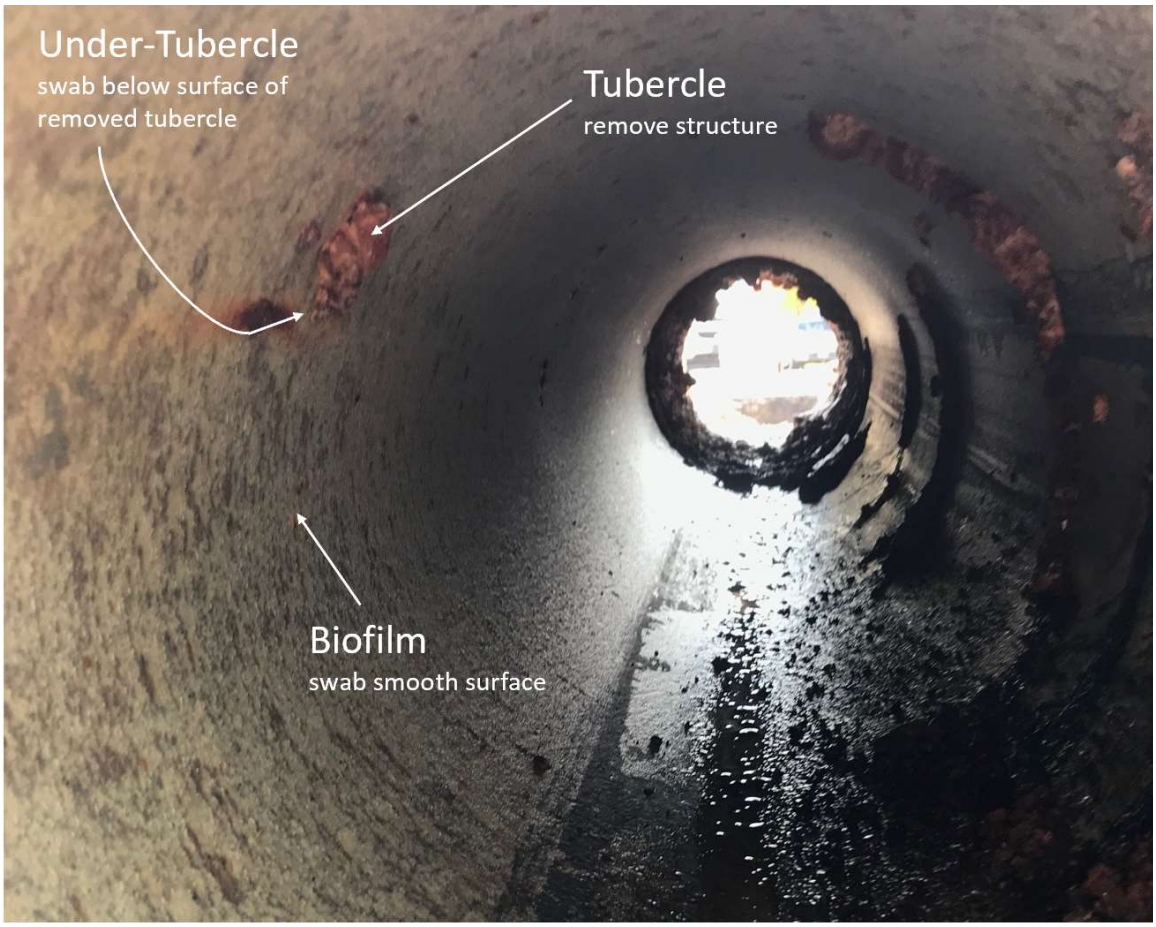

Figure A-1 Sample types collected from cast iron and ductile iron pipes, when available. Biofilm samples were obtained by swabbing the smooth surface of each pipe. Tubercle samples were obtained by removing the structure with a spatula. Under-tubercle samples were obtained by swabbing the surface below tubercles following tubercle removal.

Table A-1 Log(Gene Copies/cm^2) of 16S rRNA of Milwaukee's Biofilm Samples

Copper			
Sampling Event	**Mean**	**SD**	**n**
MKE 1	5.810	0.352	15
MKE 9	5.621	0.099	10
MKE 13	5.504	0.190	12
Lead			
Sampling Event	**Mean**	**SD**	**n**
MKE 2	5.987	0.277	13
MKE 3	5.504	0.171	13
MKE 8	5.711	0.079	14
Cast Iron			
Sampling Event	**Mean**	**SD**	**n**
MKE 5-cFe	7.025	0.106	6
MKE 4	6.398	0.317	14
MKE 7	6.648	0.297	8
Ductile Iron			
Sampling Event	**Mean**	**SD**	**n**
MKE 5-dFe	6.260	0.278	14
MKE 16	6.517	0.311	12
MKE 14	6.132	0.269	12

Table A-2 Alpha Diversity Metrics for Dataset of Sequenced Samples

	Shannon Diversity Index	**Richness (Observed OTUs)**	**Pielou's Evenness Index**
Minimum	0.057	5	0.0355
Maximum	3.832	107	0.9396
Median	2.444	37.5	0.7047
Mean	2.351	40.2	0.6508
Standard Deviation	0.920	21.6	0.2209

Table A-3 Range of Relative Abundances of the Top Twelve Dominant Genera in Milwaukee's Biofilm Samples

	Cast Iron		**Ductile Iron**		**Copper**		**Lead**	
	Min	**Max**	**Min**	**Max**	**Min**	**Max**	**Min**	**Max**
burkholderia	0%	59%	0%	88%	0%	71%	0%	55%
undibacterium	0%	11%	0%	46%	0%	19%	0%	47%
mycobacterium	0%	1%	0%	90%	0%	0%	0%	4%
desulfovibrio	0%	0%	0%	44%	0%	4%	0%	14%
sphingomonas	0%	13%	0%	0%	0%	9%	0%	32%
candidatus nitrotoga	0%	29%	0%	3%	0%	1%	0%	2%
corynebacterium	0%	26%	0%	6%	0%	19%	0%	15%
cupriavidus	0%	6%	0%	0%	0%	47%	0%	0%
sideroxydans	0%	40%	0%	3%	0%	0%	0%	1%
microvirga	0%	1%	0%	0%	0%	56%	0%	0%
ralstonia	0%	1%	0%	3%	0%	23%	0%	9%
gallionella	0%	17%	0%	1%	0%	0%	0%	11%

Table A-4 Range of Relative Abundances of the Top Twelve Dominant Genera in the Connected Pipes from Sampling Events MKE 5-cFe and MKE 5-dFe

	MKE 5-cFe		**MKE 5-dFe**	
	Min	**Max**	**Min**	**Max**
burkholderia	0%	59%	0%	88%
corynebacterium	0%	26%	0%	6%
mycobacterium	0%	1%	0%	26%
undibacterium	1%	3%	0%	21%
nitrosomonas	0%	16%	0%	0%
sphingomonas	0%	12%	0%	0%
abiotrophia	0%	2%	0%	12%
escherichia	0%	3%	0%	7%
pandoraea	0%	8%	0%	0%
lautropia	0%	8%	0%	0%
candidatus nitrotoga	0%	7%	0%	0%
kocuria	0%	0%	0%	7%

Table A-5 Range of Relative Abundances of the Top Twelve Dominant Genera in the Connected Pipes from Sampling Events WK 2-cFe and WK 2-Pb

	WK 2-cFe		**WK 2-Pb**	
	Min	**Max**	**Min**	**Max**
ralstonia	0%	86%	0%	0%
burkholderia	0%	0%	0%	50%
cupriavidus	0%	47%	0%	0%
flavobacterium	0%	0%	0%	10%
sphingomonas	0%	0%	0%	7%
corynebacterium	0%	11%	0%	0%
solitalea	0%	6%	0%	5%
xylophilus	0%	3%	0%	4%
methylobacterium	0%	9%	0%	0%
bradyrhizobium	0%	8%	0%	0%
bacillus	0%	8%	0%	0%
shigella	0%	5%	0%	3%

Table A-6 Range of Relative Abundances of the Top Twelve Dominant Genera in Copper Biofilm Samples

	Milwaukee		**Oak Creek**	
	Min	**Max**	**Min**	**Max**
ralstonia	0%	84%	0%	84%
burkholderia	0%	71%	0%	44%
cupriavidus	0%	47%	0%	0%
microvirga	0%	56%	0%	0%
corynebacterium	0%	19%	0%	26%
brevibacillus	0%	21%	0%	0%
shigella	0%	18%	0%	0%
tepidimonas	0%	33%	0%	33%
pseudomonas	0%	8%	0%	8%
lautropia	0%	25%	0%	0%
alkanindiges	0%	15%	0%	4%
undibacterium	0%	19%	0%	2%

Table A-7 Range of Relative Abundances of the Top Twelve Dominant Genera in Lead Biofilm Samples

	Milwaukee		**Waukesha**	
	Min	**Max**	**Min**	**Max**
burkholderia	0%	55%	0%	50%
undibacterium	0%	47%	0%	3%
sphingomonas	0%	32%	0%	7%
flavobacterium	0%	4%	0%	10%
ralstonia	0%	9%	0%	0%
desulfovibrio	0%	14%	0%	2%
gallionella	0%	11%	0%	1%
halorhodospira	0%	11%	0%	2%
thermodesulfovibrio	0%	15%	0%	1%
hydrogenophaga	0%	8%	0%	1%
mycobacterium	0%	4%	0%	4%
corynebacterium	0%	15%	0%	0%

Table A-8 Range of Relative Abundances of the Top Twelve Dominant Genera in Cast Iron Biofilm Samples

	Milwaukee		**Waukesha**	
	Min	**Max**	**Min**	**Max**
burkholderia	0%	59%	0%	84%
candidatus nitrotoga	0%	29%	0%	10%
ralstonia	0%	1%	0%	86%
sideroxydans	0%	40%	0%	1%
corynebacterium	0%	26%	0%	11%
cupriavidus	0%	6%	0%	47%
sphingomonas	0%	13%	0%	5%
bradyrhizobium	0%	19%	0%	8%
nitrosomonas	0%	16%	0%	0%
gallionella	0%	17%	0%	0%
pandoraea	0%	14%	0%	0%
acidovorax	0%	10%	0%	2%

Table A-9 Range of Relative Abundances of the Top Twelve Dominant Genera in Ductile Iron Biofilm Samples

	Milwaukee		**Waukesha**		**Oak Creek**	
	Min	**Max**	**Min**	**Max**	**Min**	**Max**
burkholderia	0%	88%	0%	0%	0%	53%
ralstonia	0%	3%	0%	97%	0%	23%
mycobacterium	0%	90%	0%	0%	0%	2%
undibacterium	0%	46%	0%	0%	0%	1%
bacillus	0%	6%	0%	28%	0%	36%
desulfovibrio	0%	44%	0%	0%	0%	2%
corynebacterium	0%	6%	0%	0%	0%	35%
cupriavidus	0%	0%	0%	38%	0%	0%
leptotrichia	0%	4%	0%	0%	0%	12%
paracoccus	0%	4%	0%	0%	0%	12%
dethiobacter	0%	0%	0%	1%	0%	14%
massilia	0%	23%	0%	0%	0%	1%

Table A-10 Range of Relative Abundances of the Top Twelve Dominant Genera in Waukesha's Samples

	Waukesha	
	Min	**Max**
ralstonia	0%	97%
burkholderia	0%	84%
cupriavidus	0%	47%
novosphingobium	0%	36%
thioalkalispira	0%	60%
bacillus	0%	28%
flavobacterium	0%	16%
nevskia	0%	22%
methylobacterium	0%	12%
paenibacillus	0%	23%
sphingomonas	0%	8%
klebsiella	0%	26%

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Table A-11 Range of Relative Abundances of the Top Twelve Dominant Genera in Oak Creek's Samples

	Oak Creek	
	Min	**Max**
ralstonia	0%	84%
burkholderia	0%	53%
corynebacterium	0%	35%
bacillus	0%	36%
tepidimonas	0%	33%
dietzia	0%	17%
pseudomonas	0%	8%
dethiobacter	0%	14%
paracoccus	0%	12%
skermanella	0%	13%
leptotrichia	0%	12%
flavobacterium	0%	13%

Table A-12 Range of Relative Abundances of the Top Twelve Dominant Genera in Milwaukee's Samples (Including Biofilm, Tubercle, and Under-Tubercle Samples)

	Milwaukee	
	Min	**Max**
desulfovibrio	0%	99%
burkholderia	0%	88%
undibacterium	0%	47%
gallionella	0%	38%
mycobacterium	0%	90%
sideroxydans	0%	52%
corynebacterium	0%	26%
simplicispira	0%	70%
candidatus nitrotoga	0%	29%
pandoraea	0%	36%
sphingomonas	0%	32%
cupriavidus	0%	47%

Table A-13 Statistical Analysis of Microbial Community Dissimilarity based on Pipe Material

	Adonis/PERMANOVA			Betadisper	
	p-value	**R^2**	**Significant?**	**p-value**	**Homogeneous Variance?**
Milwaukee Biofilm Samples					
All Genera	0.004	0.1451	Yes	0.2882	Yes
Top 12	0.002	0.17701	Yes	0.1839	Yes
Top 20	0.001	0.16207	Yes	0.2048	Yes
MKE 5-cFe vs. MKE 5-dFe					
All Genera	1	0.13169	No	0.2177	Yes
Top 12	0.1535	0.7	No	0.06204	Yes
Top 20	0.8	0.1442	No	0.08579	Yes
WK 2-cFe vs. WK 2-Pb					
All Genera	0.2	0.21357	No	0.4941	Yes
Top 12	0.4	0.23211	No	0.5479	Yes
Top 20	0.2	0.21463	No	0.5561	Yes

Table A-14 Statistical Analysis of Microbial Community Dissimilarity based on Utility

	Adonis/PERMANOVA			Betadisper	
	p-value	**R^2**	**Significant?**	**p-value**	**Homogeneous Variance?**
Copper					
All Genera	0.548	0.06564	No	0.6753	Yes
Top 12	0.288	0.08263	No	0.3256	Yes
Top 20	0.219	0.08667	No	0.7185	Yes
Lead					
All Genera	0.581	0.07879	No	0.6413	Yes
Top 12	0.535	0.08143	No	0.6067	Yes
Top 20	0.577	0.07424	No	0.6439	Yes
Cast Iron Biofilm					
All Genera	0.076	0.10457	No	0.07463	Yes
Top 12	0.13	0.10161	No	0.1016	Yes
Top 20	0.142	0.0977	No	0.06416	Yes
Ductile Iron Biofilm					
All Genera	0.04	0.15446	Yes	0.2846	Yes
Top 12	0.025	0.17324	Yes	0.3151	Yes

Top 20	0.034	0.16489	Yes	0.3508	Yes

Table A-15 Detection Rate of the *Pseudomonas aeruginosa*, *Legionella pneumophila*, *Burkholderia cepacia*, and *Ralstonia pickettii* in Various Sample Groupings

Utility Grouping				
	Pseudomonas aeruginosa	*Legionella pneumophila*	*Burkholderia cepacia*	*Ralstonia pickettii*
Blanks (n=3)	33%	0%	0%	33%
All (n=86)	13%	3%	97%	88%
Milwaukee (n=55)	15%	5%	95%	85%
Waukesha (n=18)	17%	0%	100%	100%
Oak Creek (n=13)	0%	0%	100%	85%
Pipe Material Grouping				
	Pseudomonas aeruginosa	*Legionella pneumophila*	*Burkholderia cepacia*	*Ralstonia pickettii*
Blanks (n=3)	33%	0%	0%	33%
Cast Iron (n=38)	16%	8%	100%	84%
Ductile Iron (21)	10%	0%	100%	86%
Copper (n=15)	20%	0%	100%	93%
Lead (n=12)	0%	0%	75%	100%
Sample Type Grouping				
	Pseudomonas aeruginosa	*Legionella pneumophila*	*Burkholderia cepacia*	*Ralstonia pickettii*
Blanks (n=3)	33%	0%	0%	33%
Biofilm Swabs (n=60)	15%	2%	95%	90%
Tubercles (n=14)	7%	14%	100%	79%
Under-Tubercles (n=12)	8%	0%	100%	92%