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THE IMPACT OF CORROSION INHIBITORS AND METALS ON ANTIBIOTIC  
RESISTANCE IN DRINKING WATER DISTRIBUTION SYSTEMS

By

Lee Kyle Kimbell

A Dissertation submitted to the Faculty of the Graduate School,  
Marquette University,  
in Partial Fulfillment of the Requirements  
for the Degree of Doctor of Philosophy

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## ABSTRACT

### THE IMPACT OF CORROSION INHIBITORS AND METALS ON ANTIBIOTIC RESISTANCE IN DRINKING WATER DISTRIBUTION SYSTEMS

Lee Kyle Kimbell

Marquette University, 2022

Drinking water distribution systems are important for transporting clean drinking water. Antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in drinking water may pose risks to human and environmental health. Aging infrastructure such as metal pipes can experience significant corrosion, which can have impacts on the chemical and microbial water quality inside of drinking water systems. Chemicals are commonly added to drinking water to prevent corrosion (i.e., corrosion inhibitors). The metal pipe materials and corrosion inhibitors containing metals may have impacts on the bacteria and ARGs in drinking water systems. This research investigated the presence of ARGs in real-world drinking water systems and analyzed the impacts of corrosion inhibitors on bacteria and ARGs in laboratory-scale experiments. Results from analysis of full-scale water main sampling indicated the presence of multiple types of ARGs and diverse bacterial communities in samples collected inside of a cast iron water main. Microbial communities in corrosion deposits were dominated by metal-tolerant bacterial genera such as *Geobacter*, *Gallionella*, *Sphingomonas*, and *Mycobacterium*. ARGs and metal resistance genes (MRGs) including *bla*TEM, *bla*SHV, *czcD*, *copA*, and the integrase gene of class 1 integrons (*intI1*) were positively correlated to the presence of several bacterial genera. In laboratory microcosm experiments, metal-containing corrosion inhibitors (zinc orthophosphate) selected for increased abundance of ARB resistant to multiple antibiotics and several different ARGs including *sul1*, *sul2*, *qacEΔ1*, and *intI1* compared to untreated controls. Metagenomic sequencing of microbial DNA from the drinking water microcosms was conducted to determine the impact of corrosion inhibitors on the resistome in a source drinking water. Analysis of the microbial resistome indicated that zinc orthophosphate selected for increased abundance of total ARGs compared to sodium silicate, sodium orthophosphate, and untreated controls. Future drinking water management decisions should consider the role of pipe materials and corrosion inhibitors to determine how engineering decisions can mitigate the spread of antibiotic resistance in drinking water systems.

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## DEDICATION

This dissertation is dedicated to my wife, Amanda, Skyler, and my family for their patience, support, and love while I completed my PhD.

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## **1 INTRODUCTION**

### **1.1 Environmental and Public Health Concerns Associated with Antibiotic Resistance and Antimicrobial Resistance**

The prevalent use of antibiotics for clinical and veterinary uses worldwide is of particular concern to public health due to resulting impacts on the spread of antibiotic resistance (Alanis 2005). Antibiotics are typically classified into several different categories such as aminoglycosides, arsenicals,  $\beta$ -lactams, macrolides, penicillins, and tetracyclines (Levy 1998; Chopra and Roberts 2001; Bouki et al. 2013; Ju et al. 2016). Antibiotic resistance is comprised of two main components, including the antibiotic or antimicrobial drug which inhibits susceptible organisms and selects for resistant ones, and the genetic resistance determinant (i.e., antibiotic resistance gene) that is selected for by a given antibiotic or antimicrobial compound (Levy 2002). The selected antibiotic resistance genes (ARGs) and their hosts spread and propagate under continuous selective pressures in natural and engineered environments to amplify and exacerbate the problem of antibiotic resistance. Widespread antibiotic resistance poses a serious threat to human health due to the associated loss of therapeutic potential for antibiotic compounds, especially in immunocompromised populations (Levy and Bonnie 2004; Ashbolt et al. 2013b). In addition, associated healthcare costs to treat antibiotic resistant infections have been estimated at \$1 billion annually and over 23,000 people die each year in the U.S. alone from infections caused by antibiotic resistant bacteria (ARB) (CDC 2013).

Antibiotic resistance is not a new phenomenon, but the number of resistant organisms and geographic locations affected by antibiotic resistance continues to increase

at an unprecedented rate (Levy and Bonnie 2004; Alanis 2005). Resistance to drugs, i.e. antibiotics such as sulfonamides, penicillin, and tetracycline, initially appeared in hospitals and clinical environments, where the majority of antibiotics were being used (Levy 1998). As early as the 1930s, sulfonamide-resistant *Streptococcus pyogenes* was observed in military hospitals (Barber 1948). Resistance to multiple drugs was first documented in enteric bacteria, i.e. *Escherichia coli*, *Shigella* and *Salmonella*, in the 1950s and 1960s (Watanabe 1963; Levy 2001). Genes for resistance traits can be transferred between bacteria in different ecological and taxonomic groups through mobile genetic elements (MGEs) such as plasmids, bacteriophages, transposons, or naked DNA (Nwosu 2001). Some transposons contain integrons, which are complex genetic elements that contain sites for integrating different antibiotic resistance genes and gene cassettes in tandem for expression from a single promoter (Hall et al. 1999). Integrons were originally discovered in Gram-negative bacteria but have since been observed in Gram-positive commensal organisms (Nandi et al. 2004). In the absence of plasmids and transposons, antibiotic resistance often occurs in bacteria through sequential chromosomal mutations (Wang and Dzik-Fox 2001; Levy 2002; Schneiders et al. 2003). For example, strains of *E. coli* and other *Enterobacteriaceae* have developed increasing resistance to fluoroquinolones, which is primarily the result of mutations to the drug target (topoisomerases) and an increase in membrane proteins (e.g. efflux pumps) that pump the drugs out of the cell (Oethinger et al. 2000; Hooper 2001; Redgrave et al. 2014).

The frequency of antibiotic resistance has increased in many different bacteria as a result of increasing antibiotic use, especially in developing countries where antibiotics are

readily available without prescription and poor sanitation conditions often exist (Levy and Bonnie 2004; Wuijts et al. 2017). Poor sanitation conditions and inadequate waste treatment allow for the spread of antibiotics, in addition to other potential stressors such as pharmaceutical compounds, estrogens, and metals, and enteric bacteria into the environment. Similarly, wastewater treatment plants (WWTPs) have also been considered “hot spots” for the development and spread of antibiotic resistance due to densely populated biological systems and the presence of potential selective agents such as heavy metals, herbicides, disinfectants, and antibiotics (Rizzo et al. 2013; Yuan et al. 2015; Guo et al. 2017). In addition, ARB and ARGs have been detected in a wide range of environmental matrices including sediments, soils, lakes, rivers, WWTP effluents, biosolids, and in drinking water distribution systems (DWDSs) (Armstrong et al. 1981; Ghosh et al. 2009; Munir and Xagorarakis 2011; Pruden et al. 2012a; Kimbell et al. 2018). Some of the most prevalent ARGs detected in various environmental compartments include resistance determinants encoding for  $\beta$ -lactam resistance (e.g. *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>), macrolide resistance (*ermB*, *ermF*), sulfonamide resistance (*sul1*, *sul2*), fluoroquinolone resistance (*qnrA*, *qnrS*), and tetracycline resistance (*tetA*, *tetO*, *tetL*, *tetW*) (Auerbach et al. 2007; LaPara et al. 2011; Gao et al. 2012; Mao et al. 2015; Rodriguez-Mozaz et al. 2015a; Garner et al. 2018a; Su et al. 2018). Similarly, the integrase gene of class I integrons (*intI1*) is frequently detected in the environment and is commonly linked to genes conferring resistance to disinfectants, antibiotics and heavy metals (Liebert et al. 1999; Partridge et al. 2009). Class 1 integrons such as *intI1* have been observed in a wide variety of pathogenic and non-pathogenic bacteria and have been considered key factors contributing to the development and spread of antibiotic resistance (Gillings et al. 2008).

The abundance of genes promoting antibiotic resistance, such as *intI1*, can rapidly increase in response to environmental pressures, because class 1 integrons reside in diverse bacterial species and are often located on MGEs that can easily be transferred between bacteria (Gillings et al. 2015).

A significant body of literature has documented the occurrence of ARB and ARGs in a wide variety of environments. Each environmental compartment, or reservoir, consists of complex dynamics that are largely driven by microbial ecological processes that ultimately determine the level of prevailing antibiotic resistance (Pruden et al. 2018). ARB and ARGs can flow between environmental compartments through a variety of methods including classic exposure routes through air, water, and soil, irrigation with contaminated water, wastewater and industrial effluent discharges, bacteria carrying ARGs on meat or produce, and application of biosolid-derived soil amendments (Vikesland et al. 2017; Pruden et al. 2018). Consequently, it is becoming increasingly important to identify and monitor ARB and ARGs via environmental exposure routes that are directly capable of causing human illness, such as DWDSs. Indeed, recent epidemiological studies have identified DWDSs as an important exposure route for disease transmission (Park et al. 2001; Anaissie et al. 2002; Brown-Elliott et al. 2011). As a result, studies documenting the occurrence and distribution of ARB and ARGs in full-scale and laboratory-scale distribution systems can provide vital information necessary for protecting public health and preventing the spread of antibiotic resistance.

## **1.2 Impact of Metals, Corrosion Inhibitors, and Corrosion Products on Antibiotic Resistance**

After finished drinking water enters the DWDS, it may spend hours to days inside the distribution system prior to reaching consumer taps. The predominant components of DWDS include reservoirs, water towers, and pumps, in addition to thousands of miles of drinking water mains buried underground, which are comprised of various materials including plastic, ductile iron, cast iron, copper, brass, and lead. As a result of the relatively high surface area to volume ratio in the DWDS environment, previous studies have estimated that up to 95% of overall biomass in drinking water distribution networks is attached to pipe walls, while only approximately 5% is in the aqueous phase (Flemming et al. 2002). Bacteria and other microorganisms present in DWDS biofilms may exacerbate corrosion of iron pipes, produce unpleasant tastes and odors, decrease residual disinfectant concentrations (e.g. chlorine, chloramines), and shed viable bacterial cells into treated drinking water (Bal Krishna et al. 2013; Gomez-Smith et al. 2015; Douterelo et al. 2016). Furthermore, biofilms may serve as reservoirs for opportunistic pathogens and other microbes harboring ARGs that could be subsequently released into finished drinking water in the distribution system.

Corrosion control treatment is required by the US EPA Lead and Copper Rule, which is necessary to protect public health from harmful levels of dissolved metals that can cause negative health effects. Corrosion of drinking water infrastructure occurs due to chemical and biological oxidation processes of the surface of metal pipe materials and can result in the accumulation and deposition of significant amounts of corrosion products and microbial biofilms inside of drinking water pipes. Passivation of drinking

water pipes occurs over time by the formation of corrosion products on pipe surfaces, and the formation of passivating scales in DWDS pipes lowers the potential for metal ions to leach into finished drinking water, but also provides increased surface area for bacterial attachment and biofilm formation (Zhu et al. 2014; Li et al. 2015). Metals can co-select for antibiotic resistance through different mechanisms such as co-resistance (different resistance determinants present on the same genetic element) and cross-resistance (the same genetic determinant responsible for resistance to antibiotics and metals) (Baker-Austin et al. 2006). Corrosion products found in DWDS are comprised of scale minerals and possibly additional contaminants deposited from drinking water which may impact antibiotic resistance and horizontal gene transfer of ARGs in drinking water systems but has yet to be investigated.

Corrosion inhibitors are frequently added to full-scale DWDS to inhibit corrosion and reduce levels of dissolved metals that leach from pipes into tap drinking water. Phosphate-based corrosion inhibitors such as orthophosphates and polyphosphates are the most common type of corrosion inhibitor used according to a 2019 US corrosion control survey of drinking water utilities (Arnold et al. 2020). This study and others have reported that the use of orthophosphate continues to increase due to its efficacy for controlling lead release in drinking water (Woszczyński et al. 2015; Arnold et al. 2020). Silicate corrosion inhibitors have limited full-scale use in the US and were reportedly used by only 2 utilities that participated in the 2019 survey. The addition of different types of corrosion inhibitors exhibits a selective pressure on the microorganisms and biofilms present in drinking water pipes, which also impacts the levels of prevailing resistance in tap drinking water transported through these systems. However, there is a

paucity of research regarding the impacts of different corrosion inhibitors on the occurrence and abundance of ARGs or ARB in full-scale DWDS.

### 1.3 Research Objectives

The overall objective of this dissertation research was to determine the impact of metals and corrosion inhibitors on phenotypic and genotypic antibiotic resistance in drinking water microbial communities. Antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) are frequently detected in tap drinking water and biofilms inside drinking water distribution systems (DWDS) and are not readily removed during drinking water treatment processes, which poses risks to human health. The general approach was to investigate the abundance of target resistance genes inside a full-scale DWDS and to use lab-scale batch reactors dosed with known amounts of corrosion inhibitors, metals, and disinfectants to evaluate the impact on antibiotic and metal resistance with varying water quality and operational parameters. Next generation DNA sequencing technologies were also employed to characterize the microbial communities from a full-scale DWDS water main and in response to different corrosion inhibitor and disinfectant treatments. A review of relevant literature is presented in **Chapter 2** and has been published (Kimbell et al. 2020).

The presence of ARGs in full-scale DWDS pipe biofilms has not been well documented. The first research objective was to evaluate the presence and abundance of ARGs, MRGs, and bacterial communities inside a full-scale water main. Droplet digital PCR (ddPCR) was used to quantify the abundance of ARGs and MRGs in different biofilm environments inside a full-scale pipe from an active distribution system. 16S

rRNA gene amplicon sequencing was conducted to determine the microbial communities present in the water main. These experimental results are presented in **Chapter 3** and have been published (Kimbell et al. 2021).

The second objective was derived from valuable information on abundant ARGs, MRGs, and microorganisms observed in Objective 1. The second objective was to determine the impact of different corrosion inhibitors containing metals (zinc orthophosphate, sodium orthophosphate, and sodium silicate) on the absolute and relative abundance of ARB, ARGs, and MRGs in a source drinking water. The impact of different corrosion inhibitor types and corrosion inhibitor concentrations was assessed using laboratory-scale drinking water microcosms. ARB were quantified by antibiotic resistant heterotrophic plate counts from direct plating of water on media with clinically-relevant antibiotics and ARGs/MRGs were quantified using qPCR. The results of this study are presented in **Chapter 4**.

The third objective was to determine the impact of corrosion inhibitor type relative to disinfectants (which can also select for resistance) on the antibiotic resistome (i.e., entire collection of resistance genes in the microbial community) and microbiome in a source drinking water using shotgun metagenomic sequencing. Laboratory-scale drinking water microcosms were set up in batch experiments to elucidate impacts of corrosion inhibitors and disinfectants. The impact of different chemical additions were quantified by heterotrophic plate counts of ARB resistant to clinically-relevant antibiotics and by quantification of target resistance genes using qPCR. Shotgun metagenomic sequencing was used to evaluate shifts in the antibiotic resistome and the microbiome. The experimental results for this objective are in **Chapter 5**.

Finally, the overall conclusions, including a summary of key research findings and suggestions for future research, are provided in **Chapter 6**.

## **2 LITERATURE REVIEW: THE IMPACT OF METAL PIPE MATERIALS, CORROSION PRODUCTS, AND CORROSION INHIBITORS ON ANTIBIOTIC RESISTANCE IN DRINKING WATER DISTRIBUTION SYSTEMS**

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### **2.1 Introduction**

Antibiotic resistance is considered a major global threat in the 21<sup>st</sup> century, and a return to a pre-antibiotic era is predicted without serious or immediate attention (WHO 2014). Antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) occur naturally but are selected for and enriched by exposure to antibiotics (Song et al. 2017), antimicrobials (Carey and McNamara 2015; Carey et al. 2016; Hartmann et al. 2016; Harrison et al. 2020), disinfectants (Chapman 2003; Zhang et al. 2017a), and metals (Seiler and Berendonk 2012; Zhang et al. 2018b) in natural and engineered environments. ARB and ARGs have been detected in a variety of environments including soils (Knapp et al. 2011), lakes (Di Cesare et al. 2015), groundwater (Mackie et al. 2006; Koike et al. 2007), aquaculture (Seiler and Berendonk 2012), rivers (Pruden et al. 2006; Pruden et al. 2012b; Kappell et al. 2015a), treated wastewater effluent (Auerbach et al. 2007; LaPara et al. 2011; Mao et al. 2015; Di Cesare et al. 2016; Guo et al. 2017; Kappell et al. 2018a; Cacace et al. 2019), biosolids (Ma et al. 2011; Munir and Xagorarakis 2011; Kimbell et al.

2018), drinking water treatment plants (Xi et al. 2009; Lin et al. 2014; Oh et al. 2018), drinking water distribution systems (DWDS) (Xi et al. 2009; Xu et al. 2016; Garner et al. 2018a), and tap drinking water (Xi et al. 2009; Bergeron et al. 2015). Natural and engineered environments are composed of complex dynamics driven by microbial ecological processes that ultimately determine the level of prevailing antibiotic resistance (Pruden et al. 2018). ARB and ARGs can be transferred between environments via exposure routes through air, water, soil, wastewater and industrial effluent discharges, bacteria carrying ARGs on meat or produce, and application of biosolid-derived soil amendments (Vikesland et al. 2017; Pruden et al. 2018). Environmental hotspots that could directly convey ARB and ARGs to people, such as DWDS, are of primary interest because management decisions could potentially reduce public health risks.

Over 100 different ARGs were detected in drinking water from two drinking water treatment plants in China with total ARG concentrations ranging from  $10^5$  to  $10^{10}$  copies/L (Xu et al. 2016). Multiple studies correlated the presence of ARGs with MGEs, suggesting that horizontal gene transfer could occur inside the DWDS (Ciric et al. 2011; Xu et al. 2016). In general, ARGs can be transferred vertically through microbial growth or acquired by horizontal gene transfer, which occurs through the uptake of free DNA (transformation), plasmid-mediated transfer (conjugation), and phage-mediated transfer (transduction) (Van Hoek et al. 2011). Horizontal gene transfer is a major mechanism for sharing ARGs between microorganisms and has been documented between pathogens, non-pathogens, and distantly related microbes, such as Gram-positive and Gram-negative bacteria (Levy et al. 1976; Courvalin 1994; Pruden et al. 2006). Horizontal gene transfer of ARGs is of particular concern for human health if pathogens acquire resistance

(Wingender and Flemming 2011; Wang et al. 2014c). Additionally, metal resistance is a common phenotype in many microorganisms and metal resistance genes (MRGs) are present in genomes isolated from numerous different environments including humans, animals, hydrothermal vents, and ocean microplastics (Pal et al. 2017; Yang et al. 2019). Metal resistance often occurs with antibiotic resistance (Baker-Austin et al. 2006). Stagnant conditions caused by intermittent water demand, which are common in urban areas, may also promote the growth of pathogens and other microorganisms potentially harboring ARGs and MRGs in DWDS (Lautenschlager et al. 2010; Proctor and Hammes 2015; Zlatanović et al. 2017; Bédard et al. 2018; Ling et al. 2018). Additionally, locations within DWDS that support higher densities of microorganisms (e.g., biofilms, tubercles, loose deposits) may aid in the persistence of antibiotic resistance in DWDS (Hallam et al. 2001). The overall growth of microorganisms and microbial ecology in DWDS is influenced by several distribution system characteristics and may have implications on the types and abundance of resistance genes in DWDS. Even though metals are known to select for antibiotic resistance, and they are present in DWDS due to corrosion of metal pipes and addition of corrosion inhibitors, research gaps persist around the impact of metals on antibiotic resistance in DWDS.

Previous analysis of microbial ecology in DWDS has been accomplished primarily through next-generation sequencing (NGS) technologies including 16S rRNA gene amplicon sequencing and DNA-based metagenomic analysis (Berry et al. 2006; Pinto et al. 2012; Douterelo et al. 2014; Ma et al. 2019). Many of these studies have investigated the drinking water microbiome during various stages of drinking water treatment and distribution, which can be influenced by many factors including spatial and

temporal variations (Pinto et al. 2014; Prest et al. 2016; Potgieter et al. 2018), geography (Proctor and Hammes 2015), treatment processes (Ma et al. 2017b; Oh et al. 2018), and distribution system characteristics (Sanganyado and Gwenzi 2019). Laboratory-scale studies have observed relationships between several distribution system parameters and the occurrence of opportunistic pathogens and microbial ecology in simulated DWDS including disinfectants (Shen et al. 2017; Zhang et al. 2019a), water age (Wang et al. 2012b; Wang et al. 2014c), water temperature (Proctor et al. 2017), and pipe materials (Wang et al. 2012b; Wang et al. 2014c; Proctor et al. 2017). ARG profiles are impacted by the microbial communities in a given environment, and many of these previously studied parameters likely have impacts on antibiotic resistance in drinking water systems but have yet to be investigated. Additionally, few studies have utilized quantitative molecular tools to quantify the abundance of clinically-relevant ARGs or MGEs in different phases (planktonic, biofilm, particle-associated, loose deposits, etc.) of full-scale DWDS (Garner et al. 2018a).

In this review, existing information on the impacts of metal pipe materials, corrosion inhibitors, and corrosion products on the prevalence of antibiotic resistance in DWDS is critically reviewed with the goal of consolidating information to help develop more effective monitoring and mitigation strategies along with recommended future research directions. As older DWDS get upgraded, there is an urgent need for improving our understanding of engineering management decisions such as pipe material selection on the dissemination of antibiotic resistance in DWDS. Here, we provide information regarding the antimicrobial activity of metals and potential mechanisms of antibiotic resistance selection in DWDS with specific emphasis on corrosion products and

corrosion inhibitors. This review also summarizes existing information available regarding the abundance of ARGs, MRGs, and MGEs in DWDS. Additionally, recommendations are provided for future research directions with a focus on improving mitigation strategies for antibiotic resistance in DWDS.

## **2.2 Quantification of Resistance Genes in Full-Scale Drinking Water Systems**

Understanding the sources and mechanisms of antibiotic resistance dissemination in drinking water networks is critical for mitigating risks to public health. Previous studies have highlighted that several factors related to drinking water treatment (Sharma et al. 2016; Sanganyado and Gwenzi 2019) and distribution system construction and operation can influence the abundance of bacteria in drinking water and biofilms (Douterelo et al. 2014; Sun et al. 2014a; Douterelo et al. 2016). After finished drinking water enters the distribution system, it may spend hours to days inside pipes prior to reaching consumer taps, which can alter the chemical and microbiological quality of the water (Ji et al. 2015). Corrosion inhibitors, pH, dissolved inorganic carbon, disinfectant type/concentration, and other chemicals used during drinking water treatment (e.g., coagulants) may impact the presence and distribution of ARB and ARGs in DWDS (Liu et al. 2016; Zhang et al. 2017a). A previous laboratory-scale study documented increased transfer of ARGs following exposure to disinfectants (free chlorine, chloramine, and hydrogen peroxide) and suggested that mechanisms including intracellular reactive oxygen species formation, SOS response, increased cell permeability, and altered expression of conjugation-relevant genes were responsible for horizontal gene transfer (Zhang et al. 2017a). Additionally, Kappell *et al.* 2019 observed increased selection for

ARB and ARGs including *sul1*, *qacH*, and *intI1* in drinking water microcosms treated with zinc-containing corrosion inhibitors compared to controls. This study reported that low concentrations of metals such as zinc in drinking water may lead to positive selection of bacteria able to assimilate metals, rather than negatively selecting against bacteria without metal resistance (Kappell et al. 2019). Although these studies have provided some insight into the potential mechanisms of antibiotic resistance selection under controlled laboratory conditions, there is a lack of data available regarding the presence of clinically-relevant ARGs, MRGs, and MGEs commonly linked to horizontal gene transfer processes in full-scale DWDS. ARGs including beta-lactam resistance genes, *sul1*, and MGEs such as *intI1* have previously been identified as potential indicators for the overall abundance of resistance genes in different environments (WHO 2014; Gillings et al. 2015; Ma et al. 2017a). MRGs such as *arsB*, *copA*, *czcD*, and *zntA* confer resistance to metals, and may be co-selected for by bacterial exposure to antibiotics, disinfectants, and other contaminants (Pal et al. 2017). Further, a previous study documented the co-occurrence of MRGs and ARGs on plasmids in clinically-relevant genera including *Escherichia*, *Staphylococcus*, *Salmonella*, *Klebsiella*, *Pseudomonas*, and *Mycobacterium* (Pal et al. 2015). However, no studies known to the authors have documented MRGs in samples from full-scale DWDS using quantitative molecular techniques, i.e., techniques that generate copy numbers on a per L, g, or surface area basis. Few studies have employed quantitative techniques to quantify ARGs in full-scale DWDS (summarized in **Table 2.1**).

Studies documenting the occurrence and abundance of ARGs are important for developing effective monitoring strategies for controlling microbial drinking water

quality. Total bacterial biomass (e.g., 16S rRNA) can range several orders of magnitude ( $\sim 10^3 - 10^{10}$  copies/L) in drinking water (**Table 2.1**, Xi et al. 2009; Garner et al. 2018b; Garner et al. 2019). ARGs including beta-lactam resistance genes (*bla*<sub>TEM</sub>), sulfonamide genes (*sul1* and *sul2*), tetracycline resistance genes (*tet*(W), *tet*(M), *tet*(X)), and the integrase gene of class 1 integrons (*intI1*) have been quantified in DWDS at concentrations ranging from approximately  $10^1$  to  $10^7$  copies/L (**Table 1**, Xi et al. 2009; Garner et al. 2018a; Su et al. 2018; Hao et al. 2019; Rocha et al. 2019; Zhang et al. 2020). The majority of previous studies focused on measuring ARG abundance in the planktonic phase and did not measure gene concentrations in biofilms or corrosion deposits, which have been cited as important reservoirs for opportunistic pathogens and ARGs (Balcázar et al. 2015). Other studies have utilized high-throughput qPCR (HT-qPCR) for detecting ARGs in DWDS, with detections of over 100 different ARGs, transposases, and integrons in drinking water samples (Xu et al. 2016; Huang et al. 2019; Waseem et al. 2019). These studies and others have observed increases in transposase genes,  $\beta$ -lactam resistance genes, and MGEs in DWDS water compared to treated drinking water entering the DWDS.

Many environmental factors exist in DWDS that may influence the abundance of ARGs. It is well documented that disinfection practices (e.g., chlorination or chloramination), antibiotics, antimicrobials, and metals may exacerbate the prevalence of antibiotic resistance in engineered systems such as DWDS. However, the fate of ARGs, MRGs, and mechanisms of horizontal gene transfer in full-scale DWDS are not well understood. Additionally, previous studies have suggested that extracellular DNA may persist in aquatic systems for long periods of time and may represent an important

reservoir of ARGs in DWDS (Hao et al. 2019; Sakcham et al. 2019). Due to potential public health implications associated with the presence of opportunistic pathogens, ARGs, and MRGs in drinking water, this area of research warrants further investigation.

**Table 2.1** ARGs Quantified in Full-scale Drinking Water Distribution Systems (DWDS)

Reference	Garner et al 2018				Hao et al 2019	Rocha et al 2019	Su et al 2018	Xi et al 2009	Zhang et al 2020
<b>Sample Type</b>	Tap Water	Tap Water	Biofilms	Biofilms	Tap Water	Tap Water	Tap Water	Tap Water	Tap Water
<b>Method of Quantification</b>	qPCR	qPCR	qPCR	qPCR	qPCR	qPCR	qPCR	qPCR	qPCR
<b>Units</b>	copies/mL	copies/mL	copies/swab	copies/swab	copies/L	copies/mL	copies/L	copies/100 mL	copies/100 mL
<b>Replicates</b>	44	56	40	21	36	3	9	8	71
<b>Source Water</b>	SW, GW	SW, GW	SW, GW	SW, GW	NA	NA	NA	NA	SW
<b>Treatment Type(s)</b>	5-stage BF, AS, DN	4-stage BF, UV, biofilter	dual media filter, MB	dual media filter	NA	NA	Sed., SF, PAC, O <sub>3</sub>	NA	NA
<b>Pipe Material(s)</b>	NA	NA	NA	NA	NA	NA	NA	NA	NA
<b>Disinfectant</b>	NH <sub>2</sub> Cl	Cl <sub>2</sub>	NH <sub>2</sub> Cl	Cl <sub>2</sub>	Cl <sub>2</sub>	NH <sub>2</sub> Cl	NA	NH <sub>2</sub> Cl	NA
<b>Observed Mean Absolute Abundance of Target Genes (Log<sub>10</sub> scale)</b>									
16S rRNA	4.2	2.3 - 3.3	4.2	3.5	-	6.0	7.0 - 7.6	5.4 - 7.0	5.2 - 7.4
<i>bla</i> <sub>TEM</sub>	1.8	1.7	1.5	1.6	3.5 - 5.5	-	-	2.2 - 3.4	2.0 - 5.0
<i>bla</i> <sub>SHV</sub>	-	-	-	-	-	-	-	0 - 1.5	-
<i>ermB</i>	-	-	-	-	2.5 - 3.8	-	5.0 - 5.5	-	ND - 5.0
<i>intI1</i>	4.7	4.9	4.2	ND	-	-	-	-	2.0 - 4.5
<i>tet</i> (M)	-	-	-	-	2.0 - 5.4	-	5.5	-	-
<i>tet</i> (W)	-	-	-	-	-	-	5.6 - 5.8	-	ND - 4.5
<i>tet</i> (X)	-	-	-	-	2.2 - 3.8	-	ND	-	-
<i>qnrA</i>	1.2	ND	ND	2.1	2.0 - 3.6	-	ND	-	-
<i>qnrS</i>	-	-	-	-	-	-	5.0	-	-
<i>sul1</i>	2.7	1.2 - 1.7	3.2	2.1	4.6 - 6.8	2.1	7.5	0.9 - 3.2	3.0 - 6.0
<i>sul2</i>	-	-	-	-	3.5 - 5.8	-	6.0	0.1 - 4.2	ND - 5.7
<i>vanA</i>	1.4	1.9	1.5	1.3	0 - 2.5	-	-	-	-

Notes: AS = activated sludge, BF = Bardenpho, GAC = granular activated carbon, PAC = powdered activated carbon, MB = membrane bioreactor, O<sub>3</sub> = ozone, Sed. = sedimentation, SF = sand filter, UV = ultraviolet disinfection, NA = not available, ND = no detection, “-“ = no measurement or value, qPCR = quantitative PCR. Mean abundance values for target genes were adapted from source article(s) text or approximated from figures.

### **2.3 Plausible Mechanisms for Antibiotic Resistance Selection by Metals in DWDS**

Previous studies on lab-scale and full-scale DWDS have focused on the impact of a variety of factors on microbial ecology in DWDS including pipe materials (Niquette et al. 2000; Lehtola et al. 2005; Proctor et al. 2017; Douterelo et al. 2020), flow regime (Manuel et al. 2007; Lautenschlager et al. 2010), nutrients (Batte et al. 2003), and disinfectant type (chlorine/chloramines) (Wang et al. 2012b; Aggarwal et al. 2018; Dai et al. 2019; Waak et al. 2019a), but they have seldomly focused specifically on the impact of metals on antibiotic resistance. Drinking water networks are comprised of various metal pipe materials including cast-iron, ductile iron (Fe), copper (Cu), brass, and lead (Pb). Metal species such as Cu, Fe, and Pb are primarily introduced to drinking water through corrosion processes and leaching of metal ions from plumbing materials (Kang et al. 2008; Kim and Herrera 2010). Zinc (Zn) is present when added as a corrosion inhibitor attached to phosphate (Payne et al. 2016). Additional heavy metals such as aluminum (Al), arsenic (As), chromium (Cr), and uranium (U) present at concentrations below their maximum contaminant levels (MCLs) or at non-detectable levels in treated drinking water can accumulate in deposits and corrosion scales in DWDS (Lytle et al. 2004; Schock et al. 2008; Peng and Korshin 2011; Peng et al. 2012). The primary concern with the presence of trace metals in DWDS is the potential for their release back into finished drinking water, which may result in elevated dissolved metal levels in domestic tap water (Sun et al. 2017). Heavy metals present in drinking water environments at sub-inhibitory concentrations can promote antibiotic resistance and horizontal transfer of ARGs (Baker-Austin et al. 2006; Zhang et al. 2018a; Zhang et al.

2018b). In addition, positive correlations have previously been observed between multiple antibiotic resistance and metal exposure (Cu, Pb, Zn) in drinking water point of use samples compared to point of entry to the DWDS, suggesting that bacteria acquired antibiotic resistance inside the DWDS (Calomiris et al. 1984).

Microorganisms have developed a variety of methods for coping with environmental stress, such as exposure to heavy metals. General mechanisms responsible for metal resistance in microbial cells include (i) exclusion by permeability barrier, (ii) active efflux, (iii) intra- and extracellular sequestration, (iv) enzymatic detoxification, and (v) decreased sensitivity of cellular targets to metal ions (Bruins et al. 2000; Harrison et al. 2009). Some microorganisms such as *Pseudomonas aeruginosa* can upregulate the expression of extracellular polymers or siderophores (metal-chelating agents) in response to metal exposure, which contain functional groups capable of binding to metal ions (Lemire et al. 2013). The role of siderophores is mainly to scavenge Fe, but can also form complexes with other essential metals (Mo, Mn, Co, and Ni) and make them available for microbial cells (Ahmed and Holmström 2014). Extracellular polymers and siderophores can precipitate metal ions in the extracellular environment, and soluble siderophores bound to metals may be subject to reduced uptake into microbial cells or increased efflux out of the cell by membrane transporters (Braud et al. 2009; Hannauer et al. 2012). Additionally, biofilms provide an advantageous way for microorganisms to survive on metallic pipes in DWDS, and a previous study reported growth of over  $10^7$  cells per  $\text{cm}^2$  after only 30 days on metal pipe materials including stainless steel and copper (Morvay et al. 2011). Molecular mechanisms responsible for stimulating horizontal gene transfer after exposure to metals (Cu, Ag, Cr, and Zn) include intracellular reactive oxygen

species (ROS) formation, SOS response, increased cell membrane permeability, and altered expression of conjugation-relevant genes (Zhang et al. 2018a). This study and other studies regarding the potential for horizontal gene transfer following metal ion exposure have suggested that selection mechanisms such as co-resistance and cross-resistance likely play a significant role in the development of antibiotic resistance in metal-contaminated environments (Baker-Austin et al. 2006; Seiler and Berendonk 2012; Knapp et al. 2017). In addition to co-resistance and cross-resistance, co-regulatory mechanisms may also promote the co-selection process, which occurs when a single regulatory gene controls multiple resistance genes that confer resistance to different compounds (Pal et al. 2017).

Co-resistance is defined as two or more genetically linked resistance genes, indicating that the genes responsible for two or more resistances are located next to each other on one MGE (Chapman 2003; Ju et al. 2016). Hasman and Aarestrup (2002) observed a correlation between copper resistance and resistance to macrolides and glycopeptides in *Enterococcus faecium* obtained from copper-exposed pigs and documented the co-transfer of copper- and macrolide-resistant phenotypes in transconjugants (Aarestrup et al. 2002; Hasman and Aarestrup 2002; Hasman and Aarestrup 2005). The physical linkage results in the co-selection of other genes located on the same genetic element when an organism is exposed to a particular stressor, such as an antimicrobial compound or metal (Baker-Austin et al. 2006; Poole 2017). Even at environmentally-relevant and sub-inhibitory concentrations, the presence of metals such as Cu has been positively correlated with ARGs, which is an indication that sublethal levels of Cu can increase ARG prevalence (Knapp et al. 2017; Zhang et al. 2018b). The

high redox potential of Cu has also allowed it to serve as an ideal biological co-factor, particularly in aerobic microorganisms (Outten et al. 2001; Rensing and Grass 2003). Previous studies have suggested that sub-inhibitory levels of copper ions present in DWDS can stimulate growth of microorganisms (Zhang et al. 2009b), catalyze decay of disinfectant residuals (chloramines) (Nguyen et al. 2012), and cause microbial community shifts (Proctor et al. 2017; Rhoads et al. 2017). Alternatively, copper and silver ion exposure can inhibit growth of microorganisms such as *Legionella pneumophila* at concentrations ranging from 0.20 – 4.0 mg/L (Liu et al. 1994; Kusnetsov et al. 2001; Van Der Kooij et al. 2005) and a strong inhibitory effect on nitrifying bacteria has been reported following copper exposure exceeding 0.90 mg/L (Zhang et al. 2008). The enrichment of antibiotic resistant microorganisms in metal contaminated environments has been attributed to the selection of organisms harboring resistance genes for agents (antibiotics and metals) on chromosomes or plasmids (Poole 2017). Furthermore, a survey of soils in Scotland demonstrated a significant correlation between Cu levels and the occurrence of ARGs including *erm*(B), *erm*(F), *tet*(M), and *tet*(W) (Knapp et al. 2011). This study also observed positive correlations between Fe and Pb in soils and the occurrence of ARGs including *tet*(M), *tet*(W), *bla*<sub>TEM</sub>, and *bla*<sub>OXA</sub>.

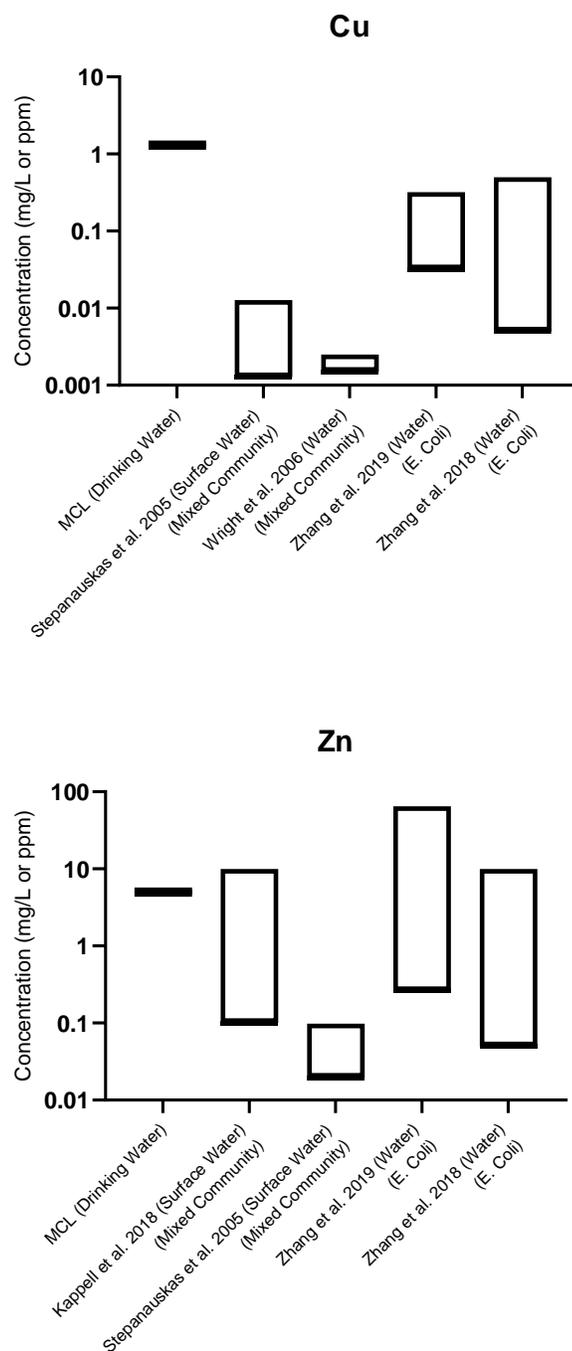
Cross-resistance is primarily observed through multidrug efflux pumps which can rapidly extrude structurally dissimilar compounds out of the cell, such as heavy metals and antibiotics (Baker-Austin et al. 2006; Martinez et al. 2009). Previous research has suggested that the abundance of class 1 integrons observed in metal contaminated environments were associated with an increase in antibiotic resistance, as class 1 integrons are closely located to genes encoding for the multidrug efflux pump *czcA*

(which can expel Zn, Cd, and Co) (Seiler and Berendonk 2012). Class 1 integrons are genetic elements capable of acquiring and exchanging DNA fragments called gene cassettes (Hall et al. 1999). The presence of integrons and other MGEs can mediate a selective advantage to microorganisms exposed to stressful environmental conditions (e.g., exposure to metals). Previous studies have observed significant positive correlations between MGEs and ARGs in aquatic environments including *bla*<sub>TEM</sub>, *ermA*, *sul1*, *tet(O)*, *tet(W)*, and *tet(X)* (Pruden et al. 2006; Szekeres et al. 2018; Dong et al. 2019).

Exposure to heavy metals may trigger co-selection responses, but can also increase the level of tolerance to antibiotics due to co-regulation of resistance genes (Seiler and Berendonk 2012). Guo et. al observed an increase in the tetracycline resistance gene (*tet(Q)*) in gut microbiota of mice exposed to Fe and suggested that iron exposure alone could potentially alter the diversity and functions of gut microbiota and the abundance of ARGs and MGEs (Guo et al. 2014). Some heavy metals (e.g., Pb) have no known function in bacterial cells but can cause oxidative stress, which could promote generation of ROS and facilitate horizontal gene transfer in the distribution system (Knapp et al. 2011). The dominant mechanisms of co-selection for metal- and antibiotic resistance remain relatively unknown, especially in drinking water environments.

Previous studies have suggested that there may be more limited opportunities for metals to drive horizontal gene transfer of antibiotic resistance; however, numerous possibilities exist for metals to select for ARB through chromosomal MRGs (Pal et al. 2014; Pal et al. 2017). Additionally, transposable elements originating in chromosomal DNA can jump to plasmids carrying ARGs and MRGs that can easily be transferred to other microorganisms through transformation or conjugation.

Metal pipe materials, including Cu, Fe, and Pb commonly used in DWDS including water mains, service connections, and premise plumbing, can influence microbial community composition and the presence of opportunistic pathogens (Rozej et al. 2015; Proctor et al. 2017; Neu et al. 2019). In fact, sub-inhibitory exposure to Cu and Zn can stimulate antibiotic resistance in water environments at concentrations below their respective maximum contaminant levels (MCLs) (**Figure 2.1**). While MCLs are based on direct impacts to human health, these data imply that indirect consequences including selection for antibiotic resistance can occur at levels below the MCLs. Co-selection of ARGs and MRGs is likely ubiquitous in full-scale DWDS due to the presence of metals, disinfectants, and antibiotics. Several bacteria genera commonly detected in DWDS contain plasmids with both ARGs and MRGs including *E. coli*, *Staphylococcus*, *Pseudomonas*, and *Mycobacterium* (Pal et al. 2015). Despite existing knowledge regarding drinking water biofilms, studies regarding the occurrence or distribution of ARGs, MRGs, or MGEs using molecular techniques such as qPCR or droplet digital PCR (ddPCR) are limited. Quantitative measurements of ARB, ARGs, and MRGs in drinking water and biofilms are essential for improving risk assessments for potable water systems. Additionally, fundamental research from laboratory-scale studies and full-scale DWDS surveys providing information on pipe materials and antibiotic resistance are both necessary to better understand how engineering management decisions impact human health.



**Figure 2.1** Studies documenting antibiotic resistance in response to metal exposure in water environments (Cu and Zn). The United States Environmental Protection Agency maximum contaminant level (MCL) for drinking water quality is plotted for reference. References for data in figure (Stepanauskas et al. 2005; Wright et al. 2006; Zhang et al. 2018b; Zhang et al. 2018c; Kappell et al. 2019; Zhang et al. 2019b).

## 2.4 Impact of Corrosion Products on Antibiotic Resistance

Corrosion of drinking water infrastructure occurs due to oxidation processes of metal materials and results in subsequent formation and accumulation of corrosion products on metal surfaces (McNeill and Edwards 2001). Various chemical oxidation processes that occur in drinking water environments can degrade pipe surfaces, valves, and connections, and gradually rust metal surfaces. Corrosion of drinking water pipes is responsible for destruction of the pipe material and also the deterioration of the drinking water quality in the distribution system (Sun et al. 2014b). Corrosion scales are formed by the accumulation of corrosion products and other suspended particles on pipe surfaces, which reduces the capacity of the system and provides habitats for potentially pathogenic microorganisms (Wang et al. 2012b; Sun et al. 2014b).

Corrosion of drinking water pipes can occur due to a variety of circumstances including increased pipe service age, water chemistry, and operational parameters such as stagnation time and flow velocity (Flemming et al. 2002; Lehtola et al. 2006; Xie and Giammar 2011). Passivation of drinking water pipes occurs over time by the formation of corrosion products on pipe surfaces, and the formation of passivating scales in DWDS pipes lowers the potential for metal ions to leach into finished drinking water, but also provides increased surface area for bacterial attachment and biofilm formation (Zhu et al. 2014; Li et al. 2015). Corrosion products are comprised of scale minerals and possibly additional contaminants deposited from drinking water which may impact antibiotic resistance and horizontal gene transfer of ARGs in drinking water systems. Corrosion products that form in iron pipes typically consist of iron(III) oxyhydroxides (e.g., goethite

( $\alpha$ -FeOOH), lepidocrocite ( $\gamma$ -FeOOH)), iron(II, III) oxides (e.g., magnetite ( $\text{Fe}_3\text{O}_4$ )), and/or iron(II) carbonates (e.g., siderite ( $\text{FeCO}_3$ )) (McNeill and Edwards 2001; Peng et al. 2010; Yang et al. 2012). Common lead corrosion products include lead(II) oxides (e.g., litharge ( $\alpha$ -PbO)), lead(II) carbonates (e.g., cerussite ( $\text{PbCO}_3$ ), hydrocerussite ( $\text{Pb}_3(\text{CO}_3)_2(\text{OH})_2$ )), lead(II) phosphates (e.g., hydroxylpyromorphite ( $\text{Pb}_5(\text{PO}_4)_3\text{OH}$ )) and lead(IV) oxides (e.g., scrutinyite ( $\alpha$ -PbO<sub>2</sub>), plattnerite ( $\beta$ -PbO<sub>2</sub>)) (Lytle and Schock 2005; Schock et al. 2005b; Schock et al. 2005a; Kim and Herrera 2010; Xie and Giammar 2011). Compared to iron and lead focused work, very few studies have investigated copper corrosion under conditions relevant to drinking water distribution, and cuprous oxide ( $\text{Cu}_2\text{O}$ ) and/or cupric oxide ( $\text{CuO}$ ) are considered the dominant copper corrosion products (Feng et al. 1996; McNeill and Edwards 2004; Xiao et al. 2007; Kang et al. 2008; Lytle and Nadagouda 2010). Further, there is a paucity of research regarding the impacts of these metal corrosion products on microbial growth, ecology, and potential selection for antibiotic resistance in DWDS.

Formation of drinking water corrosion products is extremely heterogeneous and is strongly affected by water chemistry parameters, such as pH, dissolved inorganic carbon, dissolved oxygen, disinfectant type, natural organic matter, and use of corrosion inhibitors (Volk et al. 2000; Sarin et al. 2004; Xie et al. 2010b; Noel et al. 2014). For instance,  $\text{PbO}_2$  has been observed as a common lead corrosion product in various distribution systems that use free chlorine as the disinfectant, while lead(II) phosphates have been identified as the predominant corrosion products in systems using phosphate corrosion inhibitors (Lytle and Schock 2005; Kim and Herrera 2010). Furthermore, corrosion products in DWDS can exhibit significantly different morphological and

structural composition and be sinks for various inorganic contaminants such as As, Cd, Cr, manganese (Mn), nickel (Ni), and vanadium (V) (Schock et al. 2008; Peng and Korshin 2011; Gerke et al. 2016). For example, some common iron corrosion products, such as goethite, lepidocrocite and magnetite have strong affinities to adsorb and concentrate trace heavy metals present in drinking water (Sarin et al. 2001; Peng et al. 2010). Consequently, the corrosion products and additional contaminants that accumulate from drinking water on biofilms and pipe surfaces may subsequently impact the distribution and abundance of ARB and ARGs present in drinking water systems.

Similar to dissolved and particulate metals in aquatic environments, exposure of bacteria to corrosion products in DWDS may alter microbial communities and increase the potential for horizontal gene transfer of ARGs and MRGs. Previous studies have suggested that exposure of microbial populations to metal pipe materials and corrosion scales that accumulate in DWDS may eliminate some microorganisms while allowing metal-tolerant organisms to survive, which can potentially disseminate bacteria resistant to both metals and antibiotics in finished drinking water (Calomiris et al. 1984; Baker-Austin et al. 2006). Furthermore, a previous study demonstrated that exposure to cuprous oxide resulted in bacteriostatic effects against gram positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus faecalis*) and gram negative bacteria including *Pseudomonas aeruginosa* and *Enterobacter cloacae* (Pang et al. 2009). Exposure of bacterial populations to copper corrosion products such as cuprous oxide and cupric oxide could increase selection for copper-related resistance genes and ARGs in organisms harboring resistance genes for both agents (Aruoja et al. 2009; Pang et al. 2009; Hans et al. 2013; Poole 2017). For example, the presence of  $\text{Cu}^{2+}$  ions and  $\text{CuO}$

nanoparticles (1-100  $\mu\text{mol/L}$ ) increased the conjugative transfer of ARGs across bacterial genera, increased cell membrane permeability, and caused the overproduction of ROS (Zhang et al. 2019b). Bacterial exposure to CuO nanoparticles at sub-inhibitory levels resulted in expression of stress response genes including *dps*, *sodA*, *sodB*, *trxC*, *katE*, and *katG* (Zhang et al. 2019b). Additionally, corrosion scales provide bacteria with organic matter and nutrients, consume residual disinfectant concentrations, provide increased surface area for bacterial adsorption, and provide iron oxides as potential electron acceptors that may increase bacterial activity (Appenzeller et al. 2002; Jang et al. 2012). Consequently, bacterial exposure to corrosion scales in DWDS containing copper and iron oxides may increase bacterial growth and selection for bacteria harboring ARGs and MRGs in finished drinking water.

There has been limited research conducted regarding different corrosion products and their effects on the abundance of ARB, ARGs, or MRGs in biofilms and drinking water in DWDS. Laboratory-scale studies are needed to understand the fundamental impacts of different corrosion products such as copper oxides and iron oxides on microbial communities and the antibiotic resistome. Full-scale studies are necessary to characterize the distribution of different corrosion products, resistance genes, and microbial communities in full-scale DWDS. Understanding the relationship of corrosion products and antibiotic resistance could provide critical information necessary to improve decisions regarding the selection of pipe materials for potable water systems.

## 2.5 Impact of Corrosion Inhibitors on Antibiotic Resistance

Optimized corrosion control treatment (OCCT) is a specific requirement of the Lead and Copper Rule (LCR) introduced in 1991 by the US Environmental Protection Agency (US EPA) (Brown et al. 2013). Drinking water utilities have three options for corrosion control treatment including adjusting the pH and alkalinity of drinking water, developing Pb(IV) scale by maintaining free chlorine residuals throughout the distribution system, and using corrosion inhibitors such as orthophosphate or sodium silicates (Brown et al. 2013). Corrosion inhibitors commonly applied in drinking water systems include polyphosphates, orthophosphate, zinc orthophosphate, and sodium silicates (Edwards and McNeill 2002; Schock et al. 2005a; Cartier et al. 2012). Recent studies have cited the potential for phosphate-containing corrosion inhibitors to serve as a nutrient source for bacterial growth, which has raised concerns in some U.S. drinking water utilities (Edwards and McNeill 2002; Fang et al. 2009). Therefore, it is important to gain a better understanding of the impacts of different corrosion inhibitors on microbial ecology and the abundance of ARGs and MRGs in full-scale DWDS.

A national survey of water utilities revealed that over 50% of utilities used phosphate inhibitors, and zinc orthophosphates were the most common phosphates applied (McNeill and Edwards 2002) with over half of utilities reporting doses between 0.7 – 2 mg/L as phosphate (McNeill and Edwards 2002). Bacteria typically require a ratio for C:N:P of approximately 100:10:1, and many DWDS are P deficient (LeChevallier et al. 2011; Brown et al. 2013). Research regarding the effects of phosphate addition on drinking water microbial communities has yielded mixed results. Several studies have

reported increased bacterial growth in drinking water systems with phosphate exposure levels ranging from 0.001 to 0.03 mg/L P (Lehtola et al. 2002; Chu et al. 2005; Fang et al. 2009; Payne et al. 2016). Phosphorus addition to drinking water can promote biofilm cell growth, decrease EPS production, and induce biofilms with increased thickness and biomass (Fang et al. 2009). By stimulating microbial growth in drinking water biofilms, phosphorus addition may also lead to increased abundance of ARB and ARGs.

Polyphosphate addition to drinking water may impact biofilm growth resulting in softer, thicker biofilms that are more prone to detachment (Shen et al. 2018). Further, the addition of polyphosphate can supply nutrients to bacteria for biofilm development, play an important role in bacteria metabolic regulation, and increase bacterial resistance to environmental stress (Rao and Kornberg 1996; Gangaiah et al. 2009). Zinc orthophosphate addition has been shown to increase microbial community diversity in drinking water biofilms (Payne et al. 2016). Another study reported that phosphate addition did not increase total biomass in biofilm communities, but did cause shifts in microbial community composition (Batté et al. 2003). Similarly, Jang *et al.* observed increased community richness and diversity in biofilms that developed on stainless steel and ductile cast-iron coupons with phosphate addition compared to controls without phosphate addition (Jang et al. 2012). The use of zinc orthophosphate for corrosion control may also result in the selection of resistant microorganisms in DWDS due to the potential for zinc to select for ARGs and MRGs (Peltier et al. 2010; Kappell et al. 2019). As a result, the use of orthophosphate and zinc orthophosphate as corrosion inhibitors influences microbial ecology and may also impact the abundance and distribution of resistance genes in DWDS. However, limited research has been conducted regarding the

effects of different corrosion inhibitor treatments on the presence of ARB, ARGs, and MRGs in full-scale DWDS.

Sodium silicates are an alternative to phosphate-based corrosion inhibitors and have been reported to be an effective strategy for lead control in some systems since the 1920s (Butler and Ison 1966; Schock et al. 2005a; Lintereur et al. 2010). Similar to polyphosphates, the molecular composition of sodium silicates are indeterminate, with the chemical formula ( $\text{Na}_2\text{O} : n(\text{SiO}_2)$ ), where  $n$  is a variable ratio (Crittenden et al. 2012). Typical dosages of silicates range from 4 to 30 mg/L as  $\text{SiO}_2$ , and higher doses are required for drinking water with higher hardness, higher chlorides, and/or higher dissolved solids (Lane et al. 1977). Silicates and phosphates act as anodic inhibitors when used at low doses for corrosion control treatment of drinking water, which reduces corrosion by forming a protective layer of oxide film on pipe surfaces. The Illinois State Water Survey conducted extensive testing with corrosion inhibitors and recommended that silicates were the best option for corrosion inhibition in galvanized steel and copper-based piping commonly used in domestic hot-water systems (Lane et al. 1977). The use of sodium silicates may have advantages over phosphate-based treatments due to the lack of phosphorus and zinc, which could potentially limit microbial growth and levels of ARGs in finished drinking water. A previous study observed decreased average bacterial growth (e.g., lower ATP concentrations) in biofilms subjected to sodium silicate treatment compared to phosphate-containing corrosion inhibitors, but the differences were not statistically significant (Kogo et al. 2017a). Additionally, Rompré et al. (2000) compared heterotrophic plate counts (HPC) between sodium silicate and phosphate treatments in a study conducted with laboratory-scale annular reactors and in a confined

section of a full-scale DWDS, but reported no significant differences between corrosion control treatments (Rompré et al. 2000). This study also concluded that surface material (unlined grey iron vs. polycarbonate) was a larger factor influencing biofilm development in DWDS pipes compared to corrosion inhibitor type. Despite previous analysis of corrosion inhibitors in DWDS, the impact of corrosion inhibitor type on the abundance of ARB, ARGs, and MRGs has yet to be elucidated in full-scale systems. Laboratory-scale and full-scale studies investigating the impacts of corrosion inhibitors and their impacts on the development of corrosion products and microbial communities are needed to better understand mechanisms of antibiotic resistance selection in DWDS.

Little information exists regarding the impacts of sodium silicates on microbial ecology and the proliferation of antibiotic resistance in full-scale drinking water systems. A previous study investigated the effects of sodium silicate addition on cucumber seedling growth and resistance to the soil-borne pathogen *Fusarium oxysporum* f.sp. *cucumerinum* Owen (Zhou et al. 2018). The study reported changes in bacterial and fungal community structure in response to two mM sodium silicate addition in addition to decreased abundance of microbial taxa containing pathogens (Zhou et al. 2018). Consequently, the addition of sodium silicates to drinking water for corrosion control may also impact bacterial community composition but has yet to be investigated. In order to mitigate public health impacts related to corrosion of drinking water infrastructure, future studies should consider the impacts of different corrosion inhibitor regimes on microbial ecology and the abundance of ARGs and MRGs in drinking water systems.

## 2.6 Conclusions and Research Gaps

Several studies have documented the presence, though not necessarily the quantity, of ARB and ARGs in drinking water environments. Factors such as metal pipe materials, corrosion inhibitors, and corrosion products that develop in full-scale DWDS may select for bacteria harboring resistance to both metals and antibiotics. There is a lack of information regarding the abundance and fate of ARB, ARGs, MRGs, and MGEs in different phases of DWDS including planktonic, biofilm, suspended solids, and loose corrosion deposits. Previous research has demonstrated that water quality deterioration in DWDS can result in increased levels of ARB and ARGs in consumer tap water, which poses a risk to human health.

There is a specific need for information regarding presence and distribution of AR in DWDS and research should prioritize monitoring the occurrence, fate, and distribution of ARB, ARGs, MRGs, and MGEs in multiple phases in full-scale systems. This information is critical for gaining a better understanding of the prevalence of antibiotic resistance in engineered systems capable of directly impacting human health. Previous characterization of DWDS has primarily focused on microorganisms in the planktonic phase and studies documenting and quantifying ARB, ARGs, or MRGs in drinking water biofilms are lacking. Drinking water biofilms have been cited as important reservoirs for opportunistic pathogens and horizontal gene transfer of ARGs (Wingender and Flemming 2011; Balcázar et al. 2015), however, the dominant pathways of antibiotic resistance selection in drinking water are not well documented. In addition to full-scale studies, laboratory-scale studies are needed to distinguish the impacts of different pipe materials,

corrosion inhibitors, and corrosion products on the abundance of resistance genes in drinking water and biofilms. These studies will provide critical information regarding the relationship of different system parameters and mechanisms of antibiotic resistance selection in engineered systems. Locations with high densities of bacteria, such as drinking water biofilms, provide conditions which are suitable for proliferation and exchange of resistance genes, and selective pressures (e.g., metals) may increase the abundance of resistance genes in these communities. Additional research is needed to address the research gaps related to the fate and transport of clinically-relevant resistance genes in DWDS.

Fundamental information regarding the impacts of pipe materials, metals, and corrosion inhibitors on pathogens and abundance of resistance genes in DWDS is necessary to reduce the prevalence of antibiotic resistance in drinking water. Different pipe materials used in full-scale DWDS may influence the composition of microbial communities and associated resistance genes that can subsequently be transferred to humans through dermal contact, consumption of contaminated drinking water, or inhalation of aerosols during bathing (Falkinham 2009; Garner et al. 2018b). Further research including quantitative microbial risk assessments (QMRA) are needed to determine the concentration of ARB or ARGs that may translate to human health risks (Ashbolt et al. 2013b). Research that links ARGs to hosts and distinguishes extracellular DNA from intracellular DNA is also needed (Rice et al. 2020). Further, limited availability of exposure assessments and dose-response data regarding ARB and ARGs for different scenarios hinder the implementation of QMRA approach for evaluating human health risks in aquatic environments (Amarasiri et al. 2019).

The selection of target genes is also important for improving routine monitoring of ARGs, MRGs, and MGEs in full-scale distribution systems. Future research and microbial surveillance efforts should consider monitoring clinically-relevant ARGs and other genes commonly linked to horizontal gene transfer processes including beta-lactam resistance genes, *intI1* and *sul1* (WHO 2014; Gillings et al. 2015; Ma et al. 2017a). The prevalence of dissolved, particulate, and solid phase metals in DWDS warrants further investigation regarding their potential for selection of resistance genes in drinking water biofilms. Additionally, the collection of samples from multiple locations (biofilm/pipe surfaces, corrosion tubercles, under tubercles) within the same distribution system or even the same pipe would increase knowledge regarding impacts of biogeographical heterogeneity in drinking water biofilms on antibiotic resistance (Liu et al. 2014; Gomez-Smith et al. 2015; Neu et al. 2019; Cruz et al. 2020). Laboratory and full-scale studies regarding pipe materials, corrosion inhibitors, and corrosion products are important for gaining insights into microbial functions and could be used to provide guidance to water utilities for making engineering decisions in DWDS that could reduce human health risks.

In this dissertation research, the impact of metals and corrosion inhibitors on antibiotic resistance was investigated. Specifically, the three research objectives addressed were:

*1) Determine the abundance and types of ARGs, MRGs, and microbial communities in a full-scale drinking water main.* The primary goal of this objective was to determine the types and quantities of resistance genes and bacteria in biofilms inside of a full-scale water main. The impact of sample location and sample type was evaluated to

determine the variability of antibiotic and metal resistance indicator genes, as well as bacterial communities, within a single drinking water pipe. This type of information adds to the basic scientific literature and knowledge in understanding the abundance and distribution of bacterial communities, ARGs, and MRGs in DWDS. Surveillance of resistance genes in engineered systems such as DWDS is critical for protecting public health and providing data which can be used for future risk assessments for urban water systems. These experimental results are presented in Chapter 3.

*2) Evaluation of the impact of corrosion inhibitors on antibiotic and metal resistance in a source drinking water.* The specific goals for this objective were to develop an understanding of the impact of corrosion inhibitor type and concentration on the abundance of target resistance genes conferring resistance to antibiotics and metals. First, the impact of different types of corrosion inhibitors on antibiotic resistance was assessed. Second, the impact of increasing concentration of each type of corrosion inhibitor was evaluated. Understanding the impact of chemicals added to drinking water for corrosion control and prevention is critical for preserving drinking water infrastructure and protecting public health. These experimental results are presented in Chapter 4.

*3) Determine the impact of corrosion inhibitor and disinfectant type on the antibiotic resistome in a source drinking water.* This research objective aimed to assess the impact of corrosion inhibitor type on the antibiotic resistome in a source drinking water using shotgun metagenomic sequencing. The impact of different corrosion inhibitors on the resistome was evaluated by analyzing the changes in abundance of ARB and resistance genes in bacterial communities. Both targeted (e.g., qPCR) and non-

targeted (e.g., metagenomic sequencing) molecular methods of analysis are critical for providing information on the occurrence and abundance of AMR in drinking water and freshwater microbial communities. Research regarding the impact of corrosion inhibitors and disinfectants on the antibiotic resistome will assist in developing a better understanding of how engineering decisions impact the drinking water microbiome and prevalence of antibiotic resistance in engineered systems and the natural environment. These experimental results are presented in Chapter 5.

### **3 CAST IRON DRINKING WATER PIPE BIOFILMS SUPPORT DIVERSE MICROBIAL COMMUNITIES CONTAINING ANTIBIOTIC RESISTANCE GENES, METAL RESISTANCE GENES, AND CLASS 1 INTEGRONS**

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#### **3.1 Introduction**

Antibiotic resistance is a major public health concern stemming from the microbial response to the widespread occurrence of antibiotics and other physiological stressors in the environment (Levy 2001; Pruden et al. 2006). Approximately 2.8 million people are diagnosed with infections caused by antibiotic-resistant bacteria (ARB), and over 35,000 deaths are attributed to antibiotic resistance annually in the U.S. alone (CDC 2019). Antibiotic resistance genes (ARGs) on mobile genetic elements can be acquired by pathogens in the human gut (Beaber et al. 2004; Salyers et al. 2004; Kohanski et al. 2007), creating risks for vulnerable populations that are exposed to ARGs (Bor and Epstein 1991). ARGs have been detected in various water environments including groundwater (Zhang et al. 2009a), surface water (Pruden et al. 2006; Kappell et al. 2015a), drinking water treatment plants (Xi et al. 2009; Xu et al. 2016), and tap drinking

water (Bergeron et al. 2015; Su et al. 2018; Garner et al. 2019) at concentrations up to  $10^{10}$  copies/L (Xu et al. 2016). Consequently, it is becoming increasingly important to quantify ARGs in exposure routes that directly convey ARGs to people, including drinking water distribution systems (DWDS) (Anaissie et al. 2002; WHO 2014; Pruden et al. 2018).

ARGs in tap water have been shown to increase from the drinking water treatment plant effluent to the tap (Xi et al. 2009). While residual disinfectants can select for antibiotic resistance (Jia et al. 2015; Zhang et al. 2017a), the actual infrastructure of DWDS, i.e. the pipe materials used, could also be an important factor that impacts microbial ecology and consequently ARG profiles (Proctor et al. 2016; Aggarwal et al. 2018; Cruz et al. 2020; Douterelo et al. 2020; Kimbell et al. 2020). DWDS are comprised of a variety of metallic pipe materials (e.g., copper, iron, and lead) and additional metals in treated drinking water can accumulate in biofilms and corrosion scales (Lytle et al. 2004; Schock et al. 2008; Peng and Korshin 2011; Peng et al. 2012). Metals select for antibiotic resistance through co-resistance and cross-resistance mechanisms (Baker-Austin et al. 2006; Zhang et al. 2018b). Additionally, microorganisms have evolved detoxification strategies, such as metal resistance genes (MRGs) and efflux pumps, to mitigate the toxic effects of metals (Pal et al. 2017; Poole 2017). Exposing bacteria to metals in DWDS may promote the survival of bacteria resistant to metals and antibiotics (Calomiris et al. 1984; Baker-Austin et al. 2006). While studies have documented the occurrence of ARGs in tap drinking water and in biofilms (Garner et al. 2018a; Hao et al. 2019), to the best of our knowledge, no research efforts have quantified ARGs, MRGs, and mobile genetic elements from different biofilm sample locations (e.g., surface

biofilms, tubercles, under tubercles) in a single pipe to understand if drinking water pipes can serve as sources of ARGs.

The objective of this research was to determine if ARGs, MRGs, and class 1 integrons (*intI1*) were quantifiable across multiple sample types in a chloraminated cast iron water main and to determine if microbial taxa were correlated to resistance gene concentrations. It was hypothesized that ARGs, MRGs, and *intI1* would be detected regardless of sample type and location. The abundance of bacterial biomass (measured by 16S rRNA gene copies), ARGs, MRGs, and the integrase gene of the class 1 integron, *intI1*, were quantified in samples collected from different microenvironments using droplet digital PCR (ddPCR) and quantitative PCR (qPCR). Microbial communities were analyzed using PCR-amplified 16S rRNA gene sequences from each pipe sample (n=24). This is the first research to determine if various types of biofilm samples from a single full-scale DWDS pipe can serve as potential sources for ARGs.

## **3.2 Materials and Methods**

### **3.2.1 Pipe collection, sampling, and DNA extraction**

A six ft section of cast-iron water main (18" ID, 105 years in operation) that transported chloraminated water was extracted, covered with sterile plastic sheeting, and immediately transported to the laboratory for sampling and analysis. The water main was collected as part of planned maintenance to replace old water mains throughout the distribution system. Pipe samples were collected from i) a visible biofilm surface, referred to as "biomass surface" (n=6), ii) a pipe surface that did not have biofilm visible

to the naked eye, referred to as “pipe surface” (n=6), iii) from three-dimensional corrosion tubercles that could be removed, referred to as “corrosion tubercles” (n=6), and iv) from the pipe surface on the location where the tubercle was removed, referred to as “under corrosion tubercles” (n=6) (see Figure 3A in Appendix 3A). Broadly speaking, all samples were microbial biofilms, not bulk water samples, and they were subcategorized into the four categories listed for comparison and statistical analysis. Biofilm swab samples were collected by firmly pressing a sterile cotton-tipped applicator (Fisher Scientific, Waltham, MA) on the biofilm surface and swabbing an area of approximately 2-5 cm<sup>2</sup>. For each of the microenvironment types sampled, top (n=3) and bottom (n=3) samples of the cast iron water main were collected. Each swab was transferred directly to a sterile DNA extraction lysing tube and the stem was snapped and severed to preserve only the sample end of the swab (Garner et al. 2018b). Tubercle samples were collected into plastic tubes using a flame sterilized spatula, and approximately 0.2 g of corrosion tubercle was sub-sampled for DNA extraction. Samples were immediately frozen at -20°C until DNA extraction was performed. DNA was extracted using the FastDNA Spin Kit (MP Biomedicals, Solon, OH). The manufacturer’s protocol was followed with the exception that initial cell lysis was conducted using liquid nitrogen freeze thaw cycling (3x) (Li et al. 2012; Kappell et al. 2018a; Kimbell et al. 2018; Harrison et al. 2020). DNA concentrations in resulting extracts were quantified by microspectrophotometry using a Nano-Drop (Nano-Drop™ Lite, Thermo Scientific, Waltham, MA) and stored at -20°C.

### 3.2.2 Quantification of Resistance Genes

Droplet digital PCR (ddPCR) assays were conducted to quantify gene copies. A subset of samples was initially analyzed at 5, 10, 50, and 100-fold dilutions to test for inhibition during gene quantification. Based on these results, a 10-fold dilution was selected to minimize inhibition for all samples. Reaction mixtures consisted of a total volume of 22  $\mu\text{L}$  with 11  $\mu\text{L}$  of QX200 ddPCR EvaGreen Supermix (final concentration 1x) (Bio-Rad Laboratories Inc., Hercules, CA), 2  $\mu\text{L}$  each of forward and reverse primers (final concentration 250 nM each), 4  $\mu\text{L}$  of diluted DNA extract, and 3  $\mu\text{L}$  molecular grade water. The ddPCR reaction mixture was added to a 96-well plate, sealed with foil, homogenized by vortexing, and centrifuged briefly to ensure that all reaction components were at the bottom of the wells. The 96-well plate was equilibrated at room temperature for 3 minutes prior to droplet generation. Aliquots of 20  $\mu\text{L}$  for each reaction were dispensed into a separate well of an eight-channel droplet generator cartridge (DG8 Cartridge, Bio-Rad) followed by 70  $\mu\text{L}$  of QX200 Droplet Generation Oil for EvaGreen into the oil wells for subsequent droplet generation using the QX100 Droplet Generator. Oil-droplet mixtures were transferred to a 96-well plate and sealed at 180°C using the PX1 PCR Plate Sealer. The 96-well plate was transferred to the C1000 Touch Thermal Cycler for PCR thermal cycling with the following conditions: 5 min at 95°C for activation of DNA polymerase, 39 cycles of 95°C for 30 s and 60°C for 60 s, followed by signal stabilization at 4°C for 5 min and 90°C for 5 min. Thermal cycling conditions were modified for genes with annealing temperatures different from 60°C (see Table 3.1 in

Appendix 3B). After thermal cycling, plates were transferred to the QX200 Droplet Reader for absolute quantification of target genes.

Data analysis was performed using the QuantaSoft Analysis Pro software and expressed as gene copies  $\mu\text{L}^{-1}$  (V 1.0.596, Bio-Rad). Positive controls were included with each ddPCR assay and were produced by ten-fold serial dilution of plasmid DNA yielding  $10^4$  to  $10^0$  copies per reaction. No-template (i.e., reagent only) controls were included with each ddPCR assay. All ddPCR negative controls failed to yield amplification above the limit of quantification for each assay. Thresholds to discriminate between positive and negative droplets were manually applied to each sample and only samples with  $\geq 3$  positive droplets were considered as positive (Di Cesare et al. 2018). Furthermore, only reactions with greater than 10,000 accepted droplets were used for subsequent analysis (Di Cesare et al. 2018). The limit of the detection (LOD) and limit of quantification (LOQ) for each tested gene were determined according to the MIQE guidelines (Bustin et al. 2009; Huggett et al. 2013). Additional MIQE guidelines were followed and are shown in Table 3.2 in Appendix 3C. DNA extract from each sample was analyzed in triplicate for each target gene, and the average value from analyzing each DNA sample three times was used for each sample for further analysis.

Target gene copies were also quantified in triplicate from DNA extracts using qPCR with previously published protocols for the 16S rRNA gene (Muyzer et al. 1993), ARGs (*bla*<sub>SHV</sub> (Xi et al. 2009), *bla*<sub>TEM</sub> (Marti et al. 2013), *sul1* (Burch et al. 2013), MRGs (*czcD*, *copA*) (Roosa et al. 2014), and the integrase gene of class 1 integrons (*intI1*) (Goldstein et al. 2001). A subset of biofilm samples was initially analyzed at 5, 10, 50, and 100-fold dilutions to determine the optimum dilution for gene quantification. A 10-

fold dilution was found to yield optimum quantitation for DNA extracts and was utilized for all samples. Each qPCR assay consisted of a total reaction volume (20  $\mu\text{L}$ ) with 10  $\mu\text{L}$  PowerUp™ SYBR® Green Master Mix, 2  $\mu\text{L}$  each of forward and reverse primers (10  $\mu\text{M}$ ), 5  $\mu\text{L}$  of diluted DNA extract, and 1  $\mu\text{L}$  molecular-grade water.

Following each qPCR assay, melt curves were generated and analyzed to verify specific target amplification based on positive controls. Gene concentrations for each sample were quantified in triplicate, and the mean value was used for subsequent statistical analysis. If only two of the three replicates yielded positive detections in the qPCR assay, then the mean value of the two positive replicates was used in subsequent analyses (Pruden et al. 2012b; Kimbell et al. 2018). Standard curves for qPCR assays were produced by ten-fold serial dilution of plasmid DNA yielding  $10^6$  to  $10^0$  copies per reaction. Standard curves and negative controls were conducted in triplicate and were included with each 96-well plate. All qPCR negative controls failed to yield amplification above the limit of quantification for each assay. Copy numbers of target genes were  $\log_{10}$  transformed to meet the assumptions of normality for statistical analysis (Burch et al. 2013). Relative abundance of each target gene was normalized to 16S rRNA gene copies for statistical analysis. All qPCR assays were conducted on a Roche LightCycler® 96 (Roche Molecular Diagnostics, Pleasanton, CA) at the Marquette University Water Quality Center in Milwaukee, WI.

Additional information on specific primer sets, amplicon sizes, annealing temperatures,  $R^2$  values, efficiencies, and quantification limits are described in the Appendix Table 3.1 in Appendix 3B.  $\beta$ -lactam resistance genes such as *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub> are grouped in the most common types of  $\beta$ -lactamases belonging to *Enterobacteriaceae*

and encode resistance to  $\beta$ -lactam antibiotics such as penicillins and cephalosporins (Bush and Jacoby 2010). Sulfonamide resistance gene (*sul1*) and the integrase gene of class 1 integrons (*intI1*) are frequently detected in various natural and engineered environments and are considered a good proxy for ARG abundance and anthropogenic pollution (Pruden et al. 2012a; Gillings et al. 2015). MRGs quantified in biofilm samples from the cast iron water main included the copper resistance gene *copA* and *czcD*, which is a part of the cation diffusion facilitator mediating resistance to cadmium, zinc, and cobalt (Outten et al. 2001; Kloosterman et al. 2007). These genes were selected based on their abundance in an initial qPCR assay conducted with over 20 different MRGs including genes encoding resistance to metals such as arsenic, copper, iron, lead, and zinc.

### **3.2.3 PCR and Illumina Sequencing of 16S rRNA gene amplicons**

Microbial communities from biofilm samples were prepared for analysis by triplicate PCR-amplifying and pooling V4 hypervariable regions of 16S rRNA genes (Caporaso et al. 2012). One extraction blank and mock community (#HM-782D, BEI Resources) were included in the sample set. PCR amplicons were sequenced with Illumina MiSeq 2x250 paired-end chemistry at the Great Lakes Genomic Center (<http://greatlakesgenomics.uwm.edu>). Primer and barcode sequences were removed from reads using cutadapt (Martin 2011). Reads were processed, including filtered, merged, error-corrected, and chimera-checked, into amplicon sequence variants (ASVs) using the R package DADA2 (Callahan et al. 2016). Taxonomy was assigned using DADA2 from the SILVA v. 132 reference database (Quast et al. 2013). ASVs that were classified as

mitochondria, chloroplast, or eukaryota were removed. Additional thresholds were set to identify and remove ASVs potentially derived from the mock community, extraction/PCR blank, and non-target samples that were included in the sequencing run. Raw sequences have been uploaded to NCBI under BioProject ID PRJNA692495.

To prepare biofilm microbial communities for DNA sequence analysis, the V4 hypervariable region of 16S rRNA genes was PCR-amplified from purified DNA using primers 515F/806R with Nextera adapters (Illumina, Inc., San Diego, CA) (Caporaso et al. 2012; Garner et al. 2019). One extraction blank and mock community (#HM-782D, BEI Resources) were included in the sample set. Reactions (25  $\mu$ L total volume) were set up as follows: 12.5  $\mu$ L KAPA HiFi HotStart ReadyMix (Roche Sequencing, Pleasanton, CA), 5  $\mu$ M 515F primer, 5  $\mu$ M 806R primer, 7.5  $\mu$ L HyClone DNA-free water, and 2  $\mu$ L diluted DNA extract. PCR thermal cycling conditions were as follows: 5 min at 95°C, 40 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 90 s, followed by 5 min at 72°C. PCR products were initially screened by electrophoresis on 1.5% agarose gels. Triplicate PCR products were pooled in ~75  $\mu$ L samples, with amplicons less than 100 bp removed using 56  $\mu$ L Agencourt AMPure XP beads in each (Beckman Coulter, Brea, CA). Final products were resuspended in 40  $\mu$ L 10 mM Tris-EDTA. Sample library preparation was prepared according to the Illumina MiSeq protocol in the Nextera XT Index kit (Illumina, Inc., San Diego, CA) using 2X KAPA HiFi HotStart ReadyMix (Roche, Basel, Switzerland). Indexed [\[LL1\]](#) [\[LL1\]](#) PCR amplicons were cleaned with AMPure XP beads and the SequelPrep Normalization kit (Thermo Fisher Scientific, Waltham, MA). Sequencing was conducted on the Illumina MiSeq platform using a 2x250-cycle paired-

end protocol at the University of Wisconsin-Milwaukee Great Lakes Genomics Center in Milwaukee, WI (<http://greatlakesgenomics.uwm.edu>).

Primer and barcode sequences were removed from reads using cutadapt (Martin, 2011). Processing of sequence reads was conducted using the open statistical program ‘R’ utilizing the ‘DADA2’ package, which is a model-based approach for correcting amplicon errors without sequence similarity clustering to construct amplicon sequence variants (ASVs). (Rosen et al. 2012) Based on quality profiles, the last 20 base pairs of forward and reverse reads were removed. Two maximum expected errors were permitted. Any reads with ambiguous bases (N) were removed and reads with quality scores lower than 10 were removed. Chimeric reads were checked using the “consensus” method and removed. Taxonomic classification for the resulting ASVs was assigned using the SILVA v132 reference database. (Quast et al. 2013).

ASVs that were classified as mitochondria, chloroplast, or eukaryota were removed. Additional thresholds were set to identify and remove ASVs potentially derived from the mock community, extraction/PCR blank, and non-target samples that were included in the sequencing run: 1) any ASVs that were exact matches to those in the mock community were removed; 2) any ASVs that were three times more abundant in the blank sample (negative control) than across the dataset were removed; 3) any ASVs that were ten times more abundant in the non-target dataset than the drinking water pipe biofilm samples were removed.

Code for these analyses can be found on GitHub:

[https://github.com/NewtonLabUWM/DrinkingWaterPipe\\_Ecology](https://github.com/NewtonLabUWM/DrinkingWaterPipe_Ecology).

### 3.2.4 Corrosion Tubercle Characterization

X-ray diffraction (XRD) analysis was conducted on corrosion tubercles that were sampled from the cast iron water main (n=6) to identify the dominant crystalline phases. XRD was performed on a Bruker D8 Discover A25 diffractometer using copper  $K\alpha$  radiation with step scanning from  $2\theta$  of 15 – 70°. The scan speed and step size were 3° per min and 0.02°, respectively. XRD patterns of each corrosion tubercle were compared to standard patterns from International Centre for Diffraction Data (ICDD).

The inorganic elemental composition of corrosion tubercles was determined using inductively coupled plasma mass spectrometry (ICP-MS) (Kappell et al. 2019). Approximately 0.1 g of each corrosion tubercle was subsampled for elemental analysis. Each tubercle sample was homogenized using a sterile mortar and pestle prior to acid digestion with nitric acid (2%) and hydrochloric acid (1%) (American Public Health Association (APHA) 1975). An Agilent Technologies 7700 Series ICP-MS (Agilent Technologies Inc., Santa Clara, CA) was used for elemental composition determination. Standard reference materials for elements including Ag, Al, As, Ba, Be, Ca, Cd, Co, Cr, Cu, Fe, Hg, Mg, Mn, Mo, Na, Ni, Pb, Sb, Se, Th, Tl, U, V, and Zn were purchased from Agilent Technologies.

### 3.2.5 Statistical analysis

Statistical analyses were conducted using RStudio in the open-sourced statistical program R (V 3.6.1) (Racine 2012; R Core Team 2018). One-way analysis of variance (ANOVA) was conducted using the ‘aov’ function to determine statistical differences between abundances of target genes across groups of samples. A significant cutoff of  $\alpha =$

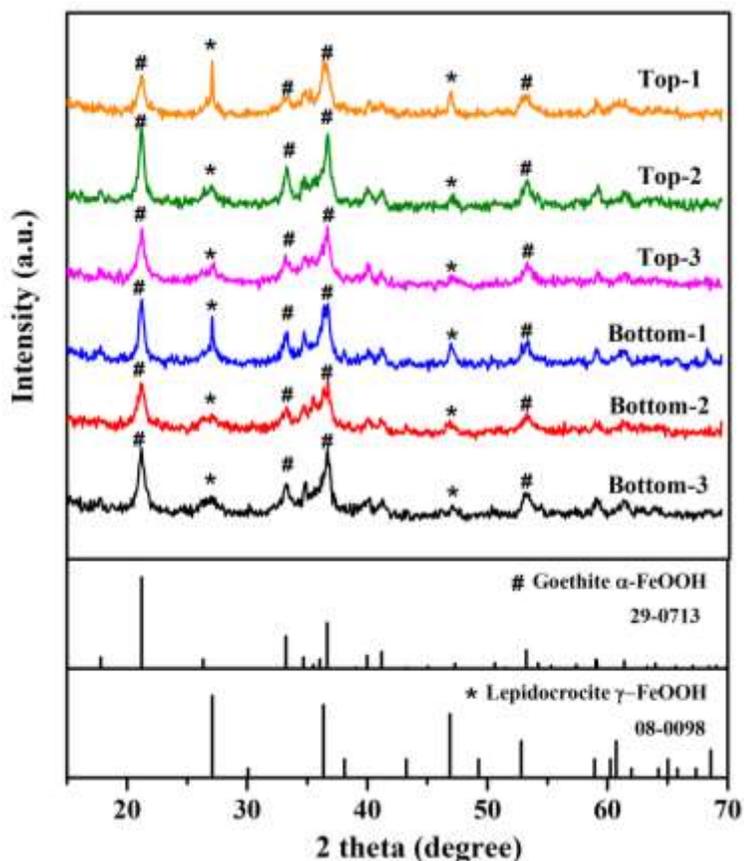
0.05 was used for all analyses. For sequence analysis, the BIOM file generated via DADA2 was imported into R using the phyloseq package (McMurdie and Holmes 2013). R packages ‘phyloseq’ and ‘ggplot2’ were utilized for general visualization of sequence data. Alpha and beta diversity metrics and plots were generated using the ‘vegan’ and ‘ggplot2’ packages. ANOVA was used to determine significance among the alpha diversity metrics. Principal coordinate analysis (PCoA) was performed using the ‘ape’ package to visualize differences between samples using the Bray-Curtis dissimilarity matrix generated in ‘phyloseq’. Canonical correspondence analysis (CCA) was conducted in R using the vegan package to identify correlations between the bacterial community structure and biofilm sample location. Spearman’s rank sum correlation coefficients were calculated in R to assess correlations between ARGs, MRGs, abundant taxa, and biofilm sample location. Indicator taxa were identified for each sample location using the multi-level pattern (indicator species) analysis in the package ‘indicspecies’ (Dufrene and Legendre 1997). Rarefaction curves were generated using the ‘ggrare’ function from the phyloseq-extended package of scripts (Mariadassou 2017).

### **3.3 Results and Discussion**

#### **3.3.1 Physical Characterization of Corrosion Tubercles**

XRD analysis indicated that the mineral phases present in the interior surfaces of the cast iron pipe primarily consisted of goethite ( $\alpha$ -FeOOH) and lepidocrocite ( $\gamma$ -FeOOH) (**Figure 3.1**). Goethite has been widely found as a main corrosion product in cast iron pipes (Wang et al. 2012a; Chen et al. 2013; Wang et al. 2014b; Zhu et al. 2014;

Wang et al. 2015; Wang et al. 2018) and several previous studies also reported the observation of lepidocrocite in iron pipes from full-scale DWDS (Sarin et al. 2001; Yang et al. 2012). The inorganic elemental composition of corrosion tubercles was characterized by ICP-MS. Iron was the dominant element in corrosion tubercles, representing approximately 98.6% of the measured mass. Other elements detected in quantities ranging from 0.1% to 1.0% in the tubercles included Ca (0.64%), Al (0.38%), Mg (0.12%), and Mn (0.10%). Elements detected below 0.1% included Na, K, Co, Cu, Pb, Zn, V, As, Se, Mo, Ag, Cd, Hg, Ni, and Be. Metals present in corrosion scales and tubercles in the cast iron water main may have originated from the pipe material itself, especially Fe, but other trace elements were likely deposited over time from the bulk drinking water.



**Figure 3.1** X-Ray Diffraction (XRD) patterns of biofilm tubercle samples (n = 6) collected from the chloraminated cast-iron water main. The reference XRD patterns for goethite (JCPDS 29-0713) and lepidocrocite (JCPDS 08-0098) are also shown for comparison.

### 3.3.2 Quantification of Bacterial Biomass

16S rRNA genes were detected above quantification limits in all 24 samples from the cast iron water main (see Figure 3D in Appendix 3D). The mean concentration in corrosion tubercles was  $4.4 \times 10^7$  16S rRNA gene copies/g tubercle. The mean concentration in biofilms collected under corrosion tubercles was  $3.5 \times 10^5$  16S rRNA copies  $\text{cm}^{-2}$ . While these mean values differed by over two orders of magnitude, the differences were not significantly different between sample types due to large variability

within each sample type (One-way ANOVA,  $p = 0.38$ ). Previous studies have reported similar levels of 16S rRNA in biofilms from chloraminated water mains with averages ranging from  $3.2 \times 10^5$  to  $2.5 \times 10^7$  copies  $\text{cm}^{-2}$  (Gomez-Smith et al. 2015; Waak et al. 2018; Waak et al. 2019a).

### **3.3.3 Quantification of Resistance Genes in Pipe Samples**

#### **3.3.3.1 Detection Frequency by ddPCR and qPCR**

The frequency of gene detects for every gene analyzed across the 24 samples using ddPCR was equal to or higher than that for qPCR (**Table 3.1; Figure 3E in Appendix 3E**). The presence of inhibiting substances such as metals or humic acids in the biofilm samples are known to impact amplification and primer annealing in qPCR assays (Cavé et al. 2016; Taylor et al. 2017). Previous studies also have demonstrated that ddPCR as compared to qPCR can have increased precision and accuracy for quantifying low concentrations of DNA in variably contaminated samples (Cavé et al. 2016; Verhaegen et al. 2016; Taylor et al. 2017). Our findings in conjunction with previous studies suggest that ddPCR is favorable for detecting ARGs in DWDS, particularly because these samples often contain low concentrations of DNA and contaminants that may interfere with qPCR. Reporting limit of detection and limit of quantification will be key for comparing across studies, as these values can differ significantly among quantification methods.

**Table 3.1** Summary of detections of ARGs, MRGs, and *intI1* with ddPCR and qPCR.

Gene	Mechanism/ Mode of Action	ddPCR detections	ddPCR LOQ (CN $\mu\text{L}^{-1}$ )	ddPCR LOQ (CN $\text{cm}^{-2}$ )	qPCR detections	qPCR LOQ (CN $\mu\text{L}^{-1}$ )	qPCR LOQ (CN $\text{cm}^{-2}$ )
16S rRNA	NA	24 (100%)	5	$5 \times 10^3$	24 (100%)	500	$5 \times 10^5$
<i>bla</i> <sub>SHV</sub>	Beta lactam resistance	24 (100%)	2	$2 \times 10^3$	20 (83%)	50	$5 \times 10^4$
<i>bla</i> <sub>TEM</sub>	Beta lactam resistance	24 (100%)	2	$2 \times 10^3$	24 (100%)	5	$5 \times 10^3$
<i>copA</i>	Copper resistance	11 (45%)	4	$4 \times 10^3$	3 (12.5%)	5	$5 \times 10^3$
<i>czcD</i>	Cobalt, zinc, cadmium efflux	20 (83%)	3	$3 \times 10^3$	19 (79%)	5	$5 \times 10^3$
<i>intI1</i>	Integrase gene of class 1 integrons	10 (42%)	4	$4 \times 10^3$	8 (33%)	5	$5 \times 10^3$
<i>sul1</i>	Enzymatic modification	17 (71%)	3	$3 \times 10^3$	4 (17%)	5	$5 \times 10^3$
<i>tet(L)</i>	Tetracycline efflux	0 (0%)	6	$6 \times 10^3$	0 (0%)	50	$5 \times 10^4$

Notes: CN – copy numbers, LOQ – limit of quantification, NA – not applicable.

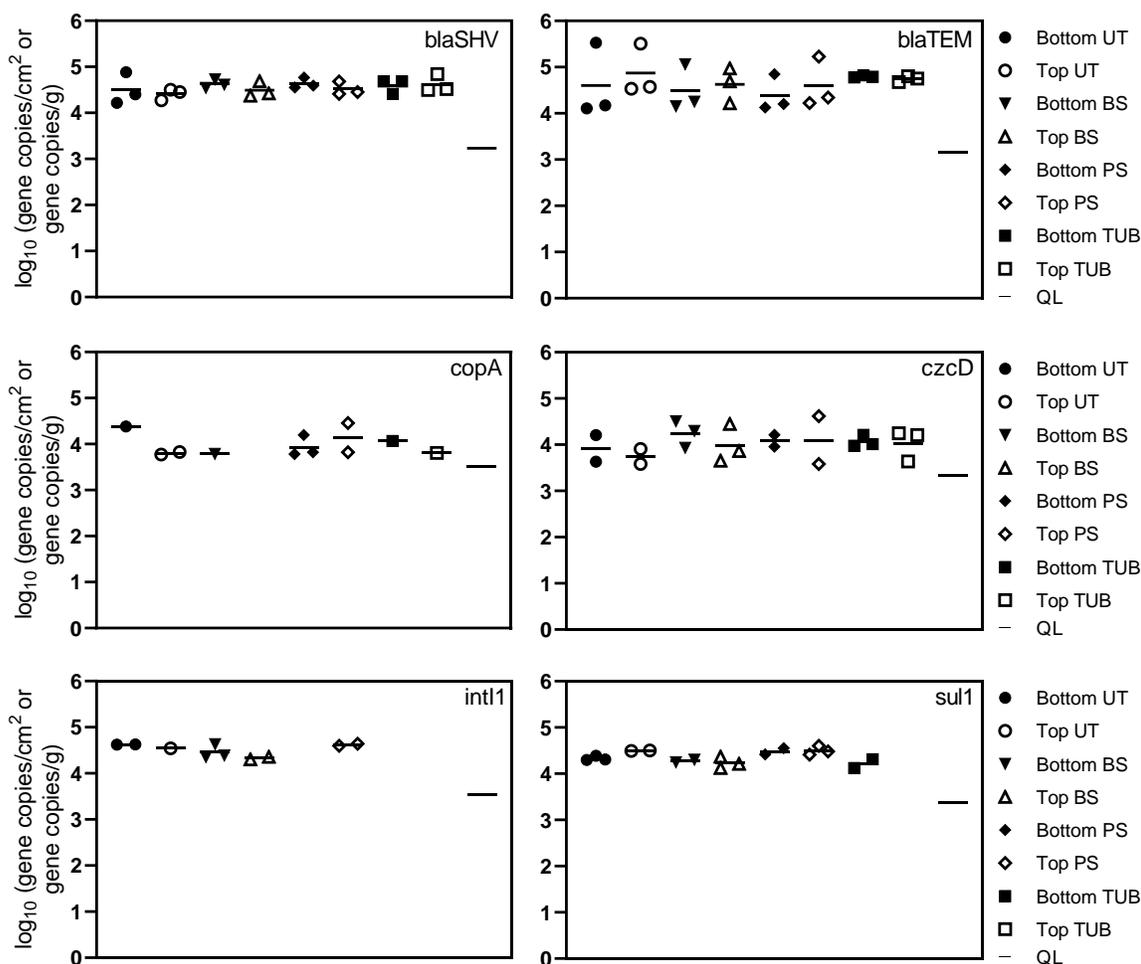
### 3.3.4 Abundance of Antibiotic Resistance Genes (ARGs) and *intI1*

The ARGs *bla*<sub>SHV</sub>, *bla*<sub>TEM</sub>, and *sul1*, and *intI1* were detected in biofilm samples from the chloraminated cast-iron drinking water main at concentrations up to nearly 6 log gene copies  $\text{cm}^{-2}$ , with the highest mean value belonging to gene *bla*<sub>TEM</sub> at approximately 4.8 log gene copies  $\text{cm}^{-2}$  (**Figure 3.2**). A previous study reported the mean absolute abundance of ARGs (*bla*<sub>TEM</sub>, *sul1*, *qnrA*, *vanA*) and *intI1* in biofilms from undefined

pipe materials to range from <LOQ to 4.2 log copies swab<sup>-1</sup> (Garner et al. 2018a). Mean absolute abundances of ARGs and *intI1* in different biofilm microenvironments varied by over one log unit, but the mean differences of the sample types were not significantly different from each other (One-way ANOVAs, p values > 0.05), indicating that sample type did not impact absolute gene abundances. Relative gene abundances (absolute normalized to 16S rRNA gene copies) demonstrated higher variability (>2-log units) between the different microenvironments sampled, but the mean relative abundance values were not significantly different based on sample type (One-way ANOVA, p > 0.05) (see **Figure 3F in Appendix 3F**). The variability in relative abundance values for ARGs in the different microenvironments was primarily due to differences in levels of bacterial 16S rRNA genes between sample locations rather than changes in ARG abundance. Indeed, these results indicate that various pipe samples and thus pipe infrastructure can serve as sources of ARGs into tap drinking water.

Absolute concentrations of ARGs remained relatively consistent in the different biofilm sample locations. ARGs were detected more frequently in surficial biofilm environments but demonstrated similar absolute abundance compared to sub-surface environments. One explanation could be that corrosion deposits and tubercles can provide relief from disinfectants, advective flow, and shear stress which allows additional biofilm development to occur (Lehtola et al. 2006). Observed relative abundance values for ARGs in the current study suggest that microbes in sub-surface communities may harbor ARGs at similar levels compared to surficial microbes. The presence of ARGs in each of the different biofilm sample locations suggests that potential selection for antibiotic resistance exerted by disinfectants, metals and other dissolved contaminants exists

throughout the cast iron biofilm communities inside the pipe. Additionally, the detection of ARGs and class 1 integrons in the biofilm communities suggests that horizontal gene transfer may be one plausible explanation for the proliferation of ARGs inside the cast iron biofilm communities due to the close proximity of microbial species within biofilms (Baker-Austin et al. 2006; Balcázar et al. 2015). Average and median chloramine concentrations inside the distribution system were 1.0 to 1.3 mg/L. Residual disinfectants such as chloramines present in DWDS at subinhibitory levels have been shown to stimulate horizontal gene transfer of ARGs through multiple pathways including reactive oxygen species response systems and the SOS response (Zhang et al. 2017a).



**Figure 3.2** Absolute abundance of antibiotic resistance genes, metal resistance genes, and *int11* in different biofilm microenvironments from a cast iron drinking water main as measured with ddPCR. The biofilm microenvironments include biomass surface (BS), pipe surface (PS), tubercle (TUB), and under tubercle (UT). Each biofilm sample is also categorized by top or bottom pipe sample location. Sample categories (e.g., Bottom UT, Top BS) are plotted when 1-3 of the samples yielded a quantifiable result. Sample categories with no positive detections were left blank. Each symbol represents the average of 3 ddPCR technical replicates from a single biofilm sample. All biofilm swab samples are plotted as  $\log_{10}$  gene copies/cm<sup>2</sup>. Corrosion tubercle samples are plotted as  $\log$  gene copies/g. The quantification limit (QL) is also plotted for each gene as the dash on the right side of the plot.

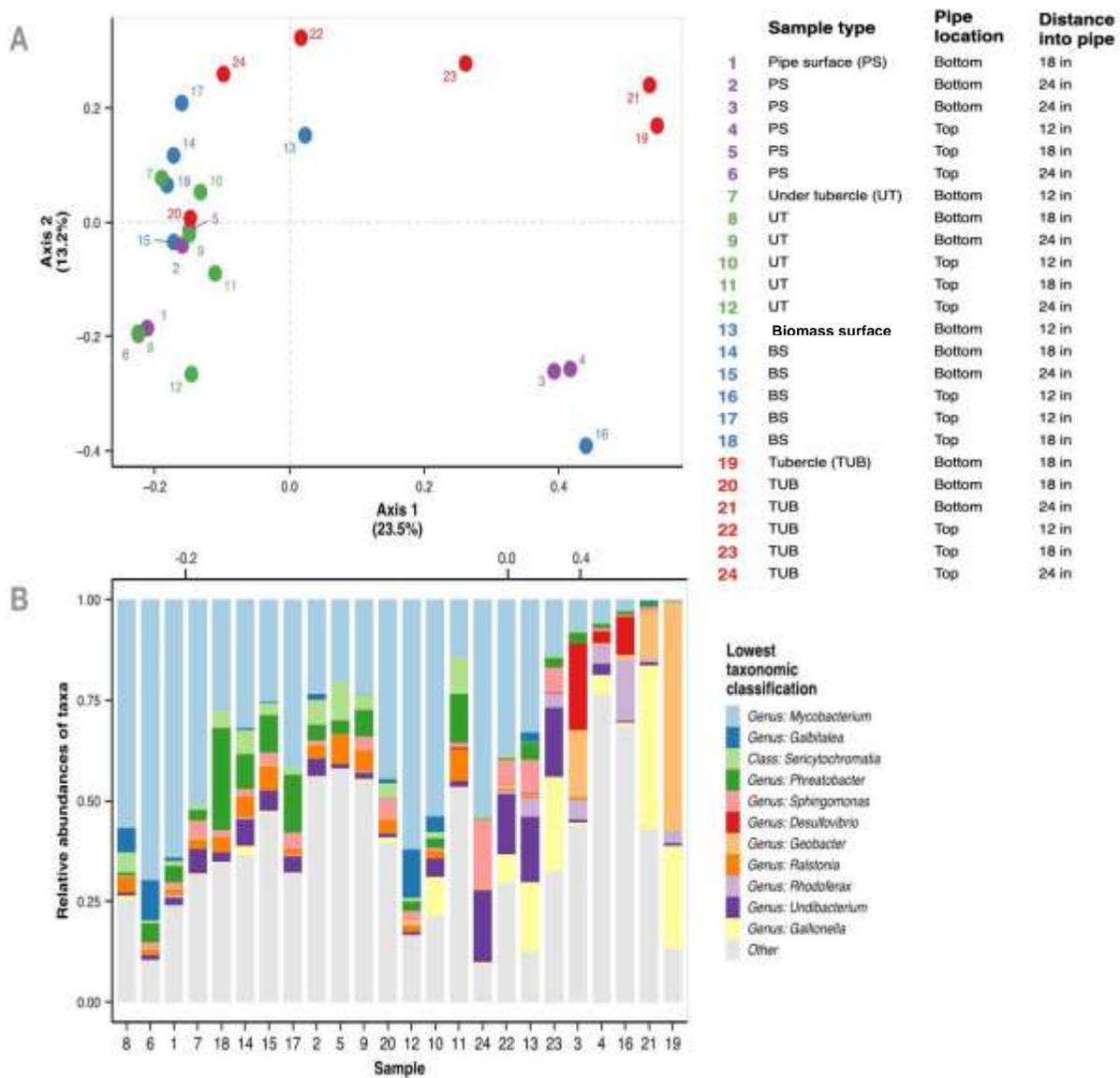
Beta-lactam resistance genes, *bla<sub>SHV</sub>* and *bla<sub>TEM</sub>*, were detected in all 24 biofilm samples ranging from 4.1 to 5.3 log gene copies cm<sup>-2</sup>. A previous study detected *bla<sub>TEM</sub>* in drinking water biofilms at lower absolute abundance (mean = 1.5 log gene copies swab<sup>-1</sup>) but with similar relative abundance (mean = -2.3 log ARG copies/16S rRNA copies) as in this study (Garner et al. 2018a). These results demonstrate that different microenvironments or niches in pipes can serve as reservoirs for bacteria harboring resistance genes, indicating that biofilms on DWDS pipes can serve as sources of ARGs when biofilms shed viable cells into tap drinking water (Balcázar et al. 2015). The sulfonamide resistance gene (*sul1*) and the integrase gene of class 1 integrons (*intI1*) are frequently detected in various natural and engineered environments and are considered potential indicators of horizontal gene transfer of ARGs (Pruden et al. 2012a; Gillings et al. 2015). The *intI1* gene was detected in 10 biofilm samples at an average concentration of 4.5 log gene copies cm<sup>-2</sup>. The *intI1* gene was detected at a higher frequency in biofilm surface samples (83%) and was not detected in tubercle samples. The frequency of *sul1* detections for biofilm surface, pipe surface, and under tubercle samples was 83%, compared to 33% for corrosion tubercles. Additional variation in ARG abundance was observed between samples collected from the top and bottom of the water main, but the differences were not statistically different (One-way ANOVA, p values > 0.05). The distance of biofilm sample collection inside the water main (12 in., 18 in., and 24 in.) also did not have a significant effect on ARG concentrations.

### 3.3.5 Abundance of Metal Resistance Genes (MRGs)

MRGs quantified in biofilm samples from the cast iron water main included the copper resistance gene *copA* and *czcD*. The *czcD* gene was detected in 83% of biofilm samples at concentrations ranging from above LOQ to 4.6 log gene copies cm<sup>-2</sup>. The mean *czcD* absolute abundance in surficial samples (BS, PS) was not significantly different compared to sub-surface samples (TUB, UT) according to One-way ANOVA results ( $p > 0.05$ ). The *czcD* gene is part of the cation diffusion facilitator mediating resistance to cadmium, zinc, and cobalt (Outten et al. 2001; Kloosterman et al. 2007) and has previously been documented in source drinking water treated with the corrosion inhibitor zinc orthophosphate (Kappell et al. 2019). The copper resistance gene *copA* was detected above the LOQ in 45% of samples with ddPCR assays. The *copA* gene encodes an ATPase efflux pump that extrudes copper ions, making it one of the main mechanisms mediating copper resistance (Yin et al. 2017). The detection of *czcD* and *copA* may be related to the presence of copper, zinc, cadmium, and cobalt ions in the drinking water and corrosion deposits. Previous studies have demonstrated that exposure to sub-inhibitory levels of Cu(II) and Zn(II) can contribute to horizontal gene transfer of ARGs (Zhang et al. 2018b). This is the first study to quantify clinically-relevant ARGs and MRGs in biofilm samples from a chloraminated cast iron water main collected from a full-scale distribution system.

### 3.3.6 Microbial Community Composition of Pipe Samples

Corrosion tubercles, especially from the bottom of the water main, exhibited the most unique microbial community composition compared to the biomass surface, pipe surface, and under tubercle samples (**Figure 3.3a**). Biofilm microbial communities exhibited similar Shannon diversity (1.8 – 4.4) to previous observations of biofilm communities in cast iron drinking water mains (Gomez-Smith et al. 2015) (see **Figure 3G in Appendix 3G**). A total of 469 microbial genera corresponding to 47 different phyla were identified in the biofilm microbial communities from the cast iron water main. The most abundant genera observed in the biofilm communities included *Mycobacterium* (0.2-70%), *Geobacter* (0-57%), *Gallionella* (0-40%), *Phreatobacter* (0-25%), *Desulfovibrio* (0-21%), *Undibacterium* (0-18%), *Streptococcus* (0-17%), and *Sphingomonas* (0-17%) (**Figure 3.3b**). Previous studies have also observed high abundance of *Mycobacterium*, *Geobacter*, *Gallionella*, *Sphingomonas*, and *Undibacterium* in corrosion deposits and biofilms in DWDS (Sun et al. 2014b; Wu et al. 2014; Zhu et al. 2014; Waak et al. 2019b). Other abundant genera observed in the cast iron biofilm communities included *Hydrogenophaga* (0-15%), *Rhodoferrax* (0-15%), *Galbitalea* (0-12%), *Corynebacterium* (0-11%), *Ralstonia* (0-8%), and *Geothrix* (0-6%).

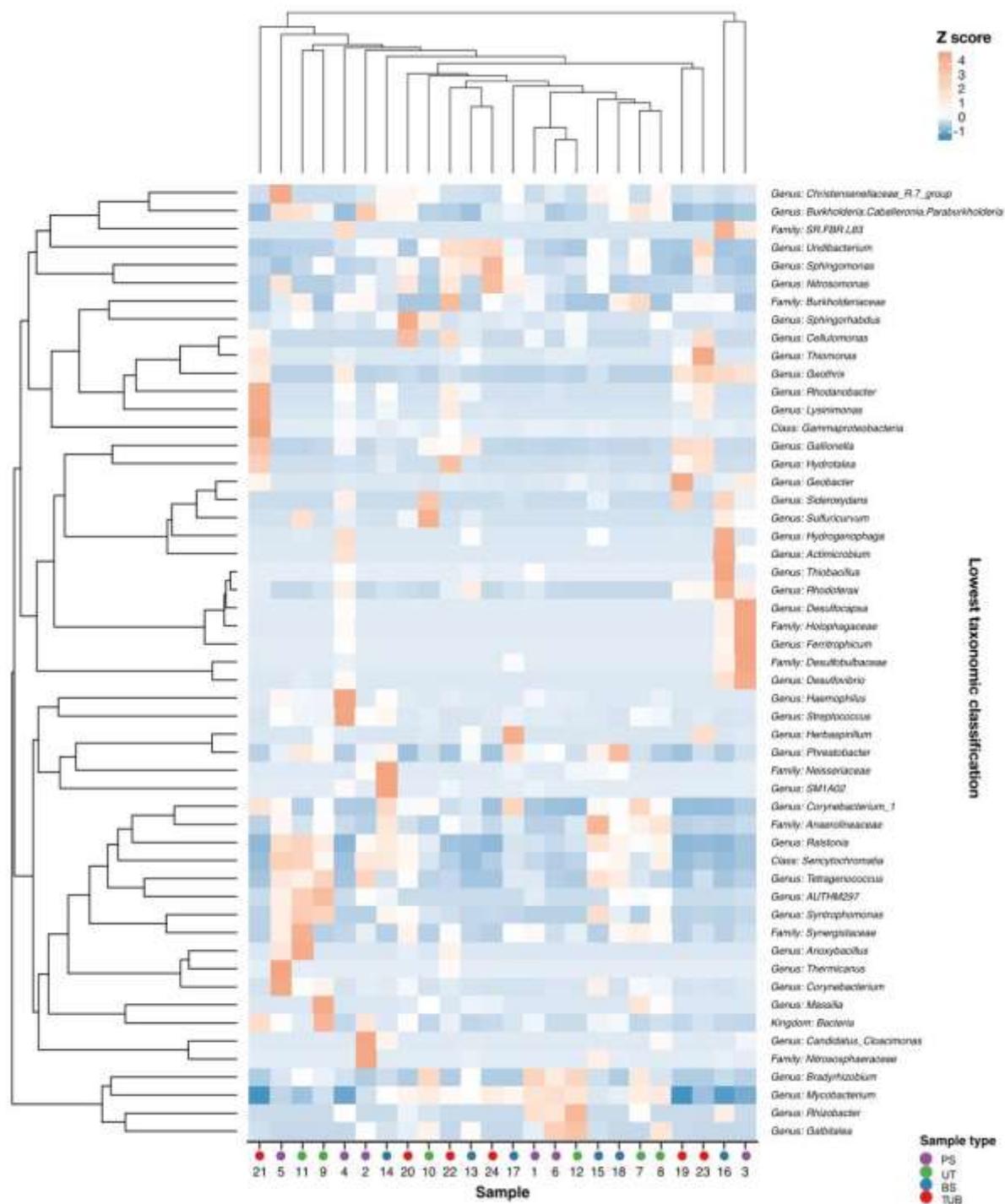


**Figure 3.3** (A) Principal coordinate analysis (PCoA) using Bray-Curtis dissimilarity of microbial communities in biofilm samples from the chloraminated cast-iron drinking water main ( $n = 24$ ). Color of points denote sample type and are as described in Table on top right. Point labels refer to sample number in table. (B) Relative abundance of 11 most abundant taxa combined down to lowest classification. Samples on x-axis ordered along Axis 1 of PCoA.

*Mycobacterium* represented the most abundant genus in the current study with 22 unique *Mycobacterium* ASVs detected in the biofilm communities. These ASVs comprised 32% of the total sequences in the biofilm samples and were detected in all 24 biofilm samples. Mean relative abundance of *Mycobacterium* spp. was highest in under tubercle samples (43%) compared to other sample locations (25-32%), but the differences were not statistically significant (One-way ANOVA,  $p > 0.05$ ). Although the mean values were not significantly different, relative abundance of *Mycobacterium* exhibited wide variability across individual samples, ranging from 0.2 to 70%. These findings are consistent with previous studies that have reported *Mycobacterium* as the dominant genus in drinking water biofilms from chloraminated cast iron water mains (Gomez-Smith et al. 2015; Waak et al. 2019a; Waak et al. 2019b). *Mycobacterium* spp. are frequently detected in DWDS (Torvinen et al. 2004; Bautista-De los Santos et al. 2016; Sevillano et al. 2020) and have several characteristics that give them a competitive advantage in chloraminated DWDS including the ability to form biofilms, resistance to residual disinfectants (Lin et al. 2014), and the ability to survive in nutrient-deficient environments (Hall-Stoodley et al. 1999; Primm et al. 2004). The prevalence of *Mycobacterium* in full-scale DWDS is a potential concern because the genus contains several opportunistic pathogens (Primm et al. 2004; Brown-Elliott et al. 2011; Haig et al. 2018; Dowdell et al. 2019).

The most abundant taxa were clustered into dendrograms using Bray-Curtis dissimilarity (**Figure 3.4**). Several corrosion-related bacterial genera including *Sphingomonas*, *Desulfovibrio*, *Gallionella*, *Geobacter*, *Hydrogenophaga*, and *Rhodobacter* were observed in the biofilm communities and have been previously documented in cast-iron pipe biofilms and corrosion scales (Li et al. 2010; Li et al. 2014;

Sun et al. 2014a; Wang et al. 2014b; Zhu et al. 2014; Gomez-Smith et al. 2015). Bacteria inhabiting distribution systems primarily perform functions related to carbon source utilization and nitrification, as well as microbial induced corrosion processes (Berry et al. 2006). Corrosion tubercles contained increased abundance of microorganisms related to microbial induced corrosion and redox cycling processes in cast iron pipes including genera such as *Gallionella*, *Geobacter*, *Geothrix*, and *Undibacterium* (Wang et al. 2014b; Zhu et al. 2014; Gomez-Smith et al. 2015). Previous studies have demonstrated that the abundance of microorganisms in DWDS may be impacted by the presence of corrosion tubercles, suspended solids, and other loose corrosion deposits (Liu et al. 2014; Liu et al. 2018). Additionally, the bottom of water mains can accumulate higher densities of bacteria and corrosion deposits, which may also support increased abundance of nitrifying bacteria (Liu et al. 2020). Similarly, the biofilm samples from the bottom of the cast iron water main displayed increased relative abundance of bacterial genera related to iron and nutrient cycling.



**Figure 3.4** Heatmap of normalized (Z score) abundances of most abundant (maximum relative abundance >2% in at least one sample) taxa combined to lowest classification. Samples along x-axis and taxa along y-axis were clustered into dendrograms using Bray-Curtis dissimilarity. Colors of points on x-axis denote sample type.

Spearman correlation analysis revealed significant relationships between the co-occurrence of several corrosion-related bacteria in the biofilm communities (**Figure 3H, Appendix 3H**). The genus *Ralstonia* demonstrated the most significant relationships with other taxa, and it is considered an emerging global opportunistic pathogens in municipal water supplies (Ryan and Adley 2014). Positive relationships were observed between the co-occurrence of corrosion-related bacteria from *Geobacter* and *Gallionella* genera, which also made up a large proportion of the communities in corrosion tubercles. *Geobacter* were most prevalent in corrosion tubercles (11.7%) but were also identified in lower abundance in pipe surface (3.1%), biofilm surface (0.4%), and under tubercle environments (0.4%). The *Geobacter* genus contains iron-reducing species, and previous studies have reported that *Geobacter* spp. were among the most resistant bacteria to monochloramine disinfection in lab-scale disinfection experiments (Chiao et al. 2014). Similarly, *Gallionella* spp. were more abundant in corrosion tubercles (16.5%) compared to biofilm surface (3.6%), pipe surface (1.0%), and under tubercle environments (1.9%). *Gallionella* spp. are neutrophilic iron oxidizing bacteria (IOB) that can promote the precipitation of iron oxides by converting ferrous iron to ferric iron and have previously been observed in association with severe iron corrosion release or “red water” events in distribution systems (Volk et al. 2000; Li et al. 2010). Bottom tubercle samples demonstrated increased relative abundance of *Gallionella* and *Geobacter* (22.5% and 23%) compared to top tubercle samples (10.5% and 0.4%), but these differences were not significant (One-way ANOVA, p values >0.05).

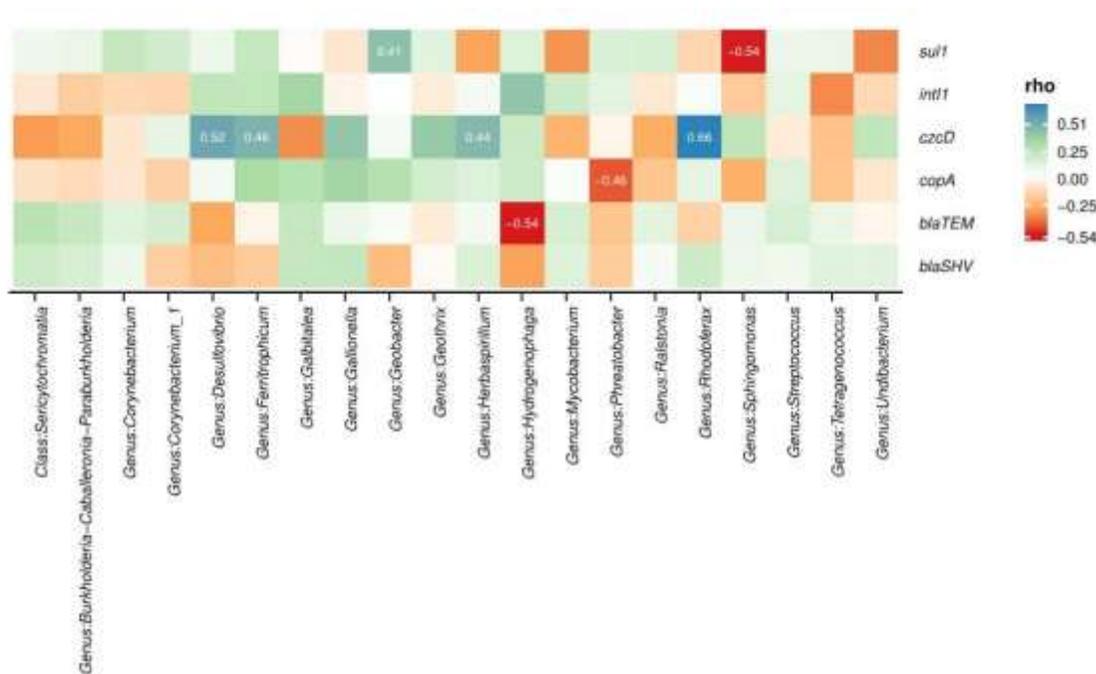
Indicator species analysis was employed to identify the most abundant indicator organisms in each sample location from the water main. Several corrosion-related genera were significant indicator organisms for corrosion tubercle samples including *Geothrix*, *Gallionella*, *Phreatobacter*, *Thiomonas*, and *Rhodovastum* (all p values < 0.05). *Methylobacterium* were identified as significant indicators for biofilm surface communities, while *Bradyrhizobium* were identified as indicators of pipe surface and under tubercle communities. *Phreatobacter* and *Ralstonia* genera were identified as indicators of communities in samples collected from pipe surfaces, biofilm surfaces, and under tubercles. Several of the identified indicator organisms in our study have been reported in previous studies analyzing cast iron biofilm communities (Sun et al. 2014b; Zhu et al. 2014; Ma et al. 2018).

### **3.3.7 Relationship between Sample Location, Resistance Genes, and Microbial Community Composition**

Microbial communities and abundances of resistance genes were compared between sample locations to determine potential host bacteria harboring ARGs, MRGs, and *intI1* (Rice et al. 2020). Additionally, the relationships among bacterial community composition and different sample types were evaluated using CCA. Biofilm sample type significantly influenced microbial community composition and explained 32% of the community variability (CCA, p = 0.009). Biofilm samples were taken from 12, 18, and 24 in. inside of the water main to assess impact of lateral sampling distance. CCA indicated that sampling distance into the pipe explained 36% of the variability in bacterial

community composition ( $p = 0.001$ ). These results indicate that biofilm sample location was a significant variable influencing microbial communities.

Spearman correlation analysis was conducted to determine relationships between bacterial community composition and abundance of resistance genes. Significant correlations were observed between several microbial genera and the abundance of resistance genes in the biofilm samples (**Figure 3.5**). The occurrence of *czcD* was significantly correlated with genera including *Desulfovibrio*, *Ferritrophicum*, *Herbaspirillum*, and *Rhodoferax* (All  $p$  values  $< 0.05$ ). Positive relationships were also observed between *czcD* and corrosion-related genera such as *Geothrix*, *Gallionella*, *Sphingomonas*, and *Undibacterium*.



**Figure 3.5** Relationships between relative abundance of most abundant taxa and absolute abundance of ARGs, MRGs, and *intI1* in biofilm samples from cast iron water main. Color denotes the result from correlation analysis using Spearman's rank sum correlation with Spearman's rho value plotted for each comparison. The Rho value for statistically significant relationships are also included ( $p$  values  $< 0.05$ ). The lowest available taxonomic classification for each observed ASV is provided.

The copper resistance gene *copA* was positively associated with the occurrence of several genera including *Galbitalea*, *Gallionella*, *Geobacter*, *Geothrix*, and *Ferritrophicum*. A significant negative relationship was observed between *copA* and *Phreatobacter*.  $\beta$ -lactam resistance genes *bla<sub>TEM</sub>* and *bla<sub>SHV</sub>* were positively correlated with taxa including *Burkholderia*, *Galbitalea*, and *Mycobacterium*. A significant negative relationship was observed between *bla<sub>TEM</sub>* and the genus *Hydrogenophaga* ( $p < 0.05$ ). The integrase gene of class 1 integrons (*intI1*) was positively correlated with genera including *Desulfovibrio*, *Galbitalea*, *Hydrogenophaga*, and *Mycobacterium*. The sulfonamide resistance gene *sul1* demonstrated a significant positive relationship with the

*Geobacter* genus (Wang et al. 2014a). The presence of statistically significant correlations between microbial taxa and resistance genes in the biofilm samples implies that at least some of the shifts observed in gene abundance could have resulted from shifts in abundances or types of host bacteria (Garner et al. 2018a).

### 3.4 Conclusions

This research established that drinking water main biofilms in a chloraminated cast iron water main can serve as sources of resistance genes, regardless of location within the pipe. This is the first research to use ddPCR and qPCR to quantify ARGs, MRGs, and class 1 integrons in drinking water biofilms from a full-scale distribution system. Future research should be conducted to determine the distribution and concentrations of resistance genes in different pipe materials and in different locations of the same distribution system. Additional research is needed to quantify resistance genes and microbial communities in other pipe materials including copper, lead, and plastic that are commonly used in full-scale DWDS to understand how engineering management decisions can impact sources of antibiotic resistance (Kimbell et al. 2020). Further, full-scale and laboratory scale studies should be conducted to determine the impacts of corrosion inhibitors and corrosion products on the prevalence of antibiotic resistance in DWDS. Previous studies have suggested that drinking water biofilms may facilitate horizontal transfer of the ARGs from one host to another due to the presence of nutrients and high bacterial density and diversity (Zhang et al. 2009a). In the current study, genera containing opportunistic pathogens detected in the biofilm samples included *Mycobacterium*, *Ralstonia*, *Staphylococcus*, and *Sphingomonas*. Further research

targeting these specific bacterial genera would be necessary to determine the presence of any potential opportunistic pathogens. Human exposure routes relevant for potable water include consumption of tap drinking water, dermal contact, and inhalation of aerosolized drinking water during showering or bathing (Anaissie et al. 2002). Studies documenting the occurrence and distribution of ARGs and MRGs in DWDS are critical for human health risk assessments evaluating the potential for the transfer and development of antibiotic resistance in engineered systems and in the environment (Ashbolt et al. 2013a; Rice et al. 2020). Given the potential for bacterial growth in DWDS, it is essential to continue to seek water treatment and management options that minimize levels of antibiotic resistance. Specific conclusions from key findings are as follows:

- ARGs (*bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *sul1*), MRGs (*copA*, *czcD*), and class 1 integrons (*intI1*) were detected in every biofilm sample type studied within a chloraminated cast iron drinking water main, indicating that pipes could serve as sources for ARGs.
- ddPCR assays resulted in more positive detections and lower detection limits for target genes compared to qPCR assays. Future studies should consider ddPCR for environmental samples containing inhibiting substances such as metals, humic acids, and other contaminants.
- Microbial communities varied between different biofilm sample locations and were dominated by corrosion-related genera including *Mycobacterium*, *Geobacter*, *Gallionella*, and *Sphingomonas*.
- Significant relationships were observed for the co-occurrence of ARGs, MRGs, *intI1* and several microbial taxa.

- Further research is warranted to determine the impacts of different pipe materials on the abundance of ARGs, MRGs, and *intI1* in biofilms inhabiting full-scale DWDS.

## **4 CORROSION INHIBITORS INFLUENCE ANTIBIOTIC RESISTANCE AND METAL RESISTANCE PROFILES IN A SOURCE DRINKING WATER**

### **4.1 Introduction**

The proliferation of antibiotic resistance in natural and engineered environments is a serious threat to human health (CDC 2019). Bacteria become resistant to antibiotics through genetic mutations or by acquiring antibiotic resistance genes (ARGs) from their surrounding environment (Levy and Bonnie 2004). ARGs are considered emerging contaminants in aquatic ecosystems (Pruden et al. 2006), and the occurrence of ARGs in drinking water is a potential risk to human health (Amarasiri et al. 2019). Groundwater and surface waters such as lakes and rivers are often used as source waters to drinking water treatment plants and are considered important reservoirs for antibiotic resistant bacteria (ARB) and associated ARGs (Baquero et al. 2008; Kappell et al. 2015b; Szekeres et al. 2018). Drinking water treatment processes do not completely remove ARB and ARGs, which are subsequently transported to drinking water distribution systems (DWDS) (Sharma et al. 2016; Kimbell et al. 2021). Drinking water disinfection processes using chlorine and secondary disinfectants (i.e., chloramines) are used to inhibit microbial growth in drinking water and to prevent regrowth during distribution. However, studies have demonstrated that chlorine disinfection and exposure to metals can also select for antibiotic resistance in water environments such as DWDS (Peltier et al. 2010; Zhang et al. 2017a; Zhang et al. 2018b).

The Lead and Copper Rule (LCR) was introduced by the US Environmental Protection Agency (EPA) in 1991, which requires drinking water utilities to perform

optimized corrosion control treatment to reduce dissolved copper and lead levels in drinking water (Brown et al. 2013). Drinking water utilities have three options for corrosion control treatment, including pH adjustment, maintenance of a disinfectant residual to develop Pb(IV) scale, and using corrosion inhibitors such as orthophosphates and sodium silicates (Schock 1996; Lytle and Schock 2005). The most common method of corrosion control in the U.S. is the use of phosphate-based corrosion inhibitors including orthophosphates and polyphosphates, with 56% of systems reporting orthophosphate dosages from 1-3 mg/L (McNeill and Edwards 2002; Arnold et al. 2020). Corrosion inhibitors containing phosphate may serve as a nutrient source for bacterial growth and increase microbial community diversity in DWDS (Fang et al. 2009; Payne et al. 2016). A recent study demonstrated that zinc orthophosphate addition to drinking water can select for increased abundance of ARB and ARGs compared to untreated controls (Kappell et al. 2019).

Sodium silicates have the general formula of  $\text{Na}_2\text{O} \cdot x\text{SiO}_2$ , where “x” varies from 1.6 to 3.22 for silicates commonly used for drinking water treatment (Schock 1996; Thompson et al. 1997). The recommended dosages from some silicate manufacturer’s include an initial dose of 24 mg/L as  $\text{SiO}_2$  for up to 60 days followed by a maintenance dosage of approximately 8-12 mg/L  $\text{SiO}_2$  (Thompson et al. 1997). Silicates are an alternative to phosphate-based inhibitors for corrosion control in DWDS and have been reported as an effective treatment for dissolved lead control under some conditions (Schock et al. 2005a; Lintereur et al. 2010). For instance, silicates may have higher efficacy for control of lead release in drinking water with high alkalinity and pH (>7.5) (Schock et al. 2005a). Meanwhile, studies have reported sodium silicate dosages ranging

from 10 to 48 mg/L as an inferior corrosion control treatment compared to 1 mg/L orthophosphate treatment in circumneutral pH water with low to moderate alkalinity (Kogo et al. 2017b; Li et al. 2021b). Similar to polyphosphate for corrosion control, excess sodium silicate in drinking water may cause increases in lead release (Li et al. 2021a). The effectiveness of sodium silicates for corrosion control may be primarily due to their ability to increase the pH, which also decreases solubility of lead and copper in drinking water (Aghasadeghi et al. 2021). Although the mechanism of corrosion control by sodium silicates is not well documented, the use of sodium silicates may be advantageous compared to phosphate-based treatments due to the lack of phosphorus (Kimbell et al. 2020). However, management requirements associated with LCR compliance have raised concerns with utilities regarding the use of sodium silicates for lead corrosion control (Li et al. 2021a). Consequently, it is imperative to gain a better understanding of the impacts of corrosion inhibitors on microbial ecology and antibiotic resistance proliferation in distribution systems and household plumbing networks.

ARB and ARGs in DWDS are selected for or against by the physical, chemical, and biological conditions in their surrounding environment and have been quantified in DWDS at concentrations ranging from  $10^1$  to  $10^{10}$  copies/L (Zhang et al. 2009a; Xu et al. 2016; Garner et al. 2018a; Su et al. 2018; Chen et al. 2020; Zhang et al. 2020). Bacterial resistance to antibiotics and metals are often genetically linked, indicating mechanisms for co-selection of antibiotic resistance during exposure to metals such as copper or zinc (Poole 2017). Several studies have reported opportunistic pathogens in drinking water systems that are chlorine-resistant and contain ARGs encoding resistance to multiple types of antibiotics, which poses risks to human health and drinking water safety (Dai et

al. 2019; Sevillano et al. 2020; Hu et al. 2021). The presence of corrosion inhibitors such as orthophosphates may also have impacts on antibiotic resistance and microbial communities in DWDS. However, there is little information on the relative effect of zinc orthophosphate, sodium orthophosphate, and sodium silicates on the abundance of antibiotic resistance and metal resistance genes within drinking water systems.

The objective of this study was to quantify the impact of corrosion inhibitor type (zinc orthophosphate, sodium orthophosphate, and sodium silicates) on microbial communities and the abundance of antibiotic resistance and metal resistance within the bacterial community of a source water for drinking water. It was hypothesized that increasing concentrations of corrosion inhibitors would increase the absolute abundance and relative abundance of ARB and ARGs within the bacterial communities. This relationship was tested through laboratory-scale microcosms of lake water exposed to corrosion inhibitors and direct plating on R2A media containing clinically relevant concentrations of antibiotics from seven different classes consisting of ampicillin, ciprofloxacin, rifampicin, tetracycline, trimethoprim, sulfamethoxazole, and vancomycin. The quantification of genetic determinates was determined via qPCR for  $\beta$ -lactam resistance (*bla*<sub>TEM</sub>), sulfonamide resistance (*sul1* and *sul2*), zinc efflux/resistance (*czcC* and *czcD*), copper resistance (*copA*), quaternary ammonium compound resistance (*qacE $\Delta$ 1*), and the integrase gene of class 1 integrons (*intI1*).

## 4.2 Materials and Methods

### 4.2.1 Microcosm Setup

Microcosm experiments were set up to test the impacts of three different corrosion inhibitors, zinc orthophosphate, sodium orthophosphate, and sodium silicate, on the abundance of ARB, ARGs and MRGs in lake water that is used as a source water for multiple drinking water treatment plants. Drinking water treatment significantly alters microbial communities during treatment processes such as filtration and chlorination (Pinto et al. 2012; Sharma et al. 2016). Therefore, a source water for drinking water was used in these experiments to observe selection for ARB and ARGs by the tested chemicals and to reduce the confounding effects of different treatment processes, disinfectant residuals, and corrosion inhibitors, such as those found in tap drinking water (Kappell et al. 2019). Additionally, the lake water used for experiments allowed for the observation of a broader potential for selection of antibiotic resistance compared to using treated drinking water.

Microcosm experiments with corrosion inhibitors (CI) consisted of 1 L glass bottles with 1 L of source water. Experiments were performed in triplicate at the following conditions: Set 1 (Normal CI concentration, lake water collected from drinking water intake pipe), Set 2 (Normal CI concentration, lake water collected at recreational beach), and Set 3 (10X CI concentration, lake water collected at recreational beach). The lake water for Set 1 was collected from a local drinking water treatment plant (DWTP) directly from the intake pipe to the DWTP. The recreational beach lake water for Sets 2 and 3 were collected from off a breakwater structure at Atwater park north of Milwaukee

harbor (Milwaukee, WI) approximately 50 meters into Lake Michigan. All samples were collected with a 45 L disinfected carboy for water homogeneity. The sample was transported immediately to the laboratory where 1 L subsamples were aseptically added to sterile 1 L clear glass bottles.

Corrosion inhibitors including 99% purity zinc orthophosphate ( $Zn_3(PO_4)_2$ ) (Fisher Scientific, Waltham, MA), monobasic sodium orthophosphate ( $NaH_2PO_4$ ) (98-102% purity, Fisher Scientific, Waltham, MA), and sodium metasilicate ( $Na_2SiO_3$ ) (49.5-52.5%  $Na_2O$ , Sigma Aldrich, St. Louis, MO) were added to the microcosms at two different concentrations to simulate conditions in DWDS. Sodium metasilicate was used as a representative silicate-based corrosion inhibitor. Previous studies have suggested that different sodium silicate formulations may have different efficacy for lead corrosion control (Li et al. 2021a). A national survey of U.S. drinking water utilities reported concentrations of phosphate corrosion inhibitors ranging from 0.2 to 3.0 mg/L in DWDS, with over 50% of utilities dosing between 0.7 and 2.0 mg/L as  $PO_4$  (McNeill and Edwards 2002). Microcosms with corrosion inhibitor concentrations to simulate full-scale DWDS conditions (Sets 1 and 2) consisted of the following: 1 mg/L zinc orthophosphate as  $PO_4$ , 1 mg/L sodium orthophosphate as  $PO_4$ , and 10 mg/L sodium silicate as  $SiO_2$ . Typical recommended dosages of sodium silicates for water treatment purposes range from 8 to 55 mg/L as  $SiO_2$  (Thompson et al. 1997; Schock et al. 2005a). Experiments conducted with high levels of corrosion inhibitors (Set 3) consisted of the following: 10 mg/L zinc orthophosphate as  $PO_4$ , 10 mg/L sodium orthophosphate as  $PO_4$ , and 100 mg/L sodium silicate as  $SiO_2$ . These experiments were conducted to observe the potential selection for antibiotic resistance under high nutrient conditions. Microcosms were

operated for a total duration of 7 days. While residence time of water in a DWDS is not expected to be 7 days, the additional time allowed for identification of slow-growing microorganisms that were selected for in the community via plating. Water samples were collected on Days 0, 3, and 7 for microbiological and chemical analysis. The microcosms were placed on an orbital mixer at 150 rpm, to keep cells suspended and water homogenized, at room temperature ( $25.0\pm 2.0^{\circ}\text{C}$ ) in darkness.

#### **4.2.2 Quantification of Antibiotic Resistant Heterotrophic Bacteria**

Water from the microcosms was collected on days 0, 3, and 7 and then plated on R2A media with and without antibiotics to determine relative abundance of antibiotic resistant heterotrophic bacteria. Subsamples and dilutions in R2A media from day 0 at were plated directly onto R2A containing 100  $\mu\text{g/mL}$  of cycloheximide to inhibit fungal growth. Antibiotics were added to R2A at diagnostic concentrations inferring failure of treatment or clinical resistance based on CLSI dilution method (CLSI 2015). The antibiotics were ampicillin (AMP; 32  $\mu\text{g/mL}$ ), ciprofloxacin (CIP; 4  $\mu\text{g/mL}$ ), rifampicin (RIF; 4  $\mu\text{g/mL}$ ), sulfamethoxazole (SULF; 100  $\mu\text{g/mL}$ ), tetracycline (TET; 16  $\mu\text{g/mL}$ ), trimethoprim (TRIM; 16  $\mu\text{g/mL}$ ), and vancomycin (VAN, 32  $\mu\text{g/mL}$ ). Subsamples were taken after 3 and 7 days of exposure to corrosion inhibitors and plated. The plates were incubated for 5 days at  $30^{\circ}\text{C}$  in an incubator with a container of tap water to keep relative humidity constant and not allow the plate to dehydrate. Counts were performed manually.

#### 4.2.3 Quantification of Antibiotic Resistance Genes, Metal Resistance Genes, and Class 1 Integrons

The remaining water from each microcosm was aseptically quantified by graduated cylinder and harvested by vacuum filtration onto a 0.22  $\mu\text{m}$  Millipore Express PLUS Membrane filter (MilliporeSigma, Burlington, MA). Filters were placed in sterile 2 mL tubes and stored at  $-20^{\circ}\text{C}$  until DNA was extracted. FastDNA Spin Kits (MP Biomedicals, Solon, OH) were used for DNA extraction, in accordance with the manufacturer's protocol, with the exception of the initial cell breakage, as described below. Tubes were placed in liquid nitrogen and filters homogenized by grinding with a sterile pipet tip. The CLS-TC lysis buffer (1.0 mL) was added and the tube was subjected to freeze-thaw cycles (x3) in liquid nitrogen and thawing at room temperature with vortexing between cycles (Kimbell et al. 2018; Kappell et al. 2019; Harrison et al. 2020; Kimbell et al. 2021). Yield of DNA was determined by microspectrophotometer analysis with Nanodrop One (Thermo Scientific, Waltham, MA).

Genes in the extracted DNA were enumerated by quantitative PCR (qPCR) using primers previously published for bacterial 16S rRNA (Muyzer et al. 1993), *bla*<sub>TEM</sub> (Marti et al. 2013), *copA* (Roosa et al. 2014), *czcC* (Roosa et al. 2014), *czcD* (Roosa et al. 2014), *intI1* (Goldstein et al. 2001), *sul1* (Burch et al. 2013), *sul2* (Kappell et al. 2019), and *qacE $\Delta$ 1* (Stedtfeld et al. 2018). All qPCR reactions were performed with 20  $\mu\text{L}$  total reaction volumes with 1x PowerUp SYBR Green Master Mix (Applied Biosystems, USA), F/R primers at a final concentration of 1.0  $\mu\text{M}$  each, and 5  $\mu\text{L}$  of DNA template. DNA templates were diluted to 1:10 for all genes except for 16S rRNA that was diluted to 1:100 to achieve concentrations within the standard curve. Each sample was run in

duplicate on qPCR and the results were averaged. The replicates from microcosms were averaged ( $n = 3$  from a triplicate experiment, and each of the three values stemmed from the average of duplicate qPCR runs). Microcosm samples with results below the quantification limit were reported as the quantification limit. No template controls and standards containing target gene DNA between  $10^0$  and  $10^7$  copies were performed in duplicate with each qPCR assay. Cycling conditions were conducted as previously described (Kappell et al. 2018b; Kimbell et al. 2018; Kimbell et al. 2021). Specificity of amplification of target genes was confirmed by melt curves consistent with that of each standard. Amplification efficiency was determined by the resulting standard curve and was considered acceptable between 0.9 and 1.1. Reactions were performed using a LightCycler 96 (Roche Molecular Systems Inc., USA). Results were reported as copies per L and normalized to 16S rRNA gene copies to observe trends in relative abundance. The qPCR limit of quantification was determined for each gene according to MIQE guidelines (Bustin et al. 2009). Specific primer sets, annealing temperatures, efficiencies, and detection limits are described in **Table 4.1A in Appendix 4A**.

#### **4.2.4 Water Quality Analysis**

Water quality for the source drinking water was characterized on the day of collection (Day 0) for the following parameters: alkalinity, pH, hardness, dissolved oxygen, ammonia, phosphate, dissolved metals, silicate, and total organic carbon (TOC). Water was aseptically removed from the microcosms on Days 3 and 7 and analyzed for pH, dissolved metals, TOC, phosphate, and silicate. A Thermo Fisher Scientific pH probe was used for measuring pH for the microcosms. Sodium silicate, alkalinity, hardness,

dissolved oxygen, and ammonia were measured according to appropriate standard methods using Hach kits (Hach Company, Loveland, CO). Phosphate was measured using the ascorbic acid method (Standard Method 4500-P) (APHA et al. 1998). Dissolved metal concentrations were quantified using an Agilent 7700s inductively coupled plasma mass spectrometer (ICP-MS) (Agilent Technologies, Santa Clara, CA). Water samples analyzed for TOC were filtered with 0.45  $\mu$ M PTFE filters prior to acidification to <2 pH with HCl (36.5-38.0%) and quantification using a Shimadzu TOC analyzer (Shimadzu, Kyoto, Japan). Water quality measurements are included in Appendix 4B.

#### **4.2.5 Statistical analysis**

Statistical analysis was performed using Graphpad Prism V 9.3.1 ( $\alpha < 0.05$ ). Two-way Analysis of variance (ANOVA) was used to determine significant differences among the concentrations and treatment types for absolute and relative abundance measurements of ARB, ARGs, MRGs, and *intI1* on Days 0, 3, and 7. Post hoc multiple pairwise comparisons were conducted by using Tukey's honest significant differences test.

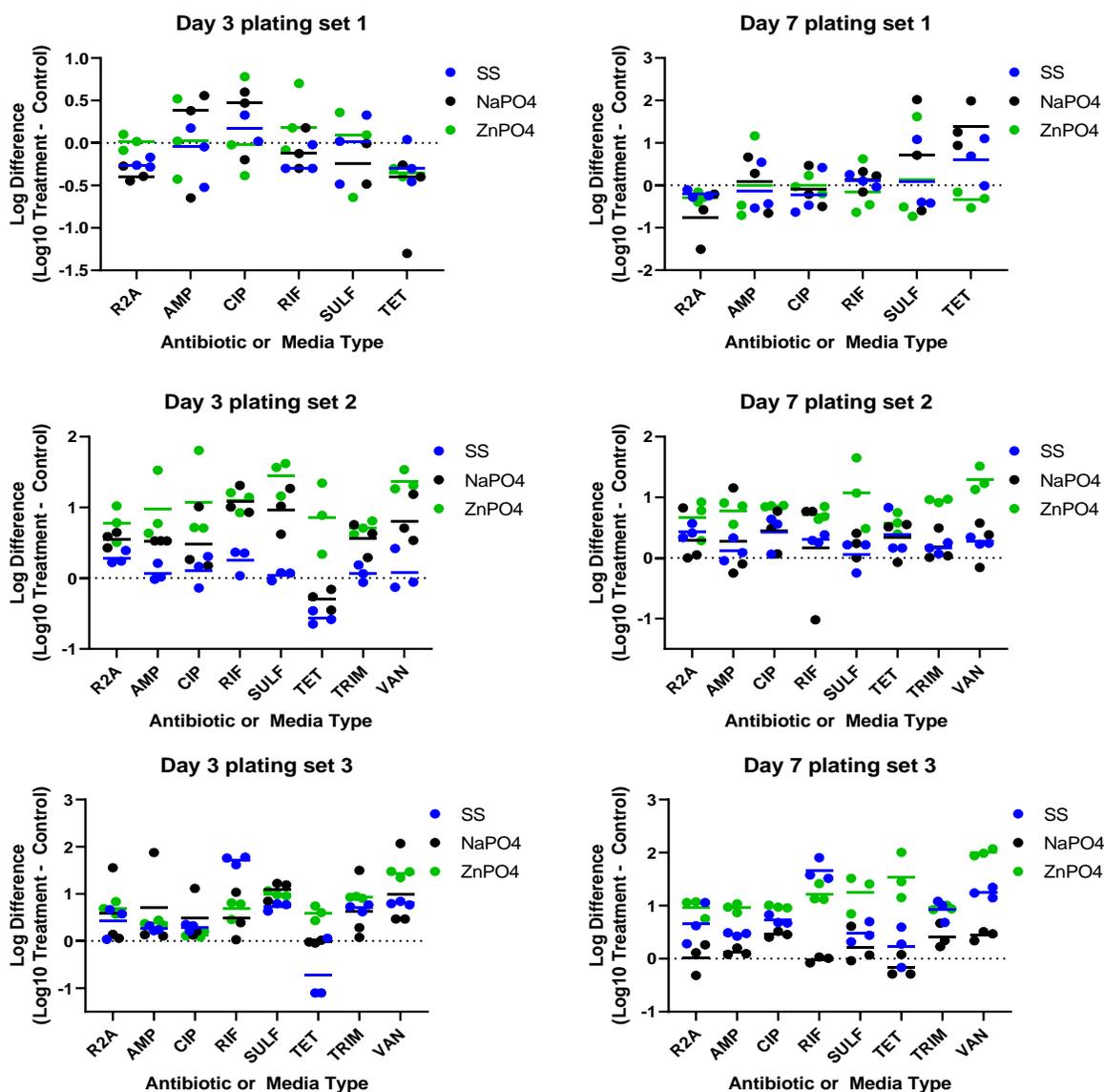
### **4.3 Results and Discussion**

#### **4.3.1 Quantification of Heterotrophic Antibiotic Resistant Bacteria (ARB)**

##### **4.3.1.1 Impact of Corrosion Inhibitor Type and Concentration**

ARB were quantified in water samples collected from each of the microcosms on days 3 and 7 to determine the effect of corrosion inhibitors on the abundance of phenotypic antibiotic resistance. There was a significantly greater absolute abundance of

ARB per liter in the zinc orthophosphate treatment at 1 mg/L compared to untreated controls on day 3 for R2A and all seven antibiotic types tested (Two-way ANOVA; all p values < 0.05) (**Figure 4.1**). Sodium orthophosphate treatment at 1 mg/L also resulted in significantly greater absolute abundance of R2A, RIF, SULF, TRIM, and VAN compared to untreated controls by day 3 (Two-way ANOVA; all p values < 0.05). At high concentrations, sodium orthophosphate treatment at 10 mg/L also resulted in significantly greater absolute abundance of ARB resistant to SULF compared to untreated controls (Two-way ANOVA; p value < 0.05). Similarly, there was a significantly greater absolute abundance of ARB in the zinc orthophosphate treatment at 10 mg/L compared to untreated controls on day 3 for bacteria resistant to SULF (Two-way ANOVA; p values < 0.05). Sodium silicate treated communities demonstrated significantly greater resistance to RIF and SULF resistant bacteria at 100 mg/L as SiO<sub>2</sub> by day 3. At normal concentrations found in DWDS (10 mg/L as SiO<sub>2</sub>), sodium silicate treatment did not select for increased absolute or relative abundance of ARB relative to untreated controls by day 3.



**Figure 4.1.** Log difference in absolute abundance of total heterotrophic bacteria (R2A) and antibiotic resistant bacteria on Day 3 and Day 7 based on direct plating of from microcosms containing different types and concentrations of corrosion inhibitors. The difference in counts of colony forming units (CFU) in Log<sub>10</sub> per liter of water for each treatment microcosm was determined relative to the control microcosm on Days 3 and 7 are shown on the y-axis. The type of media or antibiotic is denoted on the x-axis. Corrosion inhibitor type is indicated by color. Different experimental conditions are denoted with shapes including circle (Set 1 – Normal concentration (1x) of CI’s, lake water from DWTP intake pipe), triangle (Set 2 – Normal concentration (1x) of CI’s, lake water collected at recreational beach), and square (Set 3 – High concentration (10x) of CI’s, lake water collected at recreational beach).

By day 7, absolute abundance of ARB was significantly greater in 1 mg/L zinc orthophosphate treatment for CIP, SULF, TRIM, and VAN resistant bacteria compared to untreated controls (Two-way ANOVA; all p values < 0.05) (**Figure 4.1**). The addition of 10 mg/L zinc orthophosphate resulted in the highest ARB growth out of all of the tested conditions on day 7, with significantly higher absolute abundances of total heterotrophic bacteria (R2A) and ARB resistant to AMP, CIP, RIF, SULF, TET, TRIM, and VAN compared to untreated controls by day 7 (Two-way ANOVA; all p values < 0.05). Sodium orthophosphate addition at 10 mg/L exhibited significantly greater absolute abundance of ARB resistant to CIP and VAN compared to untreated controls by day 7 (Two-way ANOVA; p values < 0.05). Sodium silicate treatment at 10 mg/L did not result in any selection for ARB by day 7 compared to untreated controls. However, absolute abundance of ARB was significantly greater for the sodium silicate treatment at 100 mg/L for AMP, CIP, RIF, and VAN compared to untreated controls by day 7 (Two-way ANOVA; p values < 0.05).

The increase in abundance of total heterotrophic bacteria and ARB indicate that the addition of zinc orthophosphate and sodium orthophosphate may be providing limiting nutrients to bacterial communities. Zinc is an essential trace metal and can stimulate microbial growth in aquatic systems, as shown previously (Kappell et al. 2019). Bacterial exposure to zinc at increased concentrations may stimulate metal resistance mechanisms and co-select for antibiotic resistance within bacterial populations (Baker-Austin et al. 2006). Orthophosphate can be readily used by heterotrophic bacteria and previous studies have documented increased microbial growth after phosphate addition

ranging from 0.03 to 3 mg/L (Fang et al. 2009; Kogo et al. 2017a). Similarly, zinc orthophosphate addition at 1-3 mg/L as PO<sub>4</sub> increased microbial community diversity and richness in drinking water biofilms grown on lead and copper coupons (Payne et al. 2016). The results observed in this study indicate that the addition of zinc orthophosphate and sodium orthophosphate resulted in increased positive selection for heterotrophic bacteria resistant to multiple types of antibiotics at concentrations typically used for corrosion control in DWDS (1 mg/L as PO<sub>4</sub>) when compared to sodium silicate treatment and untreated controls. Interestingly, the addition of orthophosphates at normal concentrations (1 mg/L) resulted in larger increases in absolute abundance of ARB on day 3 compared to higher concentrations in the 10 mg/L treatment. However, by day 7 the absolute abundance of the high dosages of corrosion inhibitors resulted in the highest levels ARB growth compared to untreated controls. Sodium silicate addition at 100 mg/L also resulted in increased positive selection for ARB resistant to multiple types of antibiotics.

Analyzing the change in relative abundance of ARB provided an indication of phenotypic selection for ARB in the presence of different corrosion inhibitor types and concentrations. When corrected for total heterotrophic bacteria there was a significantly greater relative abundance of VAN-resistant bacteria in the 10 mg/L zinc orthophosphate treatment compared to untreated by day 3 (Two-way ANOVA; p value < 0.05) (see **Figure 4C in Appendix 4C**). Similar selection for VAN resistance was observed at 1 mg/L zinc orthophosphate but the differences were not statistically significant. There was also a significant increase in relative abundance of VAN-resistant bacteria in the zinc orthophosphate compared to the sodium silicate treatment at 1 mg/L (Two-way ANOVA;

p value < 0.05). Sodium silicate addition at higher dosages (100 mg/L) significantly increased the relative abundance of RIF-resistant bacteria compared to untreated controls and both phosphate corrosion inhibitor treatments by day 3 (Two-way ANOVA; p values < 0.05). By day 7, the changes in relative abundance for each of the corrosion inhibitor treatments were not significantly different from the untreated controls (see **Figure 4D in Appendix 4D**). Similarly, a previous study suggested that the selection of ARB was transient in nature due to the initial influx of zinc and assimilation through population growth (Kappell et al. 2019). The increased time of exposure of bacterial populations to the different corrosion inhibitor treatments may have caused the resistant populations that developed by day 3 through intrinsic and/or acquired resistance mechanisms to be outcompeted by other bacteria by day 7.

#### **4.3.1.2 Impact of Source Water on Antibiotic and Metal Resistance**

The impact of source water selection was evaluated through comparing results from microcosm experiment sets 1 and 2, which were both operated under normal corrosion inhibitor concentrations found in full-scale DWDS (i.e., 1 mg/L as PO<sub>4</sub> and 10 mg/L as SiO<sub>2</sub> dosages). The influent source water collected from the DWTP did not display the same patterns of selection for phenotypic resistance compared to the surface water collected from the Atwater Beach (**Figure 4.1**). There were no observed significant changes in absolute or relative abundance for ARB for the samples collected from the DWTP in experiment set 1 relative to untreated controls. The DWTP intake pipe extends more than 2 miles from the shore and collects lake water at a depth of approximately 40 feet. The bacterial community and concentrations of contaminants present in this water

sample were lower in comparison to the source water collected for microcosm set 2 at Atwater Beach. The source water from Atwater Beach is likely more representative of the surrounding landscape with increased amounts of nutrients, bacteria, and other contaminants from stormwater runoff entering the lake and continuous mixing with the sand and sediment at the beach shore.

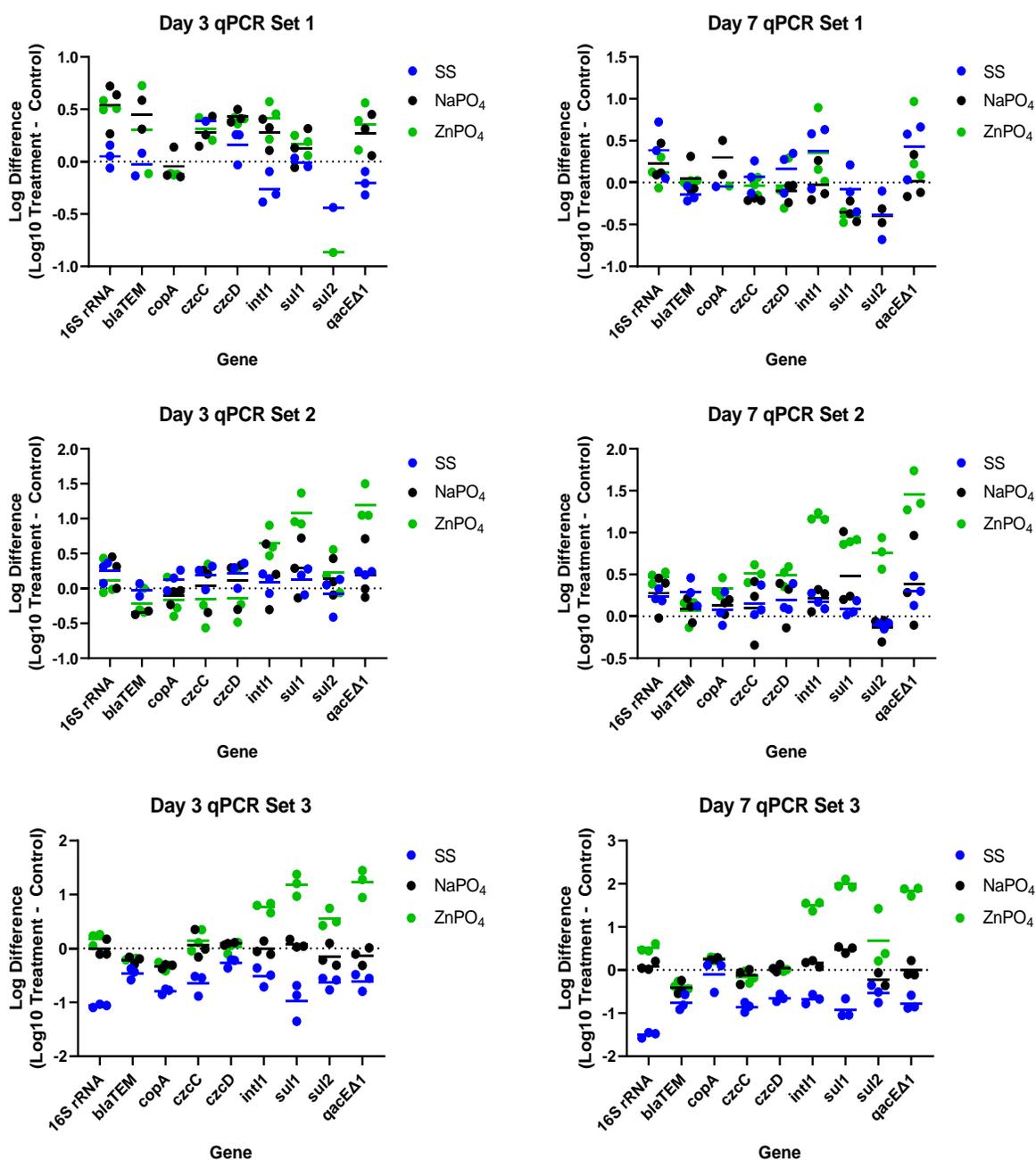
The source water collected for microcosm experiment set 2 contained greater than 0.05 mg/L of orthophosphate on day 0, but the day 0 sample collected from microcosm Set 1 was below the detection limit for orthophosphate (<0.05 mg/L as PO<sub>4</sub>). The silicate concentration for set 1 was below the detection limit (<1 mg/L as SiO<sub>2</sub>); however, the silicate concentration for set 2 on day 0 was approximately 2 mg/L as SiO<sub>2</sub>. The source water for set 2 also had slightly higher TOC values ranging from 2.05 to 2.99 mg/L compared to 2.16 to 2.92 mg/L for set 1. TOC values for surface waters used for drinking water range from approximately 0.1 to 20 mg/L (Volk et al. 2002).

## **4.3.2 Quantification of Genotypic and Phenotypic Antibiotic Resistance**

### **4.3.2.1 Impact of Corrosion Inhibitor Type and Concentration**

ARGs, MRGs, and the integrase gene of class 1 integrons (*intI1*) were quantified in water samples collected from each of the microcosms on days 3 and 7 to determine the effect of corrosion inhibitors on the abundance of genotypic antibiotic resistance and metal resistance. There was a significantly greater absolute abundance of ARGs per liter in the zinc orthophosphate treatment at 1 mg/L compared to all other treatments for *sul1* and *qacEΔ1* on day 3 (Two-way ANOVA; p values < 0.05) (**Figure 4.2**). Zinc

orthophosphate addition at 10 mg/L selected for significantly greater absolute abundance of ARGs including *copA*, *intI1*, *sul1*, *sul2*, and *qacEΔ1* compared to untreated controls on day 3 (Two-way ANOVA; all p values < 0.05). Sodium orthophosphate addition at 1 mg/L did not result in significant increases in growth by day 3, but 10 mg/L addition increased the absolute abundance of *bla<sub>TEM</sub>* and *copA* to levels significantly higher than untreated controls (Two-way ANOVA; p values < 0.05). Sodium silicate addition at 10 mg/L did not result in any significant changes in ARG or MRG abundance relative to untreated controls by day 3. Sodium silicate addition at high concentrations (100 mg/L) selected for significantly lower absolute abundance of 16S rRNA and ARGs including *bla<sub>TEM</sub>*, *copA*, *czcC*, *sul1*, *sul2*, and *intI1* by day 3 compared to untreated controls, sodium orthophosphate and zinc orthophosphate treatments.



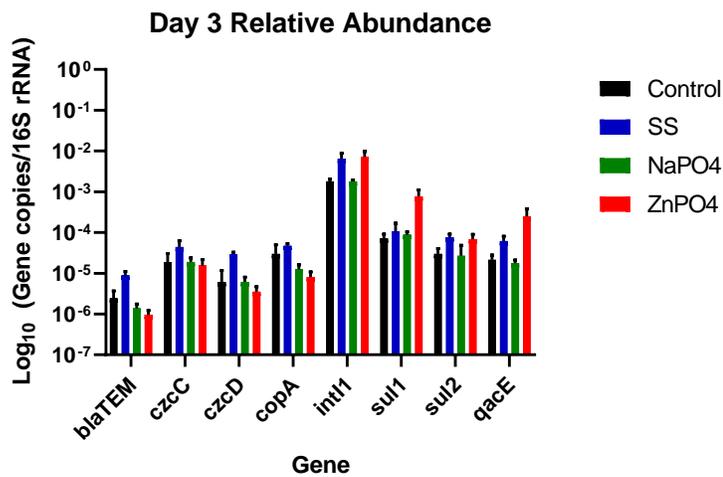
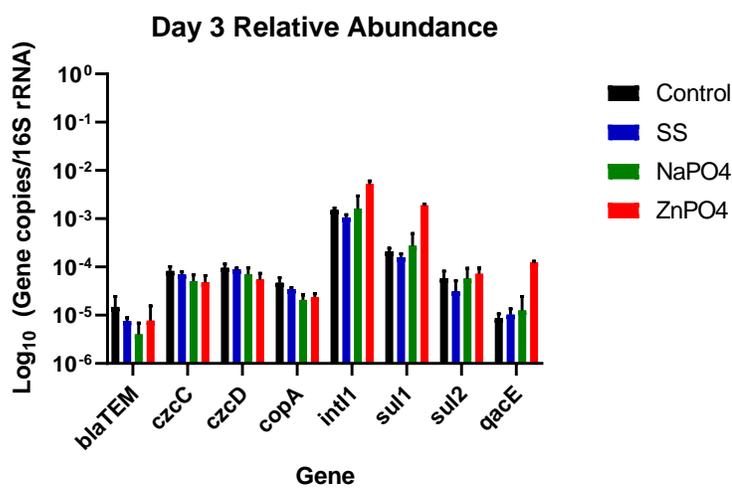
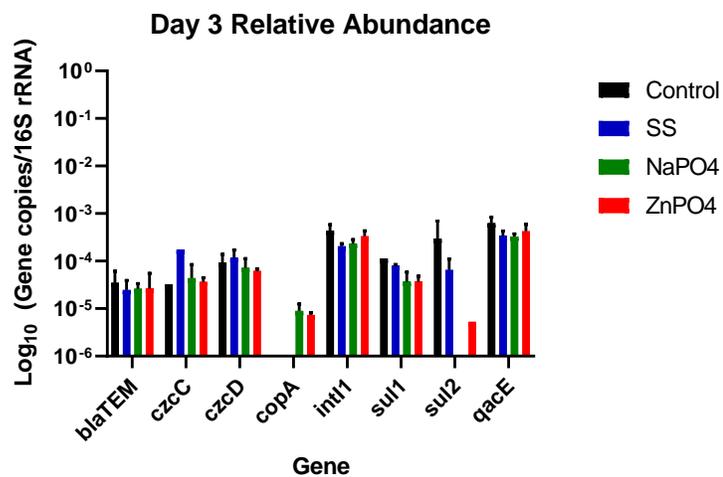
**Figure 4.2.** Log difference in absolute abundance of total bacterial biomass (16S rRNA), antibiotic resistance genes (ARGs), metal resistance genes (MRGs), and *int11* on Day 3 and Day 7 based on qPCR analysis from microcosms containing different types and concentrations of corrosion inhibitors. The difference in counts of gene copies in Log<sub>10</sub> per liter of water for each treatment microcosm was determined relative to the control microcosm on Days 3 and 7 are shown on the y-axis. The type of gene is denoted on the x-axis. Corrosion inhibitor type is indicated by color. Different experimental conditions

are denoted with shapes including circle (Set 1 – Normal concentration (1x) of CI's, lake water from DWTP intake pipe), triangle (Set 2 – Normal concentration (1x) of CI's, lake water collected at recreational beach), and square (Set 3 – High concentration (10x) of CI's, lake water collected at recreational beach).

By day 7, zinc orthophosphate treatment at 1 mg/L resulted in significantly greater absolute abundance of *sul1*, *sul2*, *qacEΔ1*, and *intI1* compared to untreated controls (Two-way ANOVA; all p values < 0.05) (**Figure 4.2**). Zinc orthophosphate treatment at 10 mg/L also resulted in significantly greater absolute abundance of 16S rRNA and ARGs including *sul1*, *qacEΔ1*, and *intI1* compared to untreated controls (Two-way ANOVA; p value < 0.05). Sodium orthophosphate addition did not exhibit similar growth patterns compared to zinc orthophosphate at the same concentrations tested, indicating that the addition of zinc may be promoting increased abundance of antibiotic resistance within the microbial communities. Zinc is an essential micronutrient required for proper structure and function of many proteins in bacterial cells (Dow and Priscic 2018). The addition of zinc may also promote P scavenging in bacterial communities since bacterial cells require zinc for production of phosphatase enzymes (e.g., alkaline phosphatase) (Coleman 1992). A previous study observed similar operons in several different bacterial species containing genes for heavy metal translocating ATPases and phosphatases (Hynninen et al. 2009). Communities without zinc orthophosphate addition may have been lacking sufficient zinc for phosphatase formation, thus limiting P scavenging and bacterial growth. Similarly, Kappell *et al.* 2018 observed similar selection for ARB and ARGs with both zinc chloride and zinc orthophosphate addition, indicating zinc as the selecting agent for observed increased

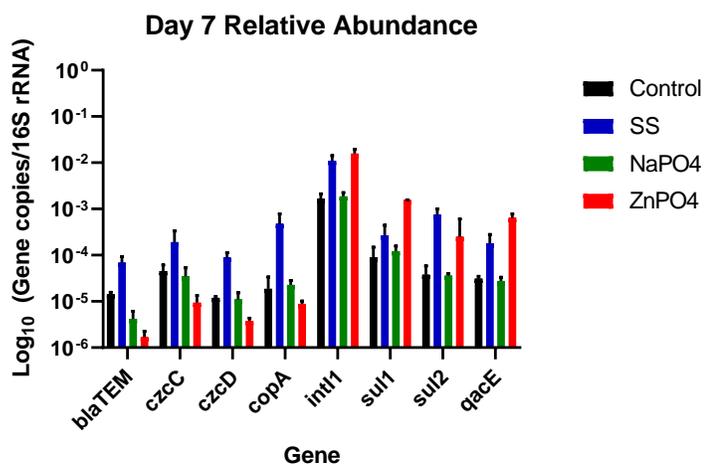
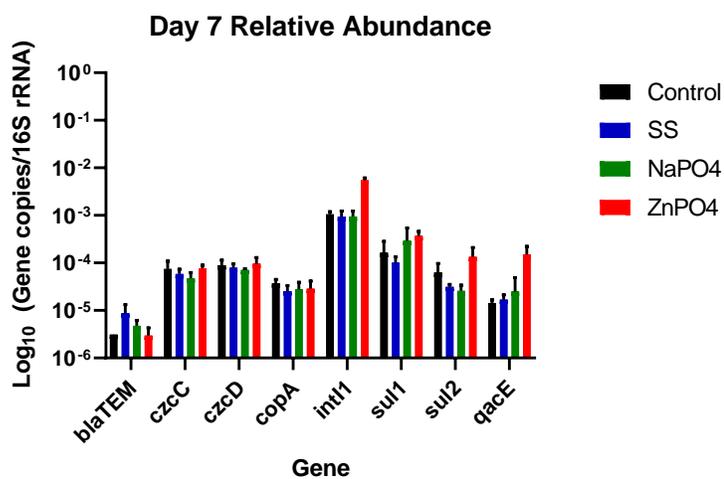
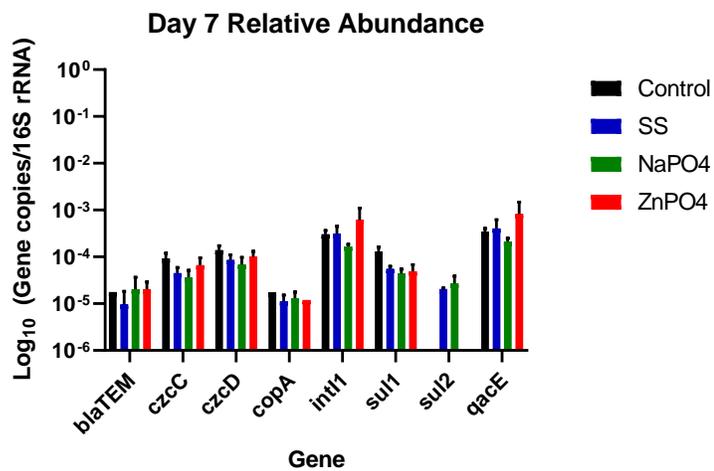
resistance in the microbial communities. Similar to results on day 3, sodium silicate addition at 100 mg/L significantly decreased the absolute abundance of several genes including 16S rRNA and ARGs including *czcC*, *czcD*, *sul1*, *sul2*, and *intI1* compared to untreated controls, sodium orthophosphate and zinc orthophosphate treatments.

Analyzing the change in relative abundance of resistance genes provided an indication of genotypic selection in the presence of different corrosion inhibitor types and concentrations. When normalized to 16S rRNA gene copies, there was a significantly greater relative abundance of *sul1*, *qacEΔ1*, and *intI1* gene copies in the zinc orthophosphate treatment at 1 mg/L compared to untreated controls, sodium silicate, and sodium orthophosphate by day 3 (Two-way ANOVA; p values < 0.05) (**Figure 4.3**). Similar selection for ARGs was observed at 10 mg/L zinc orthophosphate, which also exhibited significantly greater relative abundance of *sul1*, *qacEΔ1*, and *intI1* compared to untreated controls and sodium orthophosphate by day 3 (Two-way ANOVA; p values < 0.05). Sodium orthophosphate addition at 1 and 10 mg/L did not result in any significant changes in relative abundance of ARGs, MRGs, or *intI1* relative to untreated controls by day 3. Sodium silicate addition at higher dosages (100 mg/L) significantly increased the relative abundance of *intI1* compared to untreated controls and sodium orthophosphate by day 3 (Two-way ANOVA; p values < 0.05).



**Figure 4.3.** Relative abundance of ARGs, MRGs, and *intI1* on Day 3 of microcosm experiments as determined by qPCR. The average relative abundance is based on triplicate measurements from microcosms containing different types and concentrations of corrosion inhibitors. The relative abundance of each target gene is shown on the y-axis. The type of gene is denoted on the x-axis. Treatment type is indicated by color. Different experimental conditions are plotted on different graphs including Set 1 – Normal concentration (1X) of CI's, lake water collected from DWTP (top), Set 2 – Normal concentration (1X) of CI's, lake water collected at beach (middle), and Set 3 – High concentration (10X) of CI's, lake water collected at beach (bottom).

By day 7, there was a significantly greater relative abundance of *intI1* and *qacEΔ1* in the zinc orthophosphate compared to untreated controls and sodium orthophosphate addition (**Figure 4.4**) at 1 mg/L (Two-way ANOVA; p values < 0.05). Similar selection was observed at 10 mg/L zinc orthophosphate, which exhibited significantly greater relative abundance of *intI1*, *sul1*, and *qacEΔ1* by day 7 (Two-way ANOVA; p values < 0.05). Similar to results from day 3, sodium orthophosphate addition at 1 and 10 mg/L did not result in any significant changes in relative abundance of ARGs, MRGs, or *intI1* relative to untreated controls. Interestingly, by day 7 sodium silicate addition at 100 mg/L resulted in significantly increased relative abundance of several genes including *bla<sub>TEM</sub>*, *czcD*, *copA*, *intI1*, and *sul2* compared to untreated controls and sodium orthophosphate addition (Two-way ANOVA; p values < 0.05).



**Figure 4.4.** Relative abundance of ARGs, MRGs, and *intI1* on Day 7 of microcosm experiments as determined by qPCR. The average relative abundance is based on triplicate measurements from microcosms containing different types and concentrations of corrosion inhibitors. The relative abundance of each target gene is shown on the y-axis. The type of gene is denoted on the x-axis. Corrosion inhibitor type is indicated by color. Different experimental conditions are plotted on different graphs including Set 1 – Normal concentration (1X) of CI's, lake water collected from DWTP (top), Set 2 – Normal concentration (1X) of CI's, lake water collected at beach (middle), and Set 3 – High concentration (10X) of CI's, lake water collected at beach (bottom).

Overall, the absolute and relative abundance of zinc orthophosphate at concentrations ranging from 1 to 10 mg/L exhibited increased selection for ARB and ARGs compared to sodium orthophosphate addition. The sub-inhibitory concentrations of zinc allowed a growth or survival advantage for bacteria harboring the *sul1*, *sul2*, *qacEΔ1*, and *intI1* genes. Similar to the changes observed in abundance of antibiotic resistant heterotrophic bacteria, the presence of zinc and not orthophosphate was determined to be the major factor contributing to selection of ARGs and *intI1*. The integrase gene of class 1 integrons (*intI1*) plays an important role in the proliferation of acquired resistance to antibiotics, metals, and disinfectants through the ability to incorporate gene cassettes (Gillings et al. 2008; Ma et al. 2017a). The *sul1* and *sul2* genes encode dihydropteroate synthase that are not inhibited by sulfonamides (Antunes et al. 2005). The *sul1* gene is normally linked to other resistance genes in class 1 integrons, while *sul2* is usually located on small non-conjugative plasmids or large transmissible multi-resistance plasmids (Enne et al. 2001; Antunes et al. 2005). The *qacEΔ1* gene encodes resistance to quaternary ammonium compound disinfectants and is commonly observed on the same mobile resistance gene cassettes as *sul1* and *intI1* (Nandi et al. 2004).

The metal resistance genes targeted in this study, *czcC*, *czcD*, and *copA*, were targeted because they indicate the presence of zinc and other related metal resistance mechanisms (Kloosterman et al. 2007). The lack of significant changes in gene abundance for zinc-related resistance genes may support the evidence that low environmental zinc concentrations were limiting microbial growth for communities not treated with zinc orthophosphate. The addition of zinc led to positive selection for bacteria able to influx zinc, rather than zinc negatively selecting against bacteria without metal resistance (Kappell et al. 2019). Zinc is an essential nutrient for bacterial cells and plays an important role in phosphatase regulation (Coleman 1992). Protein phosphatases are involved in metabolic homeostasis, stress response, and other essential biological mechanisms. Zinc orthophosphate treated communities were likely more efficient at scavenging P, which increased bacterial growth compared to sodium orthophosphate or sodium silicate treated communities.

#### **4.4 Conclusions**

Environmental factors such as metal pipe materials, corrosion inhibitors, and corrosion products that develop in DWDS may be preferentially selecting for bacteria harboring resistance to both metals and antibiotics (Calomiris 1984; Stepanauskas et al. 2005; Kimbell et al. 2020). The results in this study indicate that corrosion inhibitors such as zinc orthophosphate, which are commonly used in drinking water systems, can alter resistance profiles and promote the occurrence of ARB, ARGs, and *intI1* in bacterial communities. Significant increases in absolute and relative abundance of ARB resistant to multiple clinically-relevant antibiotics including CIP, SULF, TRIM, and VAN and

ARGs including *sul1*, *sul2*, *qacEΔ1*, and *intI1* were observed in response to zinc orthophosphate addition at concentrations typically found in distribution systems (~1 mg/L). The presence of zinc orthophosphate may lead to positive selective pressure for ARB, which causes increases in the abundance and types of resistance genes they harbor (Kappell et al. 2019). Zinc orthophosphate resulted in the highest selection of ARB and associated ARGs compared to controls, sodium orthophosphate, and sodium silicate treatment. Sodium silicate dosage at 10 mg/L resulted in decreased bacterial growth as measured by heterotrophic plate counts and qPCR, when compared to zinc and sodium orthophosphate treatments. High doses of silicates at 100 mg/L increased the growth of ARB resistant to RIF and VAN and several ARGs and MRGs including *bla<sub>TEM</sub>*, *czcD*, *copA*, *intI1*, and *sul2* relative to untreated controls. High levels of zinc orthophosphate (10 mg/L) and sodium silicate (100 mg/L) exhibited similar selection of ARGs including *intI1*, *sul2*, and *qacE* by day 7.

The results of this study suggest that utilities using zinc orthophosphate as a corrosion inhibitor may be promoting increased occurrence of bacteria and ARGs in tap drinking water. According to a 2019 AWWA survey, zinc orthophosphate is the most common form of corrosion control with over 38% of utilities reporting its use for LCR compliance (Arnold et al. 2020). Implementation of sodium silicate as an alternative corrosion inhibitor may be beneficial for limiting bacterial growth, however, silicates may have reduced ability to mitigate lead release and protect against galvanic corrosion compared to orthophosphates (Li et al. 2021b). Others have noted that sodium silicate addition can result in the formation of a passivation layer on the interior surface of pipes, which may help to inhibit corrosion (Thompson et al. 1997; Schock et al. 2005a). Studies

documenting silicate usage have reported higher lead release when compared to orthophosphates for lead and copper control (Rompré et al. 2000; Lintereur et al. 2010; Woszczyński et al. 2015; Aghasadeghi et al. 2021). Future research should be conducted to determine the potential implications and unintended consequences of corrosion inhibitor usage on bacterial communities and antibiotic resistance in DWDS, in addition to lead and copper corrosion control.

Management practices should be considered that minimize the impact of chemicals added for water treatment purposes on the spread of antibiotic resistance. Corrosion inhibitors that simultaneously minimize dissolved metal concentrations and bacterial growth in drinking water should be prioritized for protection of public and environmental health. The experiments in this study were conducted with a source drinking water without the presence of disinfectants such as chlorine to analyze the impact of specific corrosion inhibitors on antibiotic resistance selection patterns. It is widely documented that disinfectants including free chlorine and chloramines can increase the occurrence of ARGs and ARB in bacterial communities (Shi et al. 2013; Dai et al. 2019). Research that considers the combined effects of disinfectants and corrosion inhibitors would be beneficial for deciphering the complex interactions between chemicals added for water treatment purposes and their potential impacts on antibiotic resistance. Metagenomic analysis of bacterial communities treated with different corrosion inhibitors could provide valuable information regarding selection for antibiotic resistance through analyzing the effects on the microbiome and resistome.

## **5 METAL-CONTAINING CORROSION INHIBITORS AND DISINFECTANTS INCREASE OCCURRENCE AND TYPES OF ANTIBIOTIC RESISTANCE GENES IN THE ANTIBIOTIC RESISTOME OF A DRINKING WATER SOURCE**

### **5.1 Introduction**

Antimicrobial resistance (AMR) is a global health problem which may result in a financial burden of over 100 trillion USD by 2050 (O'Neill 2016). Resistance can be intrinsic in bacterial cells, occur through point mutations conferring target modification, or by acquiring antibiotic resistance genes (ARGs) from the environment (Levy 2002; Levy and Bonnie 2004). Antibiotic resistant bacteria (ARB) and associated ARGs are ubiquitous in the natural environment (Berglund 2015) and in engineered systems such as drinking water treatment plants (Xi et al. 2009; Jia et al. 2020) and wastewater treatment plants (LaPara et al. 2011; Munir et al. 2011; Mao et al. 2015). ARGs and ARB are transported between different environments and can be enriched for through horizontal gene transfer (HGT) and reproduction of host ARB. Mobile genetic elements (MGEs) such as plasmids, transposons, and integrons enable ARGs to be spread rapidly between bacteria, which poses risks to human health (Guo et al. 2017). Bacterial cells are exposed to many environmental factors that may contribute to the development and proliferation of antibiotic resistance, including antibiotics (Rodriguez-Mozaz et al. 2015b), antimicrobials (Carey and McNamara 2016; Harrison et al. 2020), disinfectants such as chlorine and chloramines (Zhang et al. 2017b), and metals (Poole 2017; Zhang et al. 2018b). Engineered systems such as drinking water distribution systems (DWDS) are primarily composed of metal pipe materials and employ disinfectants such as chlorine

and chloramines, which may contribute to the proliferation of ARB and ARGs in tap drinking water (Xi et al. 2009; Kimbell et al. 2020).

The presence of chlorine, metals, antibiotics, and other antimicrobial compounds in DWDS may preferentially select for bacterial communities resistant to both antibiotics and metals (Calomiris et al. 1984; Baker-Austin et al. 2006). Several studies have reported pathogens in drinking water systems that are chlorine-resistant and contain ARGs encoding resistance to multiple types of antibiotics, which potentially poses risks to human health and drinking water safety (Dai et al. 2019; Sevillano et al. 2020; Hu et al. 2021). Additionally, previous studies have demonstrated that the use of phosphate-containing corrosion inhibitors such as zinc orthophosphate can increase total and relative abundance of ARB and ARGs in a source water used for drinking water (Kappell et al. 2019). The use of sodium silicates as an alternative corrosion inhibitor to phosphate-based inhibitors has been considered due to the potential for limiting bacterial growth and biofilm development (Kogo et al. 2017a; Aghasadeghi et al. 2021). However, the mechanism of lead and copper sequestration by sodium silicates is not well documented and some researchers have suggested sodium silicate addition does not offer any additional benefits compared to pH adjustment for corrosion control (Li et al. 2021a). Compliance with the Lead and Copper Rule (LCR) is the primary goal of corrosion control in DWDS, but there may be unintended consequences stemming from the addition of corrosion inhibitors such as phosphates and sodium silicates to drinking water.

Non-targeted methods of analysis such as shotgun metagenomics have revealed insights into the effects of drinking water treatment processes such as chlorination (Jia et

al. 2015) and filtration (Pinto et al. 2012) on bacterial communities in drinking water. Characterizing the antibiotic resistome (i.e., collection of all resistance genes in a community) is vital for understanding the spread of antibiotic resistance and identifying novel resistance elements (Dias et al. 2020). It is widely documented that chlorine disinfection causes systemic changes in the microbiome and resistome in microbial communities in DWDS (Shi et al. 2013; Jia et al. 2015). The addition of phosphate-based corrosion inhibitors can supply nutrients to bacteria for growth and biofilm development, play key roles in metabolic regulation, and increase bacterial resistance to environmental stressors (Rao and Kornberg 1996; Gangaiah et al. 2009; Payne et al. 2016). Phosphate may also select for microorganisms with enhanced capabilities related to phosphorus metabolism and heavy metal resistance in chlorinated systems (Del Olmo et al. 2020). However, there is a paucity of research related to the impacts of corrosion inhibitors including sodium silicates, sodium orthophosphate, and zinc orthophosphate on the prevalence and transmission of AMR in DWDS.

The objective of this study was to quantify the impact of disinfectants (free chlorine/chloramines) and corrosion inhibitors (zinc orthophosphate, sodium orthophosphate, and sodium silicates) on the microbial community structure and resistome within the bacterial community of a source water used for drinking water. This relationship was tested through laboratory-scale microcosms of lake water exposed to corrosion inhibitors and direct plating on R2A media containing clinically relevant concentrations of antibiotics from 7 different classes: ampicillin, ciprofloxacin, rifampicin, tetracycline, trimethoprim, sulfamethoxazole, and vancomycin. The quantification of genetic determinates including 16S rRNA, quaternary ammonium

compound resistance (*qacEΔ1*), and the integrase gene of class 1 integrons (*intI1*) were determined using qPCR. The microbiome and antibiotic resistome in each of the communities was analyzed using shotgun metagenomics.

## 5.2 Materials and Methods

### 5.2.1 Microcosm Setup

Microcosm experiments were set up to test the impacts of three corrosion inhibitors, zinc orthophosphate, sodium orthophosphate, and sodium silicate, on the abundance of ARB, ARGs, and microbial communities in lake water that is used as a source water for multiple drinking water treatment plants. Drinking water treatment significantly alters microbial communities during treatment processes such as filtration and chlorination (Pinto et al. 2012; Sharma et al. 2016). A source water for drinking water was used in these experiments to observe selection for ARB and ARGs by the tested chemicals and to reduce the confounding effects of different treatment processes, corrosion inhibitors, and disinfectant residuals, such as those found in tap drinking water (Kappell et al. 2019). Additionally, lake water contains a more diverse microbial community which allowed for a broader potential for selection compared to using treated drinking water for microcosm experiments.

Corrosion inhibitors including 99% purity zinc orthophosphate ( $Zn_3(PO_4)_2$ ) (Fisher Scientific, Waltham, MA), monobasic sodium orthophosphate ( $NaH_2PO_4$ ) (98-102% purity, Fisher Scientific, Waltham, MA), and sodium metasilicate ( $Na_2SiO_3$ ) (49.5-52.5%  $Na_2O$ , Sigma Aldrich, St. Louis, MO) were added to the microcosms to simulate

conditions in DWDS using different types of corrosion control. Sodium metasilicate was used as a representative silicate-based corrosion inhibitor. Previous studies have suggested that different sodium silicate formulations may have different efficacy for lead corrosion control (Li et al. 2021a). A previous national survey of U.S. drinking water utilities reported concentrations of phosphate corrosion inhibitors ranging from 0.2 to 3.0 mg/L in DWDS, with over 50% of utilities dosing between 0.7 and 2.0 mg/L as  $\text{PO}_4$  (McNeill and Edwards 2002).

The source drinking water was collected from off a breakwater structure at Atwater park north of Milwaukee harbor (Milwaukee, WI) approximately 50 meters into Lake Michigan. All samples were collected with a 45 L disinfected carboy for water homogeneity. The samples were transported immediately to the laboratory where 1 L subsamples were aseptically added to sterile 1 L clear glass bottles. Microcosm experiments with different disinfectants and corrosion inhibitors consisted of 1 L glass bottles with 1 L of source water performed in triplicate. Experimental conditions for the disinfectant experiment included the addition of free chlorine at 1.6 mg/L as  $\text{Cl}_2$ , chloramine ( $\text{NH}_2\text{Cl}$ ) at 1.6 mg/L, and ammonia ( $\text{NH}_4$ ). Free chlorine was added as 5.65-6.0% sodium hypochlorite ( $\text{NaClO}$ ) (Fisher Scientific, Waltham, MA). Monochloramine formation was achieved through the addition of sodium hypochlorite and ammonia chloride ( $\text{NH}_4\text{Cl}$ ), as described previously (Xie et al. 2010a). Ammonia experiments were conducted by adding ammonium chloride to achieve the same molar ratio of nitrogen as the chloramine experiment. The second set of experimental microcosms were conducted with each of the corrosion inhibitors and free chlorine to simulate full-scale DWDS conditions, which consisted of the addition of 1 mg/L zinc orthophosphate as  $\text{PO}_4$ , 1

mg/L sodium orthophosphate as PO<sub>4</sub>, and 10 mg/L sodium silicate. Free chlorine was dosed at 1.6 mg/L as Cl<sub>2</sub> in each of the microcosms testing different types of corrosion inhibitors. Additional microcosm experiments were conducted to determine the impact of natural organic matter (NOM) in addition to corrosion inhibitors used in DWDS, without the presence of chlorine or other disinfectants. These experiments were conducted with the same corrosion inhibitor concentrations as previously stated with the addition of 5 mg/L Suwanee River NOM (International Humic Substances Society, Minneapolis, MN). Microcosms were operated for a total duration of 7 days. Water samples were collected on Days 0, 3, and 7 for microbiological and chemical analysis. The microcosms were placed on an orbital mixer at 150 rpm, to keep cells suspended and water homogenized, at room temperature (25.0±2.0°C) in darkness.

### **5.2.2 Quantification of Antibiotic Resistant Bacteria**

Water from the microcosms collected on days 0, 3, and 7 was plated on R2A media with and without antibiotics to determine the absolute and relative abundance of antibiotic resistant heterotrophic bacteria. Subsamples and dilutions in R2A media from day 0 were plated directly onto R2A containing 100 µg/mL of cycloheximide to inhibit fungal growth. Antibiotics were added to R2A at diagnostic concentrations inferring failure of treatment or clinical resistance based on CLSI dilution method (CLSI 2015). The antibiotics were ampicillin (AMP; 32 µg/mL), ciprofloxacin (CIP; 4 µg/mL), rifampicin (RIF; 4 µg/mL), sulfamethoxazole (SULF; 100 µg/mL), tetracycline (TET; 16 µg/mL), trimethoprim (TRIM; 16 µg/mL), and vancomycin (VAN, 32 µg/mL). Subsamples were taken after 3 and 7 days of exposure to corrosion inhibitors and

disinfectants and plated. R2A plates with sample were incubated for 5 days at 30°C in an incubator with a container of tap water to keep relative humidity constant and not allow the plated to dehydrate. Counts were performed manually.

### 5.2.3 DNA Extraction and Quantification of Resistance Genes

The remaining water from each microcosm was aseptically quantified by graduated cylinder and harvested by vacuum filtration onto a 0.22 µm Millipore Express PLUS Membrane filter (MilliporeSigma, Burlington, MA). Filters were placed into sterile 2 mL tubes and stored at -20°C until DNA was extracted. For DNA extraction, the manufactures protocol of FastDNA Spin Kit (MP Biomedicals, Solon, OH) was used with the exception of the initial cell breakage. Tubes were placed in liquid nitrogen and filters homogenized by grinding with a sterile pipet tip. The CLS-TC lysis buffer (1.0 mL) was added and the tube was subjected to freeze-thaw cycles (x3) in liquid nitrogen and thawing at room temperature with vortexing between cycles (Kimbell et al. 2018; Kappell et al. 2019; Harrison et al. 2020; Kimbell et al. 2021). DNA yield was determined by microspectrophotometer analysis with a Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA).

Target genes in the extracted DNA were enumerated by qPCR using primers previously published for bacterial 16S rRNA (Muyzer et al. 1993), *intI1* (Goldstein et al. 2001), and *qacEΔ1* (Stedtfeld et al. 2018). The *intI1* gene was targeted with qPCR due to its use as an indicator gene for horizontal gene transfer, and the *qacEΔ1* gene was quantified because it confers resistance to disinfectants such as chlorine. All qPCR reactions were performed with 20 µL total reaction volumes with 1x PowerUp SYBR

Green Master Mix (Applied Biosystems, USA), F/R primers at a final concentration of 1.0  $\mu\text{M}$  each, and 5  $\mu\text{L}$  of DNA template. DNA templates were diluted to 1:10 for all genes except for 16S rRNA that was diluted to 1:100 to achieve concentrations within the standard curve. Each sample was run in duplicate on qPCR and the results were averaged. The replicates from microcosms were averaged ( $n = 3$  from a triplicate experiment, and each of the three values stemmed from the average of duplicate qPCR runs). Microcosm samples with results below the quantification limit were reported as the quantification limit. No template controls and standards containing target gene DNA between  $10^0$  and  $10^7$  copies were performed in duplicate with each qPCR assay. Cycling conditions were conducted as previously described (Kappell et al. 2018b; Kimbell et al. 2018; Kimbell et al. 2021). Specificity of amplification of target genes was confirmed by melt curves consistent with that of each standard. Amplification efficiency was determined by the resulting standard curve and was considered acceptable between 0.9 and 1.1. Reactions were performed using a LightCycler 96 (Roche Molecular Systems Inc., USA). Results were reported as copies per L and normalized to 16S rRNA to observe trends in relative abundance. The qPCR limit of quantification was determined for each gene according to MIQE guidelines (Bustin et al. 2009). Specific primer sets, annealing temperatures, efficiencies, and detection limits are described in **Table 5.1A in Appendix 5A**.

#### **5.2.4 Shotgun Metagenomic Sequencing**

To profile the microbiome and antibiotic resistome, shotgun metagenomic sequencing was conducted on DNA extracted from the microcosms on each collection date as described with sequencing conducted on an Illumina HiSeq with  $2 \times 100$ -cycle

paired end reads at the Microbial Genome Sequencing Center (Pittsburgh, PA). Metagenomic reads were trimmed using Trimmomatic v0.36 (Bolger et al. 2014). Taxonomy was assigned to trimmed reads using Kaiju v1.7.3 (Menzel et al. 2016). Quality of merged metagenomic reads was assessed using FastQC. Reads were assembled using MetaSPAdes version 3.15.3 (Nurk et al. 2017). Assembled contigs were binned using MaxBin2 v2.2.4 (Wu et al. 2016). The abundance of known ARGs was determined by annotating metagenomic reads against the CARD (Comprehensive Antibiotic Resistance Database) (McArthur et al. 2013).

### **5.2.5 Water Quality Analysis**

Water quality for the source drinking water was characterized on the day of collection (Day 0) for the following parameters: alkalinity, pH, hardness, dissolved oxygen, ammonia, chloride, phosphate, dissolved metals, silicate, and total organic carbon (TOC). Water was aseptically removed from the microcosms on Days 3 and 7 and analyzed for pH, dissolved metals, TOC, phosphate, free and total chlorine, and silicate. A Thermo Fisher Scientific pH probe was used for measuring pH for the microcosms. Silicate, alkalinity, hardness, dissolved oxygen, and ammonia were measured according to appropriate standard methods using Hach kits (Hach Company, Loveland, CO). Phosphate was measured using the ascorbic acid method (Standard Method 4500-P) (APHA et al. 1998). Dissolved metals were quantified using an inductively coupled plasma mass spectrometer (ICP-MS) in the Marquette Water Quality Center. TOC was quantified using a Shimadzu TOC analyzer in the Marquette University Water Quality

Center. Free chlorine and total chlorine were measured according to the standard method (APHA et al. 1998).

### **5.2.6 Statistical analysis**

Statistical analysis of heterotrophic plate counts and qPCR gene abundances were performed using Graphpad Prism V 9.3.1. Analysis of variance (ANOVA) was used to determine significant differences among the concentrations and treatment types for absolute and relative abundance measurements of ARB and targeted resistance genes on Days 0, 3, and 7. Post hoc multiple pairwise comparisons were conducted by using Tukey's honest significant differences test. Significant differences were defined as p-values less than 0.05.

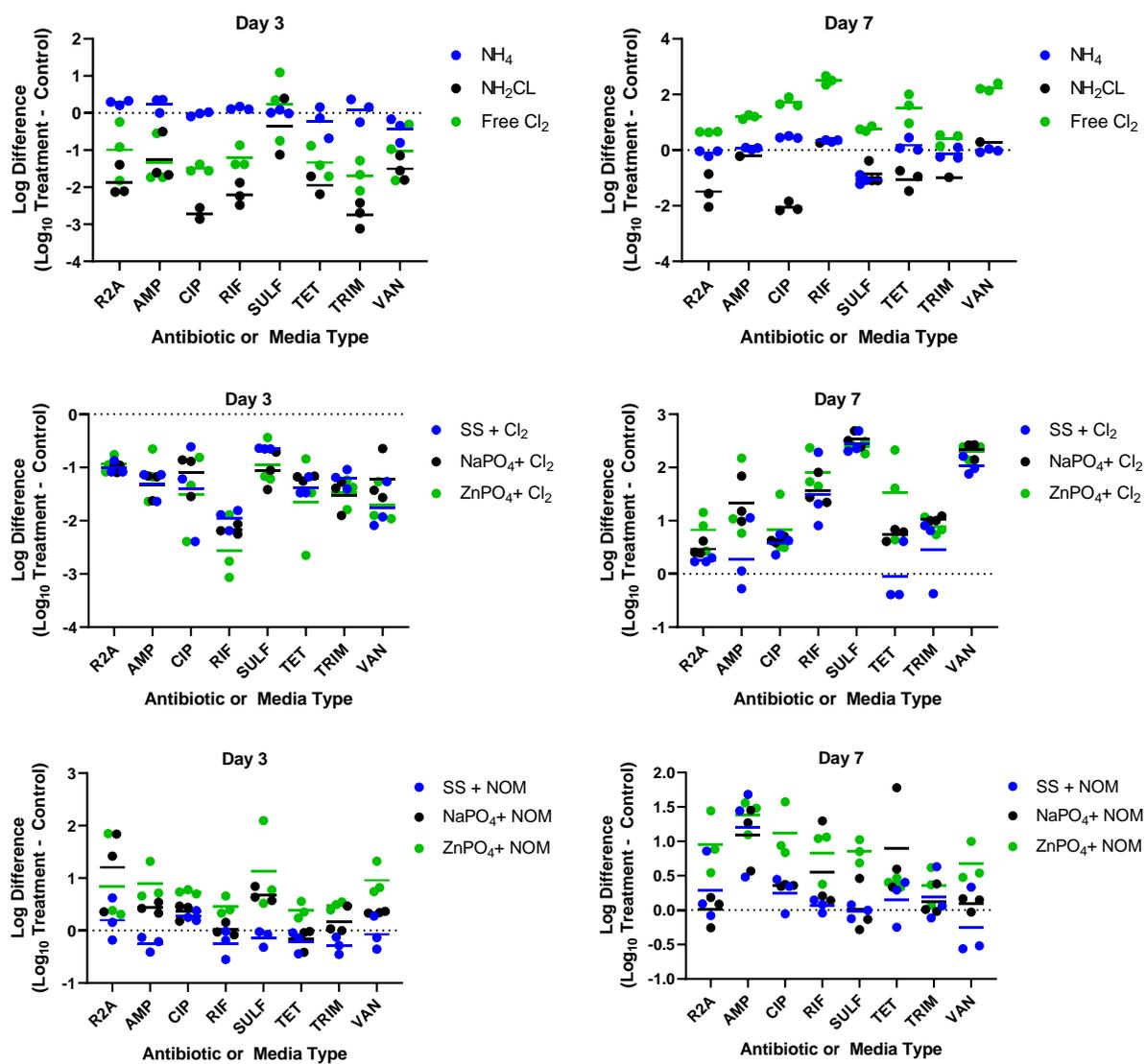
## **5.3 Results and Discussion**

### **5.3.1 Quantification of Phenotypic Antibiotic Resistance**

#### **5.3.1.1 Impact of Disinfectant Type**

ARB were quantified in samples collected from each of the microcosms on days 3 and 7 to determine the effect of different corrosion inhibitors and disinfectants on the abundance of phenotypic antibiotic resistance. By day 3, free chlorine disinfection resulted in significantly lower observed absolute abundance for ARB resistant to AMP, CIP, TET, and TRIM compared to untreated controls (**Figure 5.1**; all p values < 0.05). The average concentration of free chlorine in the microcosms by day 3 was approximately 0.2 – 0.3 mg/L (initially dosed at 1.6 mg/L as Cl<sub>2</sub> on day 0). Disinfection

with chloramine resulted in significantly lower absolute abundance of total heterotrophic bacteria (R2A) and ARB resistant to AMP, CIP, RIF, TET, TRIM, and VAN compared to untreated controls by day 3 (all p values < 0.05). The residual chloramine concentration in the microcosms on day 3 ranged from 0.8 to 0.9 mg/L as total Cl<sub>2</sub>, which is above the minimum selectable concentration (MSC) for bacteria such as *Bacillus* sp., *Acidovorax* sp., and *Micrococcus* sp. (Khan et al. 2017). Chloramine treatment resulted in a more stable residual chlorine concentration compared to free chlorine and was more effective at limiting microbial growth in the drinking water microcosms. These results are consistent with previous research that has demonstrated the ability of disinfectants such as chlorine and free chlorine for inactivation of microorganisms and ARB in water environments (Zhang et al. 2017b; Zhang et al. 2019a). ARB resistant to SULF exhibited the highest levels of resistance to chlorine and chloramine disinfection. The addition of nitrogen as ammonia at 0.6 mg/L exhibited similar growth patterns compared to untreated controls by day 3.



**Figure 5.1.** Log difference in absolute abundance of total heterotrophic bacteria (R2A) and antibiotic resistant bacteria on Day 3 and 7 based on direct plating of from microcosms containing different types and concentrations of corrosion inhibitors and disinfectants. The difference in counts of colony forming units (CFU) in  $\text{Log}_{10}$  per liter of water for each treatment microcosm was determined relative to the control microcosm on each day is shown on the y-axis. The type of antibiotic or media is denoted on the x-axis. Corrosion inhibitor and disinfectant type is indicated by color and shape.

By day 7, microcosms disinfected with free chlorine exhibited increased absolute abundance of total heterotrophic bacteria (R2A) and ARB resistant to AMP, CIP, RIF, TET, and VAN compared to the addition of chloramines, ammonia, and untreated controls (**Figure 5.1**; all  $p$  values  $< 0.05$ ). The subinhibitory concentrations of chlorine present in the microcosms selected for bacterial communities with increased resistance to multiple types of clinically-relevant antibiotics. Previous research has demonstrated that subinhibitory levels of disinfectants such as free chlorine and chloramines can stimulate intragenera conjugative transfer by increasing reactive oxygen species formation, SOS response, increased cell membrane permeability, and altered expressions of conjugation-relevant genes (Zhang et al. 2017b). By day 7, chloramine-treated microcosms exhibited significantly lower growth of total heterotrophic bacteria and ARB resistant to CIP, TET, and TRIM compared to untreated controls ( $p$  values  $< 0.05$ ). The absolute abundance of ARB in ammonia-treated communities were not significantly different from untreated controls by day 7.

### **5.3.1.2 Impact of Corrosion Inhibitor Type**

The impact of different types of corrosion inhibitors on the prevalence of ARB in drinking water in combination with free chlorine was conducted to simulate conditions in chlorinated distribution systems. By day 3, all corrosion inhibitor and chlorine-treated microcosms exhibited significantly lower absolute abundance of total heterotrophic bacteria (R2A) and ARB resistant to AMP, RIF, SULF, TET, TRIM, and VAN (**Figure 5.1**; all  $p$  values  $< 0.05$ ) compared to untreated controls. The chlorine concentrations on

day 3 ranged from below detection to 0.1 mg/L as Cl<sub>2</sub> for free chlorine and 0.2 to 0.3 mg/L as Cl<sub>2</sub> for total chlorine.

By day 7, communities treated with all three types of corrosion inhibitors and free chlorine exhibited significantly higher absolute abundance of ARB resistant to RIF, SULF, and VAN compared to untreated controls (**Figure 5.1**; p values < 0.05). The addition of phosphate-based inhibitors (sodium orthophosphate and zinc orthophosphate) at 1 mg/L also resulted in significantly higher absolute abundance of ampicillin-resistant bacteria compared to untreated controls and sodium silicate addition at 10 mg/L (p values < 0.05). Additionally, zinc orthophosphate selected for significantly higher absolute abundance of TET-resistant bacteria by day 7 compared to sodium silicate addition and untreated controls (p values < 0.05). Sodium orthophosphate stimulated increased growth of TRIM-resistant bacteria by day 7 to levels that were significantly greater than untreated controls (p value < 0.05). These results indicate that the nutrients added in the form of corrosion inhibitors enabled the growth of bacteria resistant to subinhibitory levels of metals and antibiotics. These results are consistent with previous research that has documented increased growth of bacteria and ARB in response to addition of corrosion inhibitors including zinc orthophosphate and sodium orthophosphate at concentrations ranging from 0 to 10 mg/L (Kogo et al. 2017a; Kappell et al. 2019).

### **5.3.1.3 Impact of NOM**

The impact of NOM addition to each of the types of corrosion inhibitors was evaluated to simulate conditions with excess nutrients such as carbon, nitrogen, and phosphorus. The addition of zinc orthophosphate and NOM resulted in significantly

higher absolute abundance of total heterotrophic bacteria (R2A) and ARB resistant to AMP, CIP, SULF, and VAN compared to untreated controls by day 3 (**Figure 5.1**; all p values < 0.05). Sodium orthophosphate addition in the presence of NOM resulted in significantly higher absolute abundance of total heterotrophic bacteria (R2A) compared to untreated controls (p value < 0.05). Sodium silicate addition with NOM did not significantly change the absolute abundance of ARB by day 3 relative to untreated controls but did exhibit significantly lower absolute abundance of TRIM and VAN resistant bacteria compared to zinc orthophosphate (p values < 0.05).

By day 7, communities treated with zinc orthophosphate resulted in significantly higher absolute abundance of total heterotrophic bacteria (R2A) and ARB resistant to AMP, CIP, and SULF compared to untreated controls (**Figure 5.1**; all p values < 0.05). Sodium orthophosphate addition also selected for increased absolute abundance of AMP and TET-resistant bacteria compared to untreated controls by day 7 (p values < 0.05). The absolute abundance of ARB resistant to CIP, SULF, and VAN was significantly lower in communities treated with sodium silicate compared to zinc orthophosphate by day 7 (all p values < 0.05). Sodium silicate addition resulted in consistently lower absolute abundance of ARB and R2A compared to phosphate-based inhibitors on days 3 and 7. Previous studies have demonstrated that orthophosphate can stimulate microbial growth and increase microbial community diversity (Fang et al. 2009; Payne et al. 2016).

Zinc orthophosphate selected for the highest levels of ARB in the presence of NOM compared to other corrosion inhibitors (sodium orthophosphate and sodium silicate). However, in the presence of free chlorine – the levels of ARB between the different corrosion inhibitor treatments were not significantly different from each other.

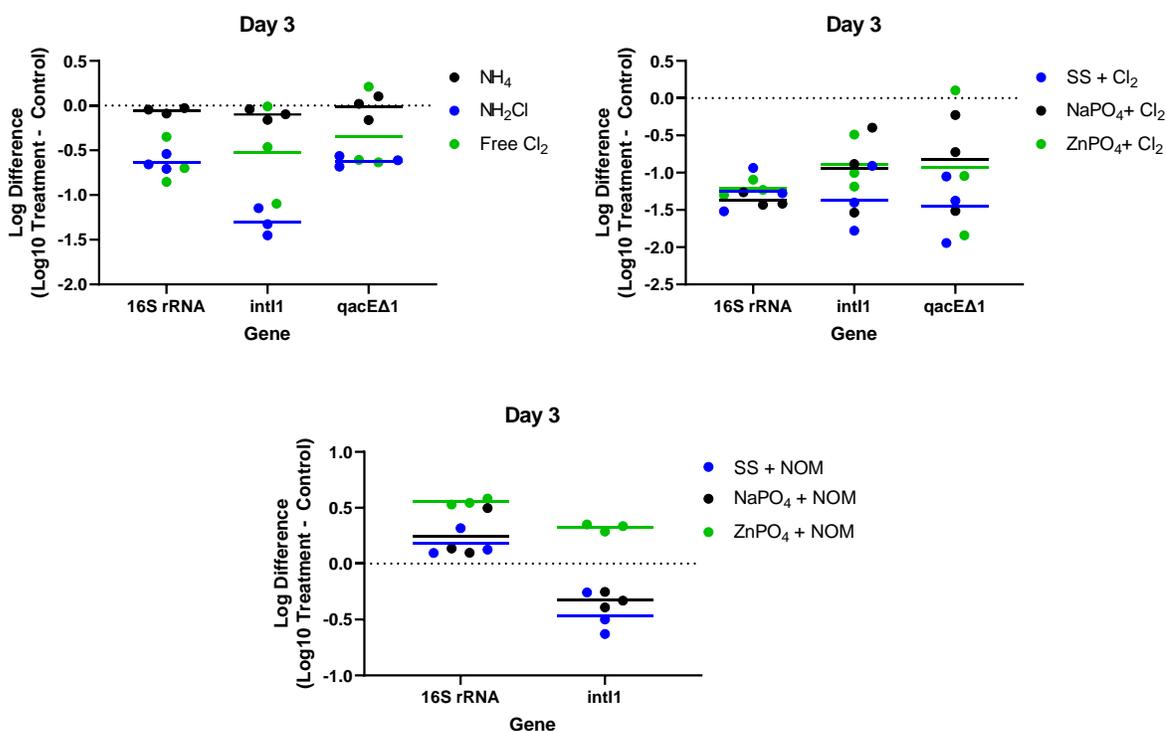
This suggests that chlorine disinfection likely exhibits a stronger impact on the development of antibiotic resistance compared to corrosion inhibitor addition on bacterial communities in drinking water. This is supported by the observed increased growth of ARB in free chlorine-treated microcosms compared to the addition of corrosion inhibitors and NOM alone by day 7. The subinhibitory levels of chlorine induced the growth of ARB resistant to multiple types of antibiotics tested. Similarly, previous research has demonstrated that disinfectant type and dose exert the strongest influence on microbial communities in simulated DWDS (Wang et al. 2012b). This study and others have noted that the interactions between pipe materials and disinfectants also play a pivotal role in governing microbial proliferation and antibiotic resistance in DWDS (Niquette et al. 2000; Lehtola et al. 2005; Wang et al. 2012b; Aggarwal et al. 2018).

### **5.3.2 Quantification of Genotypic Antibiotic Resistance**

#### **5.3.2.1 Impact of Disinfectant Type**

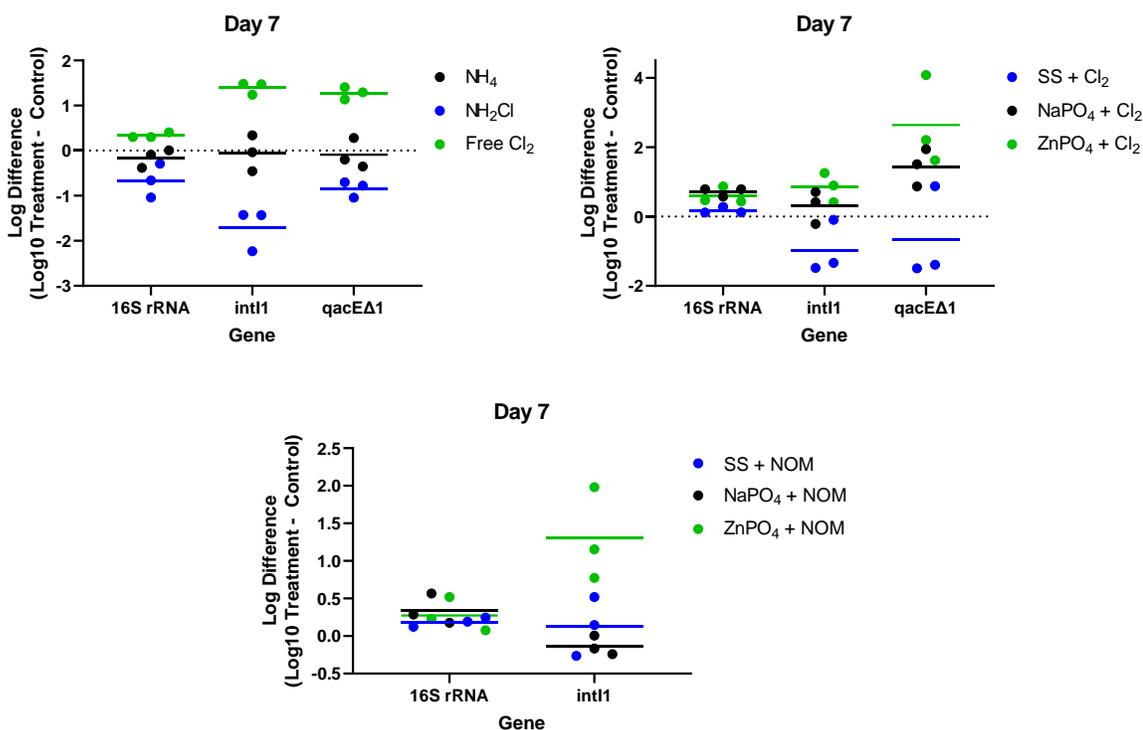
Quantification of total bacterial biomass (16S rRNA gene copies), an ARG encoding resistance to disinfectants and quaternary ammonium compounds (*qacEΔ1*) and the *intI1* gene, as an indicator of potential for horizontal gene transfer, was conducted on DNA extracted on days 0, 3, and 7. There was a significant difference in the absolute abundance of 16S rRNA, *qacEΔ1*, and *intI1* genes in response to the addition of different disinfectant and corrosion inhibitor types on days 3 and 7. Free chlorine disinfection significantly decreased the absolute abundance of the 16S rRNA, *qacEΔ1*, and *intI1* genes by day 3 (**Figure 5.2**; all p values < 0.05). However, by day 7 the absolute

abundance of *intI1* and *qacEΔ1* in the free chlorine treated communities was significantly greater compared to untreated controls, ammonia, and chloramine treatments ( $p$  values  $< 0.05$ ). Ammonia addition at 0.6 mg/L did not result in any significant changes in gene abundance relative to untreated controls. Chloramine disinfection resulted in the greatest changes in absolute abundance with significantly lower absolute abundance of 16S rRNA, *intI1*, and *qacEΔ1* detected in communities on day 3 and day 7 ( $p$  values  $< 0.05$ ).



**Figure 5.2.** Log difference in absolute abundance of total bacterial biomass (16S rRNA), *qacEΔ1*, and *intI1* on Day 3 based on qPCR analysis from microcosms containing different types and concentrations of corrosion inhibitors. The difference in counts of gene copies in Log<sub>10</sub> per liter of water for each treatment microcosm was determined relative to the control microcosm on Day 3 is shown on the y-axis. The type of gene is denoted on the x-axis. Corrosion inhibitor and disinfectant types are indicated by shape and color.

The combined impact of free chlorine and each of the three corrosion inhibitors was evaluated to simulate conditions in chlorinated distribution systems. By day 3, the absolute abundance of 16S rRNA, *intI1*, and *qacEA1* genes were significantly lower for all communities treated with corrosion inhibitors and free chlorine compared to untreated controls (p values < 0.05). By day 7, there was a significant increase in the absolute abundance of *intI1* and *qacEA1* in the communities amended with free chlorine and zinc orthophosphate compared to untreated controls and sodium silicate addition (Figure 5.3; p values < 0.05).



**Figure 5.3.** Log difference in absolute abundance of total bacterial biomass (16S rRNA), *qacEA1*, and *intI1* on Day 7 based on qPCR analysis from microcosms containing different types and concentrations of corrosion inhibitors. The difference in counts of gene copies in Log<sub>10</sub> per liter of water for each treatment microcosm was determined relative to the control microcosm on Day 7 is shown on the y-axis. The type of gene is denoted on the x-axis. Corrosion inhibitor and disinfectant types are indicated by shape and color.

### 5.3.2.2 Impact of Corrosion Inhibitor Type

By day 3, the addition of different corrosion inhibitors in the presence of free chlorine did not exhibit significant differences in gene abundance from each other but did exhibit significantly lower absolute abundance compared to untreated controls (all p values < 0.05). The chlorine concentrations on day 3 ranged from below detection to 0.1 mg/L as Cl<sub>2</sub> for free chlorine and 0.2 to 0.3 mg/L as Cl<sub>2</sub> for total chlorine. By day 7, there was a significantly greater absolute abundance of gene copies per liter in the zinc orthophosphate treatment compared to untreated controls and sodium silicate addition for *intI1* and *qacEΔ1* (**Figure 5.3**; p values < 0.05). Sodium orthophosphate addition did not result in significant increases in absolute abundance of target genes relative to untreated controls. These results are congruent with previous data suggesting that addition of zinc may promote an increase in abundance of resistance genes related to horizontal gene transfer and conferring resistance to disinfectants (Kappell et al. 2019).

### 5.3.2.3 Impact of NOM

By day 3, the addition of sodium silicate and sodium orthophosphate in the presence of NOM did not exhibit significant increases in target gene abundance compared to untreated controls (**Figure 5.2**). Zinc orthophosphate addition resulted in the highest levels of all target genes by day 3 but were not significantly different from untreated controls. However, by day 7 there was a significantly greater absolute abundance of gene copies per liter in the zinc orthophosphate treatment compared to untreated controls and sodium silicate addition for *intI1* and *qacEΔ1* (**Figure 5.3**; p

values  $< 0.05$ ). Similar to results from previous experiments, sodium orthophosphate and sodium silicate addition did not result in significant increases in absolute abundance of target genes relative to untreated controls by day 7. These results reiterate the potential for zinc as a selecting agent for antibiotic resistance in DWDS.

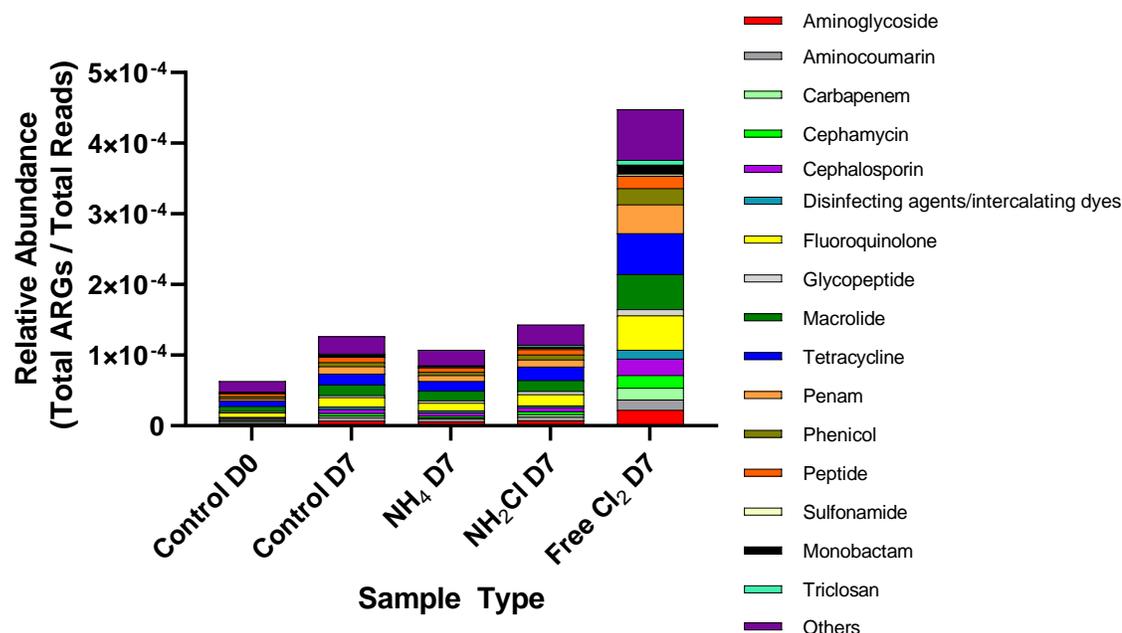
### 5.3.3 Metagenomic Characterization of the Antibiotic Resistome

#### 5.3.3.1 Impact of Disinfectant Type on Antibiotic Resistome

Shotgun metagenomic sequencing was conducted to investigate the abundance of known ARGs by annotating reads against the CARD database. Thirty-eight different classes of antibiotic resistance were identified across all samples ( $n = 45$ , **Figure 5.4**). Across the data set,  $>500$  different AMR genes were annotated. The most abundant ARGs detected in all of the samples included an aminoglycoside resistance gene (*ranA*), a fosfomycin resistance gene (*abaF*), a bacitracin resistance gene (*bcrA*), a tetracycline resistance gene (*tetA*), a macrolide resistance gene (*macB*), a vancomycin resistance gene (*vanR*), and several multidrug resistance genes (*adeL*, *evgS*, *yajC*, *mexT*, *parS*). The most frequently detected antibiotic drug classes included macrolides, fluoroquinolones, tetracyclines, penams, aminoglycosides, cephalosporins, carbapenems, phenicols, and peptides. The average number of metagenomic sequence reads per sample was 13,637,280 (see Table 5F in Appendix 5F).

Chlorine disinfection resulted in a significant increase of ARGs classified with the antibiotic efflux resistance mechanism compared to untreated controls, ammonia, and chloramine disinfection by day 7 ( $p$  values  $< 0.05$ ). Similarly, previous studies have demonstrated that chlorine exposure can induce over expression of the MexEF-OPrN

efflux pump in *Pseudomonas aeruginosa* (Hou et al. 2019). The most common ARGs detected in the chlorine-treated communities included *qacEΔ1*, *sul4*, *yajC*, *adeF*, *rsmA*, and *soxR*. Free chlorine selected for significantly higher abundance of ARGs corresponding to several different classes including aminoglycoside, carbapenem, cephamycin, cephalosporin, fluoroquinolone, macrolide, penam, phenicol, tetracycline, and peptide compared to untreated controls (all p values < 0.05). Subinhibitory levels of chlorine can stimulate horizontal gene transfer of ARGs through multiple pathways, including ROS formation, SOS response, increased cell membrane permeability, and altered gene expression of conjugation-relevant genes (Zhang et al. 2017a). The addition of chloramine and ammonia alone did not exhibit significant impacts on the abundance of ARGs classified with CARD on day 7.

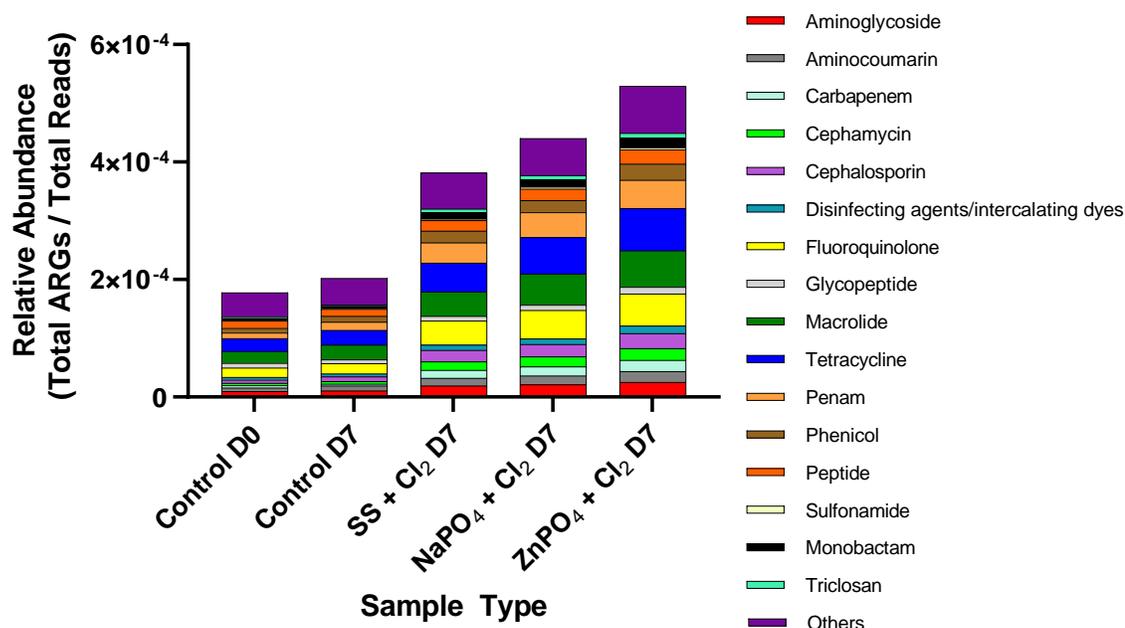


**Figure 5.4.** Relative abundance of ARGs classified by antibiotic drug class as determined by CARD for each type of disinfectant or nutrient condition tested. ARG abundance was normalized to total metagenomic reads for each sample. ARGs are grouped by antibiotic drug class to which they confer resistance. The y-axis represents the mean relative abundance of ARGs classified for each sample type for triplicate samples. The x-axis represents the different experimental conditions including untreated controls, ammonia (0.6 mg/L), chloramine (1.6 mg/L as Cl<sub>2</sub>), and free chlorine (1.6 mg/L as Cl<sub>2</sub>). The sample collection day is denoted by D0 (day 0) and D7 (day 7).

### 5.3.3.2 Impact of Corrosion Inhibitor Type on Antibiotic Resistome

Corrosion inhibitor type had a significant impact on ARGs classified with the antibiotic efflux resistance mechanism with zinc orthophosphate exhibiting the highest levels compared to other corrosion inhibitors and untreated controls by day 7 (p values < 0.05). Zinc orthophosphate also selected for significantly higher ARGs related to antibiotic target alteration compared to untreated controls (p value < 0.05). ARGs conferring resistance through this mechanism modify the target for a particular antibiotic in the bacterial cell. The addition of zinc was linked to an increase in the total number of

ARGs detected and ARGs related to antibiotic efflux (Figure 5G, Appendix 5G), which enables bacterial cells to extrude antibiotics and metals through the cell membrane (Pal et al. 2017). Significant increases in the abundance of efflux-related ARGs in the sodium silicate and sodium orthophosphate-treated communities were also observed compared to untreated controls on day 7 (p values < 0.05).



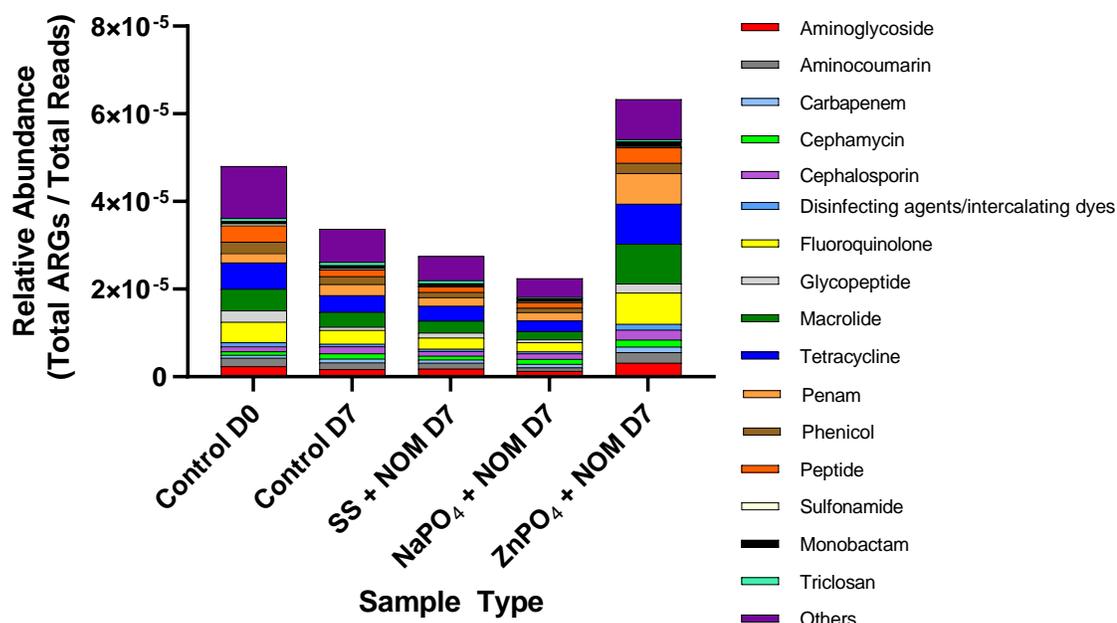
**Figure 5.5.** Relative abundance of ARGs classified by antibiotic drug class as determined by CARD for each type of corrosion inhibitor. ARG abundance was normalized to total metagenomic reads for each sample. ARGs are grouped by antibiotic drug class to which they confer resistance. The y-axis represents the mean relative abundance of ARGs classified for each sample type for triplicate samples. The x-axis represents the different experimental conditions including sodium silicate (10 mg/L as SiO<sub>2</sub>) + free chlorine (1.6 mg/L as Cl<sub>2</sub>), sodium orthophosphate (1 mg/L as PO<sub>4</sub>) + free chlorine (1.6 mg/L as Cl<sub>2</sub>), and zinc orthophosphate (1 mg/L as PO<sub>4</sub>) + free chlorine (1.6 mg/L as Cl<sub>2</sub>). The sample collection day is denoted by D0 (day 0) and D7 (day 7).

Zinc orthophosphate addition in the presence of free chlorine resulted in significantly higher abundance of ARGs belonging to classes including aminoglycoside, aminocoumarin, carbapenem, cephamycin, cephalosporin, fluoroquinolone, macrolide, tetracycline, penam, phenicol, peptide, and monobactam compared to untreated controls on day 7 (**Figure 5.5**; all p values < 0.05). Sodium orthophosphate also selected for increased ARGs belonging to the aminoglycoside, carbapenem, cephamycin, cephalosporin, fluoroquinolone, macrolide, tetracycline, penam, and phenicol classes compared to untreated controls (all p values < 0.05). Sodium silicate resulted in less overall selection for ARGs compared to corrosion inhibitors containing orthophosphate. However, sodium silicate addition in the presence of chlorine did promote increased abundance of ARGs corresponding to the fluoroquinolone, macrolide, tetracycline, and penam classes compared to untreated controls (all p values < 0.05). These results indicate that corrosion inhibitors can select for increased types and abundances of ARGs and may play a key role in the proliferation of AMR, especially in chlorinated DWDS.

### **5.3.3.3 Impact of NOM + Corrosion Inhibitor Type on Antibiotic Resistome**

The results of the addition of Suwannee River NOM to each of the three types of corrosion inhibitors indicated a significant increase in antibiotic efflux ARGs in the zinc orthophosphate amended communities compared to sodium orthophosphate, sodium silicate, and untreated controls on day 7 (p values < 0.05). Sodium orthophosphate addition at 1 mg/L resulted in the lowest total abundance of ARGs as classified by CARD on day 7. Sodium silicate addition at 10 mg/L exhibited similar resistance profiles compared to untreated controls by day 7. The addition of NOM did not have a significant

impact on the antibiotic resistance profiles of the bacterial communities in the drinking water microcosms by day 7. In fact, there were significantly less total ARGs and efflux-related ARGs in all of the NOM-amended microcosms compared to untreated controls on day 0 with the exception of the zinc orthophosphate treatment (all  $p$  values  $< 0.05$ ).



**Figure 5.6.** Relative abundance of ARGs classified by antibiotic drug class as determined by CARD for each type of corrosion inhibitor with NOM. ARG abundance was normalized to total metagenomic reads for each sample. ARGs are grouped by antibiotic drug class to which they confer resistance. The y-axis represents the mean relative abundance of ARGs classified for each sample type for triplicate samples. The x-axis represents the different experimental conditions including sodium silicate (10 mg/L as SiO<sub>2</sub>) + NOM (5 mg/L), sodium orthophosphate (1 mg/L as PO<sub>4</sub>) + NOM (5 mg/L), and zinc orthophosphate (1 mg/L as PO<sub>4</sub>) + NOM (5 mg/L). The sample collection day is denoted by D0 (day 0) and D7 (day 7).

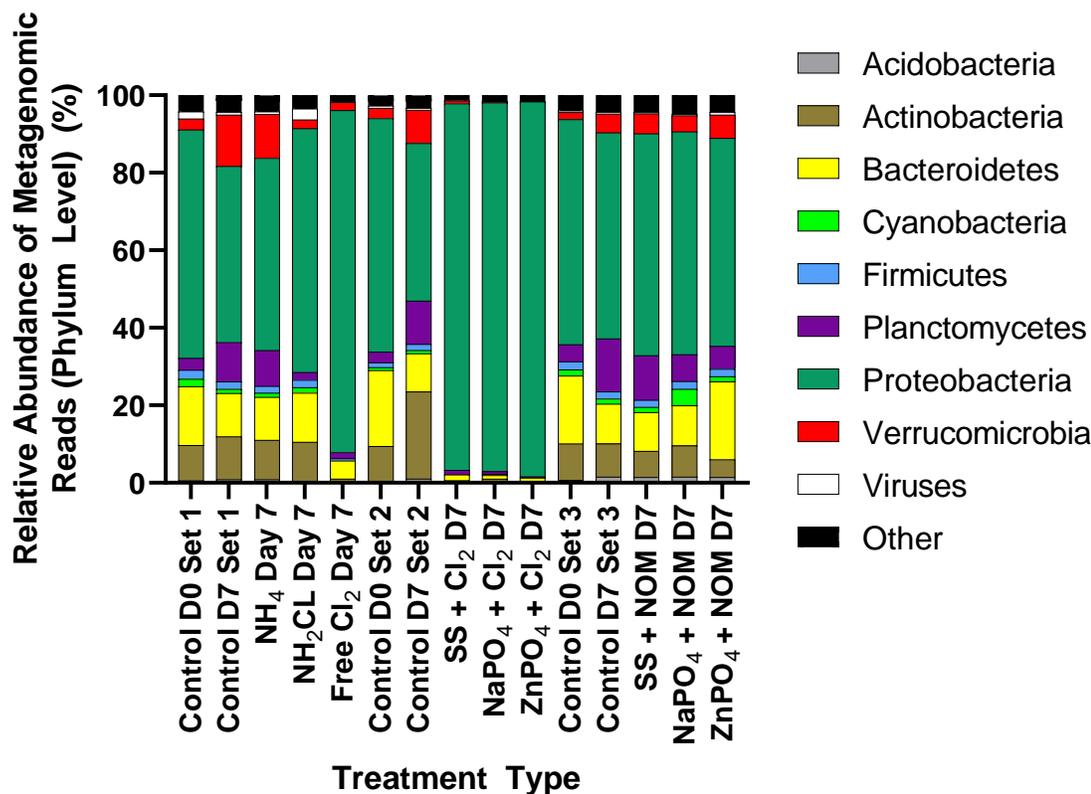
Zinc orthophosphate addition in the presence of NOM resulted in significantly higher abundance of ARGs belonging to classes including fluoroquinolone, macrolide, tetracycline, and penam compared to untreated controls, sodium silicate, and sodium

orthophosphate treatment on day 7 (**Figure 5.6**; all p values < 0.05). Sodium orthophosphate and sodium silicate addition in the presence of NOM did not significantly increase the abundance of ARGs.

### 5.3.4 Metagenomic Characterization of the Microbiome

Metagenomic characterization of microbial communities in the drinking water samples revealed a core community dominated by *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Planctomycetes* at the phylum level (**Figure 5.7**). Bacterial communities present in day 0 from each of the three different microcosm experiments exhibited similar core microbial communities. The addition of free chlorine resulted in a significant increase in the relative abundance of bacteria belonging to the *Proteobacteria* phylum compared to untreated controls and non-disinfected microcosms (p values < 0.05). Chloramine disinfection significantly reduced the relative abundance of microorganisms in the phylum *Planctomycetes* compared to untreated controls and ammonia addition by day 7 (p values < 0.05). *Proteobacteria* are common inhabitants of drinking water systems and have several members that are resistant to disinfectants such as chlorine and chloramine (Douterelo et al. 2013; Mi et al. 2015). Additionally, the addition of zinc orthophosphate in the presence of NOM significantly increased the abundance of the phylum *Bacteroidetes* compared to sodium orthophosphate, sodium silicate, and untreated controls on day 7 (p values < 0.05). The phylum *Bacteroidetes* is primarily composed of gram-negative bacteria and are frequently detected in metal pipe biofilms and drinking water samples (Revetta et al. 2010; Yu et al. 2010). Sodium silicate addition with NOM did not result in significant changes to the bacterial community

relative to untreated controls. These results demonstrate that the addition of corrosion inhibitors and disinfectants can exhibit significant impacts on microbial community structure.



**Figure 5.7.** Relative abundance of metagenomic reads classified at the phylum level by sample day and treatment type. Each stacked bar represents the mean of triplicate samples. Bacterial phylum is indicated by color. Day of sample collection is denoted by D0 (day 0) and D7 (day 7).

At the genus level, the most detected genera across the data set included *Limnohabitans*, *Flavobacterium*, *Rhodoferrax*, *Polaromonas*, *Hydrogenophaga*, *Pseudomonas*, *Sphingomonas*, *Roseimicrobium*, *Luteolibacter*, and *Microbacterium*. The most abundant genera detected in the microbial communities on day 0 included

*Limnohabitans*, *Flavobacterium*, and *Rhodospirillum rubrum*, which are commonly found in freshwater ecosystems and in DWDS (Newton and McLellan 2015; Liu et al. 2017). Chlorine disinfection resulted in a significant increase in microbial genera including *Hydrogenophaga*, *Pseudomonas*, and *Sphingomonas* compared to untreated controls and ammonia addition on day 7 (p values < 0.05). These bacterial genera contain several members classified as opportunistic pathogens and resistant to chlorine disinfection (Jia et al. 2015; Hou et al. 2019). The addition of chloramines also significantly increased the relative abundance of the genus *Pseudomonas* compared to untreated controls on day 7 (p value < 0.05). The addition of ammonia did not exhibit significant changes to the microbiome compared to untreated control communities.

The addition of corrosion inhibitors in the presence of free chlorine resulted in increased selection for several microbial genera. Sodium silicate addition in the presence of free chlorine selected for increased relative abundance of genera including *Pseudomonas*, *Limnobacter*, *Porphyrobacter*, *Methylophilus*, and *Hydrogenophaga* compared to untreated controls (p values < 0.05). Similarly, a previous study observed impacts from chlorine exposure on *Pseudomonas aeruginosa* including increased ROS production, which promoted the development of resistance through over expression of drug efflux pumps (e.g., MexXY-OprM multidrug efflux system) (Hou et al. 2019). Sodium orthophosphate and zinc orthophosphate addition also resulted in increased selection of genera including *Pseudomonas*, *Limnobacter*, *Porphyrobacter* compared to untreated controls on day 7 (p values < 0.05). These results suggest that the combination of corrosion inhibitors and free chlorine may promote increased growth of bacterial genera containing opportunistic pathogens commonly found in DWDS. Previous research

has demonstrated that chlorination can enrich the prevalence of ARB in drinking water (Bai et al. 2015). In fact, Shi *et al.* observed a higher proportion of ARB resistant to cephalothin, chloramphenicol, and trimethoprim following chlorination (Shi et al. 2013). Bacterial exposure to free chlorine in the current study resulted in the enrichment of ARB resistant to multiple types of antibiotics and several genera that contain pathogenic bacteria relevant to DWDS. Further, the addition of corrosion inhibitors, particularly phosphates, can promote formation of heterogeneous biofilms with increased number of microorganisms (Liu et al. 2016).

The addition of NOM and corrosion inhibitors resulted in the least significant changes to the microbial community structure by day 7. Sodium silicate addition in the presence of NOM selected for increased abundance of the microbial genera *Tabrizicola*. Sodium orthophosphate in the presence of NOM selected for increased abundance of microorganisms belonging to the genus *Hydrogenophaga*. Zinc orthophosphate and NOM addition selected for increased abundance of genera including *Halicomenobacter* and *Lacunisphaera*. These results highlight the importance of considering the microbiological impacts of corrosion inhibitor addition in DWDS. The proportions of bacterial subclasses in DWDS vary widely depending on many factors including biofilm age (Martiny et al. 2003), pipe materials (Ji et al. 2015), and phosphate treatment (commonly used for corrosion control) (Appenzeller et al. 2001), as well as disinfection practices (Batté et al. 2003; Gomez-Alvarez et al. 2012). Corroded pipe materials and biofilms can retain nutrients including carbon, nitrogen, and phosphorus which can subsequently be utilized by bacteria inhabiting DWDS (Morton et al. 2005). Gaining a better understanding of the complex interactions between corrosion inhibitors, corroded

pipe materials, and growth of microorganisms is vital for effective and safe management of DWDS.

This is the first research to quantify the impacts of different corrosion inhibitors on the antibiotic resistome and microbiome in a source water for drinking water. ARB and ARGs in natural freshwater systems can withstand drinking water treatment processes and, in turn, be transported with finished drinking water to consumer taps. Previous studies have highlighted DWDS as important routes for disease transmission (Anaissie et al. 2002) and ARB/ARGs are increasingly being considered as emerging contaminants due to their potential to impact public health (Pruden et al. 2006). There remains a paucity of research regarding the interactions between freshwater and drinking water microbial communities and the transmission of ARB and ARGs in drinking water supplies.

#### **5.4 Conclusions**

The results presented in this study provide evidence that corrosion inhibitors such as zinc orthophosphate and disinfectants such as free chlorine can lead to significant changes to the antibiotic resistome and microbiome in a source for drinking water. This study provides a head-to-head comparison of corrosion inhibitors and disinfectants and their impact on AMR. Targeted and non-targeted methods of molecular analysis were employed to characterize the antibiotic resistome and microbial communities. Disinfectants and corrosion inhibitors increased the occurrence of phenotypic resistance to multiple clinically-relevant antibiotics observed through direct plating of antibiotic-resistant heterotrophic bacteria and genotypic antibiotic resistance including genetic

determinants related to disinfectant resistance (*qacEΔ1*) and horizontal gene transfer (*intI1*). Metagenomic characterization of the resistome indicated increased types and abundances of ARGs related to antibiotic efflux in communities treated with free chlorine and zinc orthophosphate.

Free chlorine disinfection selected for significantly higher abundance of ARGs corresponding to several different classes including aminoglycoside, carbapenem, cephamycin, cephalosporin, fluoroquinolone, macrolide, penam, phenicol, tetracycline, and peptide compared untreated controls. Similarly, phosphate-based corrosion inhibitors promoted increased abundances of ARGs belonging to the aminoglycoside, carbapenem, cephamycin, cephalosporin, fluoroquinolone, macrolide, tetracycline, penam, and phenicol classes. Chlorine exhibited the strongest selection for ARB, ARGs, and microbial communities according to HPC, qPCR, and metagenomic characterization of samples collected from the microcosms on day 7. Out of the corrosion inhibitors tested, zinc orthophosphate resulted in highest selection of ARB and associated ARGs compared to controls, sodium orthophosphate, and sodium silicate treatments. Sodium silicate dosage at 10 mg/L resulted in decreased bacterial growth as measured by heterotrophic plate counts and qPCR when compared to zinc and sodium orthophosphate treatments. The addition of corrosion inhibitors in the presence of chlorine selected for increased relative abundance of several microbial genera including *Pseudomonas*, *Limnobacter*, *Porphyrobacter* compared to untreated controls.

The results of this study suggest that utilities using phosphate-based corrosion inhibitors and disinfectants may be preferentially selecting for bacterial communities with the increased occurrence of ARGs, ARB, and microbial genera containing opportunistic

pathogens. This is the first research to quantify the impacts of different corrosion inhibitors on AMR gene indicators and the antibiotic resistome. This area of research warrants further investigation due to the potential risk of humans acquiring antibiotic-resistant infections related to the consumption of drinking water containing ARB and ARGs. However, there is currently no method to directly translate ARG abundance to human health risks. Another challenge is that ARGs occur naturally in aquatic ecosystems, and levels of prevailing resistance across different seasons and geographic locations can vary substantially. This highlights the need for further research to identify and quantify ARG targets and concentrations that may pose risks to human health in full-scale DWDS and in source waters used for drinking water.

Profiling the resistome and microbiome in aquatic environments is vital to gaining a better understanding of AMR dynamics and developing mitigation approaches that protect human and environmental health. Characterizing microbial communities and the resistome in DWDS and freshwater environments is critical to help track the dissemination and spread of AMR. Additionally, this type of research can help identify new variants of AMR genes and identify potential novel microorganisms. As bacterial communities are influenced by temporal and biogeographical factors, it is necessary to analyze the transport and fate of ARB and AMR genes in many different environments and geographical locations to help better understand the complex dynamics of AMR proliferation in natural and engineered systems. Engineering practices related to selection of pipe materials, corrosion inhibitors, and disinfectant regimes in DWDS should consider impacts on microbial communities and AMR. System parameters should be evaluated to determine their impacts on AMR indicator genes and the abundance of ARB,

which can contribute to horizontal gene transfer of resistance genes and the emergence of novel AMR genes. With increased monitoring and data related to AMR it is possible to identify a baseline of current AMR conditions for each DWDS, for example, and to evaluate its role in the dissemination and spread of ARB and ARGs in the environment. Further, improved surveillance of AMR's genetic indicators in environmental reservoirs such as DWDS or freshwater sources would enable a more comprehensive understanding of the problem at a global scale.

## **6 CONCLUSIONS**

The possible risks to humans due to exposure of ARB and ARGs as a result of their passage through conventional drinking water has driven the effort to develop safe and effective DWDS. ARB and ARGs are considered emerging contaminants and are important factors in the spread of AMR. Increased monitoring of resistance determinants and microorganisms in the natural environment and engineered systems is required to gain a better understanding of AMR at a global scale, identify and track new resistance variants, and to develop engineering management programs that consider impacts on bacterial communities and the spread of AMR. The overall objective of this dissertation was to determine the impact of select metals and corrosion inhibitors on phenotypic and genotypic antibiotic resistance. The evaluation was completed by testing the impact of different corrosion inhibitors containing metals and disinfectants on the abundance of ARB and ARGs. The relationships were evaluated under varying water quality conditions, which provided essential information regarding the effects of different corrosion inhibitor addition and their specific impact on the antibiotic resistance profile in the microbial communities tested.

### **6.1 Key findings**

The first objective was to evaluate the presence and abundance of ARGs, MRGs, and bacterial communities inside a full-scale water main. Droplet digital PCR (ddPCR) was used to quantify the abundance of ARGs and MRGs in different biofilm environments inside a full-scale pipe from an active distribution system. 16S rRNA gene

amplicon sequencing was conducted to determine the microbial communities present in the different biofilm environments.

The results demonstrated that ARGs and class 1 integrons were detected in every biofilm sample type studied from a chloraminated cast iron drinking water main, indicating that pipes could serve as potential sources and important reservoirs for ARGs. Assays conducted with ddPCR resulted in more positive detections and lower detection limits for target genes compared to qPCR assays. Future studies should consider ddPCR for environmental samples containing inhibiting substances such as metals, humic acids, and other contaminants. Microbial communities varied among different biofilm sample locations and were dominated by corrosion-related genera including *Mycobacterium*, *Geobacter*, *Gallionella*, and *Sphingomonas*. Significant relationships were observed for the co-occurrence of ARGs, MRGs, *intI1* and several microbial taxa.

The second objective was to determine the impact of different corrosion inhibitors containing metals (zinc orthophosphate, sodium orthophosphate, and sodium silicate) on the absolute and relative abundance of ARB, ARGs, and MRGs in a source water for drinking water. The impact of different corrosion inhibitor types and corrosion inhibitor concentrations was assessed using laboratory-scale drinking water microcosms. ARB were quantified by heterotrophic plate counts from direct plating of water on media with clinically-relevant antibiotics and ARGs/MRGs were quantified using qPCR.

The results in this study indicate that corrosion inhibitors such as zinc orthophosphate, which are commonly used in drinking water systems, can alter resistance profiles and promote the occurrence of ARB, ARGs, and *intI1* in bacterial communities. Significant increases in absolute and relative abundance of ARB resistant to multiple

clinically-relevant antibiotics including CIP, SULF, TRIM, and VAN and ARGs including *sul1*, *sul2*, *qacEΔ1*, and *intI1* were observed in response to zinc orthophosphate addition at concentrations typically found in distribution systems (~1 mg/L). The presence of zinc orthophosphate can lead to positive selective pressure for ARB, which increases the abundance and types of resistance genes they harbor. Zinc orthophosphate resulted in highest selection of ARB and associated ARGs compared to corrosion inhibitor-free controls, sodium orthophosphate, and sodium silicate treatment. Sodium silicate dosage at 10 mg/L resulted in decreased bacterial growth as measured by heterotrophic plate counts and qPCR, when compared to zinc and sodium orthophosphate treatments. High doses of silicates at 100 mg/L increased the growth of ARB resistant to RIF and VAN and several ARGs and MRGs including *bla<sub>TEM</sub>*, *czcD*, *copA*, *intI1*, and *sul2* relative to untreated controls. High levels of zinc orthophosphate (10 mg/L) and sodium silicate (100 mg/L) exhibited similar selection of ARGs including *intI1*, *sul2*, and *qacEΔ1* by day 7. The observed increases in total and relative abundance of ARB and ARGs suggests that enrichment for multiple types of antibiotic resistance occurred within the bacterial communities as a result of the addition of the selected corrosion inhibitors.

The third objective was to determine the impact of corrosion inhibitor type and disinfectant type on the microbiome and entire collection of resistance genes in the microbial community (i.e., the resistome) in a source drinking water as determined by shotgun metagenomic sequencing. The approach to this study was to determine the impact of different types of corrosion inhibitors and disinfectants on the antibiotic resistome using laboratory-scale drinking water microcosms. The impact of different chemical additions were quantified by heterotrophic plate counts of ARB resistant to

clinically-relevant antibiotics and by quantification of target resistance genes using qPCR. Shotgun metagenomic sequencing was used to evaluate the impact of disinfectants and corrosion inhibitors on the antibiotic resistome and microbiome.

The results presented in this study provide evidence that corrosion inhibitors such as zinc orthophosphate and disinfectants such as free chlorine exhibit significant changes to the antibiotic resistome and microbiome of a source water for drinking water. Disinfectants and corrosion inhibitors increased the occurrence of phenotypic resistance to multiple clinically-relevant antibiotics, and genotypic antibiotic resistance including genetic determinants related to disinfectant resistance (*qacEΔ1*) and horizontal gene transfer (*int1*). Metagenomic characterization of the resistome indicated increased types and abundance of ARGs related to antibiotic efflux in communities treated with free chlorine and zinc orthophosphate. Free chlorine disinfection selected for significantly higher abundance of ARGs corresponding to several different classes including aminoglycoside, carbapenem, cephamycin, cephalosporin, fluoroquinolone, macrolide, penam, phenicol, tetracycline, and peptide compared untreated controls. Similarly, phosphate-based corrosion inhibitors promoted increased abundances of ARGs belonging to the aminoglycoside, carbapenem, cephamycin, cephalosporin, fluoroquinolone, macrolide, tetracycline, penam, and phenicol classes. Zinc orthophosphate resulted in highest selection of ARB and associated ARGs compared to controls, sodium orthophosphate, and sodium silicate treatments.

This was the first research conducted on the impacts of corrosion inhibitors on antibiotic resistance using targeted and non-targeted molecular methods of analysis. This is an important development in understanding the dissemination of AMR in aquatic

environments such as DWDS. This research establishes that zinc and other metals such as iron can promote increased occurrence of ARB and ARGs in DWDS and the natural environment. This research can better the lives of many by providing the data needed to guide management and engineering decisions related to decreased risk of occurrence of bacteria and AMR genes in potable drinking water. Improved monitoring and surveillance of microbial communities and AMR indicator genes is required to develop a more comprehensive understanding of the fate and transport of ARB and ARGs through engineered systems such as DWDS. Engineering management decisions such as pipe material selection, corrosion inhibitor selection, and disinfectant type and dosage should be evaluated for their impact on the abundance of specific ARGs and/or ARB. Each DWDS is unique and contains microbial communities that have been preferentially selected for based on the geographical, temporal, spatial, and physicochemical factors inside of the system. In order to better identify potential areas for improvement or mitigation of ARB and ARGs in DWDS, improved monitoring of the transport and fate of bacteria and genes across different reservoirs such as freshwater source waters and drinking water is imperative.

## **6.2 Future work recommendations and implications**

Future research on antibiotic resistance in DWDS should focus on monitoring the occurrence, fate, and distribution of ARB, ARGs, MRGs, and MGEs in multiple phases in full-scale systems. This information is critical for gaining a better understanding of the prevalence of antibiotic resistance in engineered systems capable of directly impacting human health. Factors such as metal pipe materials, corrosion inhibitors, and corrosion

products that develop in full-scale DWDS may select for bacteria harboring resistance to both metals and antibiotics. However, there is a lack of information regarding the abundance and fate of ARB, ARGs, MRGs, and MGEs in different phases of DWDS, including planktonic cells, biofilm, suspended solids, and loose corrosion deposits. Previous characterization of DWDS has primarily focused on microorganisms in the planktonic phase and studies documenting and quantifying ARB, ARGs, or MRGs in drinking water biofilms are lacking.

In addition to full-scale studies, laboratory-scale studies such as those performed in this study are needed to distinguish the impacts of different pipe materials, corrosion inhibitors, and corrosion products on the abundance of resistance genes in drinking water and biofilms. These studies will provide critical information regarding the relationship of different system parameters and mechanisms of antibiotic resistance selection in engineered systems. Locations with high densities of bacteria, such as drinking water biofilms, provide conditions which are suitable for proliferation and exchange of resistance genes, and selective pressures (e.g., metals) may increase the abundance of resistance genes in these communities. Additional research is needed to address the research gaps related to the fate and transport of clinically-relevant resistance genes in DWDS.

Fundamental information regarding the impacts of pipe materials, metals, and corrosion inhibitors on pathogens and abundance of resistance genes in DWDS is necessary to reduce the prevalence of antibiotic resistance in drinking water. Further research including QMRA is needed to determine the concentration of ARB or ARGs that may translate to human health risks (Ashbolt et al. 2013b). Additionally, research

that links ARGs to potential hosts and distinguishes extracellular DNA from intracellular DNA is also needed (Rice et al. 2020). The limited availability of exposure assessments and dose-response data regarding ARB and ARGs for different scenarios hinders the implementation of QMRA approach for evaluating human health risks in aquatic environments (Amarasiri et al. 2019).

Lastly, the selection of target genes is also important for improving routine monitoring of AMR in full-scale distribution systems and aquatic environments. Future research and microbial surveillance efforts should consider monitoring clinically-relevant ARGs and other genes commonly linked to horizontal gene transfer processes. Laboratory and full-scale studies regarding pipe materials, corrosion inhibitors, and corrosion products are important for gaining insights into microbial functions and could be used to provide guidance to water utilities for making engineering decisions in DWDS that could reduce human health risks.

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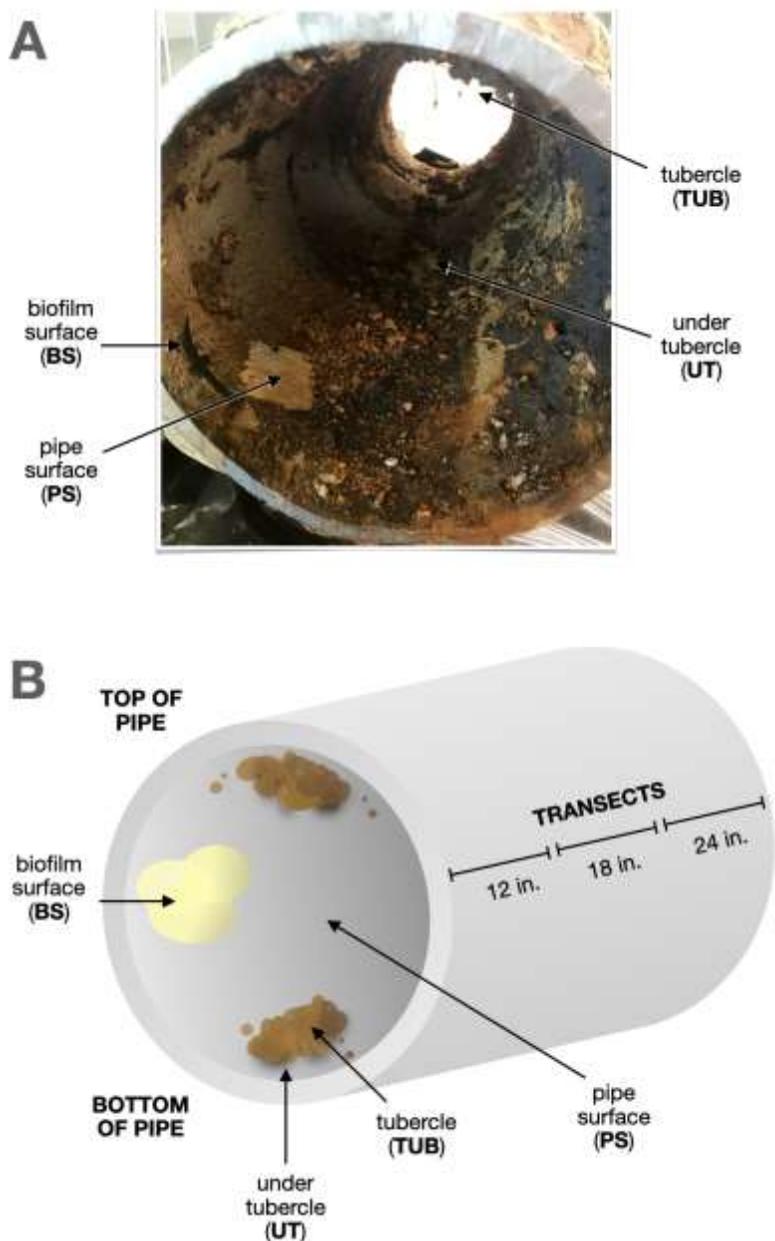
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**Appendices**  
**Appendix 3A. Pipe Diagram**



**Figure 3A.** Diagram of different biofilm sample locations from chloraminated cast iron drinking water main. Sample locations included biomass surface (BS), pipe surface scrape (PS), tubercles (TUB), and under tubercles (UT). Biomass surface samples were collected in areas with increased corrosion deposits and/or biofilm development. Pipe surface samples were collected by swabbing areas of the pipe with the least amounts of biofilm development.

### Appendix 3B. qPCR Methodology

**Table 3.1. Primers, annealing temperatures, amplification efficiencies, and R<sup>2</sup> values for qPCR analysis of target genes**

Gene	Annealing Temp. (°C)	Forward Primer & Reverse Primer	Size (bp)	Efficiency Avg. (%)	R <sup>2</sup>	Reference
16S rRNA	60	F- (5'-CCTACGGGAGGCAGCAG-3') R- (5'-ATTACCGCGGCTGCTGG-3')	202	98.5%	0.98	(Muyzer et al. 1993)
<i>bla<sub>SHV</sub></i>	64	F- (5'-CGCTTTCCCATGATGAGCACCTTT-3') R- (5'-TCCTGCTGGCGATAGTGGATCTTT-3')	94	95%	1.0	(Xi et al. 2009)
<i>bla<sub>TEM</sub></i>	60	F- (5'-GCKGCCAACTTACTTCTGACAACG-3') R- (5'-CTTTATCCGCCTCCATCCAGTCTA-3')	257	107%	0.98	(Marti et al. 2013)
<i>copA</i>	63	F- (5'-ATGTGGAACSARATGCGKATGA-3') R- (5'-AGY TTCAGCCSGGAATACG-3')	193	101%	0.99	(Roosa et al. 2014)
<i>czcD</i>	55	F- (5'-TCATCGCCGGTGCATCATCAT-3') R- (5'-TGTCATTCACGACATGAACC-3')	272	99%	0.98	(Roosa et al. 2014)
<i>intI1</i>	60	F- (5'-CCTCCCGCACGATGATC-3') R- (5'-TCCACGCATCGTCAGGC-3')	280	96.5%	1.0	(Goldstein et al. 2001)
<i>sul1</i>	60	F- (5'-CCGTGGCCTCCTGTAAAG-3') R- (5'-TIGCCGATCGCGTGAAGT-3')	67	97%	1.0	(Burch et al. 2013)
<i>tet(L)</i>	60	F- (5'-TCGTTAGCGTGCTGTCATTC-3') R- (5'-GTATCCCCACCAATGTAGCCG-3')	276	99%	0.99	(Ng et al. 2001)

## Appendix 3C. MIQE Guidelines/Checklist

Table 3.2. MIQE Guidelines/Checklist

Item to check	Importance*	Comments
<b>Experimental Design</b>		
<b>Definition of experimental and control groups</b>	E	Yes
<b>Number within each group</b>	E	Yes
<b>Assay carried out by core lab or investigator's lab?</b>	D	Yes
<b>Power analysis</b>	D	NA
<b>Sample</b>		
<b>Description</b>	E	Yes
<b>Volume or mass of sample processed</b>	E	Yes
<b>Microdissection or microdissection</b>	E	NA
<b>Processing Procedure</b>	E	Yes
<b>If frozen – how and how quickly?</b>	E	Yes
<b>If fixed – with what, how quickly?</b>	E	NA
<b>Sample storage conditions and duration</b>	E	Yes
<b>Nucleic acid extraction</b>		
<b>Quantification – instrument/method</b>	E	Yes
<b>Storage conditions: temperature, concentrations, duration, buffer</b>	E	Yes
<b>DNA or RNA quantification</b>	E	Yes
<b>Quality/integrity, instrument/method, e.g. RNA integrity/R quality index and trace or 3':5'</b>	E	NA
<b>Template structural information</b>	E	Yes
<b>Template modification (digestion, sonication, preamplification., etc.)</b>	E	NA
<b>Template treatment (initial heating or chemical denaturation)</b>	E	NA
<b>Inhibition dilution or spike</b>	E	NA
<b>DNA contamination assessment of RNA sample</b>	E	NA
<b>Details of DNase treatment where performed</b>	E	NA
<b>Manufacturer of reagents used and catalogue number</b>	D	Yes
<b>Storage of nucleic acid: temperature, concentration, duration, buffer</b>	E	Yes
<b>RT (if necessary)</b>		
<b>cDNA priming method + concentration</b>	E	NA
<b>One- or 2-step protocol</b>	E	NA
<b>Amount of RNA used per reaction</b>	E	NA
<b>Detailed reaction components and conditions</b>	E	NA
<b>RT efficiency</b>	D	NA

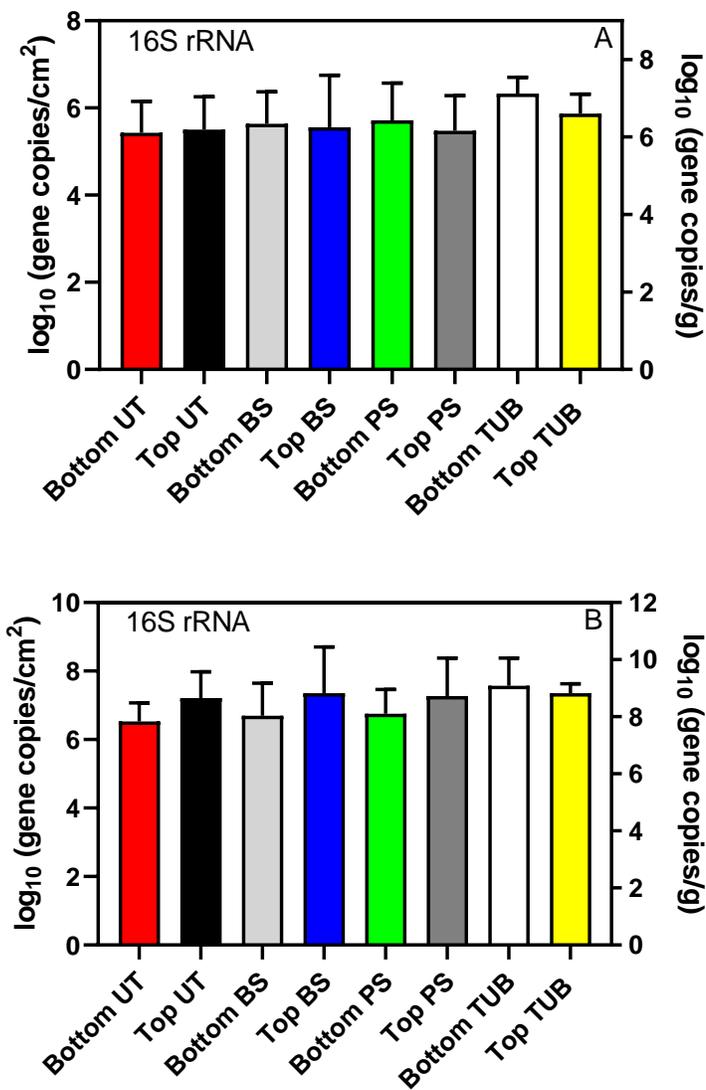
<b>Estimated copies measures with and without addition of RT</b>	D	NA
<b>Manufacturer of reagents used and catalogue number</b>	D	NA
<b>Reaction volume (for 2-step RT reaction)</b>	D	NA
<b>Storage of cDNA: temperature, concentration, duration, buffer</b>	D	NA
<b>dPCR target information</b>		
<b>Sequence accession number</b>	E	Yes
<b>Amplicon location</b>	D	No
<b>Amplicon length</b>	E	Yes
<b>In silico specificity screen (BLAST, etc.)</b>	E	No
<b>Pseudogenes, retro-pseudogenes or other homologs</b>	D	NA
<b>Sequence alignment</b>	D	NA
<b>Secondary structure analysis of amplicon and GC content</b>	D	NA
<b>Location of each primer by exon and intron</b>	E	Yes
<b>Where appropriate, which splice variants are targeted?</b>	E	No
<b>dPCR oligonucleotides</b>		
<b>Primer sequences and/or amplicon context sequence</b>	E	Yes
<b>RTPrimerDB identification number</b>	D	NA
<b>Probe sequences</b>	D	NA
<b>Location and identity of any modifications</b>	E	NA
<b>Manufacturer or oligonucleotides</b>	D	Yes
<b>Purification method</b>	D	NA
<b>dPCR protocol</b>		
<b>Complete reaction conditions</b>	E	Yes
<b>Reaction volume and amount of RNA/cDNA/DNA</b>	E	Yes
<b>Primer, (probe), Mg<sup>++</sup> and dNTP concentrations</b>	E	Yes
<b>Polymerase identity and concentration</b>	E	Yes
<b>Buffer/kit catalogue no. and manufacturer</b>	E	Yes
<b>Exact chemical constitution of the buffer</b>	D	NA
<b>Additives (SYBR green I, DMSO, etc.)</b>	E	Yes
<b>Plate/tubes catalogue No and manufacturer</b>	D	Yes
<b>Complete thermocycling parameters</b>	E	Yes
<b>Reaction setup</b>	D	Yes
<b>Gravimetric or volumetric dilutions</b>	D	Yes
<b>Total PCR reaction volume prepared</b>	D	Yes
<b>Partition number</b>	E	NA
<b>Individual partition</b>	E	NA
<b>Total volume of the partitions measured (effective reaction size)</b>	E	NA
<b>Partition volume variance/SD</b>	D	Yes

<b>Comprehensive details and appropriate use of controls</b>	E	Yes
<b>Manufacturer of dPCR instrument</b>	E	Yes
<b>dPCR validation</b>		
<b>Optimization data for the assay</b>	D	Yes
<b>Specificity (when measuring rare mutations, pathogen sequences etc.)</b>	E	NA
<b>Limit of detection of calibration control</b>	D	Yes
<b>If multiplexing, comparison with singleplex assays</b>	E	NA
<b>Data analysis</b>		
<b>Mean copies per partition</b>	E	Yes
<b>dPCR analysis program</b>	E	Yes
<b>Outlier identification and disposition</b>	E	NA
<b>Results of no-template controls</b>	E	Yes
<b>Examples of positive(s) and negative experimental results as supplemental data</b>	E	Yes
<b>Where appropriate, justification of number and choice of reference genes</b>	E	NA
<b>Where appropriate, description of normalization method</b>	E	NA
<b>Number and concordance of biological replicates</b>	D	Yes
<b>Number and stage (RT of dPCR) of technical replicates</b>	E	NA
<b>Repeatability (intraassay variation)</b>	E	NA
<b>Reproducibility (interassay/user/lab etc. variation)</b>	D	Yes
<b>Experimental variance or CI</b>	E	Yes
<b>Statistical methods used for analysis</b>	E	Yes
<b>Data submission using RDML (real-time PCR data markup language)</b>	D	No

\* Essential information (E) submitted with the manuscript if applicable to the study. Desirable information (D) submitted if possible. NA = not applicable, Yes = provided in manuscript, No = not performed or provided in manuscript.

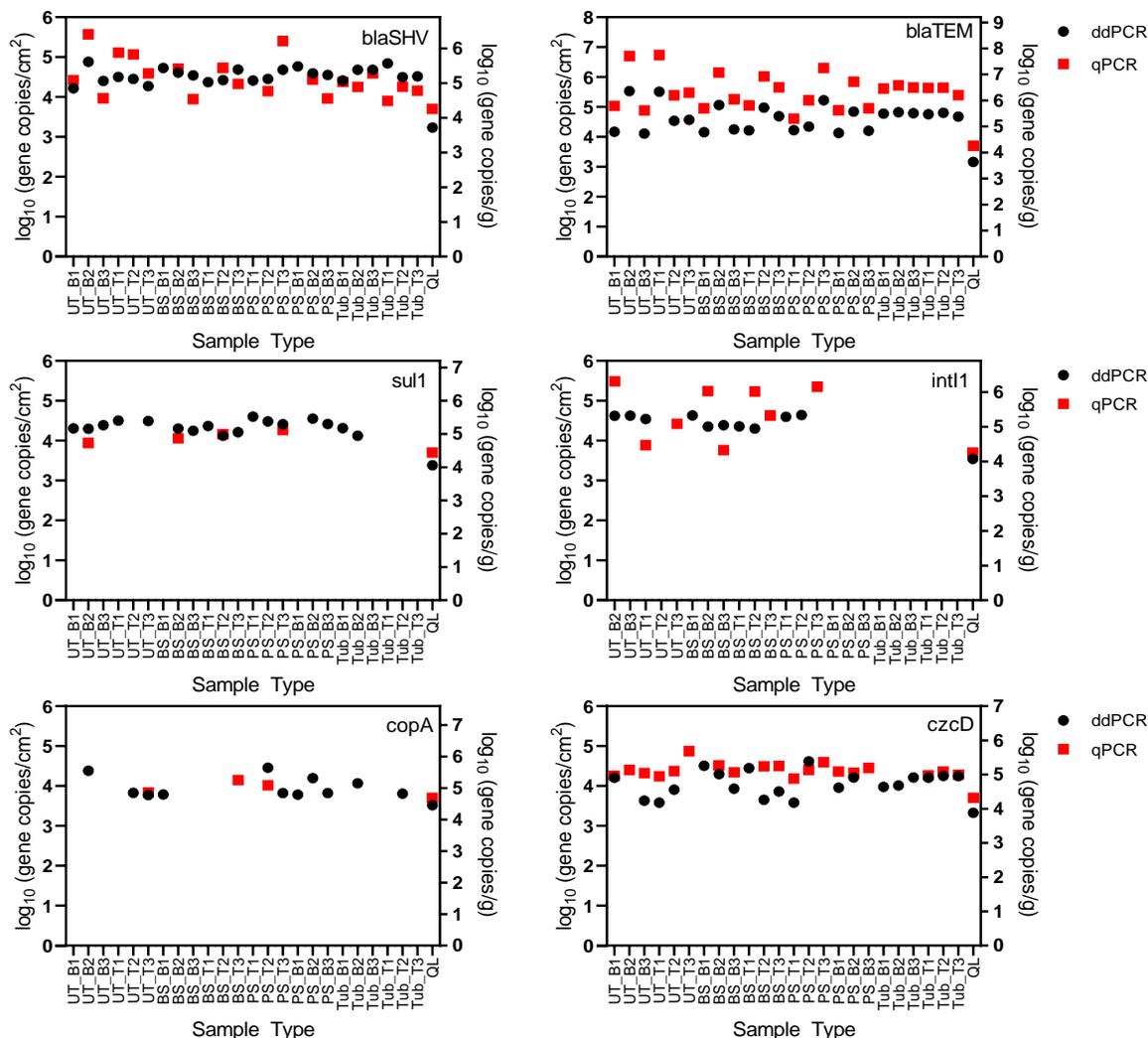
\*Table adapted from (Bustin et al. 2009).

### Appendix 3D. 16S rRNA qPCR results



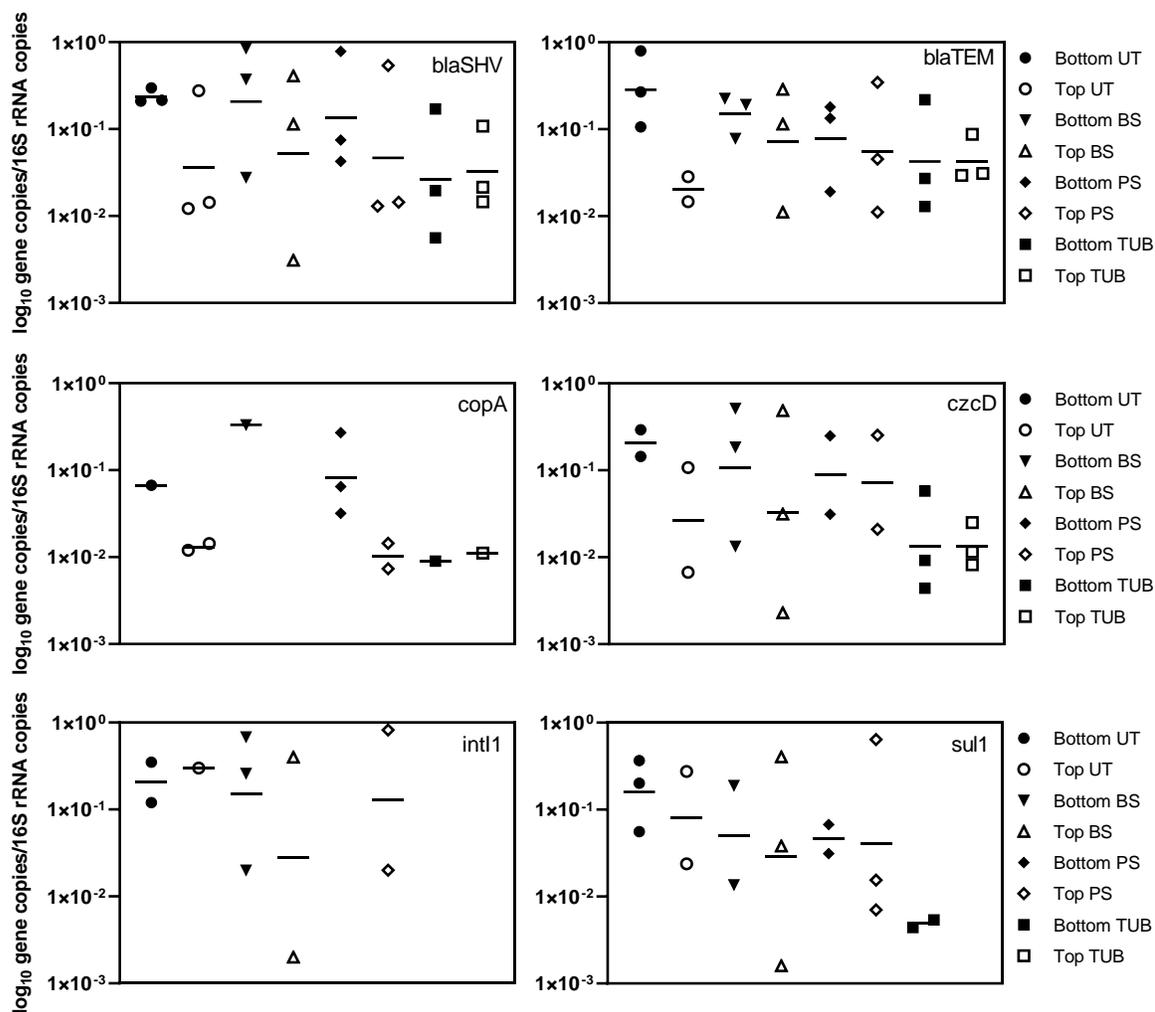
**Figure 3D.** Absolute abundance of 16S rRNA genes in different biofilm microenvironments from a cast-iron drinking water main as measured with droplet digital PCR (ddPCR) (Figure A) and quantitative PCR (qPCR) (Figure B). The biofilm microenvironments include biomass surface (BS), pipe surface (PS), tubercle (TUB), and under tubercle (UT). Each bar represents a different sample category which includes triplicate biofilm samples (n=3). The mean absolute gene abundance (left y-axis:  $\log_{10}$  gene copies/cm<sup>2</sup>, right y-axis:  $\log_{10}$  gene copies/g) for each of the biofilm samples is plotted on the y-axis with one standard deviation. The right y-axis only applies to corrosion tubercle (TUB) samples, which were analyzed by weight.

## Appendix 3E. ddPCR and qPCR comparison



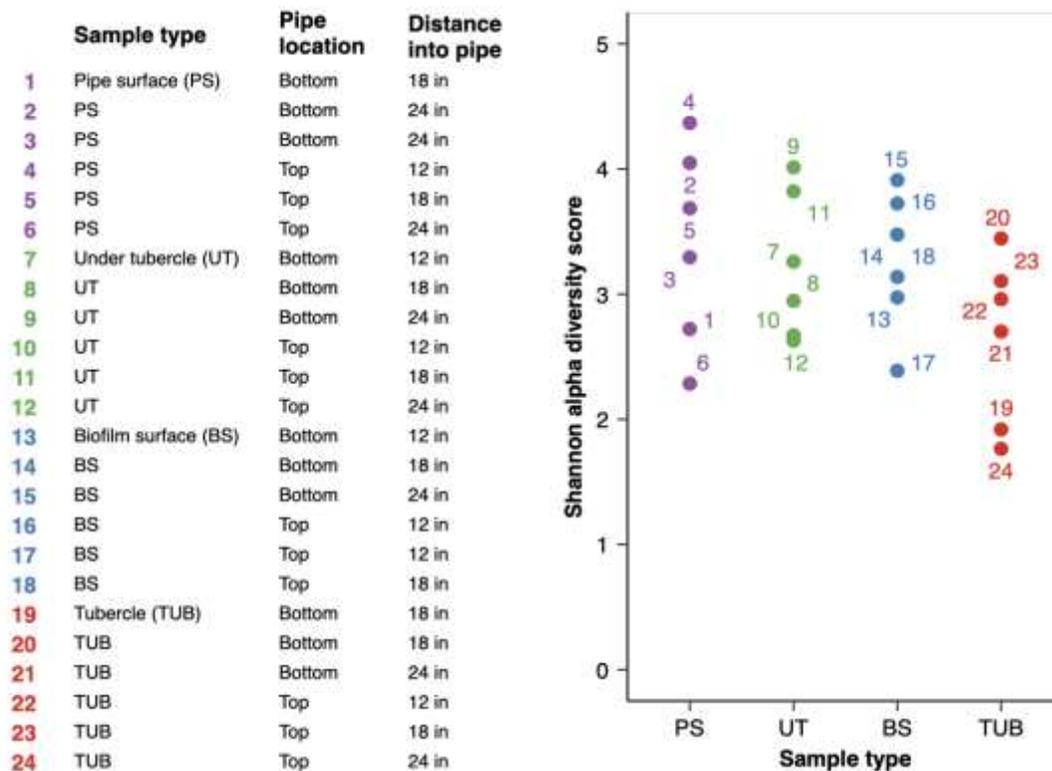
**Figure 3E.** Comparison of absolute gene abundance of ARGs, MRGs, and *int11* as measured with ddPCR and qPCR assays. The biofilm microenvironments include biomass surface (BS), pipe surface (PS), tubercle (TUB), and under tubercle (UT). Each biofilm sample is also categorized by top (T) or bottom (B) pipe sample location. Each point represents the average of technical replicates for each biofilm sample (n=3). The mean absolute gene abundance (left y-axis: log<sub>10</sub> gene copies/cm<sup>2</sup>, right y-axis: log<sub>10</sub> gene copies/g) for each of the biofilm samples is plotted. The right y-axis only applies to corrosion tubercle (TUB) samples, which were analyzed by weight. ddPCR concentrations are denoted with solid black circles and qPCR concentrations are denoted with red squares. The quantification limit (QL) is also plotted for each gene.

### Appendix 3F. Relative abundance of ARGs, MRGs, and *int1*



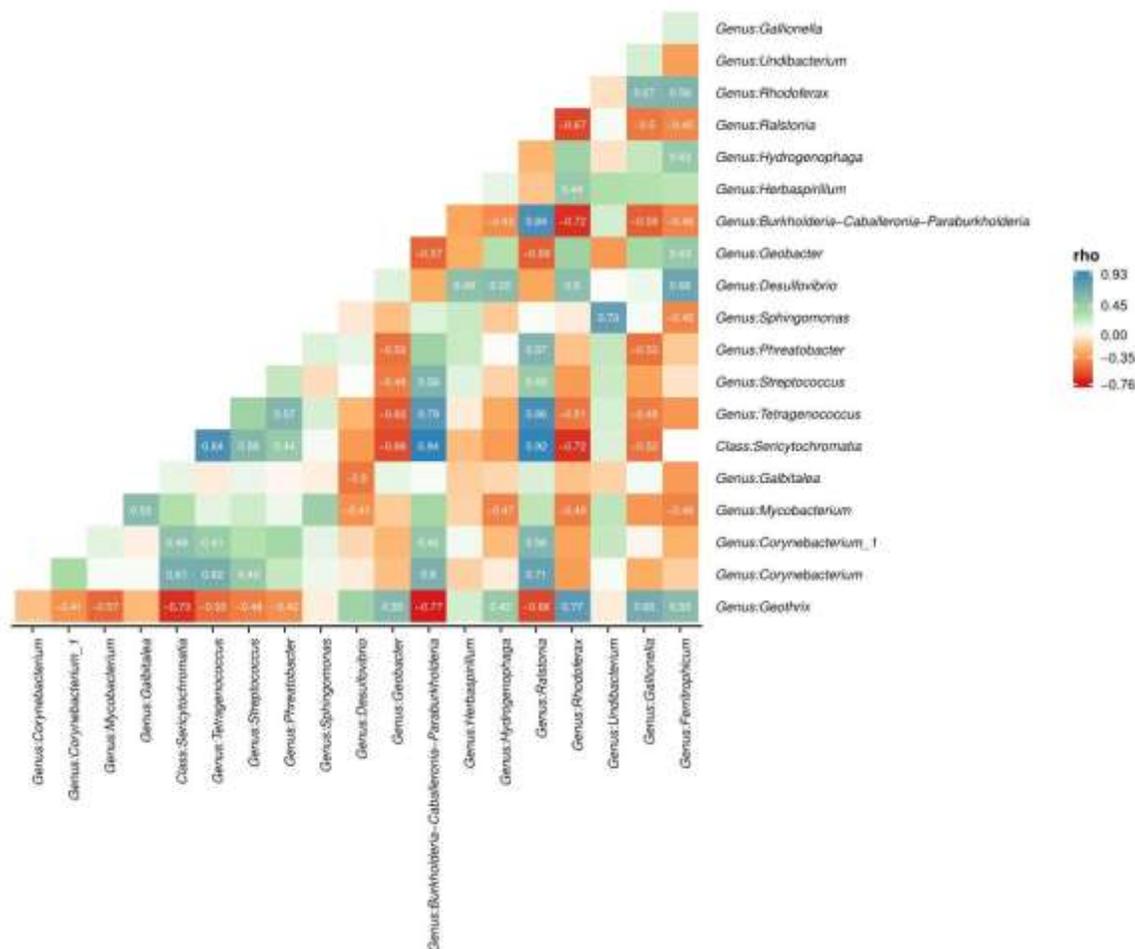
**Figure 3F.** Relative abundance of antibiotic resistance genes, metal resistance genes, and *int1* in different biofilm microenvironments from a cast-iron drinking water main as measured with droplet digital PCR (ddPCR). The biofilm microenvironments include biomass surface (BS), pipe surface (PS), tubercle (TUB), and under tubercle (UT). Each biofilm sample is also categorized by top or bottom pipe sample location. Each point represents the average of technical replicates for each biofilm sample ( $n=3$ ).

### Appendix 3G. X-Ray Diffraction Patterns



**Figure 3G.** Sample information for drinking water pipe samples (left). Shannon alpha diversity measurements of microbial communities characterized by 16S rRNA gene sequencing (right). Color of points denote biofilm microenvironments (sample type). Point labels indicate sample number as in table on left.

### Appendix 3H. Spearman correlation between microbial genera



**Figure 3H.** Relationships between relative abundance of most abundant bacterial genera observed in biofilm samples from cast iron water main. Color denotes the result from correlation analysis using Spearman's rank sum correlation with Spearman's rho value plotted for each comparison. The Rho value for statistically significant relationships are also included (p values < 0.05). The lowest available taxonomic classification for each observed ASV is provided.

## Appendix 4A. qPCR Methodology

**Table 4.1A** Primers, annealing temperatures, amplification efficiencies, and  $R^2$  values for qPCR analysis of target genes

Gene	Anneal Temp. (°C)	Forward Primer & Reverse Primer	Size (bp)	Efficiency Avg. (%)	$R^2$	Reference
16S rRNA	60	F- (5'-CCTACGGGAGGCAGCAG-3') R- (5'-ATTACCGCGGCTGCTGG-3')	202	98%	0.98-1.0	(Muyzer et al. 1993)
<i>bla<sub>TEM</sub></i>	60	F- (5'-GCKGCCAACTTACTTCTGACAACG-3') R- (5'-CTTTATCCGCCTCCATCCAGTCTA-3')	257	92%	0.99-1.0	(Marti et al. 2013)
<i>copA</i>	63	F- (5'-ATGTGGAACSARATGCGKATGA-3') R- (5'-AGYTTTCAGGCCSGBAATACG-3')	193	90%	0.98-0.99	(Roosa et al. 2014)
<i>czcC</i>	62	F- (5'-AGCCGYCAGTATCCGGATCTGAC-3') R- (5'-GTGGTCGCCGCTGATAGGT-3')	418	90%	0.98-1.0	(Roosa et al. 2014)
<i>czcD</i>	55	F- (5'-TCATCGCCGGTGCGATCATCAT-3') R- (5'-TGTCATTCACGACATGAACC-3')	272	95%	0.98-0.99	(Roosa et al. 2014)
<i>int1</i>	60	F- (5'-CCTCCCGCACGATGATC-3') R- (5'-TCCACGCATCGTCAGGC-3')	280	95%	0.98-1.0	(Goldstein et al. 2001)
<i>sul1</i>	60	F- (5'-CCGTTGGCCTTCCTGTAAAG-3') R- (5'-TTGCCGATCGCGTGAAGT-3')	67	92%	0.99-1.0	(Burch et al. 2013)
<i>sul2</i>	60	F- (5'- TCCGATGGAGGCCGGTATCTGG-3') R- (5'- CGGGAATGCCATCTGCCTTGAG-3')	191	90%	0.98-1.0	(Muziasari et al. 2014)
<i>qacEΔ1</i>	60	F- (5'-CCCCTTCCGCCGTTGT-3') R- (5'-CGACCAGACTGCATAAGCAACA-3')	101	95%	0.98-1.0	(Zhu et al. 2013)

## Appendix 4B. Day 0 Water Quality

**Table 4B.** Day 0 Water Quality Characteristics for Microcosm Experiments

Experiment Set	Microcosm Set 1	Microcosm Set 2	Microcosm Set 3
Sample Location	DWTP Intake Pipe	Atwater Beach	Atwater Beach
Description	Day 0	Day 0	Day 0
pH (standard units)	8.20	8.15	8.05
Temp (°F)	62	63	50
Silica (mg/L as SiO <sub>2</sub> )	<1	2	4
H <sub>2</sub> S (mg/L)	<0.1	<0.1	<0.1
Chloride (mg/L as Cl)	20	30	25
Free Chlorine (mg/L as Cl)	<0.1	<0.1	<0.1
Total Chlorine (mg/L as Cl)	<0.1	<0.1	<0.1
Dissolved Oxygen (mg/L as O <sub>2</sub> )	13	9	13
Ammonia (mg/L)	0.1	0.1	0.2
Calcium Hardness (mg/L as CaCO <sub>3</sub> )	100	120	120
Total Hardness (mg/L as CaCO <sub>3</sub> )	180	180	160
Total Alkalinity (mg/L as CaCO <sub>3</sub> )	140	160	140
Phenolphthalein Alkalinity (mg/L)	0	0	0
Orthophosphate (mg/L as PO <sub>4</sub> )	<0.05	0.05	0.25
DOC (mg/L as C)	2.57	2.80	1.85

**Notes:** DOC = dissolved organic carbon, mg/L = milligrams per liter, DWTP = drinking water treatment plant, CaCO<sub>3</sub> = calcium carbonate, C = carbon, °F = degrees Fahrenheit

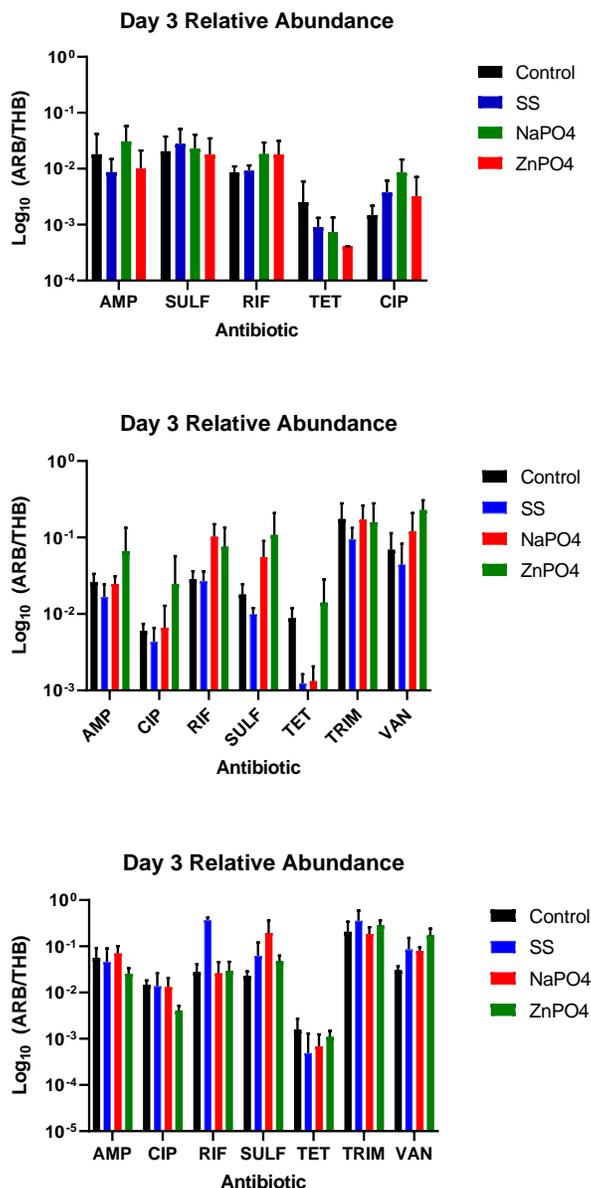
## Appendix 4C. Day 3 and 7 Water Quality

**Table 4C.** Day 3 and 7 Water Quality for Microcosm Experiments

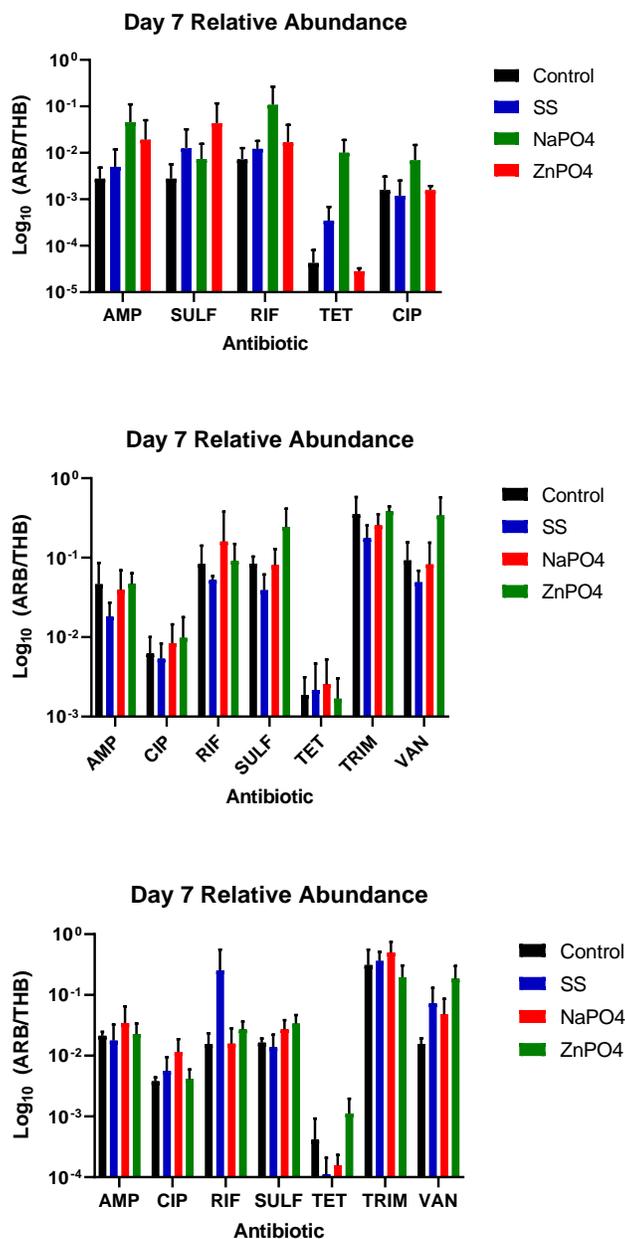
Sample Name	Day	Initial Dose	pH (S.U.)	PO <sub>4</sub> (mg/L as PO <sub>4</sub> )	Silica (mg/L as SiO <sub>2</sub> )	DOC (mg/L as C)
Control (Set1)	3	NA	7.92-8.22	0.01-0.05	<1	2.72-2.87
Sodium Silicate	3	10 mg/L	8.01-8.33	0.02-0.04	10.0-12.0	1.69-3.43
Sodium Orthophosphate	3	1 mg/L	8.12-8.17	0.99-1.0	<1	2.58-2.74
Zinc Orthophosphate	3	1 mg/L	8.22-8.26	0.72-1.15	<1	2.59-2.71
Control (Set1)	7	NA	8.16-8.26	0.01-0.02	<1	2.16-2.92
Sodium Silicate	7	10 mg/L	8.13-8.33	0.02-0.03	10.0-12.0	1.76-2.83
Sodium Orthophosphate	7	1 mg/L	8.16-8.22	0.99-1.11	<1	2.79-2.99
Zinc Orthophosphate	7	1 mg/L	8.18-8.21	0.20-0.83	<1	2.89-3.04
Control (Set2)	3	NA	7.92-8.22	0.05-0.07	2	2.83-2.99
Sodium Silicate	3	10 mg/L	8.01-8.30	0.08-0.11	10.0-12.0	2.65-2.89
Sodium Orthophosphate	3	1 mg/L	8.14-8.17	0.7-1.25	2	2.70-3.18
Zinc Orthophosphate	3	1 mg/L	8.22-8.26	0.7-0.80	2	2.06-2.40
Control (Set2)	7	NA	7.86-8.06	0.1-0.13	2	2.05-2.33
Sodium Silicate	7	10 mg/L	7.81-8.05	0.13-0.14	10.0-12.0	1.97-2.11
Sodium Orthophosphate	7	1 mg/L	7.62-7.76	0.84-1.05	2	1.96-2.14
Zinc Orthophosphate	7	1 mg/L	7.8-8.14	0.84-1.30	2	2.17-2.29
Control (Set3)	3	NA	7.80-7.93	0.16-0.22	4	1.85-2.12
Sodium Silicate	3	100 mg/L	8.16-8.26	0.23-0.27	95-100	1.81-8.88
Sodium Orthophosphate	3	10 mg/L	7.65-7.97	10.0-13.0	4	1.88-2.00
Zinc Orthophosphate	3	10 mg/L	7.79-8.03	10.0-14.0	4	1.95-2.00
Control (Set3)	7	NA	7.69-7.82	0.24-0.33	4	1.76-1.89
Sodium Silicate	7	100 mg/L	8.5-8.62	0.17-0.36	95-100	1.79-1.89
Sodium Orthophosphate	7	10 mg/L	8.06-8.2	10.3-12.5	4	1.83-1.90
Zinc Orthophosphate	7	10 mg/L	7.98-8.15	10.5-12.5	4	1.89-1.95

**Notes:** DOC = dissolved organic carbon, mg/L = milligrams per liter, NA = not applicable, PO<sub>4</sub> = orthophosphate

## Appendix 4D. Relative abundance of ARB



**Figure 4D.** Relative abundance of heterotrophic plate counts observed on Day 3 for microcosm experiments. The average relative abundance of antibiotic resistant bacteria (ARB) based on direct plating of from microcosms containing different types and concentrations of corrosion inhibitors. The relative abundance of ARB is shown on the y-axis. The type of antibiotic is denoted on the x-axis. Corrosion inhibitor type is indicated by color. Different experimental conditions are plotted on different graphs including Set 1 – Normal concentration (1X) of CI's, lake water collected from DWTP (A - top), Set 2 – Normal concentration (1X) of CI's, lake water collected at beach (B - middle), and Set 3 – High concentration (10X) of CI's, lake water collected at beach (C - bottom).



**Figure 4E** Relative abundance of heterotrophic plate counts observed on Day 7 for microcosm experiments. The average relative abundance of antibiotic resistant bacteria (ARB) based on direct plating of from microcosms containing different types and concentrations of corrosion inhibitors. The relative abundance of ARB is shown on the y-axis. The type of antibiotic is denoted on the x-axis. Corrosion inhibitor type is indicated by color. Different experimental conditions are plotted on different graphs including Set 1 – Normal concentration (1X) of CI's, Drinking water collected from DWTP (top), Set 2 – Normal concentration (1X) of CI's, surface water collected at beach (middle), and Set 3 – High concentration (10X) of CI's, surface water collected at beach (bottom).

**Table 4F.** Summary of differences of absolute abundance (Log10 scale) of total heterotrophic bacteria (R2A) and antibiotic resistant bacteria (ARB) measurements obtained from direct plating onto R2A-media with antibiotics. Values represent the difference between the absolute abundance of untreated controls and the average of triplicate treatment reactors for each condition tested.

Treatment Type	Conc. (mg/L)	Source Water	Day	R2A	AMP	CIP	RIF	SULF	TET	TRIM	VAN
SS	10	SW	3	-0.237	-0.130	0.174	-0.206	-0.046	-0.239	NA	NA
NaPO4	1	SW	3	-0.370	0.097	0.291	-0.082	-0.247	-0.653	NA	NA
ZnPO4	1	SW	3	0.011	0.039	0.125	0.266	-0.062	-0.349	NA	NA
SS	10	SW	7	-0.210	-0.143	-0.227	0.107	0.087	0.593	NA	NA
NaPO4	1	SW	7	-0.763	0.097	-0.080	0.130	0.710	1.393	NA	NA
ZnPO4	1	SW	7	-0.280	-0.003	0.000	-0.157	0.127	-0.333	NA	NA
			AVG	-0.308	-0.007	0.040	0.010	0.115	0.093	NA	NA
			SD	0.340	0.565	0.423	0.352	0.820	0.822	NA	NA
SS	10	SW	3	0.284	0.069	0.110	0.252	0.037	-0.564	0.064	0.078
NaPO4	1	SW	3	0.554	0.527	0.484	1.082	0.970	-0.292	0.559	0.810
ZnPO4	1	SW	3	0.771	0.979	1.080	1.093	1.449	0.858	0.713	1.369
SS	10	SW	7	0.439	0.124	0.420	0.308	0.062	0.386	0.161	0.270
NaPO4	1	SW	7	0.292	0.269	0.442	0.172	0.216	0.332	0.179	0.268
ZnPO4	1	SW	7	0.663	0.774	0.857	0.722	1.069	0.572	0.950	1.290
			AVG	0.501	0.457	0.565	0.605	0.634	0.215	0.438	0.681
			SD	0.291	0.475	0.457	0.542	0.629	0.569	0.363	0.570
SS	100	SW	3	0.425	0.261	0.296	1.719	0.732	-0.713	0.704	0.800
NaPO4	10	SW	3	0.585	0.706	0.481	0.483	1.087	-0.016	0.619	1.000
ZnPO4	10	SW	3	0.697	0.383	0.133	0.683	0.999	0.593	0.927	1.429
SS	100	SW	7	0.654	0.462	0.727	1.667	0.486	0.235	0.923	1.243
NaPO4	10	SW	7	0.020	0.126	0.458	-0.013	0.214	-0.167	0.412	0.438
ZnPO4	10	SW	7	0.962	0.958	0.979	1.222	1.256	1.535	0.956	2.000
			AVG	0.557	0.483	0.512	0.960	0.796	0.245	0.757	1.152
			SD	0.468	0.453	0.345	0.684	0.421	0.787	0.351	0.602

**Notes:** NA = not analyzed, SW = surface water, SS = sodium silicate, ZnPO4 = zinc orthophosphate, NaPO4 = sodium orthophosphate, mg/L = milligrams per liter, AVG = average of absolute abundance measurements, SD = standard deviation of absolute abundance measurements. Yellow highlight indicates any change in relative abundance that was greater than the observed standard deviation. Bold values indicate each result that was statistically different compared to the untreated control (all p values < 0.05).

**Table 4G.** Summary of differences of absolute abundance (Log10 scale) of total bacterial biomass (16S rRNA), antibiotic resistance genes (ARGs), and metal resistance genes (MRGs) measurements obtained from qPCR. Values represent the difference between the average (n=3) absolute abundance of untreated controls and the average absolute abundance of treatment reactors for each condition tested (n=3).

				Average Log <sub>10</sub> Difference in Absolute Abundance for Target Genes with qPCR (Treatment - Control)								
Type	Conc. (mg/L)	Source Water	Day	16S rRNA	<i>blaTE M</i>	<i>czcC</i>	<i>czcD</i>	<i>copA</i>	<i>int1</i>	<i>su1</i>	<i>su2</i>	<i>qacEΔ1</i>
SS	10	SW	3	0.050	-0.028	0.385	0.160	0.000	-0.264	-0.007	-0.52	-0.206
NaPO4	1	SW	3	<b>0.541</b>	0.448	0.278	<b>0.429</b>	-0.045	0.279	0.13	0	0.274
ZnPO4	1	SW	3	<b>0.528</b>	<b>0.306</b>	0.312	<b>0.397</b>	-0.121	<b>0.414</b>	0.167	-0.867	0.355
SS	10	SW	7	0.386	-0.147	0.066	0.166	0.229	0.381	-0.083	-0.391	0.425
NaPO4	1	SW	7	0.226	0.051	-0.203	-0.104	0.029	-0.024	-0.353	-0.396	0.017
ZnPO4	1	SW	7	0.122	0.017	-0.038	-0.034	0.000	<b>0.357</b>	-0.412	0	<b>0.426</b>
			AVG	0.309	0.081	0.088	0.169	0.002	0.190	-0.078	-0.479	0.215
			SD	0.263	0.280	0.234	0.256	0.192	0.357	0.266	0.269	0.345
SS	10	SW	3	0.248	-0.021	0.185	0.220	0.125	0.091	0.125	-0.077	0.226
NaPO4	1	SW	3	0.256	-0.349	0.040	0.109	-0.103	0.178	0.292	0.144	0.193
ZnPO4	1	SW	3	0.120	-0.216	-0.152	-0.142	-0.172	<b>0.655</b>	<b>1.083</b>	0.224	<b>1.198</b>
SS	10	SW	7	0.242	0.288	0.158	0.193	0.076	0.179	0.087	-0.045	0.303
NaPO4	1	SW	7	0.277	0.085	0.104	0.194	0.129	0.216	0.484	-0.077	0.381
ZnPO4	1	SW	7	0.468	0.063	0.509	0.490	0.334	<b>1.185</b>	<b>0.889</b>	<b>0.817</b>	<b>1.452</b>
			AVG	0.268	-0.005	0.141	0.177	0.065	0.417	0.493	0.134	0.626
			SD	0.195	0.247	0.328	0.298	0.225	0.445	0.456	0.371	0.588
SS	100	SW	3	-1.063	<b>-0.464</b>	<b>-0.650</b>	-0.265	<b>-0.795</b>	<b>-0.523</b>	<b>-0.969</b>	<b>-0.636</b>	<b>-0.612</b>
NaPO4	10	SW	3	-0.012	<b>-0.218</b>	0.062	0.087	<b>-0.330</b>	-0.008	0.079	-0.143	-0.136
ZnPO4	10	SW	3	0.182	-0.195	0.138	0.023	<b>-0.329</b>	<b>0.765</b>	<b>1.182</b>	<b>0.555</b>	<b>1.224</b>
SS	100	SW	7	<b>-1.502</b>	<b>-0.767</b>	<b>-0.856</b>	<b>-0.648</b>	-0.099	<b>-0.675</b>	<b>-0.92</b>	<b>-0.206</b>	<b>-0.771</b>
NaPO4	10	SW	7	0.089	<b>-0.420</b>	-0.129	0.040	0.252	0.155	0.478	0.120	0.005
ZnPO4	10	SW	7	0.506	-0.359	-0.170	-0.002	0.267	<b>1.493</b>	<b>1.988</b>	<b>1.005</b>	<b>1.829</b>
			AVG	-0.300	-0.404	-0.268	-0.127	-0.172	0.201	0.306	-0.041	0.256
			SD	0.749	0.218	0.407	0.274	0.401	0.772	1.108	0.594	0.991

**Notes:** SW = surface water, SS = sodium silicate, ZnPO4 = zinc orthophosphate, NaPO4 = sodium orthophosphate, mg/L = milligrams per liter, AVG = average of absolute abundance measurements, SD = standard deviation of absolute abundance measurements. Yellow highlight indicates any change in relative abundance that was greater than the observed standard deviation. Bold values indicate each result that was statistically different compared to the untreated control (all p values < 0.05).

## Appendix 5A. qPCR Methodology

**Table 5A.** Primers, annealing temperatures, amplification efficiencies, and  $R^2$  values for qPCR analysis of target genes.

Gene	Annea l Temp. (°C)	Forward Primer & Reverse Primer	Size (bp)	Efficiency Average (%)	$R^2$	Reference
16S rRNA	60	F- (5'-CCTACGGGAGGCAGCAG-3') R- (5'-ATTACCGGGCTGCTGG-3')	202	103%	0.99-1.0	(Muyzer et al. 1993)
<i>int1</i>	60	F- (5'-CCTCCCGCACGATGATC-3') R- (5'-TCCACGCATCGTCAGGC-3')	280	95%	0.98-1.0	(Goldstein et al. 2001)
<i>qacEΔ1</i>	60	F- (5'-CCCCTTCCGCCGTTGT-3') R- (5'-CGACCAGACTGCATAAGCAACA-3')	101	96%	0.96-1.0	(Zhu et al. 2013)

## Appendix 5B. Day 0 Water Quality

**Table 5B.** Day 0 Water Quality Observations

Experiment Set:	Set 1	Set 2	Set 3
Sample Location	Beach	Beach	Beach
Description	Day 0	Day 0	Day 0
pH	8.22	8.12	8.14
Temp (°F)	42	68	66
Silica (mg/L)	2	2	2
H <sub>2</sub> S (mg/L)	<0.1	<0.1	<0.1
Chloride (mg/L)	30	20	20
DO (mg/L)	15	8	14
Ammonia (mg/L)	0.1	0.1	0.1
Calcium Hardness (mg/L)	80	100	100
Total Hardness (mg/L)	160	180	180
Total Alkalinity (mg/L)	120	160	100
Phenolphthalein Alkalinity (mg/L)	0	0	0
Orthophosphate (mg/L)	0.20	0.10	0.11
DOC (mg/L)	2.11	2.54	2.30

**Notes:** DOC = dissolved organic carbon, DO = dissolved oxygen, mg/L = milligrams per liter

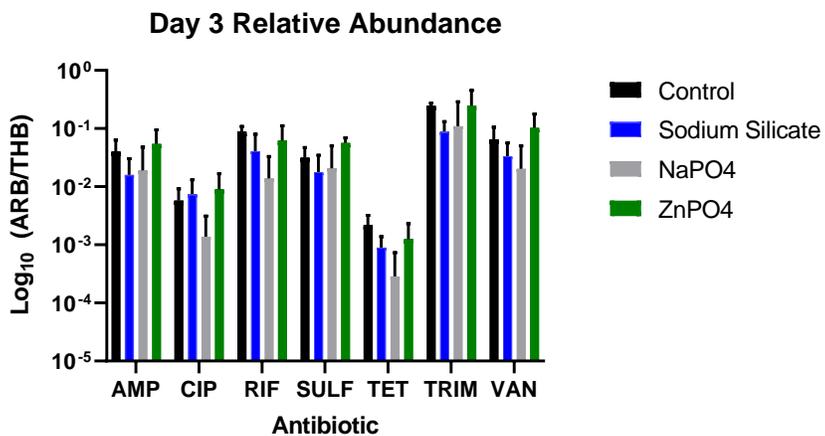
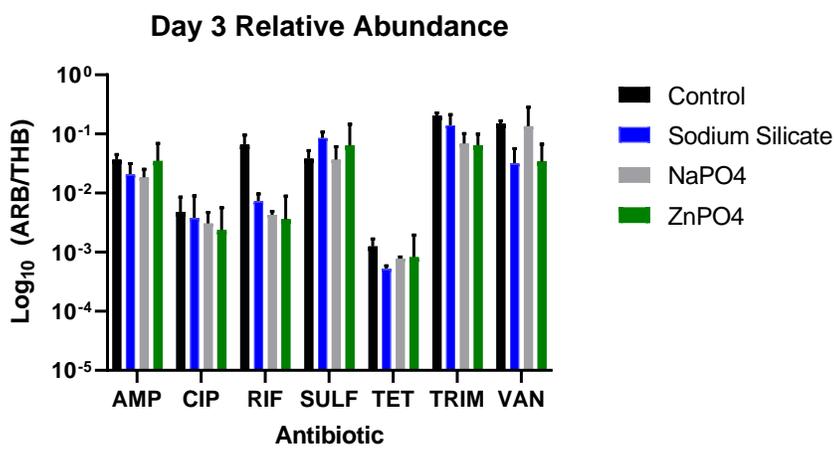
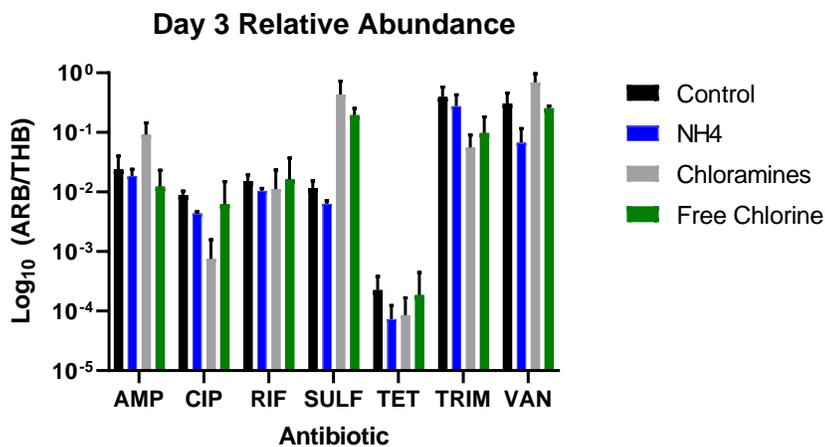
### Appendix 5C. Days 3 and 7 Water Quality

**Table 5C.** Days 3 and 7 Water Quality Observations

Sample Name	Day	pH	Total/Free Chlorine (mg/L as Cl)	PO <sub>4</sub> (mg/L)	Silica (mg/L)	DOC (mg/L)
Control (Set 1)	3	8.23 - 8.30	ND / ND	NA	NA	1.83
Ammonia	3	8.20 - 8.31	ND / ND	NA	NA	1.78
Chloramine	3	8.20 - 8.36	0.9 / 0.3	NA	NA	1.93
Free Chlorine	3	8.27 - 8.35	0.2 / 0.1	NA	NA	2.10
Control (Set 1)	7	8.19 - 8.28	ND / ND	NA	NA	1.70
Ammonia	7	8.14 - 8.25	ND / ND	NA	NA	1.75
Chloramine	7	8.28 - 8.33	0.8 / 0.3	NA	NA	1.91
Free Chlorine	7	8.22 - 8.26	0.2 / ND	NA	NA	2.09
Control (Set 2)	3	8.15 - 8.16	ND / ND	0.03	2	2.33
SS + Free Cl	3	7.92 - 7.96	0.3 / ND	0.03	12	3.03
NaPO <sub>4</sub> + Free Cl	3	8.04 - 8.06	0.25 / ND	1.05	2	3.33
ZnPO <sub>4</sub> + Free Cl	3	7.95 - 7.98	0.25 / ND	0.95	2	3.33
Control (Set 2)	7	8.10 - 8.15	ND / ND	0.04	2	2.30
SS + Free Cl	7	7.94 - 7.98	0.1 / ND	0.04	12	2.68
NaPO <sub>4</sub> + Free Cl	7	8.15 - 8.18	0.15 / ND	1.05	2	2.54
ZnPO <sub>4</sub> + Free Cl	7	8.15 - 8.21	0.1 / ND	0.90	2	2.51
Control - Set 3	3	7.80-7.93	NA	0.08	2	2.02
SS + NOM	3	8.16-8.26	NA	0.16	12	5.99
NaPO <sub>4</sub> + NOM	3	7.65-7.97	NA	1.01	2	5.84
ZnPO <sub>4</sub> + NOM	3	7.79-8.03	NA	1.01	2	6.07
Control (Set 3)	7	7.69-7.82	NA	0.2	2	1.96
SS + NOM	7	8.5-8.62	NA	0.2	12	5.78
NaPO <sub>4</sub> + NOM	7	8.06-8.2	NA	1.1	2	6.17
ZnPO <sub>4</sub> + NOM	7	7.98-8.15	NA	1.0	2	5.96

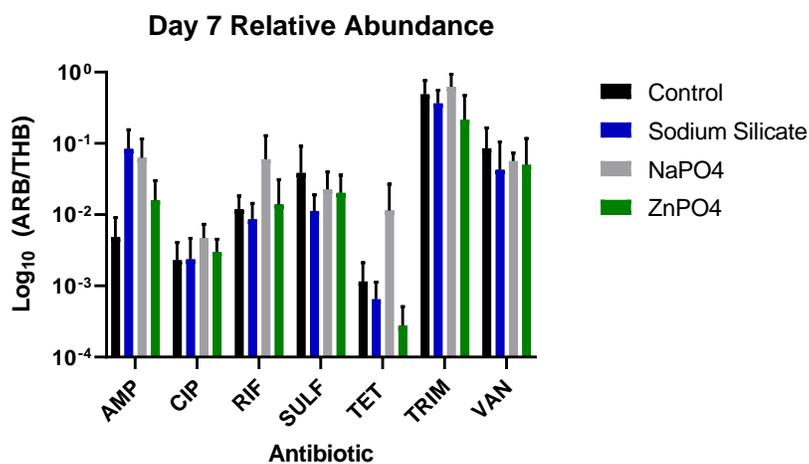
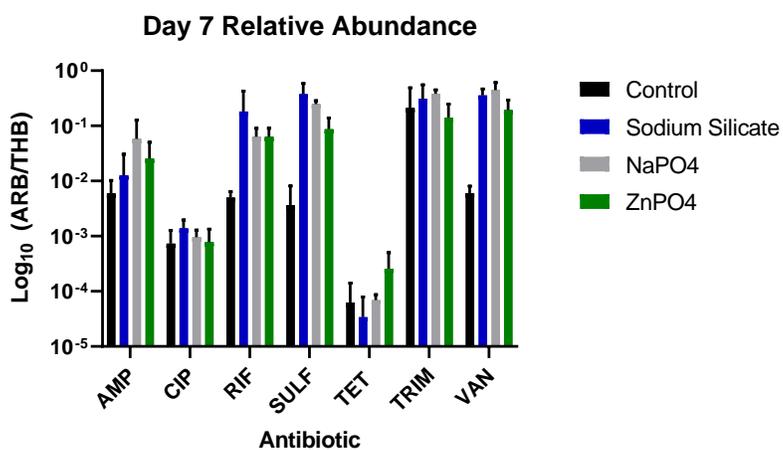
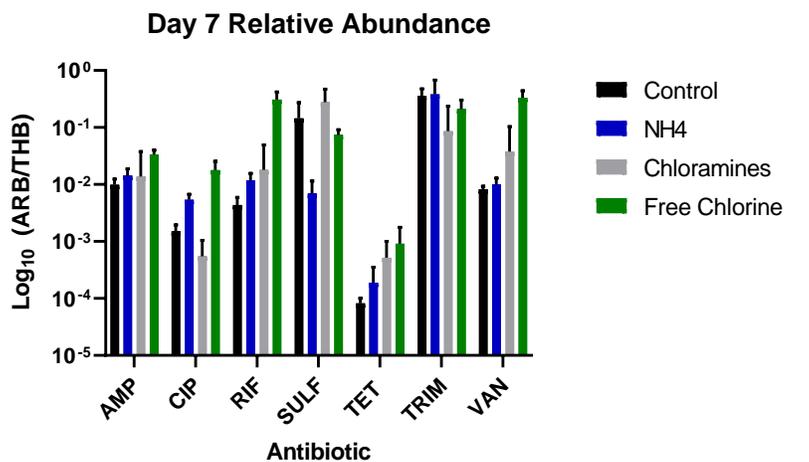
**Notes:** SS = sodium silicate, NaPO<sub>4</sub> = sodium orthophosphate, ZnPO<sub>4</sub> = zinc orthophosphate, Cl = chlorine, NOM = natural organic matter, mg/L = milligrams per liter, ND = not detected, NA = not applicable.

**Appendix 5D. Relative abundance Day 3 as determined by heterotrophic plate counts of antibiotic resistant bacteria**



**Figure 5D.** Relative abundance of heterotrophic plate counts observed on Day 3 for microcosm experiments. The average relative abundance of antibiotic resistant bacteria (ARB) based on direct plating of from microcosms containing different types and concentrations of corrosion inhibitors. The relative abundance of ARB is shown on the y-axis. The type of antibiotic is denoted on the x-axis. Corrosion inhibitor and disinfectant type is indicated by color. Different experimental conditions are plotted on different graphs including Set 1 – Disinfectants, (A - top), Set 2 – Corrosion Inhibitors + free chlorine (1.6 mg/L) (B - middle), and Set 3 – Corrosion Inhibitors + natural organic matter (NOM) (C - bottom).

### Appendix 5E. Relative abundance of ARB on Day 7 as determined by HPC



**Figure 5E.** Relative abundance of heterotrophic plate counts observed on Day 7 for microcosm experiments. The average relative abundance of antibiotic resistant bacteria (ARB) based on direct plating of from microcosms containing different types and concentrations of corrosion inhibitors. The relative abundance of ARB is shown on the y-axis. The type of antibiotic is denoted on the x-axis. Corrosion inhibitor and disinfectant type is indicated by color. Different experimental conditions are plotted on different graphs including Set 1 – Disinfectants, (A - top), Set 2 – Corrosion Inhibitors + free chlorine (1.6 mg/L) (B - middle), and Set 3 – Corrosion Inhibitors + natural organic matter (NOM) (C - bottom).

### Appendix 5F. Metagenomic sample information

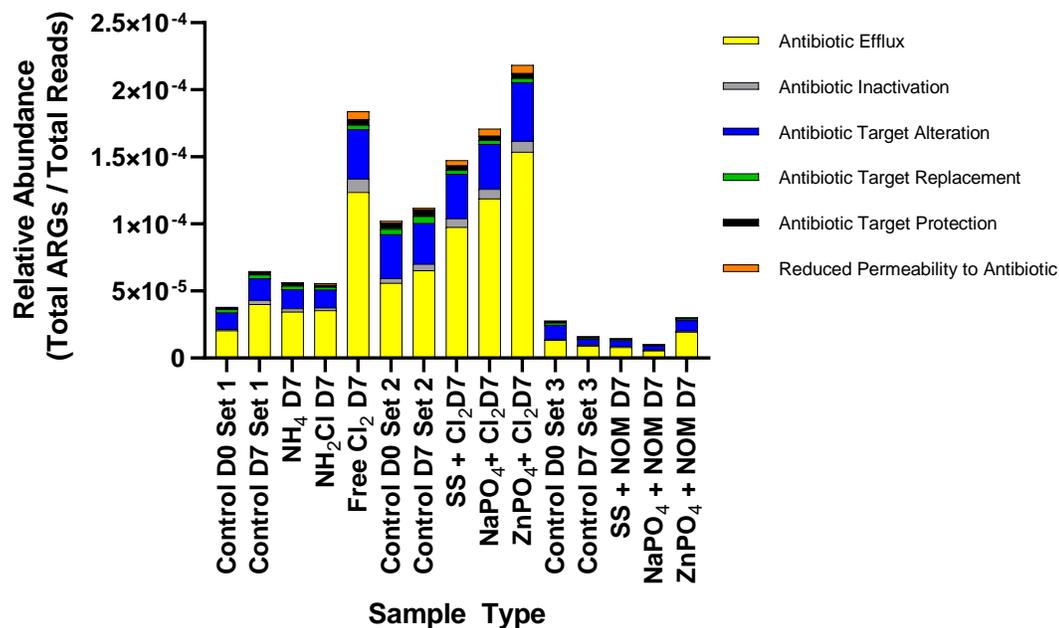
**Table 5F.** Sample information and results for samples submitted for shotgun metagenomic sequencing (n=45).

Sample Description	Number of Paired-End Reads	GC % of Trimmed reads	GC % of assembled contigs	# Contigs	N50
A1 D0 - set 1	16,297,536	50.5%	44.9%	4,196	4,840
A2 D0	16,468,630	50.7%	45.1%	4,185	5,117
A3 D0	16,590,212	51.2%	42.9%	2,376	4,060
A1 D7	16,569,164	56.1%	58.0%	5,471	5,507
A2 D7	15,794,908	56.2%	57.6%	6,636	3,764
A3 D7	17,915,522	56.4%	57.7%	6,974	4,901
NH4 D7	13,826,700	56.2%	57.0%	5,263	3,951
NH4 D7	9,413,722	57.7%	58.4%	2,242	3,652
NH4 D7	13,250,862	56.8%	57.0%	5,594	4,073
NH2CL D7	15,463,218	53.7%	52.0%	6,409	5,677
NH2CL D7	16,378,490	49.5%	43.9%	5,389	4,981
NH2CL D7	8,188,532	49.7%	42.1%	3,037	4,397
CL D7	11,526,512	58.8%	58.6%	4,133	17,082
CL D7	14,993,792	56.7%	57.5%	6,791	13,171
CL D7	17,398,916	57.6%	56.4%	6,890	14,453
A1 D0 Set 2	14,076,858	51.1%	46.3%	6,797	5,407
A2 D0	7,709,842	51.5%	45.5%	3,341	4,644
A3 D0	12,667,586	52.3%	47.6%	4,666	6,409
A1 D7	15,758,950	56.1%	53.3%	6,660	6,423
A2 D7	16,437,170	57.4%	54.8%	7,038	8,177
A3 D7	13,000,284	57.9%	54.5%	5,936	7,096
SS D7 + CL	14,775,600	59.7%	60.3%	3,984	32,488

Sample Description	Number of Paired-End Reads	GC % of Trimmed reads	GC % of assembled contigs	# Contigs	N50
SS D7 + CL	13,098,276	60.4%	62.1%	4,652	12,487
SS D7 + CL	12,227,176	60.1%	58.5%	1,894	85,091
NaPO4 D7 + CL	14,641,140	63.0%	63.6%	4,585	16,508
NaPO4 D7 + CL	12,877,932	62.1%	62.2%	5,098	16,250
NaPO4 D7 + CL	14,819,628	63.2%	63.5%	4,381	34,159
ZnPO4 D7 + CL	13,052,686	57.5%	64.0%	4,982	14,443
ZnPO4 D7 + CL	14,344,580	62.7%	62.7%	6,419	16,584
ZnPO4 D7 + CL	14,378,552	57.4%	62.1%	4,555	27,499
A1 D0 Set 3	11,468,748	53.9%	44.8%	1,431	4,908
A2 D0	16,185,190	51.4%	43.9%	1,678	4,856
A3 D0	12,264,656	53.7%	45.1%	1,471	4,194
A1 D7	12,065,530	56.7%	54.9%	1,627	3,112
A2 D7	14,475,294	56.4%	50.2%	1,853	3,295
A3 D7	12,175,818	57.1%	54.3%	1,256	2,711
SS D7 + NOM	13,519,950	57.5%	45.1%	1,009	4,014
SS D7 + NOM	12,658,720	57.7%	51.6%	1,437	3,118
SS D7 + NOM	14,482,078	57.4%	49.7%	1,228	3,377
NaPO4 D7 + NOM	14,984,032	57.5%	48.7%	1,264	3,535
NaPO4 D7 + NOM	11,434,986	57.5%	54.0%	988	3,098
NaPO4 D7 + NOM	12,538,658	58.2%	45.8%	724	4,420
ZnPO4 D7 + NOM	10,426,364	54.9%	53.9%	1,754	3,639
ZnPO4 D7 + NOM	10,017,648	55.2%	55.6%	2,212	3,924
ZnPO4 D7 + NOM	11,037,074	54.1%	53.8%	2,902	3,895

**Notes:** NH<sub>4</sub> = ammonia, NH<sub>2</sub>Cl = chloramine, Cl = chlorine, SS = sodium silicate, NaPO<sub>4</sub> = sodium orthophosphate, ZnPO<sub>4</sub> = zinc orthophosphate, NOM = natural organic matter.

### Appendix 5G. ARG relative abundance by resistance mechanism



**Figure 5G.** Relative abundance of ARGs classified by resistance mechanism as determined by CARD. ARG abundance was normalized to total metagenomic reads for each sample. ARGs are grouped by the mechanism which they confer resistance. The y-axis represents the mean relative abundance of ARGs classified for each sample type for triplicate samples. The x-axis represents the different experimental conditions. The sample collection day is denoted by D0 (day 0) and D7 (day 7).