THE INFLUENCE OF WASTEWATER TREATMENT ON COMMUNITY DYNAMICS AMONG ANTIBIOTIC-RESISTANT BACTERIA DURING TREATMENT, STREAM RELEASE, AND BIOSOLIDS LAND APPLICATION

by

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A dissertation submitted to the faculty of
The University of North Carolina at Charlotte
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
in Biological Sciences

Charlotte

2020

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ABSTRACT

ALICIA ANN SORGEN. The influence of wastewater treatment on community dynamics among antibiotic-resistant bacteria during treatment, stream release, and biosolids land application. (Under the direction of DR. MOLLY REDMOND)

Wastewater treatment plants (WWTP) are a key link between anthropogenic activity and the environment, facilitating the development and horizontal transfer of antibiotic resistance among populations of commensal environmental microorganisms and clinical pathogens. It is vital to understand the ecological factors associated with antibiotic-resistant microbes, encompassing their origins, evolution, selection, and dissemination, focusing on WWTPs as they are suspected to be major hotspots for the evolution and propagation of antibiotic resistance. Extensive research has been conducted on clinically relevant antibiotic-resistant pathogens; however, investigations into environmental reservoirs of antibiotic-resistant determinants and their influence on clinical situations have only been considered recently. By identifying sources of antibiotic-resistant bacteria (ARB), their environmental distribution, and how anthropogenic factors affect dissemination, we can institute approaches to contend with antibiotic resistance.

We have employed culture-dependent and -independent approaches to address the following aims: (1) determine changes in resistance levels, (2) evaluate the community structure in multiple environments when exposed to treated wastewater products, and (3) identify antibiotic resistance fitness costs to those with such phenotypes.

Within this investigation of four WWTPs located in Charlotte, North Carolina, we have demonstrated that the release of treated wastewater into the Charlotte watershed does not significantly increase ARB abundance within environmental compartments. The highest concentrations of ARB were found in raw sewage and the preliminary treatment stages. These concentrations were reduced considerably with biological aeration treatment, and the

final treated effluent resembled that of natural stream communities, indicating that the Charlotte Water WWTPs are effective in the removal of ARB and their associated resistance genes. Similar findings were observed with the application of the treated biosolids as fertilizers upon agricultural crop fields where the application of ARB-rich biosolid sources resulted only in an immediate increase in resistant communities, leveling off over time to pre-application levels. We also found that the acquisition of multiple antibiotic resistance genes does not necessarily confer a fitness disadvantage in those exhibiting multi-drug resistance. More resistant strains of *Enterococcus faecium,* strains harboring a greater number of resistance genes, showed no fitness costs to heat, cold, osmotic, acid, or oxidative stressors relative to less resistant strains.

We did, however, demonstrate that the culturable fractions of total environmental source populations are higher than previously speculated. On average, 12.8% of the total bacterial population was recovered using standard plating techniques, with some proportions greatly exceeding the widely accepted 1% culturability ideology. Though higher percentages of the microbial population were cultivable, the overall makeup of the cultured fractions varied considerably from that of the natural communities with culturing biases favoring *Gammaproteobacteria*. With the increasing threat of antibiotic resistance within healthcare settings, it is imperative to gain a better understanding of the phenotypic and ecological characteristics displayed by these resistant bacteria outside of these clinical settings.

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Molly Redmond, for providing me with opportunities out of my comfort zone in both microbiology and bioinformatics. The knowledge I've gained through my graduate studies is valuable and useful for current concerns in today's society. I would also like to thank my committee members, Dr's Anthony Fodor, Cynthia Gibas, Todd Steck, Matthew Parrow, and David Vinson, for their input and support in my research. I would still be crying over my computer if it weren't for James Johnson and Dr. Kevin Lambirth. You've helped me grow into the pseudo-bioinformatician that I am today. I would also like to thank my fellow graduate students for keeping me sane by enjoying life and science in a healthy balance.

To my Chi Omega sisters, you've watched me struggle through so many biological fields so that I could find my passion in microbial ecology while cheering me on the whole way. John, you've been my rock through some of my toughest moments, and I always come out smiling because of you. You've supported me in every way possible and been my every source of comfort. I am forever grateful to my family for their support and unwavering belief in me and my goals. I've grown into the determined woman I am today because you never let me believe I couldn't achieve my goals.

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ADI	Arginine deiminase
AgDI	Agmatine deiminase
Ahp	Alkyl hydroperoxidase
AME	Aminoglycoside modifying enzyme
ANOVA	Analysis of variance
ARB	Antibiotic-resistant bacteria
ARG	Antibiotic resistance gene
ATE	Aeration tank effluent
BIGSdb	Bacterial Isolate Genome Sequence Database
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BT	Body temperature
CAP	Constrained Analysis of Principal Coordinates
CARD	Comprehensive Antibiotic Resistance Database
CDC	Centers for Disease Control and Prevention
CDS	Protein-coding sequence
CFU	Colony forming unit
CL	Clinical
CLSI	Clinical and Laboratory Standards Institute
CSP	Cold shock protein
DI	Deionized
DS	Downstream
DUF	Domain of unknown function
EPA	Environmental Protection Agency
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FCE	Final clarifier effluent
FDR	False discovery rate
HGT	Horizontal gene transfer
HOS	Hospital sewage
HPC	Heterotrophic plate count

HSP	Heat shock protein
INF	Influent
kb	Kilobase
LB	Lysogeny broth
LSAP	Lincosamide-streptogramin A-pleuromutilin
MAFFT	Multiple alignment using fast Fourier transform
MCL	Markov cluster
MDR	Multi-drug resistance
MetSO	Methionine sulfoxide
MFS	Major facilitator superfamily
MGD	Million gallons daily
MGE	Mobile genetic element
MH	Mueller-Hinton
MIC	Minimum inhibitory concentration
MLS	Macrolide-lincosamide-streptogramin
MLST	Multi-locus sequence typing
NC	Nonclinical
NCBI	National Center for Biotechnology Information
Npr	NADH peroxidase
Ohr	Organic hydroperoxidase resistance
ORF	Open reading frame
OTU	Operational taxonomic unit
PCE	Primary clarifier effluent
PCI	Primary clarifier influent
PCoA	Principal coordinate analysis
PERMANOVA	Permutational multivariate analysis of variance
PMQR	Plasmid-mediated quinolone resistance
QIIME	Qualitative Insights Into Microbial Ecology
R2A	Reasoner's 2 Agar
RES	Residential sewage
RGI	Resistance Gene Finder
rMLST	Ribosomal multi-locus sequence typing

ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
SDI	Shannon diversity index
TDC	Tyrosine decarboxylase
UP	Upstream
UV	Ultraviolet
VF	Virulence factor
WWTP	Wastewater treatment plant
ZOI	Zone of inhibition

CHAPTER 1: Introduction

1.1 Antibiotics

Antimicrobials are compounds with the ability to kill or inhibit the growth of a variety of microorganisms, including bacteria, fungi, viruses, and protozoa (Grenni *et al.*, 2018). Antibiotics are a class of antimicrobials that specifically act on bacteria or fungi in a host organism for the improvement of human, animal, and plant health. Antibiotics can have bactericidal (having the ability to kill) or bacteriostatic (inhibiting growth and replication) properties and may occur naturally or through synthetic and semi-synthetic production. These compounds have been naturally produced by environmental microbiota for billions of years, yet the study and usage of antibiotics have been heavily concentrated in a clinical point of view. At low molecular weights and active at low concentrations, these organic compounds have revolutionized the field of medicine by treating and preventing infections caused by pathogenic bacteria, saving countless lives, and improving quality of life (Bouki, Venieri, & Diamadopoulos, 2013; Martens & Demain, 2017).

Upon the marketing of the first antibiotic in the United States, penicillin G, in 1943, the health benefits of antibiotics resulted in the "antibiotic era" (Aminov, 2010). During this era, many novel antibiotics were used for the treatment of bacterial infections (Aminov, 2010). According to the Centers for Disease Control (CDC), in 2015 alone, ~269 million antibiotic prescriptions were distributed from outpatient facilities in the US alone (CDC, 2017); and this annual rate continues to rise (Blaser, 2016). These antibiotics can be administered topically for the treatment of skin infections, orally for the treatment of most mild to moderate infections, and intravenously in the case of more severe infections.

Upon the discovery of the medical benefits to humans, antibiotics have been implemented extensively for agricultural purposes as well (Rizzo *et al.*, 2013). Antibiotics are

administered to livestock to treat common veterinary infections such as mastitis, skin abscesses, and enteric and pulmonary infections (Teuber, 2001). These drugs are also administered to livestock through their feed in the absence of clinical infections to get the animal to market faster (Kumar *et al.*, 2005). The use of antibiotics as feed additives helps to reduce the microbial population, limiting the production of growth-suppressing microbial metabolites and lessening the microbial uptake of nutrients, thus enhancing nutrient uptake through the intestinal walls of the antibiotic-fed animal (Kumar *et al.*, 2005). The use of antibiotic feed additives can also offset the effects of crowded living conditions and poor hygiene. Antibiotics can also be applied to crop fields as a biocide (Vidaver, 2002).

Therapeutic antibiotics can exploit the comparative differences between prokaryotic and eukaryotic biochemical machinery (Rizzo *et al.*, 2013). Certain classes of antibiotics are used to inhibit bacterial cell-wall biosynthesis, protein synthesis, or DNA replication and repair (Roose-Amsaleg and Laverman, 2016). Drug classes such as β-lactams and glycopeptides target enzymes and substrates required for the synthesis of the cell wall, thereby weakening it and predisposing the cell to lysis (Walsh, 2000; Kapoor *et al.*, 2017). Tetracyclines, lincosamides, and macrolides target subunits of the bacterial ribosome preventing the initiation, elongation, or termination of protein assembly, thus inhibiting the growth and reproduction of the cell (Gaca and Lemos, 2019). Fluoroquinolones and nitrofurans inhibit the cell's DNA replication and repair mechanisms, causing an accumulation of DNA break sites resulting in cell death (Kapoor *et al.*, 2017).

1.2 Antibiotic resistance

Being that antibiotic production is a natural phenomenon, it is only natural that bacteria have also evolved and maintained genes enabling them to resist the effects of such

compounds. For an antibiotic to be effective against a microbe, it must reach the intracellular bacterial target and accumulate to concentrations able to act upon the cell (Walsh, 2000). One such mechanism of resistance includes the overproduction of existing protein pumps to export the drug from the cell (Kumar *et al.*, 2005). These efflux pumps, which are simply variants of existing membrane pumps possessed by the bacterium, can rid the cell of the drug faster than it can accumulate, keeping cellular concentrations low and ineffective (Walsh, 2000). Similar minor structural variations of target proteins can also render the drug ineffective. Variations in the target-encoding genes can modify the structure and function of the gene product such that the target cannot bind with the drug or perform its normal functions while bound (Walsh, 2000; Kapoor *et al.*, 2017). Such alterations can occur in ribosomal subunits, cell wall precursors and transpeptidases, and DNA replication and transcription machinery (Kim *et al.*, 2002; Lambert, 2002; Tenover, 2006; Kos *et al.*, 2008). Resistance can also arise from the inactivation of the antibiotic through modifications in the drug compound by bacterial enzymes (Tolmasky, 2000; Alekshun and Levy, 2007; Strateva and Yordanov, 2009).

1.3 The threat of antibiotic resistance

Despite the natural, environmental development of antibiotic resistance, the increase in antibiotic administration over the past 50 years has imposed selective pressures on susceptible bacteria, favoring the survival of antibiotic-resistant bacteria (ARB) and the proliferation of associated antibiotic resistance genes (ARG) furthering resistance development (Novo and Manaia, 2010; Bell *et al.*, 2014). According to the CDC, 2 million Americans are infected with ARB, resulting in 23,000 deaths annually (CDC, 2017). Causes of this crisis have been attributed to the inappropriate and overuse of prescription antibiotics

and extensive agricultural use, as well as regulatory barriers and the lack of new drug developments (Ventola, 2015). The CDC estimates that 30% of all antibiotics prescribed in outpatient facilities are unnecessary and most of these prescriptions are for respiratory infections of viral origin, which are unaffected by antibiotics, or for bacterial infections that do not necessarily require antibiotics for treatment, such as many sinus and ear infections (CDC, 2017). Due to a large number of bacteria in an infection, rapid generation times, and inherent mutation rates, even one resistance mutation can allow the bacterium to grow in the space vacated by dead, susceptible bacteria becoming the dominant strain in the population (Walsh, 2000). The increased selection for resistant strains has led to the "antibiotic resistance crisis," where the therapeutic effects of these prescribed medications are diminishing (Ventola, 2015).

1.4 Dissemination of Antibiotic Resistance Genes

Some bacterial species are inherently resistant to certain antibiotics as a result of structural or functional properties (Blair *et al.*, 2015). Those not inherently resistant can acquire resistance through mutations in chromosomal genes and via horizontal gene transfer (HGT) (Blair *et al.*, 2015). Advantageous mutations, such as antibiotic resistance, are kept and maintained within the genome for future generations. This vertical transfer of ARGs is mediated by the clonal spread of a particular resistant strain (Mathur and Singh, 2005). Many of these ARGs are known to occur on mobile elements, such as plasmids, transposons, and integrons, which can be easily transferred between bacteria via HGT (Allen *et al.*, 2010; Huerta *et al.*, 2013). These mobile genetic elements (MGE) can be efficiently transferred between organisms regardless of their level of genetic relatedness (Domingues *et al.*, 2012).

Antibiotics administered to both humans and animals are often only partially metabolized (Kümmerer and Henninger, 2003), resulting in a large portion of the antibiotic and its metabolites being released into the environment through human waste and manure (Gao *et al.*, 2012; Sharma *et al.*, 2016). The release of these compounds has been regarded as a crucial factor in the evolution and selection of antibiotic resistance (Allen *et al.*, 2010). As the environment is already a natural source of ARGs from both commensal and pathogenic bacteria (D'Costa *et al.*, 2011; Vaz-Moreira *et al.*, 2014), the release of contaminant antibiotics, resistant bacteria, and ARGs further increases the accumulation and spread of antibiotic resistance across different niches (Rizzo *et al.*, 2013; Vaz-Moreira *et al.*, 2014). The horizontal transmission of resistance genes is thought to be the most important mechanism in the acquisition and spread of ARGs (Barbosa and Levy, 2000).

Bacteria are naturally capable of the uptake, incorporation, and expression of extracellular DNA fragments through transformation (von Wintersdorff *et al.*, 2016). For transformation to occur, there must be naked DNA in the extracellular environment, a state of competence in the recipient organism, and integration of the acquired DNA fragment into the recipient genome (Thomas and Nielsen, 2005). Competence is regarded as a transient "window of opportunity" for DNA internalization and is a regulated property that varies widely among bacterial species (Johnston *et al.*, 2014). The induction of competence enables the cell to respond to environmental triggers and can be induced in response to stressors that threaten cell survival, such as sublethal concentrations of antibiotics (Charpentier *et al.*, 2011, 2012). Studies have shown that exposure to antibiotics can promote a state competence in many species of bacteria, which can then use transformation to acquire ARGs; thus, antibiotics could not only select for resistant strains but also encourage the dissemination of their ARGs (Prudhomme *et al.*, 2006). As the process of transformation

is entirely conducted by the recipient cell, it provides an immense capacity for the dissemination of resistance determinants between different species (von Wintersdorff *et al.*, 2016).

Bacteriophages also contribute to the horizontal transfer of ARGs via transduction, promoting both bacteriophage survival and resistance dissemination (Modi *et al.*, 2013). Transmissible DNA sequences can include chromosomal DNA and MGEs and, once the mobilized DNA reaches the recipient, can be integrated into the host genome for the expression of resistant phenotypes (Brown-Jaque *et al.*, 2015).

Conjugation is the transfer of chromosomal or extra-chromosomal DNA via a multi-step process involving direct cell-to-cell contact through surface pili or adhesins (von Wintersdorff *et al.*, 2016). In many cases, ARGs are associated with MGEs, including plasmids, transposons, or genomic islands, and though the transfer of these MGEs can also occur via transformation and transduction, conjugation is the most likely mode of such genetic transfer (von Wintersdorff *et al.*, 2016). The process of conjugation protects the transmissible DNA from the extracellular environment and offers a more efficient method of entrance into the recipient than transformation while offering a broader host range than transduction (von Wintersdorff *et al.*, 2016). Conjugation is also a process designated for the transfer of bacterial genes, while transduction is merely an aftereffect of mistaken bacteriophage replication (Anders *et al.*, 2009). The conjugative transfer of MGEs conferring resistance has been detected in many ecological settings, with transfer between commensal bacteria found in the environment to pathogens in healthcare and food (Davison, 1999). Conjugation contributes significantly to resistance dissemination between these different reservoirs due to the broad host range of MGEs. The well-studied dissemination of resistance in human pathogens shows that once ARGs are successfully established in

plasmids, they can spread quickly across differing taxa (von Wintersdorff *et al.*, 2016). As several ARGs can be co-localized on the same plasmid, the spread of multi-drug resistance (MDR) becomes relatively easy.

1.5 Wastewater treatment plants

Wastewater treatment plants (WWTP) are major sewage repositories for many residential and medical treatment facilities, in which the continuous inflow of pre-existing ARB and antibiotic residues are important sources of resistance material (Gao *et al.*, 2012; Bouki *et al.*, 2013). The entry of antibiotics into WWTPs occurs through human and farm animal excretion, and the direct disposal of medical and industrial wastes (Bouki *et al.*, 2013). The wastewater treatment process commonly involves three stages (Bouki *et al.*, 2013). Solids are removed through physical processes in primary treatment, where the wastewater flows through a screen to filter out large solids into a grit chamber (EPA, 1998). After the initial screening is complete, the wastewater enters a sedimentation tank where remaining suspended solids sink to form a mass known as biosolids or sludge. Biological and chemical processes are employed to remove organic material during secondary treatment (Bouki *et al.*, 2013). Here, an activated sludge process is performed in aeration tanks by bringing in air and sludge densely populated with bacteria, speeding up the bacterial breakdown of organic matter into harmless by-products (EPA, 1998). In tertiary treatment, additional operations are involved in removing any components not reduced during secondary treatment, such as nutrients, toxic materials, and other organic materials (Bouki *et al.*, 2013). Before environmental release, wastewater undergoes a disinfection stage to eliminate harmful bacteria in the effluent. Commonly applied disinfection methods include chlorine, ultraviolet (UV) light, and ozone treatment. However, the UV doses typically administered in

wastewater disinfection do not entirely destroy the resistance genes in the bacteria, resulting in free ARGs in the extracellular environment with the potential for uptake by new bacteria (Destiani *et al.*, 2018). Some antibiotics can be removed via degradation and sorption to biosolids during the secondary and tertiary stages of treatment (Barbosa and Levy, 2008). However, not all antibiotics can be entirely degraded, resulting in environmental release at sub-inhibitory concentrations (Giger *et al.*, 2003).

1.6 Wastewater treatment plants in resistance dissemination

Considered a major route by which ARGs and ARB reach natural ecosystems, WWTPs are considered "hotspots" for resistance dissemination (Novo and Manaia, 2010). Three main arguments have been proposed to support the concept of WWTPs being integral in the spread of resistance. The first being that substances with the potential to select for resistance, be it antibiotic residues, ARGs, or ARB, are heavily released into municipal sewage systems (Kümmerer, 2009; Novo and Manaia, 2010; Novo *et al.*, 2013). The second argument is that WWTP conditions are favorable to microorganisms and may promote the selection and transfer of antibiotic resistance genes (Zhang *et al.*, 2011; Novo *et al.*, 2013). An abundance of nutrients and micropollutants, specifically sub-inhibitory concentrations of antibiotics and heavy metals, are continuously mixed, promoting the development and spread of resistance (Martinez, 2009). The final argument is based on the observation that, worldwide, WWTPs lead to the production of final effluents containing ARB (Novo *et al.*, 2013). Environmental contamination by either ARB or by ARGs can have an impact on the community structure and function of environmental bacterial populations (Martinez, 2009). The release of antibiotics with human-associated microbial flora into settings already enriched with resistance elements enhances the possibility for the development of novel

resistance determinants (Martinez, 2009; Zhang *et al.*, 2009; Bouki *et al.*, 2013). These determinants can, in turn, be transferred from the environment back into human-associated bacteria via water, crops sprayed with treated surface water, or fertilized with biosolids making these ARGs clinically relevant (Kümmerer, 2009; Bouki *et al.*, 2013).

1.7 Concluding remarks

Despite the observation that treated wastewater contains ARB, a general observation in literature is that the treatment process does lead to a considerable reduction in the bacterial load compared to untreated wastewater, including total numbers of ARB. However, sufficient inactivation of ARB does not eliminate all biological resistance determinants, such as ARGs and antibiotic residues, from the treated wastewater (Pruden *et al.*, 2006; Yuan *et al.*, 2015). Even with ARB removal, intact fragments of DNA remain within the cell debris and have been observed to persist long after their hosts are removed, and could potentially transfer into new hosts (Pruden *et al.*, 2006; Dodd, 2012; Yuan *et al.*, 2015; Manaia *et al.*, 2016). Many studies have demonstrated that the removal of antibiotics and their resistance determinants are incomplete in WWTPs and that they will eventually enter natural environments through the discharge of treated wastewater into aquatic ecosystems or the application of treated biosolids on agricultural land (Gao *et al.*, 2012).

CHAPTER 2: Characterization of environmental and cultivable antibiotic-resistant microbial communities associated with wastewater treatment under differing culturing conditions

2.1 Rationale

Antibiotics have revolutionized the field of medicine, but the improper and overuse of these compounds have exerted selective pressures on susceptible bacteria resulting in antibiotic resistance and the spread of antibiotic resistance genes (ARG) (Li *et al.*, 2015). Wastewater treatment plants (WWTP) are considered "hotspots" for resistance dissemination due to the sub-inhibitory concentrations of antibiotic compounds and favorable conditions that promote horizontal gene transfer (HGT). WWTPs may receive sewage from residential and medical treatment facilities, and the continuous inflow of pre-existing antibiotic-resistant bacteria (ARB) and antibiotic residues are important sources of resistance material (Gao *et al.*, 2012; Bouki *et al.*, 2013). Due to the increasing concern associated with antibiotic resistance, especially within healthcare settings, insight into ARB community trends throughout the wastewater treatment process can help us to understand the effect of the released treated wastewater on natural microbial communities.

The wastewater treatment process generally involves three stages of treatment where (1) large solids are removed through physical processes before (2) entering a sedimentation tank in which remaining suspended solids sink to form activated sludge and where biological and chemical processes are employed to remove organic matter (Bouki *et al.*, 2013). Any additional organic or chemical constituents are removed using (3) additional treatment processes (Guardabassi *et al.*, 2002). Prior to environmental release, wastewater undergoes a disinfection stage to eliminate harmful bacteria in the effluent (Destiani *et al.*, 2018).

Areas with limited water treatment infrastructure have been shown to pass human microbial resistomes into the environment via mobile genetic elements (MGE), such as

plasmids, transposons, and integrons (Pehrsson *et al.*, 2016; von Wintersdorff *et al.*, 2016). Even in locations with innovative wastewater treatment systems, the degree of contaminant removal depends on the particular technology and operating parameters used, whether it be ultraviolet (UV), chlorine, or microfiltration disinfection methods (Cydzik-Kwiatkowska and Zielińska, 2016).

In this study, we sampled sites along WWTP-associated urban streams. We sampled stream locations upstream and downstream of the WWTP treated water release point to understand the overall impact of treated water release on the stream microbial community. Inside the WWTP, we sampled untreated sewage influent as well as the effluents of each stage of the treatment process, to identify which taxa are introduced to the WWTP in influent and whether they are subsequently removed. Samples pulled at each site within the stream environment and within the WWTP were characterized using 16S ribosomal RNA (rRNA) amplicon sequencing (Lambirth *et al.*, 2018). In parallel, each sample was cultured, and heterotrophic plate count (HPC) methods were used to isolate antibiotic-resistant subpopulations from treated wastewater and stream water samples. We then used 16S rRNA amplicon sequencing to characterize these resistant subpopulations and compare them to the source microbial communities. This experiment provides a window into potential antibiotic resistance that cannot be achieved by simply screening whole-microbiome sequencing data for resistant organisms or resistance signatures.

HPC methods are often employed to culture and represent subpopulations of environmental samples, and as all commensals and opportunistic pathogens are heterotrophic, HPC methods are often performed to assess water quality (Allen *et al.*, 2004). Such an approach allows us to investigate phenotypic variations in subpopulations, including antibiotic resistance patterns. However, as described by "the great plate count anomaly," not

all heterotrophic microbial populations can be easily cultivated from environmental samples (Staley and Allan Konopka, 1985). Microbial communities are a consortium of many organisms, each requiring specific conditions for survival and replication, which cannot yet be replicated under laboratory conditions (Joint *et al.*, 2010; Rygaard *et al.*, 2017). Aside from the physical niche required for an organism's growth, it has been shown that some bacteria cannot grow unless their syntrophic counterparts are present for the exchange of essential growth factors (Nichols *et al.*, 2008; D'Onofrio *et al.*, 2010; Epstein, 2013).

The use of culture-independent techniques, such as those based on 16S rRNA gene sequencing, allows us to identify a large portion of the microbial population that cannot be observed by HPC methods (Cottrell and Kirchman, 2000), yet is limited in that it offers no insight into bacterial resistance profiles. The use of such an approach alone provides no insight into the physiological and ecological roles played within the community (Song *et al.*, 2009). Even metagenomic approaches only provide insight into the community's genetic potential, and as yet, many microbial genes remain uncharacterized (Sharpton, 2014).

By culturing and sequencing the microbial communities associated with wastewater treatment under various nutrient and temperature conditions with amendments of commonly prescribed antibiotics, we aimed to culture representative communities of ARB. All samples were grown on nutrient-poor Reasoner's 2 agar (R2A) and nutrient-rich lysogeny broth (LB) agar to mimic oligotrophic and nutrient-rich conditions experienced throughout the treatment process. By culturing samples at both room (RT) and human body temperature (BT), we sought to isolate resistant organisms that are culturable under atmospheric conditions and organisms that can potentially withstand survival in the human body. By sequencing the cultivable microbial communities using the same methodology used for culture-independent assessments of these same communities, we attempt to identify the

various fractions of the environmental communities that are cultivable while also observing resistance patterns within the treatment process.

Understanding the potential human impact on environmental surface water microbiomes in Charlotte, North Carolina, is valuable as this is a city of approximately 870,000 people within a larger metropolitan area of 2.5 million (The United States Census Bureau, 2018). We sampled bacterial communities from two WWTPs and their receiving surface water located in Charlotte. By combining traditional HPC and 16S rRNA gene sequencing methods to analyze these samples, we can (1) identify changes in resistant microbial communities throughout successive stages of the treatment process and (2) compare resistant communities identified via HPC to that of the total environmental populations observed.

2.2 Methods and Materials

2.2.1 Sampling locations

We chose to investigate two water systems associated with the wastewater treatment process: the environmental stream waters found upstream and downstream from treated effluent discharge points, and raw and subsequently treated sewage sampled at several sites inside the treatment plant. Charlotte does not have a large body of water within the city itself but does have a network of creeks and streams, many of which are important features of public greenway and park facilities. The regional water utility, Charlotte Water, manages five major wastewater treatment facilities, from which we selected Mallard Creek Water Reclamation and Sugar Creek Wastewater Treatment Plant. The Mallard Creek facility is estimated to process 12 million gallons daily (MGD) with effluent release into Mallard Creek, part of the Yadkin-Pee Dee river basin. The Sugar Creek facility is rated for 20 MGD and

discharges into Little Sugar Creek, which becomes Sugar Creek before eventually joining the Catawba river basin (Lambirth *et al.*, 2018). Both WWTPs are activated biosolids plants that use physical screens, grit removal stations, primary clarifiers with subsequent processing of activated biosolids of anoxic and aerobic zones, and secondary clarification ending with ultraviolet disinfection. Lambirth *et al.* (2018) further detail specific treatment parameters.

2.2.2 Selection of sampling sites

Sampling locations were selected from sewer lines routed to each facility, multiple sites within each WWTP, water upstream from effluent release, and the downstream receiving watershed (Figure 1). Pre-treated composite samples from hospital (HOS) and residential (RES) sewage lines to each WWTP were sampled prior to the merging of the two lines into a main sewage trunk line. The sites selected within the Mallard Creek facility included the pre-treated collective raw influent (INF), the primary clarifier influent (PCI) and primary clarifier effluent (PCE) which has undergone primary treatment, the activated sludge-processed aeration tank effluent (ATE) and final clarifier effluent (FCE). Analogous sampling sites were chosen at the Sugar Creek facility, including INF, PCE, ATE, and FCE. The location of sampling points at the Sugar Creek plant did not allow for the collection of a PCI sample but did allow for access to the ultraviolet disinfected effluent (UV), which was not accessible at the Mallard Creek facility. Stream sites upstream (UP) and downstream (DS) of each plant's effluent pipe were selected to assess changes in the stream microbiome upon introduction to effluent release.

2.2.3 Sample collection

Stream samples were manually collected by submerging sterile 1 L Nalgene bottles several inches below the surface water with the bottle mouth oriented against the

streamflow. Composite samples of the raw and treated sewage throughout the treatment plant were collected via peristaltic ISCO 6712 auto-samplers (Teledyne, Lincoln, NE, USA), pulling 150 mL every 30 minutes over 24 hours into sterile 2.5-gallon carboys on ice. After collection, sewage samples were transferred from their respective carboys into sterile 1 L Nalgene bottles through a peristaltic pump within a sterile biosafety cabinet with replacement pump tubing between each sample transfer. All samples were stored at 4°C prior to sample analysis.

2.2.4 Environmental DNA extraction

Each water sample was vacuum-filtered onto 0.45-micron cellulose filters (MO BIO Laboratories, Inc) in 100 mL aliquots until sample flow ceased for extraction of total genomic DNA. Filter material from these environmental samples was aseptically removed from the vacuum manifold and cut into strips, DNA was extracted from the filter residue using FastDNA® SPIN Kit for Soil (MP Biomedicals™) according to the manufacturer's instructions. DNA quantification was performed using a Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Inc.) and Nanodrop™ (Thermo Fisher Scientific, Inc.). All isolated DNA was stored at −80°C until sequencing was performed.

2.2.5 Heterotrophic plate counts

Antibiotics were amended to nutrient-rich LB (containing 10g NaCl per liter) and nutrient-poor R2A agar to evaluate bacterial resistance compared to total heterotrophic growth on antibiotic-free agar. The following frequently prescribed antibiotics were added to the culture media in higher concentrations than are typically prescribed: ampicillin, 1000 μg/mL; ciprofloxacin, 50 μg/mL; doxycycline, 100 μg/mL; sulfamethoxazole, 1000 μg/mL. Each of these antibiotics represents a major antibiotic class and mechanism of action:

penicillins (ampicillin) with peptidoglycan layer formation inhibition, DNA gyrase inhibition by fluoroquinolones (ciprofloxacin), protein formation inhibiting tetracyclines (doxycycline), and sulfonamides (sulfamethoxazole) inhibiting folate synthesis. All growth media were sterilized by autoclaving (30 minutes; 121°C) before the addition of sterile-filtered antibiotic stocks.

Water samples were serially diluted 10-fold in sterile deionized (DI) water and applied, in triplicate, to agar plates each amended with one antibiotic and to antibiotic-free controls using standard spread plating techniques. Both antibiotic-amended and control R2A and LB plates were incubated at room temperature for 5 to 7 days (22°C) and human body temperature (37°C) for 2 to 3 days. Upon completion of the incubation period, colony-forming units (CFU) were visually quantified, and statistical comparisons were performed.

2.2.6 Culture DNA extractions

All biomass from the cultured heterotrophic bacteria was aseptically collected for DNA extraction into sterile 1.5 mL microcentrifuge tubes. Biomass from the replicates was pooled, and DNA from each pooled sample was extracted using the UltraClean® Microbial DNA Isolate Kit (MO BIO Laboratories, Inc) according to the manufacturer's instructions and quantified using a Qubit® 2.0 Fluorometer. Isolated DNA was stored at −20°C per manufacturer's recommendation.

2.2.7 16S rRNA library preparation and Illumina sequencing

All sequencing was performed at the David H. Murdock Research Institute. Ribosomal amplicon libraries were created from collected DNA templates using universal primers of the V6 variable region of the 16S rRNA gene (forward primer, 5'-

CAACGCGAGAACCTTACC-3'; reverse primer, 5'- ACAACACGAGCTGACGAC-3'). Each sample was uniquely indexed and sequenced with 125 base pair (bp) paired-end reads on an Illumina® HiSeq 2500 flow cell.

2.2.8 16S sequencing analysis using QIIME2

Quantitative Insights Into Microbial Ecology (QIIME2; version 2018.4) was used for 16S community analysis of all samples (Bolyen *et al.*, 2019). Forward and reverse sequence reads were paired, denoised, and quality trimmed to 115 base pairs using DADA2 (Callahan *et al.*, 2016). Open reference operational taxonomic unit (OTU) clustering was performed using the Silva 128 reference database with a sequence identity threshold of 97% (Quast *et al.*, 2013; Yilmaz *et al.*, 2014). *De novo* chimera detection was performed using the UCHIME algorithm, and chimeric and borderline chimeric sequences were filtered out (Edgar *et al.*, 2011; Rognes *et al.*, 2016). All singletons were removed using the QIIME2 *feature-table filter* function. The resulting OTU table was again filtered using the same filtering function to remove OTUs with a relative abundance of <0.01% across all samples eliminating rare OTUs. This OTU table was then used for subsequent analyses.

Multiple sequence alignment was performed on the chimera-filtered sequences through multiple alignment using fast Fourier transform (MAFFT), and alignments were filtered to remove positions that were highly variable for the generation of a phylogenetic tree via FastTree. Diversity analyses were performed via the QIIME2 *diversity core-metrics-phylogenetic* function. Alpha diversity was calculated using the Shannon index. Beta diversity was determined using Bray-Curtis equilibrium distances and plotted using the Constrained Analysis of Principal Coordinates (CAP) ordination method via the *capscale* function in the Vegan package in R (Oksanen *et al.*, 2019). Permutational multivariate analysis of variance

(PERMANOVA) was used to analyze beta diversity with the *adonis* function in the Vegan package. Consensus sequence taxonomy classification was performed with VSearch using the Silva 128 16S rRNA gene sequence and taxonomic reference base. Finally, OTUs were summarized at a minimum relative abundance of 3% in each sample at the family level, and all low abundance and unclassified OTUs were reclassified as "Other" (Joseph *et al.*, 2019).

2.2.9 Statistical analysis

Statistical comparisons in microbial abundance were performed using linear regression models via the *lm* function in R (version 1.1.456) to evaluate significant differences between taxa isolated from each treatment stage and culturing condition using a log10 normalization scheme with an added pseudo-count (R Core Team, 2018). By combining the CFU count data and the relative abundance of observed 16S rRNA gene abundance, we were able to estimate the absolute abundance of taxa within the samples. Relative abundance, determined through 16S rRNA gene sequencing, was multiplied by the calculated CFU/mL to determine estimated taxonomic abundances. This *estimated* abundance should not be misconstrued as the definitive absolute abundance of the organisms found on each culturing plate as not all organisms carry the same copy numbers of the 16S rRNA gene (Pereira-Flores *et al.*, 2019). As replicate samples clustered tightly together, the sample with the deepest sequencing was retained as a representative for that measurement. A threshold frequency of a non-zero presence in at least 25% of all environmental samples was used to avoid stochastic differences in rare taxa. The Benjamini-Hochberg false discovery rate (FDR) was used for multiple hypothesis correction for community analyses (Benjamini and Hochberg, 1995).

2.3 Results

2.3.1 16S rRNA sequence analysis revealed a pattern of cultivable taxa

We first examined and compared the overall compositional characteristics of the environmental and cultured microbial communities. The Illumina HiSeq 16S rRNA sequencing runs of the cultivated heterotrophic plate count and culture-independent environmental DNA samples yielded 194,689,459 and 1,056,377 sequence reads, respectively. The 228 cultivated HPC DNA samples were represented by 363 unique OTUs, while 977 unique OTUs represented the 76 environmental samples. The number of reads in each cultured sample ranged from 149,730 to 2,190,647 with an average of 853,901 ± 316,590 reads/sample, while environmental samples ranged from 165,685 to 1,056,377 with a mean of 545,234 ± 174,861 reads/sample. Diversity analyses were performed on OTU tables rarefied to 100,000 reads. The Shannon diversity index (SDI) of the cultured samples had a mean value of 2.00 ± 1.00, while the environmental samples had a significantly higher mean value of 4.90 ± 0.91 (p<0.001).

The identified culture sequences were assigned to 68 unique genera, belonging to 47 families from four phyla of the bacterial domain. At the phylum level, Proteobacteria were found to dominate the community composition (93.89%), followed by lower proportions of Firmicutes (3.21%) and Bacteroidetes (2.76%) and a minor fraction of Actinobacteria (0.14%); representative OTUs from each phylum were present in all sample locations. At the family level, identified OTU clusters were predominantly classified as *Pseudomonadaceae* (40.70%), *Comamonadaceae* (12.81%), and *Enterobacteriaceae* (9.11%), all of which were found throughout each sample.

The environmental samples were assigned to 19 total phyla comprising 110 unique genera belonging to 90 families. These samples were dominated by OTUs identified as

Proteobacteria (53.3%) with lesser concentrations of Firmicutes (16.56%), Bacteroidetes (15.47%), and Actinobacteria (11.84%). *Comamonadaceae* (14.5%) and *Moraxellaceae* (8.99%) were the dominant families within the Proteobacteria, followed by *Lachnospiraceae* (8.68%; Firmicutes), *Sporichthyaceae* (5.04%; Actinobacteria), and *Cytophagaceae* (3.99%; Bacteroidetes) (Table A16).

2.3.2 Similar bacterial communities were observed in Mallard and Sugar Creek WWTPs

To identify any differences between the two WWTP-associated stream systems and determine whether they could be combined for subsequent analysis, we next compared both the heterotrophic plate count results and the 16S amplicon sequencing for the two systems. Using linear regression at a 5% FDR threshold, no significant differences in the abundance of total bacterial colonies (p=0.74) or the community structure identified via taxonomic diversity analysis were observed between the colonies originating from samples taken from Mallard and Sugar Creek treatment plants respectively (Figure A1). Linear regression analysis of the CFU/mL normalized total bacterial communities found within Mallard and Sugar Creek sites indicated only minor variations in the taxonomy and abundance of bacterial cultures from the two sampling sites (Figure A2).

Both Mallard and Sugar Creek sites predominantly harbored OTUs belonging to the families *Pseudomonadaceae*, *Comamonadaceae*, and *Enterobacteriaceae* (Table A3). The Sugar Creek site had a higher relative abundance of OTUs belonging to the families *Neisseriaceae* (p=0.020) and *Aeromonadaceae* (p=0.038) while *Xanthomonadaceae* (p<0.001) and *Alcaligenaceae* (p<0.001) were more abundant in the Mallard Creek samples (Table A3, Figure A2). As minimal differences were observed between the taxa cultured from each treatment site, additional analyses were performed by combining data from the two watersheds.

2.3.3 Treatment significantly reduced ARB colony counts

Analysis of antibiotic-resistant colony counts through each stage of the treatment process revealed a significant decrease in the observed survival upon treatment (Figure 2). Raw sewage and the wastewater collected from preliminary treatment stages (RES, HOS, INF, PCI, and PCE) yielded the highest number of colonies per plate of total heterotrophic and antibiotic-resistant colonies (Table A4). Antibiotic-resistant colony yields decreased significantly during the biological activated sludge treatment stage (ATE) when grown in the presence of antibiotics (Table A1): ampicillin by 90.22% (p<0.001), ciprofloxacin by 91.96% (p <0.001), doxycycline by 58.49% (p <0.001), and sulfamethoxazole by 95.88% (p=0.001). Total heterotrophic growth did not appear to be affected during this stage of treatment, with no significant decrease in colony counts from PCE to ATE (p=0.312) (Figure 2, Table A1).

As we might expect, since no active biological treatment occurs during primary treatment, no significant changes in taxonomic abundance were observed at the family level among the raw and primary treated wastewater (Figure 3), as was also observed by Lambirth *et al.* Corresponding with the decrease in the viable colonies observed in ATE samples, we also saw a decrease specifically in antibiotic-resistant *Enterobacteriaceae* in the post-treatment cultures, where there is a decrease from approximately 12% of the pre-ATE community to 6.93% post-ATE (p=0.012) and reaching less than 0.01% in the UV treated community (p=0.006) (Figure 3, Table 1 & A7). Resistant bacteria belonging to the families *Burkholderiaceae* (p=0.027) and *Pseudomonadaceae* (p=0.020) were also found to be significantly reduced by the aeration stage of the wastewater treatment (Table 1).

As observed with heterotrophic plate counts, UV treatment significantly decreased the abundance and diversity of bacterial taxa (Figure 2). The UV treated communities displayed the lowest Shannon alpha diversity (Figure A5, Table A5), and with this decrease

in taxonomic abundance, the cultivable resistant community was dominated by *Comamonadaceae* (Figure 3, Table A7). This study reveals bacterial community changes through the treatment process and that antibiotic-resistant bacteria are significantly depleted during treatment, resulting in effluent and downstream waters resembling that of natural stream communities.

2.3.4 Net community changes between upstream and downstream locations are subtle

When considering the impact of treated wastewater release on streams, the overall net change between upstream and downstream sites is of interest, irrespective of what taxa are introduced in the wastewater treatment plant as raw sewage and subsequently removed during treatment. No significant differences were observed in total HPCs or antibiotic resistance counts observed between water located upstream and downstream of the treated effluent discharge point using linear regression at a 5% FDR threshold (Figure A6, Table A1). Although these differences were not significant, of all the sampling locations in this study, downstream water exhibited the lowest resistance levels to the antibiotics selected (Table A4). Due to low biomass recovery and DNA yields, downstream samples cultured on doxycycline and sulfamethoxazole could not be sequenced for community analysis.

Analysis using Bray-Curtis distances of 16S sequences indicated no significant differences in beta diversity between upstream and downstream waters (p=0.079) (Figure A7). Community analysis via linear regression revealed little difference in the bacteria found among upstream and downstream samples (Figure A8). Antibiotic-resistant *Pasteurellaceae* were the only organisms observed to differ between stream waters with a higher abundance in the upstream samples (p<0.001). Both up and downstream antibiotic-free communities were predominantly composed of *Pseudomonadaceae*, *Comamonadaceae*, and *Sphingomonadaceae*

(Figure A9, Table A8). The majority of the antibiotic-resistant taxa found in upstream waters were absent downstream with only *Pseudomonadaceae* and *Comamonadaceae* dominating, although these differences were not significant (Figure 3). Analysis of alpha diversity did not indicate a significant difference in total sample diversity or within individual antibiotic treatments (Table A2). These findings indicate that the treatment of wastewater and the subsequent release back into stream environments has, at best, a modest effect on the total cultivable communities.

2.3.5 Minor variations between hospital and residential sewage microbial communities

The data collected allowed us to compare the composition of influent sewage from a purely residential trunk line to sewage taken from a trunk line in a neighborhood with a hospital. Significant differences in antibiotic-free and ARB colony abundances were observed between the raw sewage lines from the hospital and residential areas (Table A1). Antibiotic-free growth from hospital sewage samples was four-fold higher than that found in the residential sewage (p=0.003) (Figure A10). The combined growth of antibiotic-resistant organisms was also higher in hospital sewage samples (p=0.004).

Beta diversity analyses indicated no significant distinctions between the two sewage sources (p=0.731) (Figure A11), and no significant differences in taxonomic abundances were observed within the two microbial communities (Figure A12). The ARB communities recovered from residential and hospital sewage were primarily composed of *Pseudomonadaceae*, as were resistant *Enterobacteriaceae*, *Aeromonadaceae*, and *Comamonadaceae* (Figure 3 & A11).

2.3.6 Microbial growth in the presence of antibiotics

In a related paper by Lambirth *et al.*, we determined concentrations of 10 common antibiotics in the same environmental samples used in this study. We noted significant net

elevations in most antibiotics tested downstream of the wastewater treatment plant and high concentrations of most antibiotics within the WWTP environment. For this study, we chose four antibiotic compounds to determine the effect of ambient high-level concentrations on heterotrophic plate counts. A significant difference in bacterial counts was observed between the antibiotic-free control and each of the antibiotic amended samples ($p<0.001$) (Table A1). Growth on ampicillin was high in all water samples and significantly greater than any of the other three antibiotics tested ($p<0.001$) (Figure A14).

Corresponding with the higher bacterial abundances, the diversity of the untreated community was significantly greater than the diversity among all of the antibiotic treatments (Figure A15, Table A2 & A5). Across all sample types, *Enterobacteriaceae* and *Pseudomonadaceae* were among the core microbiome (Figure 4). Rank abundance analysis revealed that *Enterobacteriaceae* were among the top five taxa present under all antibiotic and control conditions and made up over half of the communities grown on sulfamethoxazole and doxycycline plates (Table A10).

2.3.7 Effect of culturing conditions

One factor in the culturability of microbes is the ability to replicate necessary growing conditions. While the focus of this study was not an exhaustive exploration of culture conditions, we were able to sample two standard growth media (LB and R2A agar) under two temperature conditions to determine the impact of these conditions on HPC results.

Among the incubation condition combinations, the temperature had a more significant effect on bacterial counts (Figure A17a). More growth was observed at room temperature than at body temperature on total heterotrophic and ARB colonies ($p<0.001$).

The media type had no significant effect on colony enumeration of total resistant organisms (Table A1). However, the total heterotrophic growth was influenced by the growth media ($p<0.001$), with R2A agar yields two-fold higher than LB.

In contrast to the quantitative plate counts, the media type had a greater effect on taxonomy and diversity than did temperature (Table A2). No significant change in alpha diversity was observed among communities in relation to the incubation temperatures (Figure 5). Media, however, had a significant effect on antibiotic-resistant ($p=0.002$) and total heterotrophic community diversity ($p=0.001$). The lower nutrient concentrations of R2A agar appeared to promote the cultivation of more diverse communities relative to the nutrient-rich LB agar.

2.3.8 Cultivable OTUs represent sizeable portions of the total population

Only fractions of total microbial species are routinely cultivable out of environmental samples. A comparison of 16S amplicon sequencing data from environmental and cultured samples allowed us to determine the proportion of culturable taxa under different combinations of conditions. The principal coordinate analysis (PCoA) plot generated using Bray-Curtis dissimilarity distances showed that the 76 environmental samples were tightly and distinctly clustered apart from the 228 cultivated samples ($p=0.001$) (Figure 6). Alpha diversity of the cultured communities was significantly lower than that found in the communities of the culture-independent approach ($p<0.001$) (Figure 5 & A16).

Despite the distinct clustering and variation in diversity, on average, 12.8% of the OTUs observed in each environmental sample were also observed in culture (Figure 7 & A21); however, the relative abundances of the cultivable organisms do not represent the same proportions found in the environment (Figure 8). Dominant bacterial families cultured,

such as *Pseudomonadaceae* and *Enterobacteriaceae*, often represented less than 5% of the environmental communities from which they were collected (Figure 8, Table A16). Though there was no dominant family, *Comamonadaceae* (16.9%) and *Moraxellaceae* (10.6%) were most abundant in the culture-independent samples. *Cytophagaceae* (4.76%), *Sporichthyaceae* (5.51%), and *Lachnospiraceae* (6.50%) were also common community members in all environmental samples, whereas they represent only a minor fraction of that found upon cultivation.

In general, OTUs within the phyla Firmicutes, Actinobacteria, and Bacteroidetes were significantly less abundant in the cultured communities than within the corresponding source microbial community ($p<0.001$) (Figure A19), while *Gammaproteobacteria* were greatly enriched with cultivation ($p=0.004$).

2.4 Discussion

This study of two Charlotte, North Carolina WWTPs and their associated waterways indicates three major observations regarding cultivable bacterial community changes and antibiotic resistance levels. First, community analysis reveals bacterial community changes through the treatment process where cultivable resistant bacteria are significantly depleted during treatment resulting in effluent and downstream waters resembling that of natural stream communities. Second, communities showed drastic variation from total cultivable heterotrophic growth in the presence of different antibiotics, indicating a degree of resistance in these communities to particularly high antibiotic concentrations. Finally, a clear cultivability bias was observed, though not to the extent widely accepted throughout literature.

2.4.1 ARB significantly depleted during treatment

Water located downstream from treated effluent sites displayed lower concentrations of cultivable heterotrophic ARB than even that of the equivalent water located upstream from treatment discharge. This observation corresponds with the general observation that the treatment process significantly reduces total numbers of resistant bacteria and support the findings of Lambirth *et al.* concerning total environmental bacteria and the presence of resistance gene markers in these sampling locations (Guardabassi *et al.*, 2002; Huang *et al.*, 2012; Bouki *et al.*, 2013). Though ARGs were found to be slightly more abundant in the downstream waters, only 9 of the 600 unique ARGs and MGEs were observed in higher concentrations downstream relative to upstream concentrations (Lambirth *et al.*, 2018).

Overall, organisms associated with the human microbiome were found in greater concentrations in the sewage and wastewater samples than in stream samples. The treatment process was found to reduce the abundance of the human-associated microbes, specifically those classified as Bacteroidetes and Firmicutes, both in the presence and in the absence of high concentrations of antibiotics (Figure A3). The activated sludge process reduced the total abundance of Firmicutes, while Bacteroidetes showed a decline upon UV treatment. Both of these phases of treatment are designed for microbial reduction, and such reductions were observed in the heterotrophic bacterial counts (Bouki *et al.*, 2013).

Both stream communities are characteristic of typical freshwater stream communities composed primarily of Proteobacteria. A greater abundance of OTUs assigned as Bacteroidetes (12.6%) were observed in the downstream waters relative to those upstream (2.37%) (Figure A9, Table A8), which is consistent with the introduction of human fecal matter from the treated wastewater (McLellan *et al.*, 2010). Overall, Charlotte Water's treatment process appears to be effective in removing resistant bacteria with significant

reductions in ARB concentrations upon activated sludge and UV treatments. However, elevated concentrations of the antibiotic compounds, themselves, were observed in the stream waters downstream of the treatment plants (Lambirth *et al.*, 2018).

2.4.2 Resistance to high antibiotic concentrations

Bacteria classified as *Gammaproteobacteria*, particularly those assigned to the families *Pseudomonadaceae* and *Enterobacteriaceae*, and *Betaproteobacteria* showed resistance to all antibiotics utilized. Resistant communities were found in much greater abundance among raw sewage and the pre-aeration stages of treatment within the WWTP (Figure 2). The highest antibiotic resistance counts were observed with ampicillin (Figure A14). Resistance to this β-lactamase sensitive drug, developed in the 1960s, was identified as early as 1972 and is now widespread (Denis *et al.*, 2010; Ravina, 2011). These ARB were dominated by *Gammaproteobacteria* (82.32%), of which *Pseudomonas* sp. (67.41%) and *Aeromonas* sp. (5.19%) were the most abundant with the vast majority of this growth occurring with room temperature incubations (Figure 4, Figure A16). Previous findings have indicated that these two genera are almost entirely resistant to this drug (Voolaid *et al.*, 2012). The *Pseudomonadaceae* family has been described as carrying several antibiotic resistance determinants along with the ability to grow on standard culture media (Blasco *et al.*, 2008; Voolaid *et al.*, 2012). Members of *Aeromonas* sp. are known to produce inducible, chromosomally encoded β-lactamases conveying resistance to some β-lactams, including ampicillin (Blasco *et al.*, 2008). The *Aeromonadaceae* family has also been reported to harbor a wide variety of antibiotic resistance mechanisms and acquire resistance determinants under selective pressures (Garcia-Armisen *et al.*, 2013).

The antibiotic-resistant bacterial counts with sulfamethoxazole were primarily

dominated by *Gammaproteobacteria* (96.42%). ARB recovered with this drug included *Enterobacteriaceae*, *Pasteurellaceae*, and *Pseudomonadaceae* across all culturing conditions, except for low recoveries of the latter in low-nutrient, high-temperature incubations (Figure 4, Figure A16). This bacteriostatic antibiotic, typically used in combination with trimethoprim, has been employed to treat infections in the urinary, gastrointestinal, and respiratory tracts (Huovinen, 2001). Resistance to sulfamethoxazole has been on the rise since the late twentieth century (Wright *et al.*, 1999). Plasmid-encoded resistance in both sulfamethoxazole and trimethoprim can be easily transferred among these Gram-negative bacterial families resulting in increased resistance to these drugs (Wright *et al.*, 1999).

Several rare families were observed in greater relative abundance in the presence of ciprofloxacin (Figure 4). *Flavobacteriaceae* and *Campylobacteraceae* made up 9.58% and 8.33% of the ciprofloxacin-resistant growth, respectively. *Bradyrhizobiaceae, Caulobacteraceae*, *Microbacteriaceae*, *Comamonadaceae,* and *Alcaligenaceae* were also among isolates grown with this broad-range fluoroquinolone, in which R2A agar at lower incubation temperatures yielded greater taxonomic diversity (Figure A16). Plasmid-mediated quinolone resistance (PMQR) genes are considered the primary route for the spread of fluoroquinolone resistance (Yan *et al.*, 2017). These PMQR genes are often associated with MGEs responsible for multi-drug resistance (MDR) (Osińska *et al.*, 2019). Such MDR genes encode for resistance to several drug classes, including quinolones, β-lactams, tetracyclines, and sulfonamides, promoting the transfer of resistance across a diverse range of bacteria (Osińska *et al.*, 2019). Nearly 70% of ciprofloxacin remains undegraded due to its recalcitrance, and even non-quinolone antibiotics can increase the selective pressures favoring the persistence of PMQR genes (Vien *et al.*, 2012).

The lowest antibiotic resistance numbers were observed with doxycycline. This semi-synthetic tetracycline initially overcame the resistance issues of its tetracycline predecessors through chemical modifications to improve its antimicrobial potency and spectrum (Jarolmen *et al*., 1970; Nelson and Levy, 2011). However, over time, the utility of this drug has narrowed as the products of tetracycline-resistance genes, including Tet(A), Tet(B), and Tet(K) efflux pumps, began to recognize the compound (Grossman, 2016). Doxycycline-resistant members of *Pasteurellaceae* and *Xanthomonadaceae* were observed at room temperature with R2A agar (Figure A16). However, *Enterobacteriaceae* were predominantly the most abundant family in all culturing conditions isolated with this tetracycline (Figure 4), which is not surprising considering the first description of tetracycline resistance attributed to a mutation in the drug target was observed in a *Klebsiella pneumoniae* strain (Villa *et al*., 2014).

In general, multiple phylogenetic groups showed high levels of resistance to at least one antibiotic. Whether it be acquired or intrinsic resistance, certain cultivable taxa were capable of withstanding these concentrations during the treatment process. As not all of the microbial community is cultivable, observing such resistant communities at these drug concentrations only shows us the small portion from the total population capable of being cultured. The bias observed in cultured communities compared to culture-independent methods may not be an accurate reflection of resistant community shifts as factors, irrespective of antibiotic amendment, pose selective pressures on the abundance of different phylogenetic groups. Despite this bias, community shifts paralleled those observed by Lambirth *et al.* in that the treated wastewater more closely resembles freshwater communities due to the efficacy of the processes used in treatment.

2.4.3 Cultivability bias

The temperature had a greater effect on the number of resistant colonies observed, while the media appeared to have little consequence on bacterial quantity (Figure A17). However, the media did have a significant influence on which organisms within the population were capable of growth (Figure 8). The quantitative findings are consistent with observations summarized by Allen *et al.* (2004) in that lower incubation temperatures favor the growth of water-based microbes. Though not statistically meaningful, R2A agar resulted in higher yields, likely promoted by the low-nutrient, low-ionic strength formulation of this medium (Reasoner, 1990).

The diversity of the culture-dependent communities was significantly lower than that of the culture-independent approach (Figure 5). While none of the culturing conditions resulted in growth reflective of such highly diverse source communities, sequence reads of the cultivable OTUs were observed in the culture-independent bacterial communities in higher concentrations than anticipated. OTUs found within the cultured bacterial communities made up, on average, 12.8% of the total OTUs recovered with the culture-independent methodology (Figure A21). These common, culturable OTUs made up higher proportions of the total communities than the commonly accepted 1% culturability paradigm. This widely accepted paradigm, likely conceived as early as 1995 (Amann *et al.*, 1995), is based on the concept of "the great plate count anomaly" proposed by Staley and Konopka (1985).

According to a literature search conducted by Adam Martiny (2019), there are several interpretations of this axiom, with current explanations that only *1% of the cells or taxa* in a community can be cultivated, despite the use of all available culturing methods. It is likely, though, that the original interpretation was that only *1% of the cells* in a community can be

cultured on *standard agar medium* (Martiny, 2019). At the time this paradigm was initially conceived, only about 5,000 bacterial species had been described (Amann *et al.*, 1995). Since that time, efforts in cultivating abundant bacteria and culturing methodology innovations have allowed for the growth of a greater proportion of bacterial communities, and, as of 2017, over 15,000 species have been described (Parte, 2018). As evidenced by the current findings and other such studies, this standard of thinking is no longer valid. Several other studies have also indicated culturable bacterial yields of 70% or more in several diverse environments (Staley and Allan Konopka, 1985; Browne *et al.*, 2016).

Though higher culturable yields were observed, the proportions of these shared OTUs within each sample were not always reflective of those found in the microbial source material. The sequences obtained from the culture-dependent approach were dominated by *Gammaproteobacteria* (55.9%), of which *Enterobacteriaceae*, *Pseudomonadaceae*, and *Aeromonadaceae* were among the most abundant families (Figure 8). These families made up significantly greater proportions of the cultured communities than they did in the source communities, primarily composed of *Betaproteobacteria* (31.4%). Selection of *Gammaproteobacteria* has been described as a cultivation bias in aquatic bacterial communities, likely due to the specific life strategies employed by members of this class (Eilers *et al.*, 2000). These findings are supported in a similar study by Garcia-Armisen *et al.* (2013), that observed comparable results regarding community compositions of culture-dependent and independent samples. In general, higher concentrations of OTUs were found to be culturable than previously thought, though the proportions of these OTUs within their respective communities varied from those identified using culture-independent approaches.

2.5 Conclusion

Cultivation practices in the study of bacteria are fundamental to our knowledge of their ecological roles. Even in the era of bioinformatic advancements, to understand the extent to which microbial populations have been characterized, we must quantify the fraction of bacterial cells that share physiologies with cultured organisms. Though methodological laboratory practices are still far from successfully culturing all bacterial community members from most environments, the principle that only 1% of the microbial population in all environments are culturable is outdated (Steen *et al.*, 2019). It is important to note that this study only demonstrated resistance to four individual antibiotics and that many viable organisms within the sample water are not represented due to culture biases. As we tested antibiotics individually, assumptions of MDR organisms and their prevalence cannot be drawn. However, it has been commonly observed that the harboring of a single resistance gene can confer resistance to multiple antibiotics and that several such genes are often co-localized on MGEs able to undergo HGT as a unit. In conjunction with previous work, we were able to determine that overall ARB and the associated ARGs are significantly reduced during treatment. Unfortunately, this reduction was not observed with the antibiotic compounds being released in the treated effluent, and thus further studies are required to assess the effect of these compounds, alone, on the microbial communities.

2.6 Tables

Table 1 | Differential abundance of significant taxa between treatment stages for combined ARB communities. Underlined locations indicate the locations containing the higher taxonomic abundance.

Phylum	Class	Family	*p* value	Locations
Actinobacteria	*Actinobacteria*	*Microbacteriaceae*	<0.001	UV - <u>DSA</u>
			0.017	ATE - <u>FCE</u>
		Propionibacteriaceae	0.047	ATE - <u>FCE</u>
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadaceae*	<0.001	UV - <u>DSA</u>
	Betaproteobacteria	*Burkholderiaceae*	0.027	<u>PCE</u> - ATE
	Gammaproteobacteria	*Enterobacteriaceae*	0.012	<u>PCI</u> - ATE
			0.001	<u>PCE</u> - ATE
			0.006	<u>PCE</u> - UV
		Halomonadaceae	0.017	ATE - <u>FCE</u>
		Pasteurellaceae	<0.001	<u>UPA</u> - DSA
		Pseudomonadaceae	0.020	<u>PCE</u> - ATE

UPA, upstream; RES, residential sewage; HOS, hospital sewage; INF, sewage influent; PCI, primary clarification influent; PCE, primary clarification effluent; ATE, aeration tank effluent; FCE, final clarification effluent; UV, UV treated effluent; DSA, downstream.

2.7 Figures

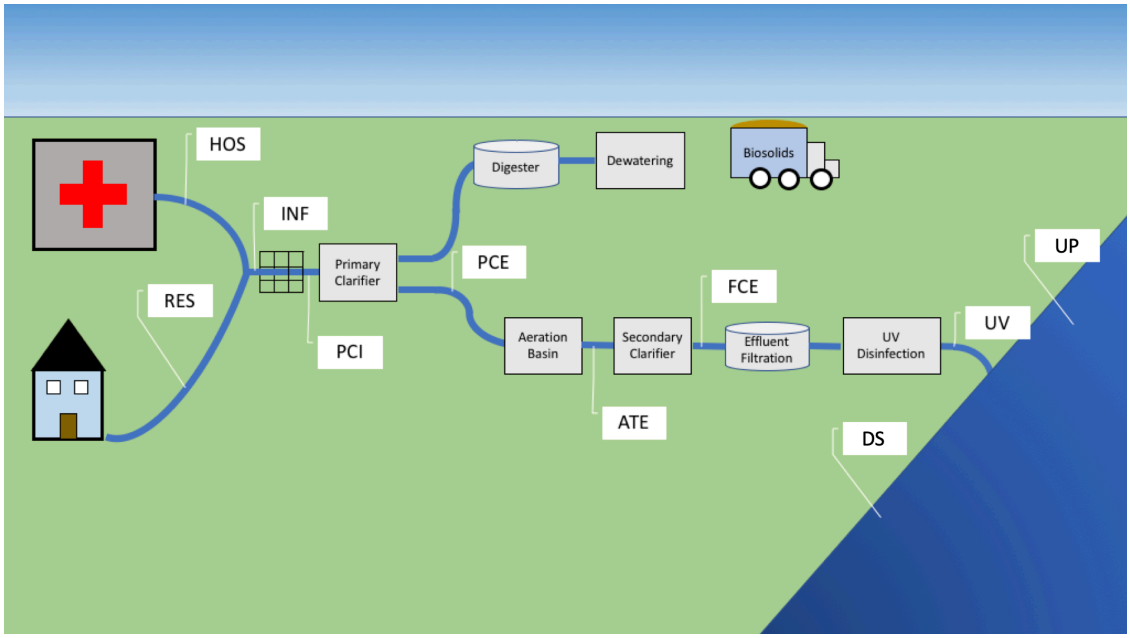

Figure 1 | Schematic of the treatment process and sampling sites of the wastewater treatment plants utilized for this study. Raw sewage from residential (RES) and hospital (HOS) sources are routed to a main sewer line. The combined raw influent is then passed through a physical screen to filter out large solids. The screen-filtered wastewater (PCI) undergoes primary clarification for an additional solid removal step. The primary clarifier effluent (PCE) is routed to an aeration basin for biological nutrient removal. The aeration tank effluent (ATE) then undergoes a secondary clarification process. The final clarification effluent (FCE) undergoes a final filtration step before UV treatment (UV) for microbial disinfection prior to stream release. The large solids removed during primary clarification are routed to a digester and dewatered for the production of biosolids. (Downstream, DS; Upstream, UP).

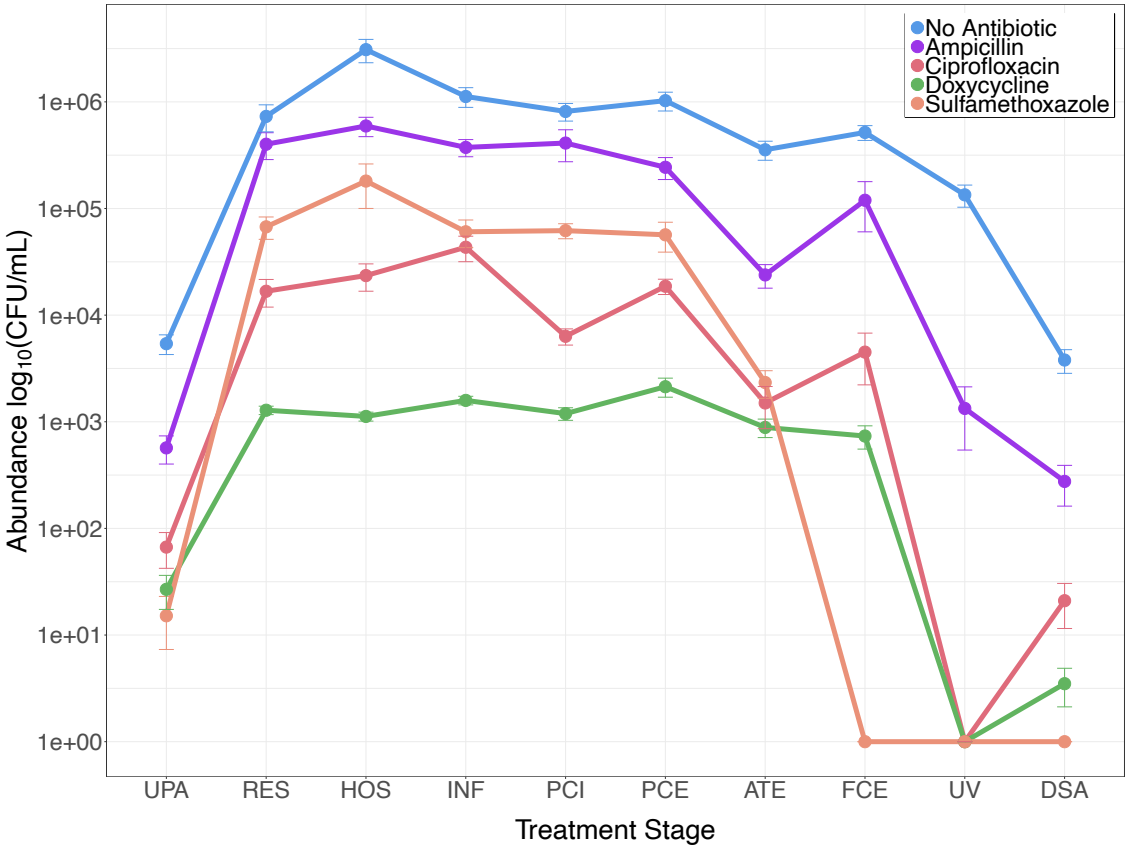

Figure 2 | Abundance of microbial colonies grown on the four antibiotics and a control at each stage of the treatment process. Total heterotrophic and resistant microbial concentrations saw significant reductions from the initial raw sewage to the final UV treated effluent. Error bars indicate the standard error of the mean for each antibiotic treatment at each site. UPA, upstream; RES, residential sewage; HOS, hospital sewage; INF, sewage influent; PCI, primary clarification influent; PCE, primary clarification effluent; ATE, aeration tank effluent; FCE, final clarification effluent; UV, UV treated effluent; DSA, downstream.

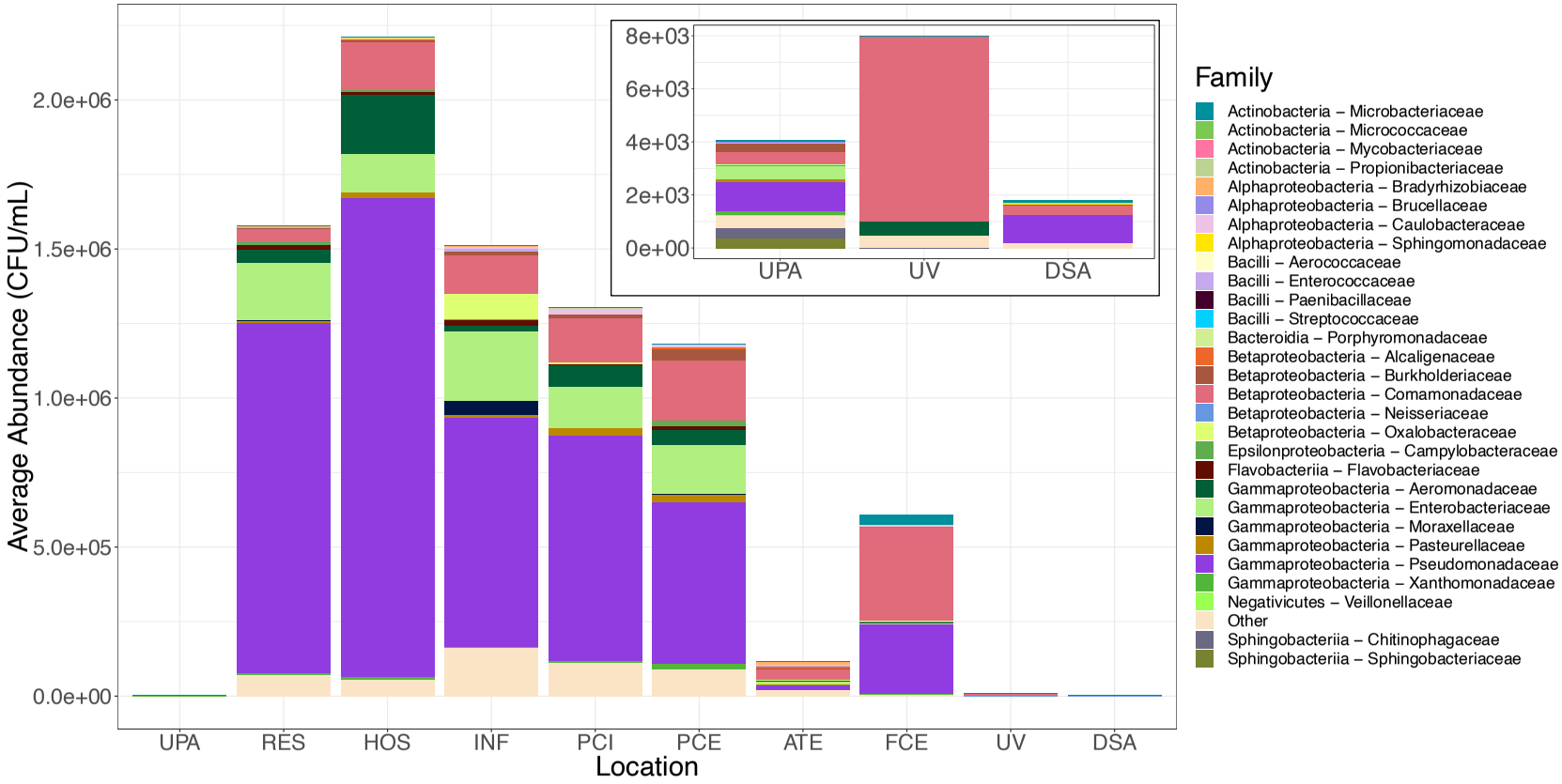

Figure 3 | CFU/mL normalized counts of 16S rRNA gene relative abundance data for antibiotic-resistant communities at each sampling location. Combined Mallard and Sugar Creek samples at each sampling location including combined antibiotic-amended cultured communities. Relative abundance determined through 16S rRNA gene sequencing was multiplied by CFU/mL at each location to determine estimated taxonomic abundances. The inset shows the enlarged taxonomic abundances for the upstream, UV treated, and downstream locations. Families making up <1% of the total community at each site were excluded. UPA, upstream; RES, residential sewage; HOS, hospital sewage; INF, sewage influent; PCI, primary clarification influent; PCE, primary clarification effluent; ATE, aeration tank effluent; FCE, final clarification effluent; UV, UV treated effluent; DSA, downstream.

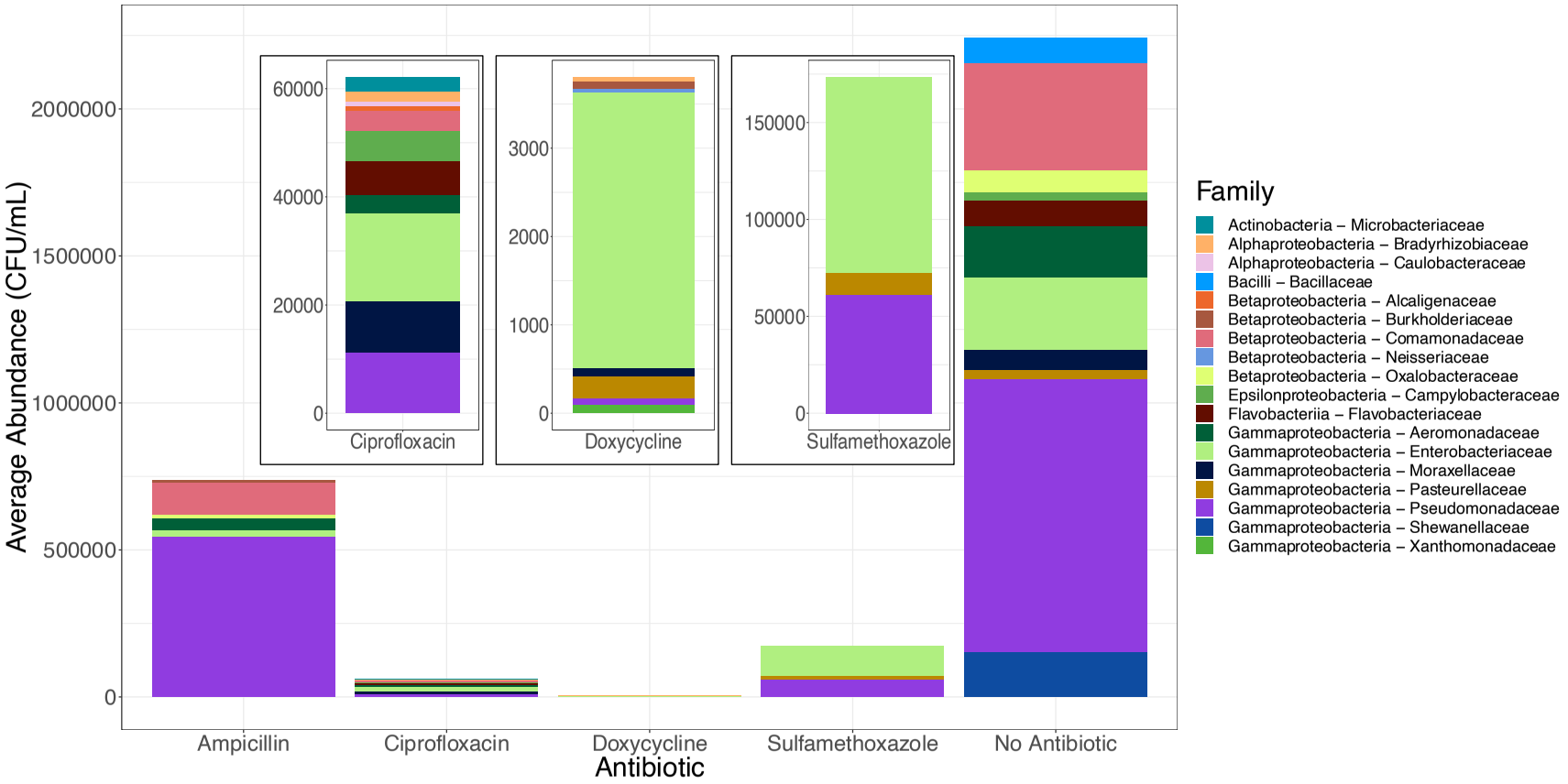

Figure 4 | CFU/mL normalized counts from relative abundance data of the taxonomic families from each cultured antibiotic treatment. The average colony counts for each location were normalized to CFUs per mL and adjusted to the calculated relative abundance determined through 16s rRNA sequences to obtain approximated absolute abundances. Families making up <1% of the total community at each site were excluded.

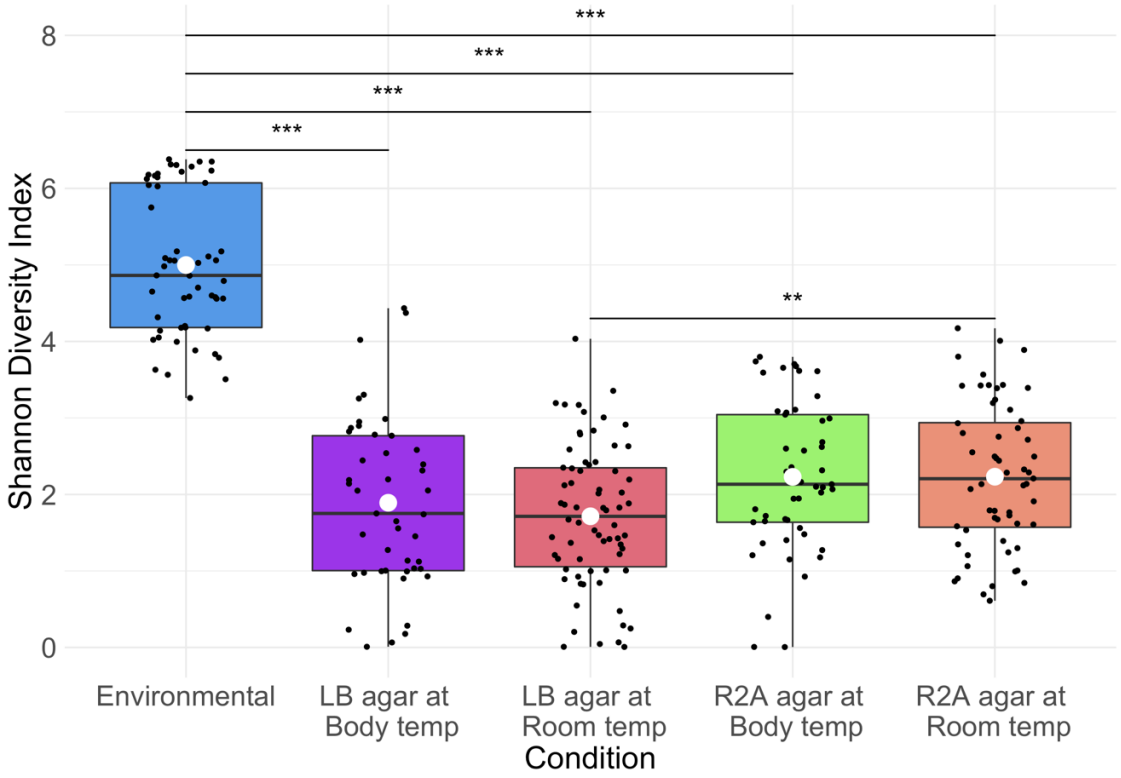

Figure 5 | Average OTU level Shannon diversity for the culture-independent and each culture-dependent community. Significant differences are indicated with bars between the locations with statistically differential diversity values. The statistical mean is represented by a white circle. "*" indicates a *p* value of 0.01 – 0.05; "**" indicates a *p* value of 0.001 – 0.01; "***" indicates a p value <0.001.

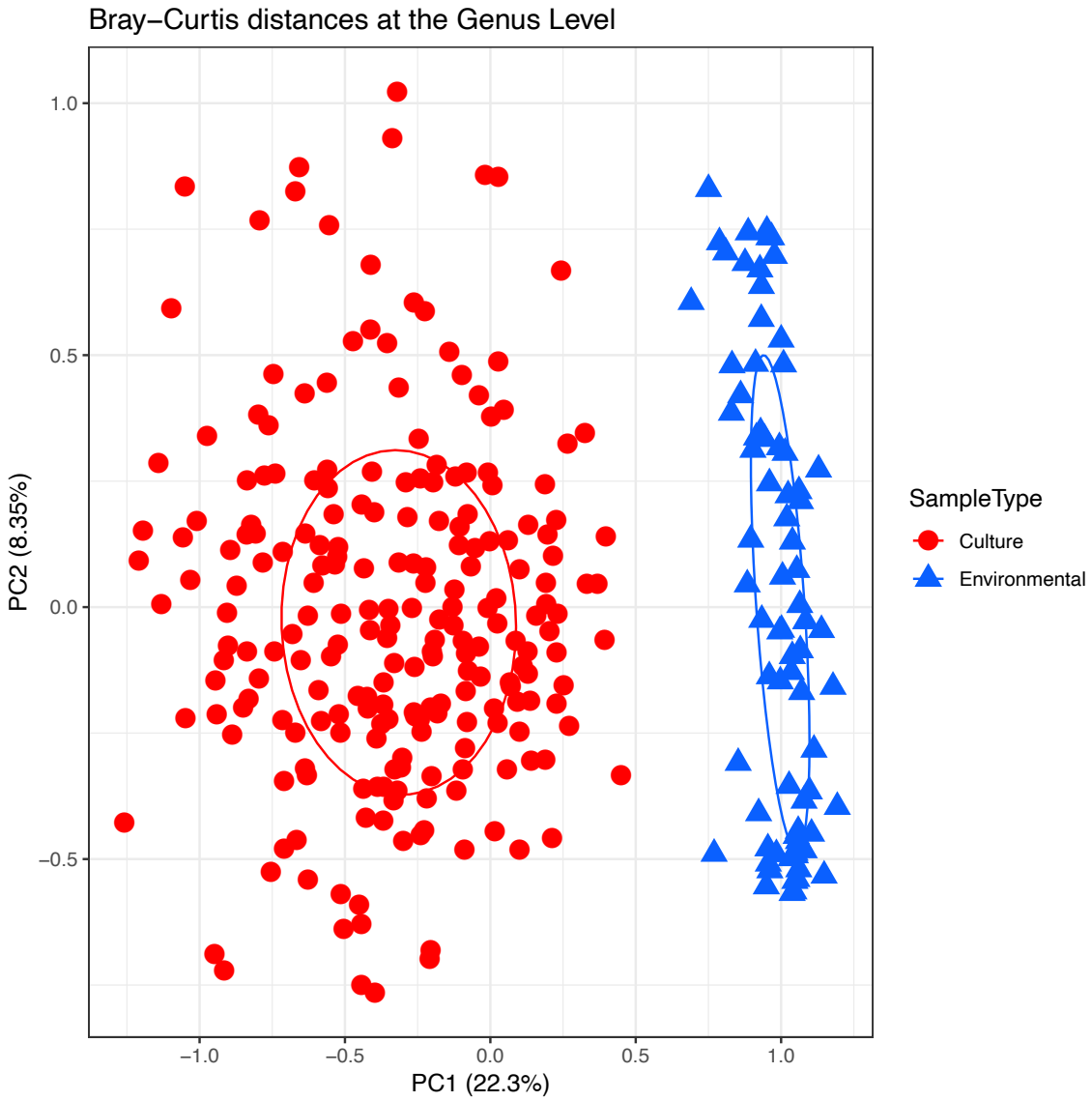

Figure 6 | Bray Curtis PCoA ordination for all culture-independent and culture-dependent samples. Beta diversities at the genus level from 16S rRNA gene sequencing are shown with PC1 and PC2 components. Data are clustered and colored by the two sequencing approaches utilized.

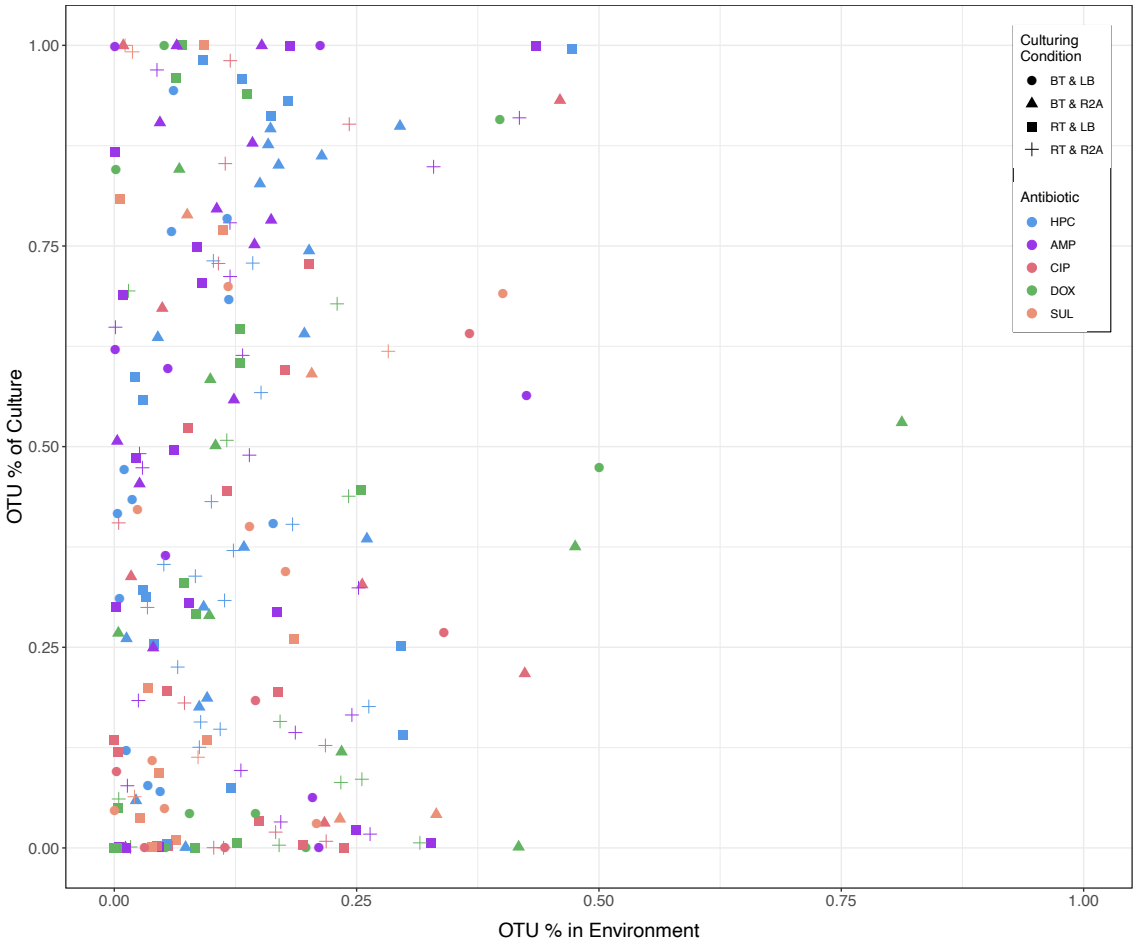

Figure 7 | Relative abundances of the shared composition within culture and environmental communities at the OTU level. BT, body temperature (37°C); RT, room temperature (22°C); LB, lysogeny broth agar; R2A, Reasoner's 2 agar.

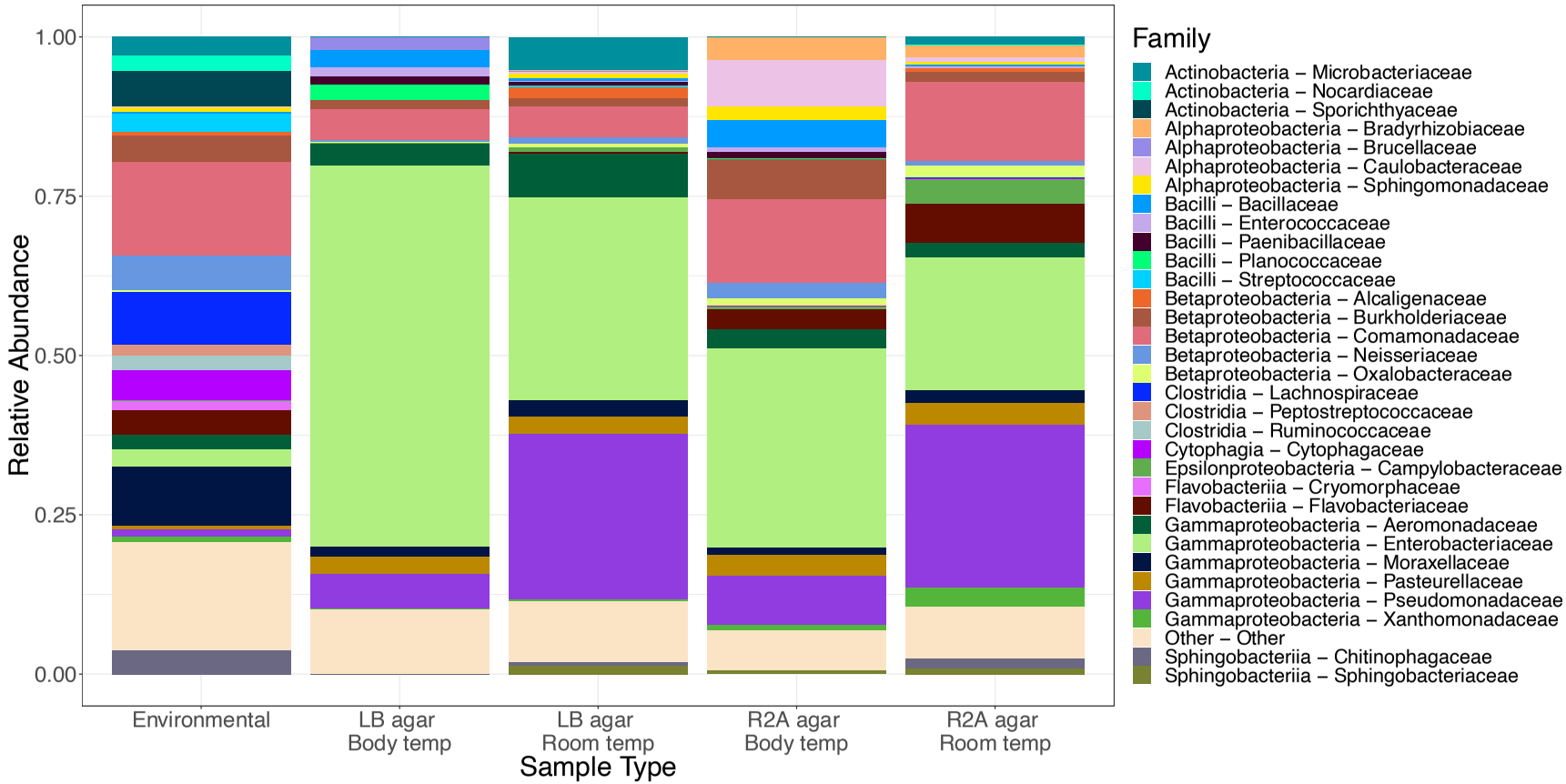

Figure 8 | Relative abundance of the taxonomic families for the culture-independent samples and each culture-dependent condition. Families making up <1% of the total community at each site were excluded.

CHAPTER 3: Assessing changes in water and sediment bacterial communities upon exposure to downstream WWTP antibiotic concentrations

3.1 Rationale

Among the many pharmaceuticals used in modern medicine, antibiotics have been of increasing concern due to increased therapeutic use and the subsequent development of antibiotic-resistant bacteria (ARB). Bacterial responses to antibiotics are concentration-dependent, where at high concentrations, antibiotics can have bactericidal and bacteriostatic effects on susceptible organisms by killing or inhibiting bacterial growth, respectively (Davies, 2006). Lower, sub-inhibitory concentrations can have inverse biological responses in bacteria (Grenni *et al.*, 2018). Sub-inhibitory levels of antibiotics are the low, non-lethal concentrations below the minimum inhibitory concentration (MIC), the lowest concentration in which a drug can inhibit bacterial growth *in vitro* (Müller *et al.*, 2002). However, lethal concentrations of antibiotics rarely occur outside of therapeutic use, resulting in the majority of environmental bacteria being exposed to these sub-lethal concentrations (French, 2006).

Municipal wastewater treatment plants (WWTP) are one of the primary sources of release of antibiotics into the environment (Baquero *et al.*, 2008). Conventional WWTPs are designed for the maximum removal of nutrients, pathogens, and toxic compounds within the wastewater before its release into the receiving environments (Chu *et al.*, 2017). However, these WWTPs are not explicitly designed to remove the antibiotic compounds themselves, resulting in the discharge of sub-inhibitory concentrations of these compounds and their metabolites into aquatic environments (Norzaee *et al.*, 2018). There is no regulation and little monitoring for antibiotics, resistant bacteria, or antibiotic resistance genes (ARG) in wastewater effluent, which is typically found to contain a variety of these substances (Chu *et al.*, 2017).

Due to the constant and stable release into the environment, both aquatic and soil organisms are regularly exposed to ecologically relevant antibiotic concentrations (González-Pleiter *et al.*, 2013). While certain antibiotics, such as β-lactams, are easily degraded due to the chemical instability of the β-lactam ring, others including fluoroquinolones (e.g., ciprofloxacin), macrolides (e.g., azithromycin), and tetracyclines (e.g., doxycycline), are more refractory, resulting in longer environmental residence times and dissemination (Cha *et al.*, 2006; Lin *et al.*, 2010; Leung *et al.*, 2012). The release of sub-inhibitory levels of antibiotics can drive changes in the structure of natural bacterial communities through the loss, inhibition, or adaptive evolution of certain bacterial groups (Grenni *et al.*, 2018).

The natural bacterial communities found in soil and aquatic ecosystems are fundamental in many ecological processes relating to the maintenance of soil and water quality (Huerta *et al.*, 2013; Llirós *et al.*, 2014). These microbes are vital for activities, such as biogeochemical cycling, due to their abundance, genetic diversity, and metabolic capability (Dai *et al.*, 2016; Grenni *et al.*, 2018; Zeng *et al.*, 2019). When antibiotic compounds are released into the environment, the microbial community structure can be affected directly or indirectly, resulting in short- and long-term community changes (Thiele-Bruhn and Beck, 2005; Aminov and Mackie, 2007; Kotzerke *et al.*, 2008).

Reductions in microbial abundance and diversity are often observed in studies assessing the direct effects of high concentrations (i.e., micrograms per liter) of antibiotics on microbial communities; whereas low, sub-inhibitory concentrations (i.e., nanograms per liter) have been found to induce long-term effects on microbial species not directly affected by the presence of antibiotics (Knapp *et al.*, 2008; Ashbolt *et al.*, 2013; Roose-Amsaleg and Laverman, 2016). Long-term, indirect impacts can include selection for resistant phenotypes by enrichment of pre-existing ARB and the acquisition of ARGs by previously susceptible

organisms (Knapp *et al.*, 2008; Ashbolt *et al.*, 2013); and it is well known that low concentrations select for and hasten the development of antibiotic resistance (Kümmerer, 2003; Chait *et al.*, 2016; Ahmed *et al.*, 2018). Resistance acquired through the horizontal transfer of resistance genes increases the quantity of these genes in the environment, and the presence of such genes can interfere with microbial interactions, which can influence the growth and enzymatic activity of communities and affect vital ecological functions, ultimately leading to a loss in the ecosystem's functional stability (Westergaard *et al.*, 2001; Allen *et al.*, 2010; Grenni *et al.*, 2018).

A corollary study found elevated antibiotic concentrations in waters downstream of the release sites of treated wastewater (Lambirth *et al.*, 2018). However, pathogenic taxa and resistance gene markers were not significantly increased in these downstream sites indicating their effective removal during treatment. Of the antibiotic compounds chosen for detection in downstream waters, azithromycin, doxycycline, ciprofloxacin, and sulfamethoxazole were among the highest detected. Despite the observed increase in antibiotic compounds detected downstream, these concentrations remained at low, sub-inhibitory concentrations. As Lambirth *et al.* also found shifts in microbial community function, and with the knowledge that resistance genes and ARB are reduced during treatment, the release of antibiotic compounds and their metabolites could be of great interest in the spread of acquired antibiotic resistance.

An understanding of the interaction between low concentration antibiotics and bacteria in the environment is necessary for risk assessment of environmental and human health. Surface water contamination by ARB and resistance genes is known to impact the community structure and activity of microbial populations (Kümmerer, 2009; Yuan *et al.*, 2015). By introducing known downstream concentrations of antibiotics, without the

associated resistance determinants, to effluent-free water collected upstream from a discharge site, we attempted to explore changes in the natural bacterial community due to the presence of sub-inhibitory antibiotic concentrations.

3.2 Methods and Materials

3.2.1 Sample collection

Surface water and sediment samples were collected from a Mallard Creek site upstream from the Mallard Creek Water Reclamation treated discharge (Lat 35.3201, Long -80.7315) in September 2016. One liter of each aqueous sample was collected individually in sterile 1 L high-density polyethylene Nalgene® bottles. Sediment samples were collected at the same time in individual 50 mL polypropylene conical tubes. All samples were stored in a cooler for transport and immediate processing.

3.2.2 Sample incubations

Bacterial cells, collected by filtration of 1 L surface water onto a 0.22 μm pore size Sterivex™ filter unit, and 50 mL of sediment were stored in sterile polypropylene conical tubes at −20°C prior to DNA extractions for the examination of the original microbial populations within each sample. Approximately 70 mL of sediment-water slurry samples were transferred to individual sterile 200 mL serum vials, while the surface water samples were maintained in the original sampling bottles. Based on the previously measured antibiotic concentrations in downstream Mallard Creek sites (Lambirth *et al.*, 2018), the following antibiotics were added to the samples to simulate effluent receiving waters: azithromycin, 34 ng/L; ciprofloxacin, 157 ng/L; doxycycline, 8 ng/L; sulfamethoxazole, 28 ng/L. All control and antibiotic amended samples were incubated in triplicate for two weeks.

Samples were incubated at room temperature in limited light. Control and antibiotic-amended samples were pulled at three points over the time series (Day 3, 7, and 14).

As samples were pulled at each time-point, aqueous bacterial cells were collected by filtration of the 1 L samples, and filters were stored in sterile polypropylene conical tubes. Water was aseptically removed from each settled sediment sample, and the remaining sediment was placed in 50 mL polypropylene conical tubes. Samples from each time-point were stored at −20°C until DNA extractions were performed.

3.2.3 DNA extractions

DNA was extracted from filters using the FastDNA® SPIN Kit for Soil (MP Biomedicals™), while sediment extractions were performed using the Powermax® Soil DNA Isolation Kit (MO BIO Laboratories, Inc) allowing for greater sample volume. All extractions were performed according to manufacturer instructions, and eluted DNA was quantified using a Qubit® 2.0 Fluorometer before storage at recommended −20°C for sequencing.

3.2.4 Community analysis via QIIIME2

Preparation of the 16S ribosomal RNA (rRNA) library and Illumina sequencing were performed as described above (Chapter 2 Methods). Sequences were denoised and truncated to 119 bp using DADA2 (Callahan *et al.*, 2016), and chimeric sequences were detected and filtered via the UCHIME algorithm (Edgar *et al.*, 2011; Rognes *et al.*, 2016). Open reference operational taxonomic unit (OTU) clustering was performed using the Silva 128 reference database with a sequence identity threshold of 97% (Quast *et al.*, 2013; Yilmaz *et al.*, 2014). Multiple sequence alignment was performed on the chimera filtered sequences through multiple alignment using fast Fourier transform (MAFFT), and alignments were filtered to

remove positions that were highly variable for the generation of a phylogenetic tree via FastTree. Alpha diversity was calculated using the Shannon index. Beta diversity was determined using Bray-Curtis equilibrium distances and plotted using the Constrained Analysis of Principal Coordinates (CAP) ordination method via the *capscale* function in the Vegan package in R (Oksanen *et al.*, 2019). Permutational multivariate analysis of variance (PERMANOVA) was used to analyze beta diversity with the *adonis* function in the Vegan package. Phylogenetic diversity metrics were then calculated to a sampling depth of 4000 reads before OTU consensus sequence classification via VSearch using the Silva 128 taxonomy reference database.

3.2.5. Statistical analysis

Changes in microbial abundance and alpha diversity (assessed at the OTU level) were assessed via linear regression models using the *lm* function in R. A threshold frequency of a non-zero presence in at least 25% of all environmental samples was used to avoid the detection of stochastic differences in rare taxa. The Benjamini-Hochberg false discovery rate (FDR) was used for multiple hypothesis correction for community analyses (Benjamini and Hochberg, 1995).

3.3 Results

3.3.1 Communities and diversity among water and sediment samples varied

We attempted to assess the short- and long-term effects of antibiotics and bacterial interactions from natural stream environments. Analysis of Shannon diversity indices indicated significant variation among microbial diversity between stream water and sediment samples ($p<0.01$) (Table B2). Multi-dimensional scaling analysis for Bray-Curtis dissimilarity

displayed distinct clustering patterns between these two sample types indicating two distinctive microbial communities (p<0.001) (Figure 9).

The identified sequences for both water and sediment samples were clustered into 207 genera, belonging to 145 families from 22 phyla of the Bacteria domain (Table B1). Representatives of the phylum Proteobacteria dominated all samples, accounting for 56.4% and 38.7% of the total reads in sediment and water samples, respectively (Table B4). This phylum was represented by four classes *Alpha-*, *Beta-*, *Delta-*, and *Gammaproteobacteria*. Rank abundance analysis indicates that a large portion of the abundant microbial taxa (those making up at least 1% of the community in each sample) could not be definitively classified into a taxonomic order resulting in a large portion of the community being unidentifiable at the lower taxonomic levels (family, genus, species). Thus, differences in taxonomy can be observed at the class level. Among the 31 identified classes, 15 were found to differ between sample types (Table 2). *Betaproteobacteria* comprised the more significant part of all proteobacterial sequences accounting for roughly 36.2% of sediment and 25.9% of water samples (Table 2, Figure B2).

Water sample communities were composed of Actinobacteria and Verrucomicrobia in higher proportions than found within sediment samples (Figure B1, Table B4). Members of the *Flavobacteriia* and *Chloroflexia* classes were also more abundant in the water column, though they made up lesser fractions of the community (Table 2, Figure B2).

Proteobacteria, Nitrospirae, and Acidobacteria were among the phyla recovered in higher proportions within the sediment samples (Figure B1, Table B4). Among these phyla, those belonging to *Blastocatellia* (p=0.001), *Nitrospira* (p=0.005), and *Betaproteobacteria* (p=0.001) were found to be significantly more abundant, as were organisms classified as *Anaerolineae* within phylum Chloroflexi (Table 2, Figure B2).

3.3.2 Incubation time had an effect on microbial water communities

Analysis of alpha diversity (p=0.146) and differential abundance within OTUs (p=0.175) in water samples revealed no significant differences between that of the samples exposed to sub-inhibitory concentrations of antibiotics and control samples (Figure B3). The alpha diversity of the water samples changed significantly over time (p<0.001), with the highest diversity at the final time-point (Figure 10a, Table B5). An overall increase in the number of OTUs was observed (p<0.001), with significant fluctuations occurring in the water communities over time (Figure 10b, Table B6). These trends were observed in both control and antibiotic amended water samples, indicating that the antibiotics were not the cause in these community shifts.

The presence or absence of antibiotics had no significant effect on the relative quantities of taxonomic groups within the water microbial communities (Figure 11, Table B7); however, taxonomic shifts were observed in the water samples relative to incubation time (Figure 12). A distinct decrease in *Cytophagia* (p=0.015) was observed within the first three days of incubation (Figure 12), while less pronounced fluctuations were also observed in *Spartobacteria*, *Acidimicrobiia*, and *Deltaproteobacteria* (Table B9).

3.3.3 Antibiotic amendment had little impact on sediment microbial communities

Alpha diversity did differ between antibiotic and control samples in the sediment communities (p=0.039), although the quantity of recovered OTUs between these samples did not differ (p=0.108) (Figure B4). The presence of sub-inhibitory concentrations of antibiotics appeared to increase the abundance of organisms assigned to the phylum Saccharibacteria (p=0.017), though this phylum only represented 0.07% and 0.35% of the control and antibiotic-amended communities, respectively (Table B11).

Changes in alpha diversity and taxonomy analyzed for the sediment control samples revealed no significant differences over time (p=0.821). However, antibiotic amended sediment samples changed in alpha diversity throughout the time series (Figure 10a, Table B10). It appears that the addition of antibiotics does affect sediment sample diversity during the first three days of incubation (Figure 10a). Despite significant shifts in alpha diversity among the antibiotic-amended sediment samples, no changes in community composition were observed in abundant taxonomic groups at the phylum or class level over time (Figure B5). The significant fluctuations in diversity could be due to changes in lower taxonomic levels undetectable at the sequencing resolution performed in this analysis.

3.4 Discussion

The principal findings of this study are as follows: 1) the microbial communities among stream water and the underlying sediment vary greatly; 2) the presence of sub-inhibitory concentrations of antibiotics do not affect water microbial communities, though changes were observed over the incubation period; and 3) changes were observed within the sediment communities upon antibiotic exposure and the exposed communities appeared to vary over time.

3.4.1 Water and sediment community variation

Discernible differences in alpha and beta diversity were observed between the bacterial communities found in the water column and sediment (Figure 9 & 10). The higher Shannon diversity found among the sediment communities has been previously described (Ye *et al.*, 2009; Chen *et al.*, 2016; Dai *et al.*, 2016, 2019). There is a growing consensus that sediment can sustain a greater number of bacterial taxa since this environment provides many microhabitats while also receiving organisms from the overlying water column,

resulting in an increased diversity (Zeng *et al.*, 2019). The predominance of organisms within bacterial phyla Proteobacteria, Actinobacteria, Bacteroidetes, and Verrucomicrobia, are typical of freshwater communities (Table B4), as are those of Proteobacteria, Acidobacteria, and Nitrospirae typical of sediment communities (Zwart *et al.*, 2002; Zhang *et al.*, 2015).

A total of 31 abundant classes (making up >1% in at least one sample) were identified, of which 15 were determined to significantly differ between the two sample types (Table 2). The hgcI clade made up a substantial portion of the water column community (Table B14), and the widespread presence of such *Actinobacteria* has been associated with reduced cell size and cell wall type (Pernthaler *et al.*, 2001; Hahn *et al.*, 2003; Tarao *et al.*, 2009). The hgcI clade is common and abundant in freshwater environments, and members of this clade have been found to metabolize carbohydrates and nitrogen-rich compounds, as well as utilize sunlight to promote carbon fixation (Warnecke *et al.*, 2004; Ghylin *et al.*, 2014; Tandon *et al.*, 2018). The Verrucomicrobia lineage, comprised of organisms with a diverse array of metabolic capabilities (Chiang *et al.*, 2018), was also more abundant in water samples (Table 2). The proportion of *Flavobacteriia* in the sediment was typically lower than that found in the water column, particularly that of *Fluviicola* sp. (p=0.004) and *Flavobacterium* sp. (p=0.027), which is consistent with previous findings (Dai *et al.*, 2016). Individuals of the phylum Nitrospirae were generally found in greater abundance in sediment (Dai *et al.*, 2016). *Nitrospira* sp. were among the prominent identified members of this phylum within the sediment (Table B14).

Numerous studies have indicated the predominance of Proteobacteria in freshwater and freshwater sediments and the large compositional shifts in the relative abundance of the major classes (Zhang *et al.*, 2015; Chen *et al.*, 2016; Dai *et al.*, 2016; Fang *et al.*, 2019). *Alpha-*, *Beta-*, and *Gammaproteobacteria* were prominent in both sample types with a sizable

concentration of *Deltaproteobacteria* in the sediment communities (Table 2). The prevalence of Proteobacteria within sediment communities indicates the phylum's active involvement in the functions of freshwater sediment ecosystems (Zhang *et al.*, 2015). Though sediment samples showed higher relative abundances of Proteobacteria, the genera within this phylum varied in abundance, an average of 78.1% of the sediment and 50.9% of the water communities could not be classified to the genus level; thus, deeper sequencing analyses should be performed to assess the specific microbes and functions performed within these closely tied communities.

3.4.2 Community shifts within the water column

The similarities in alpha diversity and community composition among antibiotic amended water samples and the analogous controls indicates that another factor is causing community changes over 14 days. Both control and antibiotic-treated communities presented typical freshwater bacterial communities consisting of a core microbiome composed of Actinobacteria, Proteobacteria, and Bacteroidetes. Similar core microbiomes have been observed previously from multiple distinct freshwater environments encompassing a wide range of physical and chemical conditions (Llirós *et al.*, 2014).

The duration of incubation periods did have a significant effect on the microbial community in the water column. The relative abundance of Nitrospirae, Planctomycetes, Proteobacteria, Saccharibacteria, and the candidate phylum TM6 (Dependentiae) increased in the water communities from the third day of incubation to the final, though only Proteobacteria were among the abundant taxa (Table B8). The changes in microbial communities observed irrespective of the antibiotic treatments could likely be attributed to the "bottle effect" wherein phenomena observed in studies of enclosed microbial

communities could be the consequence of bacterial confinement rather than experimental manipulation (Pernthaler and Amann, 2005).

Enclosure experiments, such as this, can introduce biases in microbial shifts, abundance, and processes compared to the equivalent communities observed *in situ* (Eilers *et al.*, 2000; Stewart *et al.*, 2012; Herlemann *et al.*, 2014). The separation of bacterial communities from the natural environment prevents the exchange of nutrients and organic matter and isolates the assemblage from other organisms ordinarily present in the environment (Herlemann *et al.*, 2019). Three phases of succession have been observed with enclosure experiments and are generally associated with the "bottle effect" (Pernthaler and Amann, 2005). The early phase is marked by an initial increase in microbial viability. In the middle phase, the growth of bacterivorous protists is observed along with a drop in bacterial cell numbers. In contrast, bacterial abundance has been shown to then increase again during the late phase, though the composition varies from the initial community, and likely includes less active organisms and those less susceptible to grazing (Schäfer *et al.*, 2000; Massana and Jürgens, 2003). These typical phases of succession were also observed in this study. Thus, changes in alpha diversity and community composition are likely results of the "bottle effect," rather than from exposure to sub-inhibitory concentrations of antibiotics.

3.4.3 Community shifts among treatment and control sediment samples

The community changes observed in the sediment samples when sub-inhibitory concentrations of antibiotics were introduced might result from the increased sorption of antibiotics to the sediment matrix, reducing the bioavailability of the compounds for degradation and promoting their persistence within the sediment (Clarke and Smith, 2011). Fluoroquinolones, sulfonamides, and tetracyclines, all included in this study, are strongly

adsorbed and can readily accumulate in sediment (Kümmerer, 2009). While it is not yet known at what concentrations these antibiotics are effective while adsorbed, or whether release from the sediment matrix occurs, such compounds can affect the resident microbial communities (Kümmerer, 2009). Though observed in low abundance, Saccharibacteria species, important in activated sludge treatment processes (Kindaichi *et al.*, 2016), were found to be significantly more abundant in the presence of antibiotics. Though taxonomic shifts were not observed in abundant groups at higher taxonomic levels, the fluctuations in diversity could be caused by genus or species-specific community shifts.

A possible explanation for the observed temporal trends in microbial diversity is the compositional shift often observed in closed incubation systems where the natural microbiota are overcome by opportunistic community members resulting in an increase in the heterotrophic microbes over time (Ionescu *et al.*, 2015). Regrettably, the poor resolution of 16S rRNA gene sequence identification at lower taxonomic levels makes it difficult to determine such trophic shifts. Additionally, 16S rRNA gene sequencing can only provide information on the residents of a community and little about the antibiotic resistance profiles among that community. Differences in the microbial sediment communities might be the result of antibiotic amendment; however, we cannot definitively attribute these changes to this without additional sequencing methods.

3.5 Conclusion

This study was unable to ascertain definitive evidence of short- or long-term effects of sub-inhibitory antibiotic concentrations on bacterial communities and their interactions. The development of *in situ* methods to conduct similar studies would provide more reliable depictions of community changes upon sub-inhibitory antibiotic exposure as the issues

arising from the "bottling effect," and artificial growth conditions are eliminated. Increasing evidence shows the effective removal of antibiotic-resistant organisms in wastewater treatment, but not of the actual antibiotic compounds. Studies, such as this, to determine the effect of antibiotics, alone, on ARB communities is essential for assessment of environmental and human health risks posed by these compounds.

3.6 Tables

Table 4 | Relative abundance of bacterial classes among water and sediment samples. Taxa with OTU clusters found in <25% of all samples were excluded from analysis.

Phylum	Class	Water	Sediment		*p* value
Acidobacteria	*Acidobacteria*	0.00%	0.10%		
	Blastocatellia	0.01%	1.71%	Sediment	0.001
	Subgroup 22	0.00%	1.23%	Sediment	0.004
	Subgroup 6	0.15%	1.56%		n.s.
Actinobacteria	*Acidimicrobiia*	0.30%	0.68%		n.s.
	Actinobacteria	24.65%	1.18%	Water	0.002
	Thermoleophilia	0.00%	0.29%		
Armatimonadetes	*Armatimonadia*	1.51%	0.00%		
Bacteroidetes	*Cytophagia*	4.64%	0.30%		n.s.
	Flavobacteriia	2.67%	0.08%	Water	0.001
	Sphingobacteriia	9.46%	11.65%		n.s.
Chlorobi	*Chlorobia*	0.00%	0.69%		
Chloroflexi	*Anaerolineae*	0.02%	3.51%	Sediment	0.021
	Chloroflexia	1.12%	0.00%	Water	0.0001
	KD4-96	0.00%	0.45%	Sediment	0.024
Firmicutes	*Clostridia*	0.09%	0.31%		
Gemmatimonadetes	*Gemmatimonadetes*	0.01%	2.08%	Sediment	0.019
Ignavibacteriae	*Ignavibacteria*	0.00%	2.16%	Sediment	0.001
Nitrospirae	*Nitrospira*	0.16%	5.54%	Sediment	0.005
Planctomycetes	OM190	0.21%	0.47%	Sediment	0.004
	Planctomycetacia	0.16%	0.18%		n.s.
Proteobacteria	*Alphaproteobacteria*	7.91%	6.79%		n.s.
	Betaproteobacteria	25.95%	36.23%	Sediment	0.001
	Deltaproteobacteria	0.52%	4.23%		n.s.
	Gammaproteobacteria	3.49%	8.88%		n.s.
	SPOTSOCT00m83	0.19%	0.27%		n.s.
Verrucomicrobia	OPB35 soil group	1.78%	3.11%		n.s.
	Opitutae	2.38%	0.68%	Water	0.021
	Spartobacteria	1.74%	0.49%	Water	0.027
	Verrucomicrobiae	4.31%	0.02%	Water	0.001
	Unassigned	5.11%	2.29%		
	Other	1.44%	2.84%		

n.s. – not significant

3.7 Figures

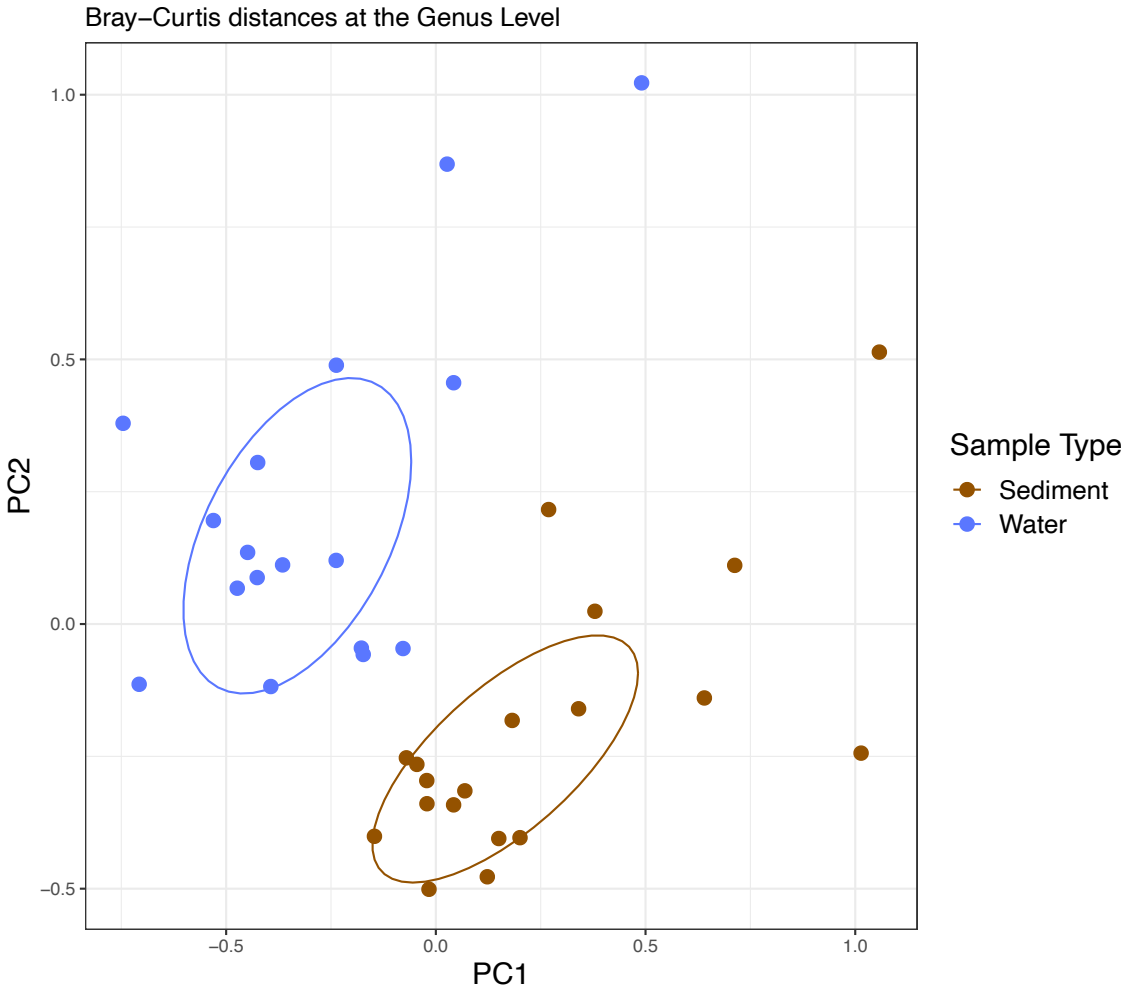

Figure 9 | Multi-dimensional scaling analysis based on Bray-Curtis dissimilarity between sediment and water communities. A clear clustering pattern appeared between communities from the two sample types (p<0.001).

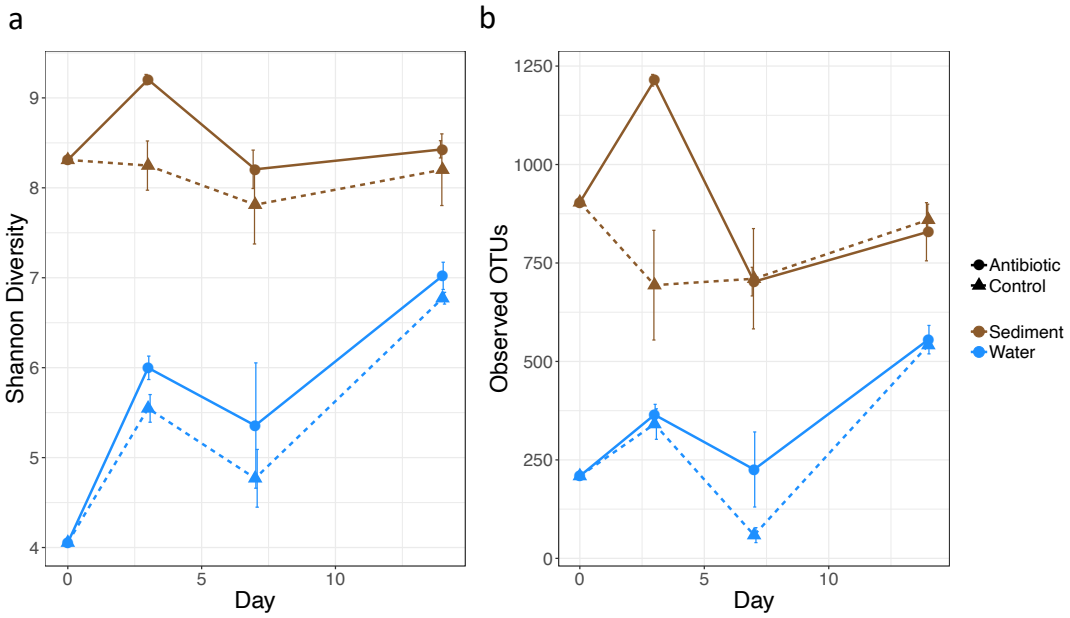

Figure 10 | Average (a) Shannon diversity and (b) number of observed OTUs recovered from antibiotic amended and control samples over 14 days.

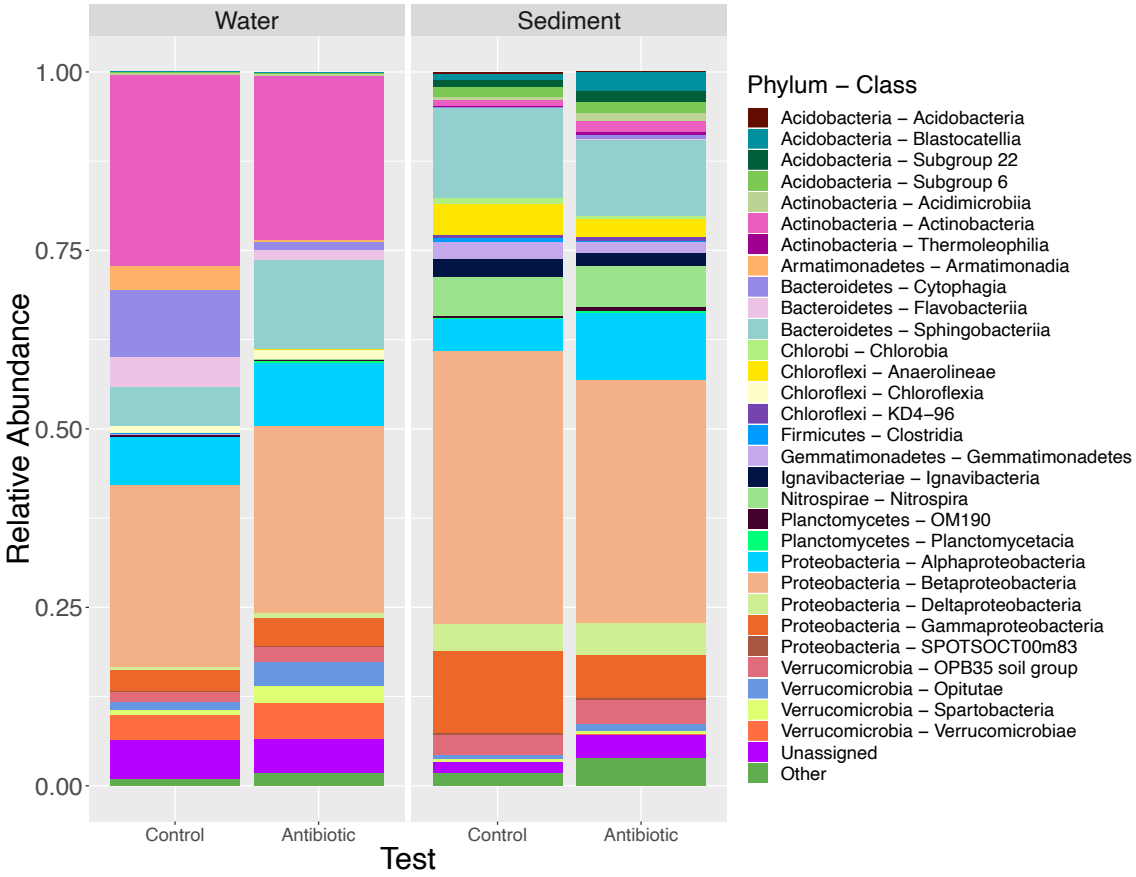

Figure 11 | Relative abundance of OTU clusters classified at the class level for water and sediment samples under control and antibiotic amended conditions.

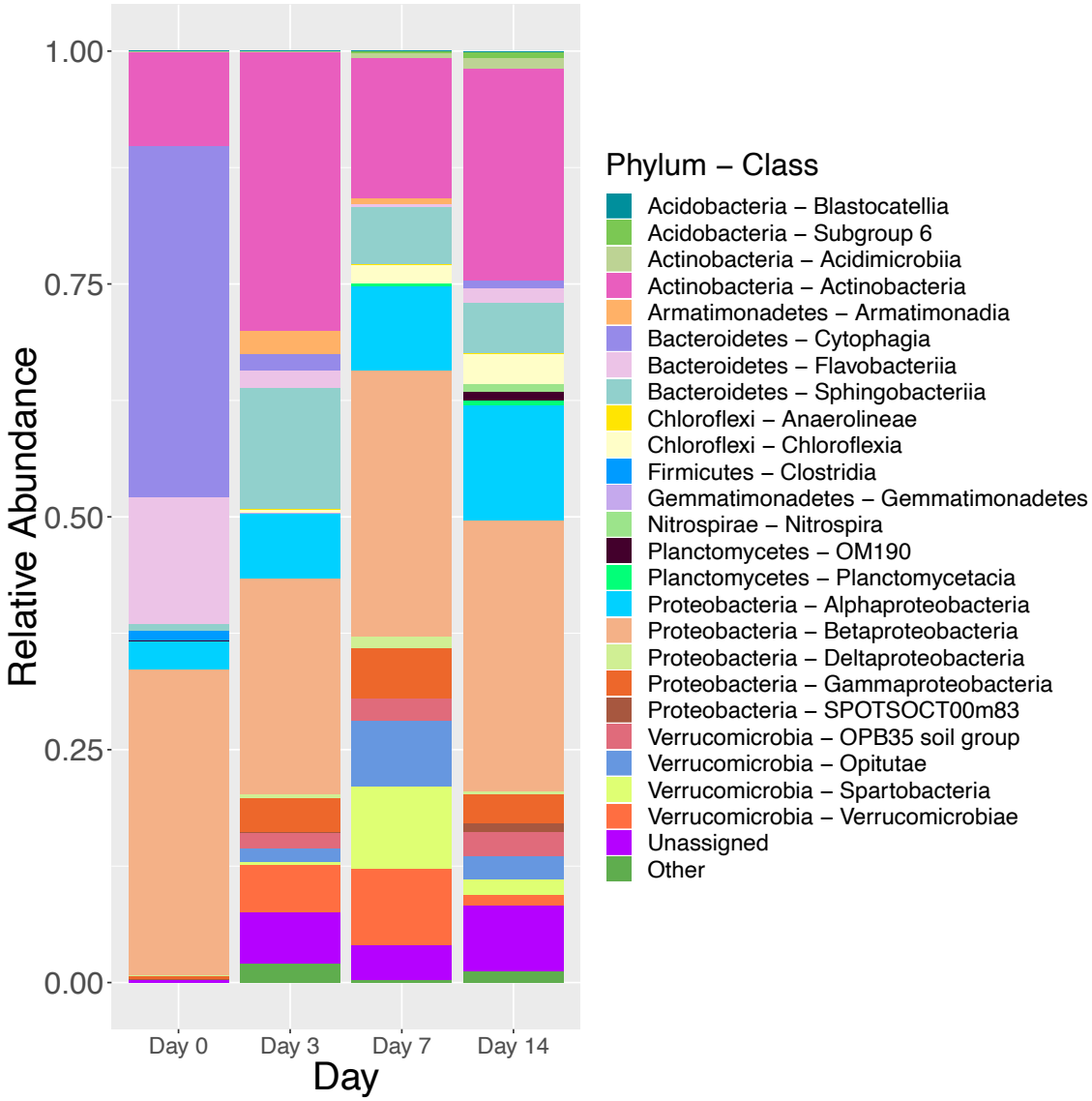

Figure 12 | Changes in relative abundance of OTUs clustered into bacterial classes within water samples over a two-week time course.

CHAPTER 4: Determining fitness costs of multi-drug resistant bacteria under antibiotic-free stress conditions

4.1 Rationale

The evolution of multi-drug resistance (MDR) has become a global issue. Antibiotic resistance has imposed a considerable strain on the human population as it has developed in pathogens, causing infections that cannot be treated with current antibiotics. There is a clear cost to human health in the development of resistance, but there are also costs to the bacteria harboring these resistance traits. While some bacterial strains possess structurally and functionally intrinsic resistance, resistance is also commonly conferred by the acquisition of antibiotic resistance genes (ARG) via associated plasmids and transposons through horizontal gene transfer (HGT) or spontaneous mutations in chromosomal DNA (Rice, 1998; Hegstad *et al.*, 2010; Hollenbeck and Rice, 2012). Bacteria in possession of acquired resistance may have a selective advantage over susceptible strains when in the presence of antibiotics, but they often have associated reductions in fitness in antibiotic-free environments (Andersson and Levin, 1999).

When in the absence of antibiotics, resistant genotypes often have lower growth rates than their susceptible counterparts in the same ecological niche (Melnyk *et al.*, 2015). Mutations conferring resistance may do so by disrupting the normal physiological processes of the cell, which can lead to detrimental side effects (Lenski, 1998). Once acquired, resistance genes can impose an energetic burden upon the cell, as additional synthesis of nucleic acids and proteins is required for function. These gene products can also interfere with normal cell physiology (Lenski, 1998). Depending on the fitness costs, resistant bacteria can become less fit than sensitive phenotypes in the absence of antibiotic selective pressures (Williams and Heymann, 1998; Andersson and Levin, 1999; Trindade *et al.*, 2009).

Despite the fitness costs to antibiotic-resistant bacteria (ARB), other investigators have found that these resistant organisms are capable of partially or wholly restoring their fitness through the accumulation of compensatory mutations and the co-selection of genetically linked genes, without compromising their resistance (Andersson and Levin, 1999; Borrell *et al.*, 2013; Melnyk *et al.*, 2015). However, the fitness costs and evolution of compensatory mutations depend on the specific resistance mutations and their interactions with other alleles, and the environmental conditions to which strains are subjected (Björkman and Andersson, 2000). The evolution of resistance to several antibiotic drugs depends on the fitness costs of harboring multiple resistance mutations and the interactions between each mutation, or epistasis (Trindade *et al.*, 2009). Epistasis occurs when the phenotypic effect of one mutation is dependent on the genetic makeup or mutations present at other loci (Borrell *et al.*, 2013; Melnyk *et al.*, 2015). As antibiotic resistance genes are known to affect bacterial fitness, the epistasis among these genes can determine the evolution of multi-drug resistance, sometimes affording MDR organisms higher fitness than strains resistant to only one drug (Weinreich *et al.*, 2005). While negative epistasis against drug resistance would result in the counter-selection of multi-drug resistant microbes in the absence of antibiotics, an alarming rate of positive epistasis between resistance mutations has driven the development of multi-drug resistance (Trindade *et al.*, 2009).

This study is designed to assess the *in vitro* fitness costs of MDR bacteria compared to strains with fewer resistance genes. Three strains of *Enterococcus faecium* were isolated from hospital and residential sewage lines routed to wastewater treatment plants (WWTP) in Charlotte, North Carolina. All three strains were isolated from communities grown on lysogeny broth (LB) agar amended with sulfamethoxazole (1000 μg/mL) and exhibited varying resistant phenotypes. Enterococci are Gram-positive, facultatively anaerobic cocci

known for their versatility and ability to survive under harsh conditions (Arias and Murray, 2012). Growth condition tolerances of enterococcal species include a broad temperature range (from 10°C to over 45°C), a pH range from 4.8 to 9.6, and the ability to survive in 6.5% NaCl solutions (Sherman *et al.*, 1937; Thammavongs *et al.*, 1996; Devriese *et al.*, 2006; Gaca and Lemos, 2019). The malleable genomes of *E. faecium* can readily accumulate mutations and exogenous genes conferring antibiotic resistance, with up to 25% of this enterococcal genome being made up of acquired elements (Hegstad *et al.*, 2010; Palmer *et al.*, 2010; van Schaik *et al.*, 2010). *E. faecium* is now a common cause of nosocomial infections, and given its ability to readily adapt to harsh conditions and accumulate resistance genes, the increasing difficulty in effectively treating this species is becoming of utmost clinical importance (Hidron *et al.*, 2008).

Microbial fitness of the isolated *E. faecium* strains in this study was evaluated by individually subjecting each strain to a mild, long-term stress condition that might be experienced naturally. Most studies on the fitness costs of antibiotic resistance achieve this by the experimental creation of mutant strains via transformation from an original parent strain, and often only focus on the fitness costs of one resistance gene. We aim to examine these same fitness costs, using naturally evolved multi-resistant phenotypes. Isolation of environmentally cultured bacteria from WWTPs subjected to constant sub-inhibitory concentrations of antibiotics takes into account the natural development of multi-drug resistance within the wastewater treatment process.

4.2 Methods and Materials

4.2.1 Sample collection and growth conditions

Sewage samples were collected according to the sampling methods described in Section 2.2.3. The growth of heterotrophic organisms was performed as per the conditions described in Section 2.2.5. Colonies were randomly selected from growth on antibiotic amended agar, and the selected colonies were cultured to isolation per standard bacterial isolation methods. All three strains of *E. faecium* were recovered from growth on sulfamethoxazole-amended LB agar at 37°C.

4.2.2 Antibiotic susceptibility testing

Antibiotic susceptibility of all three strains was tested against seven antibiotics via disc diffusion and minimum inhibitory concentration (MIC) testing for nine additional antibiotics on Mueller-Hinton (MH) agar. The antimicrobial susceptibility discs (Oxoid™) tested were ampicillin (10 μg), ciprofloxacin (5 μg), doxycycline (5 μg), vancomycin (30 μg), clindamycin (2 μg), azithromycin (15 μg), and sulfamethoxazole (25 μg), each representing a commonly prescribed member of a major antibiotic class. Briefly, inoculum suspensions of each isolate were grown overnight in LB broth and then adjusted to 0.5 McFarland turbidity in sterile deionized (DI) water before being spread onto MH agar. The antimicrobial discs were aseptically placed onto the cultures, and after 24-hour incubations, the diameter of the zone of inhibition surrounding the antimicrobial discs was measured. Minimum inhibitory concentrations were also determined for the previously mentioned antibiotics and nine additional antibiotics.

Chloramphenicol, erythromycin, three aminoglycosides (kanamycin, neomycin, and streptomycin), and four additional β-lactams of various classes (ertapenem, amoxicillin, ceftriaxone, and cephalexin) were used to determine MICs of the three isolates. Diluted

suspensions were drop-plated on to MH agar amended with individual antibiotics to concentrations ranging from 0.25-128 μg/mL. Resistance to all antibiotics was determined by interpretation of the inhibition zone diameter and MICs according to the Clinical and Laboratory Standards Institute's (CLSI) performance standards for antimicrobial susceptibility testing and, where not possible, according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoint tables (European Committee on Antimicrobial Susceptibility Testing, 2020; EM100 Connect - CLSI M100 ED30:2020, 2020).

4.2.3 Identification of isolated organisms

Initial 16S ribosomal RNA (rRNA) gene sequencing of the isolated organisms was performed by Eurofins (Luxembourg) upon DNA extractions using the MO BIO UltraClean® Microbial DNA Isolate Kit. Sequence analysis was conducted via ChromasPro sequence analysis software, and species identification was conducted using the National Center for Biotechnology Information (NCBI) nucleotide Basic Local Alignment Search Tool (BLASTn) and identifications were made at a 98% sequence identity.

4.2.4 DNA extraction and whole-genome sequencing

Genomic DNA from each strain was extracted, as described in Section 4.2.3. Quality control testing, library preparation, and sequencing were conducted by Omega Bioservices (Norcross, Georgia, USA). Each sample was sequenced with 300 base pair (bp) paired-end reads on an Illumina® MiSeq flow cell. Adapter and quality trimming were performed with Trimmomatic (version 0.39), and *de novo* genome assembly was conducted using SPAdes (version 3.11.1). Isolate identities were again confirmed via *in silico* ribosomal multi-locus sequence typing (rMLST) by indexing variations of 52 genes encoding for bacterial ribosome

protein subunits (*rps* genes) using the Bacterial Isolate Genome Sequence Database (BIGSdb) and PubMLST (Jolley and Maiden, 2010; Jolley *et al.*, 2012). Additional multi-locus sequence typing (MLST) was then performed using Galaxy (version 2.19.0) on the assembled genomes based on seven *E. faecium* housekeeping genes, *atpA*, *ddl*, *ghd*, *purK*, *gyd*, *pstS*, and *adk*, for clonal typing (Seemann, 2016).

4.2.5 Identification of E. faecium orthologous genes

In addition to the three isolates recovered in this study, nucleotide sequences from 30 *E. faecium* genomes were retrieved from Genbank (http://www.ncbi.nlm.nih.gov/GenBank/) in March of 2020 (Table C1). These reference genomes were chosen based on the in-depth analysis performed by Kim and Marco (2014). Prokka (version 1.13) was used for open reading frame (ORF) prediction, translation, and annotation of all isolates (Seeman, 2014).

Roary (version 3.11.2) was utilized for the pan-genome construction of the 33 isolates and identification of (Page *et al.*, 2015). This pipeline utilizes Prokka-generated GFF files where coding regions are extracted and converted to protein sequences. These sequences are then filtered to remove any partial sequences before iterative pre-clustering is performed with CD-HIT (Fu *et al., 2012).* An all-against-all comparison is performed on these filtered sequences using BLASTp at a 95% sequence identity threshold. The sequences are then clustered with the Markov cluster (MCL) algorithm and merged with the pre-clustered CD-HIT results (Enright *et al.*, 2002). Homologous clusters are then split into groups of true orthologous protein-coding sequences (CDS) using conserved gene neighborhood information.

4.2.6 Hierarchical isolate clustering

Hierarchical isolate clustering was conducted using the presence or absence of orthologous genes in each *E. faecium* genome using the Euclidean distance method employed in the R package (R Core Team, 2018). The assignment of the three *E. faecium* isolates into non-clinical (NC) or clinical (CL) clades was statistically examined using the R package, Pvclust, with 10,000 bootstrap resamplings (Suzuki and Shimodaira, 2006).

4.2.7 Determination of stress response and resistance genes

Antibiotic resistance genes were detected through the combined use of the Resistance Gene Identifier (RGI; version 5.1.0) feature within the Comprehensive Antibiotic Resistance Database (CARD; version 3.0.8) and ResFinder v3.2 (Zankari *et al.*, 2012; Alcock *et al.*, 2020). Virulence factors were identified using VirulenceFinder v2.0 (Joensen *et al.*, 2014), and plasmid identification was performed using PlasmidFinder v2.0 (Carattoli *et al.*, 2014). For the identification of stress response genes, multiple local BLAST databases were built from over 500 protein sequences from the Uniprot database of Bacilli matching each of the key terms: acid resistance, osmoprotectant, cold shock, heat shock, and oxidative stress. Sequences were then blasted against these local databases using the protein BLAST algorithm (BLASTp).

4.2.8 Stress tolerance assays

Stress tolerance was measured individually for each strain. The *E. faecium* inoculum suspensions were prepared according to the protocol described in Section 4.2.2. Cultures were diluted to ~10_9 colony forming units (CFU) per mL and subjected to individual stress conditions. Stress tolerance assays were performed according to procedures outlined in similar studies conducted by Ferreira *et al.* (2018) and Ma *et al.* (2019) with modifications.

Strains were subjected to oxidative stress upon exposure of 1 mL of the diluted suspensions to 9mL of hydrogen peroxide at a 3 mM concentration for 30 minutes at under atmospheric conditions. Similarly, 1 mL of each suspension was placed in 9 mL of a 0.01 N hydrochloric acid (HCl) solution at a pH 2.0 for 30 minutes to test the microbial acid tolerance. Salt tolerance was assessed with the inoculation of 1 mL of each strain into 9 mL of a 30% w/w sodium chloride (NaCl) solution for 48 hours. Temperature stress was assessed by adding 1 mL culture dilutions to 9 mL sterile DI water and placed in a 55°C water bath or ice water (~4°C) for 30 minutes. Control samples were prepared by inoculating 1 mL aliquots of each strain into 9 mL sterile DI water and incubated for the same duration as the stress assays under atmospheric conditions.

The plate count method was used for quantification of total and stress survival cultivability. Each sample was serially diluted in sterile DI water to achieve a quantifiable concentration of CFUs. Then, 0.1 mL of the diluted solutions were plated onto LB agar and incubated at 35°C for 24 hours. Colonies were then visually quantified and expressed as log10 CFU/mL reduction for statistical measurements of stress survival for each strain.

4.2.9 Statistical analysis

All experiments were carried out in triplicate. Statistical differences in stress survival were determined for each strain using a Welch two-sample t-test carried out with the *t.test* function in R. Pearson tests were conducted to determine the correlations among various genome characteristics.

4.3 Results

4.3.1 General features of E. faecium genomes

Initial BLAST analysis of the 16S rRNA gene sequences identified the three isolates, named Ef75, Ef76, and Ef77 as *E. faecium* (99%, 99%, and 98% confidence). Subsequent rMLST analysis verified the identifications with 100% confidence. Orthologous gene clustering based on sequence similarity identified 8,787 CDS orthologs among the 33 *E. faecium* genomes (Table C2). Approximately 10.5% (923 CDS) of the orthologous genes identified in the pan-genome were designated with a gene name. Of the 8,787 orthologs, 1,208 were identified as core genes (13.8%), and the genomes averaged to 2,825.3 ± 119.2 kilobases (kb) (Table 3).

The presence/absence of orthologous CDSs was used to determine if distinct lineages could be observed among the environmental/community-associated isolates and those from nosocomial infections. Of the 30 reference *E. faecium* genomes used for comparison (Table C1), ten came from non-clinical settings, and 20 strains were isolated from clinically infected human blood and tissue (Rice *et al.*, 2009). Hierarchical clustering of the 8,787 CDS orthologs revealed that NC isolates were grouped into two sub-clades (NC1 and NC2), as observed in a 2014 study conducted by Kim and Marco. Isolate Ef75 was clustered among the NC1 clade, while Ef76 and Ef77 were more closely related within the NC2 clade (Figure 13). Despite the phylogenetic separation between the NC and CL strains, two clinical strains (1,141,733 and 1,231,501) were found to cluster amongst the NC strains, as observed in previous studies (Arias *et al.*, 2009; Qin *et al.*, 2012).

On average, CL strains had genomes 132.8 kb larger than those isolated from NC settings (p=0.001). The CL genomes had roughly 154 more genes, and these gene quantities positively correlated with total genome size (r =0.97, p<0.001) (Figure 14a). Attributing to

these larger genomes were a higher number of genes coding for antibiotic resistance (p=0.004) among the CL strains (Figure 14b and Table C4). The number of virulence factors (VF) was found to be higher in the CL isolates, with 25% of these isolates harboring genes encoding for an enterococcal surface protein (*espfm*) and a putative glycosyl hydrolase (*hylEfm)* in addition to the commonly observed adhesins, *acm* and *efaAfm,* found in NC and CL strains (Table C5). The number of plasmids did not differ between the NC and CL isolates.

4.3.2 General characteristics of the cultured E. faecium isolates

Genome analysis of the cultured isolates from this study, Ef75, Ef76, and Ef77, determined total genome sizes to be approximately 2,795.7, 2,762.0, and 2,915.0 kb, respectively (Table 4). Isolate Ef75 was classified as ST1442 according to MLST analysis. The G+C content of Ef75 was roughly 38.1%, and 2,651 CDS were detected in this isolate, along with three plasmids and two virulence factors, *acm* and *efaAfm* (Table C6). A definitive clonal type could not be assigned to Ef76; however, MLST identified this isolate as either ST1527 or ST1567 (Table 4). This isolate was found to harbor five plasmids, yet only one VF (*efaAfm*), and a G+C content of 37.9%. Approximately 2,624 CDS were detected in Ef76. Accompanying the larger genome size of Ef77, this isolate also had a higher number of CDS (2,741) and was classified as clonal type ST841 with 37.9% G+C content. The VF *efaAfm* was also identified in this isolate's genome, along with four plasmids.

4.3.3 Minimum inhibitory concentrations

Upon identification of the isolates as *E. faecium*, minimum inhibitory concentrations were used to determine resistance profiles for each strain (Table C7). According to standards set in place by EUCAST and CLSI, all isolates exhibited resistance to amoxicillin and

clindamycin, in addition to the intrinsic resistance afforded to enterococci against aminoglycosides, cephalosporins, and sulfonamides (Table 5). Ef75 also showed intermediate resistance to erythromycin but was susceptible to the remaining compounds (Table 5). Ef76 showed additional resistance to erythromycin and intermediate tolerance to chloramphenicol with susceptibility to ampicillin, ciprofloxacin, doxycycline, and vancomycin. Ef77 showed the highest level of antibiotic resistance with susceptibility only to ampicillin and vancomycin.

4.3.4 Characterization of resistance genes among strains

Overall the number of ARGs was higher in CL isolates than in NC strains (p=0.004), and the quantity of these resistance genes was positively correlated with total genome size (r =0.46, p=0.007) (Figure 14b). Among the 33 isolates, the number of ARGs detected in individual isolates ranged from 3 to 16, with 63.6% of isolates harboring at least five resistant genes. Eight aminoglycoside-modifying enzymes (AME) were identified, with *aac(6')-Ii* (n = 33), *ant(6)-Ia* (n = 15), *aph(3')-III* (n = 14), and *sat4* (n = 14) being the most common (Table C4). The AME, *aac(6')-Ie-aph(2")-Ia* (n = 11), was found exclusively in strains of clinical origin (Table C4). Nine macrolide-lincosamide-streptogramin (MLS) resistance genes were detected, including *lsaE*, *lnuB*, and *vatD*. Three of these MLS resistance genes were especially predominant: *msrC* (n=33), *ermB* (n = 18) and *eatAv* (n = 17) (Table C4). Nineteen isolates carried at least one tetracycline resistance gene. The six *tet* genes identified were primarily recovered from CL isolates, with *tetM* (n = 12) being the most prevalent (Table C4). Trimethoprim-sulfamethoxazole gene homologs, *dfrF* and *dfrG*, were detected primarily in clinical strains (Table C4). A multi-drug efflux pump gene, *efmA,* was found in 26 isolates, with a presence in 90% of the clinical isolates.

The three isolates tested in this study all harbored at least three resistance genes at a sequence identity threshold of 95% or higher (Table 6). These three core genes include the AME, *aac(6')-Ii*, which encodes for a chromosomal aminoglycoside acetyltransferase inherent to *Enterococcus* spp., a chromosomally-encoded ATP-binding cassette (ABC) subfamily protein encoded by *msrC*, and the *eatA$_v$* gene, a mutated form of the wildtype ABC-F subfamily protein (Alcock *et al.*, 2020). The *eatA$_v$* gene confers resistance to several antibiotics, including lincosamides, macrolides, phenols, and tetracyclines through the protection of the antibiotic's ribosomal target and the subsequent prevention antibiotic binding (Draker *et al.*, 2003; Isnard *et al.*, 2013).

Ef76 and Ef77 also possessed 100% sequence identity matches to the major facilitator superfamily (MFS) transporter permease antibiotic efflux pump encoded by *efmA* (Table 6). This efflux pump complex is active against lincosamides and fluoroquinolones, among many other antibiotic classes (Nishioka *et al.*, 2009). Ef77 was found to have acquired four additional resistance genes, *catA8*, *tetL*, *tetM*, and *ermB*. The *catA8* gene product is responsible for the inactivation of phenicol antibiotic compounds, while ErmB and TetL/TetM offer protection and modifications to the cellular antibiotic targets against MLS and tetracyclines, respectively (Alcock *et al.*, 2020). All three tested isolates showed similar resistance genotypes to the other NC strains analyzed.

4.3.5 Stress tolerance and identification of stress response genes

Stress tolerance was evaluated by the enumeration of cultivable isolates upon stress exposure. All isolates showed significant growth inhibition with acid and salinity stressors (Table 7). Salinity stress resulted in growth reductions over 25% in all isolates, with Ef77 showing the lowest reductions in survival (Figure 15a). Regarding osmotic stress

mechanisms, each isolate encoded for components of a transport system for the cellular uptake of glycine-betaine, an osmoprotective quaternary amine (Table 8) (Gaca and Lemos, 2019). Exposure to 0.01 N HCl caused the most significant decrease in survival with logarithmic reductions of 3.06, 3.19, and 3.87 in Ef75, Ef76, and Ef77, respectively (Figure 15b and Table 7). Acid-resistance membrane proteins were identified in all samples, as were cytoplasmic buffering enzymes associated with acid tolerance (Table 8). Arginine deiminase and carbamate kinase, involved in the catabolism of arginine to counter cytoplasmic acidification, were encoded in all isolates. An additional deiminase was also identified in Ef77 for the catabolism of the decarboxylated derivative of arginine, agmatine.

Heat, cold, and oxidative stresses appeared to have no impact on the survival and growth rate of the three enterococcal isolates (Figure 15c, d, e). All isolates encoded for molecular chaperone and protease heat shock proteins (HSP) to assist in correct protein assembly and for the degradation of denatured proteins, including major chaperones, DnaK and GroL, and ATP-dependent proteases within the Clp regulon (Table 8). The three strains of enterococci each coded for two types of major cold shock proteins (CSP) identified as CspA and CspE, along with an additional CSP, CspC. Several enzymes of varying functions associated with oxidative stress responses were encoded in each isolate's genome. An NADH peroxidase (Npr) and several thiol peroxidases were identified in each isolate, along with other molecular conversion enzymes, such as superoxide dismutase and methionine sulfoxide reductase. Several transcriptional regulators associated with the regulation of oxidative stress genes were identified as well.

4.4 Discussion

Previous studies have demonstrated that many resistance mutations confer a fitness cost to the bacterial host (Melnyk *et al.*, 2015). However, fitness costs are highly variable depending on the species and the antibiotic. In this study, three isolates originating from residential (Ef75 and Ef76) and hospital (Ef77) sewage lines were collected and identified as *E. faecium* with varying antibiotic resistance profiles. Phylogenetic analysis of the tested *E. faecium* isolates with ten non-clinical and 20 clinical strains revealed that the three isolates clustered among the NC strains (Figure 13) and is supported by the similarities in the NC genome, VF, and ARG profiles. The differing resistance phenotypes of each isolate indicate the presence and absence of acquired resistance genes; thus, this study assesses the fitness costs of naturally acquired ARGs among the three environmental isolates.

4.4.1 Antibiotic resistance

As all enterococci are known to be intrinsically resistant to aminoglycosides, cephalosporins, and sulfonamides, resistance to antibiotics within these classes was to be expected, along with the associated intrinsic genes, *aac(6')-Ii* and *msrC* (Table 6). As enterococci inherently limit the penetrance of aminoglycosides into the cell (Gaca and Lemos, 2019), *aac(6')-Ii* offers additional protection from aminoglycosides by modifying the amino and hydroxyl groups of the drug, inhibiting its binding affinity to the bacterial ribosome (Mingeot-Leclercq *et al.*, 1999). The *E. faecium* specific *msrC* gene is known to provide some protection against macrolides and can be observed in the intermediate (Ef75) and resistant (Ef76 and Ef77) phenotypes found in this study (Singh *et al.*, 2001).

In addition to these two intrinsic ARGs, the three tested isolates also harbored a mutated allelic variant of the intrinsic *eatA* (for *Enterococcus* ABC transporter) gene, encoding

for an ABC homolog with 66% amino acid sequence identity to the intrinsic *E. faecalis*-specific LsaA protein conferring MLS resistance (Isnard *et al.*, 2013). This variant, named *eatA*$_v$, is thought to be responsible for resistance to lincosamides, streptogramin A, and pleuromutilins (LS$_A$P phenotype), though no changes in macrolide MICs have been attributed to this variant (Isnard *et al.*, 2013). While streptogramin and pleuromutilin resistance were not assessed in this study, lincosamide resistance was observed in each of the three tested isolates supporting the LS$_A$P phenotype afforded by this mutated gene.

A homolog (86% sequence similarity) of the *Lactococcus lactis* multi-drug efflux pump, MdtA, was also identified in Ef76 and Ef77 isolates (Perreten *et al.*, 2001). This MFS homolog is designated EfmA (for *E. faecium* multi-drug resistance) and has been shown to increase the MICs of macrolides and fluoroquinolones when present in the genomes of Gram-positive organisms (Nishioka *et al.*, 2009). The presence of *efmA* in these isolates is consistent with the macrolide (Ef76 and Ef77) and ciprofloxacin (Ef77) resistant phenotypes observed. Ef77 harbored an additional MLS resistance gene (*ermB*), tetracycline resistance genes *tetL* and *tetM,* and a chloramphenicol acetyltransferase (*catA8*). Consequently, this isolate was found to exhibit resistant phenotypes to chloramphenicol and the tetracycline, doxycycline.

The isolate designated Ef75 was found to be most susceptible to the antibiotics selected, showing resistance only to amoxicillin and clindamycin, as did the other tested isolates. Ef76 showed resistance to macrolides, while Ef77 showed the highest levels of multi-drug resistance, being susceptible only to ampicillin and vancomycin. It has been well documented that the acquisition of ARGs can hinder the fitness and survival of the bacterium in the absence of antibiotics (Andersson and Levin, 1999; Gagneux, 2006; Shcherbakov *et al.*, 2010; Borrell *et al.*, 2013). Analysis of heat, cold, osmotic, oxidative, and

acid stressors on each isolate was performed to quantify the relative changes in individual fitness in relation to the quantity of such acquired genes.

4.4.2 Response to heat, cold, and oxidative stresses

Analysis of individual stressors upon the isolates revealed similar trends in relative fitness regardless of the quantity of acquired ARGs. Heat, cold, and oxidative stress did not result in any decreases in fitness among Ef75, Ef76, or Ef77 despite the differing quantities of acquired ARGs. However, growth inhibition was observed with acid and salinity stressors; yet, reductions in survival were uniform independent of ARG numbers (Table 7).

Enterococci are known to endogenously produce reactive oxygen species (ROS), including H_2O_2 and O_2-; thus, these organisms must possess strong antioxidant capabilities and redox-sensing regulation for the control of these antioxidant pathways (Huycke *et al.*, 2001, 2002). Such protective mechanisms include the degradation of ROS and the protection and repair of solvent-exposed iron-sulfur (Fe-S) clusters that seem to be the primary targets of ROS (Imlay, 2008). Several classes of enzymes dedicated to oxidative stress protection and repair, including ROS degrading catalases/peroxidases and methionine sulfoxide reductase for Fe-S cluster repair, were observed in the genomes of the stress-tested *E. faecium* isolates (Table 8). One copy of a manganese-containing catalase and an NADH peroxidase were encoded in each isolate's genome to catalyze the dismutation of H_2O_2 to H_20 and O_2 and to reduce H_2O_2 to H_2O, respectively (Gaca and Lemos, 2019). Though it is thought that Npr's primary function is to degrade endogenous H_2O_2, this enzyme is likely also utilized for protection against exogenous sources (Seaver and Imlay, 2001). Several known enterococcal thiol peroxidases were also among the ROS degrading enzymes in the three isolates, including an alkyl hydroperoxidase (AhpCF) complex and organic hydroperoxidase

resistance (Ohr) proteins (Rincé *et al.*, 2001; La Carbona *et al.*, 2007). As methionine is one of the most sensitive amino acids to oxidation, this stress results in the formation of methionine sulfoxide (MetSO) residues, which, if left unrepaired in a protein, can cause changes in hydrophobicity and conformation, eventually rendering the protein inactive (Sharov *et al.*, 1999; Grimaud *et al.*, 2001; Stadtman *et al.*, 2003). The presence of several oxidation-associated transcriptional regulators (Table 8) and the associated enzymes, provides an arsenal of protection for enterococci (Schell, 1993; Zuber, 2004; Ravcheev *et al.*, 2012). As observed in this study, innate oxidative stress pathways are not inhibited by the acquisition of additional ARGs, enabling the organism to recover from such stress (Figure 15e).

Enterococci are known to grow in temperatures ranging 10 to 45°C and have been found to survive short exposures to temperatures as high as 62°C (Sherman *et al.*, 1937; Boutibonnes *et al.*, 1993; Thammavongs *et al.*, 1996). Coinciding with survival at such a wide temperature range is the synthesis of HSPs and CSPs (Table 8). The survival of the three *E. faecium* isolates at 55°C can be attributed to the synthesis of two major classes of HSPs: molecular chaperone/chaperonins that aid in the correct folding and assembly of nascent and partially denatured proteins, and ATP-dependent proteases for the degradation of permanently impaired proteins (Gaca and Lemos, 2019). The joint action of these HSP classes is essential for a cell to cope with aggregated and misfolded proteins brought on by thermal stress (Gottesman *et al.*, 1997). Two prominent molecular chaperones, DnaK and GroL, and their associated co-chaperones were identified in the tested isolates, and the associated operons are known to be strongly induced with increased temperatures (Silva Laport *et al.*, 2003; Laport *et al.*, 2004). Proteases within the Clp operon were also among the

identified HSPs in the enterococcal isolates along with an additional two-component protease, HslUV, associated with thermotolerance (Frees *et al.*, 2005; Grandvalet *et al.*, 2005).

Shifts in temperatures below the permissive growth range can inflict physiological changes to enterococci, including increased stability of the secondary structure of DNA and RNA that decreases transcription/translation efficiency (Inouye and Phadtare, 2004). In order to combat these physiologic issues, cells are known to induce CSPs in response to temperature downshifts. Though the identities and actions of many CSPs have yet to be determined, three such proteins were encoded in the genomes of the studied isolates, CspA, CspE, and CspC, likely contributing to the persistence of these organisms under cold stress. CspA has been identified as an RNA chaperone; the binding of CspA to RNA has been shown to facilitate the destabilization of RNA secondary structures making them susceptible to ribonucleases (Jiang *et al.*, 1997), while CspE and CspC are involved in the upregulation of stress response genes in *Escherichia coli* (Shenhar *et al.*, 2012; Czapski and Trun, 2014; Keto-Timonen *et al.*, 2016). The heat, cold, and oxidative stresses employed in this study were relatively mild for such a sturdy and versatile species, suggesting that *E. faecium* has strong adaptability to these physical and chemical pressures and that MDR strains have time to respond to such stressors (Gaca and Lemos, 2019).

4.4.3 Response to acid and osmotic stresses

Salinity and acidity stressors did result in decreased microbial survival (Figure 15a, b); however, similar reductions in fitness were observed among all isolates despite the presence/absence of added resistance genes (Table 7). Each isolate encodes at least one copy of an acid-resistance membrane protein with a 99% sequence identity to an enterococcal domain of unknown function (DUF), DUF308 (Table 11) (Yeats *et al.*, 2003). At least one

copy of a tyrosine decarboxylase (TDC) was found in each isolate. Amino acid decarboxylation pathways, induced at low pH, are thought to contribute to acid tolerance through the decarboxylation of an amino acid to its cognate amine, which is then exchanged for its amino acid precursor (Cotter and Hill, 2003). During this process, a proton is consumed, facilitating cytoplasmic pH neutralization. The TDC pathway, in particular, protects *E. faecium* against acute acid stress via extracellular pH neutralization and internal pH maintenance (Pereira *et al.*, 2009). Cytoplasmic buffering can also be facilitated through the arginine (ADI) and agmatine (AgDI) deiminase pathways by the production of ammonia (Marquis *et al.*, 1987; Casiano-Colón and Marquis, 1988). Arginine is deiminated by ADI to L-citrulline, while its decarboxylated derivative, agmatine, is deiminated to carbamoyl-putrescine by AgDI, resulting in free ammonia (NH_3) to bind with protons, producing ammonium (NH_{4+}), and thereby increasing the intracellular pH (Roon and Barker, 1972). L-citrulline and carbamoyl-putrescine are phosphorylated to carbamoyl-phosphate, which is then metabolized by a carbamate kinase producing ATP, CO_2, and another molecular of ammonia for further cytoplasmic pH buffering (Simon and Stalon, 1982). The AgDI pathway is also known to contribute to acid tolerance with additional extracellular pH neutralization (Suárez *et al.*, 2013). Each isolate contained ADI and its associated carbamate kinase; however, Ef77 was also found to encode two copies of AgDI and its carbamate kinase. Interestingly, all isolates showed similar reductions in survival under acid stress, despite the presence of an additional cytoplasmic buffering pathway in Ef77.

All isolates were also significantly inhibited by osmotic shock, with each isolate harboring two copies of an ABC transporter responsible for the uptake of the osmoprotective solute, glycine-betaine. The uptake of this quaternary amine is known to enhance enterococcal growth by countering water efflux in times of hyperosmotic stress

(Kempf and Bremer, 1998; Pichereau *et al.*, 1999). Such osmoprotective solutes can accumulate within the cell with no evident interference with vital cellular processes (Csonka, 1989). Nevertheless, acid and osmotic stressors had severe lethal effects on the three *E. faecium* isolates in this study, irrespective of the number of acquired resistance genes, indicating that this species may not be able to respond to these pressures effectively. Though enterococci can survive a broad range of pH values and hyperosmotic conditions, the stress levels used in this study, a 2.0 pH and 30% NaCl concentration, exceed the sublethal conditions typically associated with this species (Sherman *et al.*, 1937; Thammavongs *et al.*, 1996).

4.4.4 Fitness costs and antibiotic resistance genes

The results of this study suggest that MDR organisms can adapt to environmental stressors comparable to more susceptible strains of the same species. However, the acquisition of antibiotic resistance genotypes usually confers fitness costs to an organism, and meta-analyses have concluded that "no-cost" resistance mutations are rare (Melnyk *et al.*, 2015). It is more likely that organisms harboring such resistance genes maintain their fitness through the co-selection of linked genes or from additional second-site mutations elsewhere in the genome. Compensatory mutations have been suggested to arise within only a few generations upon the acquisition of a costly resistance gene and epistasis may also play a role in that the phenotypic effect of a mutation (e.g., fitness cost) depends on the genetic makeup of the individual (Björkman and Andersson, 2000; Trindade *et al.*, 2009). Furthermore, positive epistasis has been observed in ARB where the fitness costs imposed by an initial resistance mutation can be alleviated by the acquisition of additional resistance genotypes (Trindade *et al.*, 2009; Ward *et al.*, 2009; Hall and MacLean, 2011; Borrell *et al.*, 2013).

The pleiotropic costs of resistance can be so varied as to include mutations with fitness costs that are indistinguishable between MDR bacteria and their antibiotic susceptible counterparts (Sander *et al.*, 2002; Ramadhan and Hegedus, 2005). Studies have shown that with the development of resistance under antibiotic stress, bacteria can alter their surrounding environment, possibly reducing the effects of subsequent stressors through adaptations in both genotype and phenotype (Bower and Daeschel, 1999; Depardieu *et al.*, 2007; Ma *et al.*, 2019). The acquisition of resistance genes under antibiotic stress has also been shown to activate the microbial SOS response for cellular DNA repair, conferring resistance to other stressors, and greater adaptability to environmental changes (van der Veen and Abee, 2011). Similar studies on the fitness costs to *Listeria monocytogenes* and *Salmonella* found comparable results in that the drug-resistant strains were more tolerant to stressors than their susceptible counterparts (Komora *et al.*, 2017; Uddin *et al.*, 2018), while a recent meta-analysis showed that *E. faecium* showed no evidence of fitness costs conferred with the presence of antibiotic-resistant genotypes (Melnyk *et al.*, 2015).

4.5 Conclusion

This study revealed no fitness disadvantages to the *E. faecium* isolates, for the conditions tested, relative to the presence of resistance mutations and genes. A variety of factors modulate fitness costs associated with antibiotic resistance, including epistasis between a resistant genotype and an organism's genetic makeup, environmental conditions, the presence of compensatory mutations, and the genetic linkage of ARGs and other genes when under selective pressure. It is important to note that this study did not address other common enterococcal stressors, including metal, nutritional, and host-derived stress conditions. Additional investigations into the correlation between antibiotic and

environmental stress tolerance are needed to elucidate the specific mechanisms conferring these fitness advantages. Furthermore, little is known of how these fitness costs observed *in vitro* correlate with those experienced *in vivo*. With the increasing evidence of fitness compensations in antibiotic-resistant organisms, it is important to be mindful of the tolerance of these MDR bacteria to environmental stressors to prevent their survival under traditional treatment and disinfection conditions to reduce the dissemination of antibiotic resistance.

4.6 Tables

Table 3 | Genome comparisons between *E. faecium* NC and CL isolates[a].

Parameter	E. faecium (*n* = 33)	NC (*n* = 13)	CL (*n* = 20)	Significance[b]
Genome size (kb)	2,825.3 ± 119.2	2,744.8 ± 100.7	2,877.6 ± 101.0	$p = 0.001$
# of CDS	2,718.5 ± 126.2	2,625.4 ± 91.0	2,779.1 ± 108.7	$p < 0.001$
G+C content (%)	37.9 ± 0.2	38.0 ± 0.1	37.8 ± 0.2	$p = 0.011$
# of ARG	7.8 ± 3.9	5.4 ± 3.7	9.4 ± 3.2	$p = 0.004$
# of Plasmids	3.1 ± 1.7	3.1 ± 1.9	3.1 ± 1.6	$p = 0.97$
# of VF	2.2 ± 0.6	1.8 ± 0.4	2.5 ± 0.7	$p = 0.003$

[a] Values are shown as means ± standard deviations.
[b] *p* values were calculated by Welch's *t* test.

Table 4 | General characteristics of the three *E. faecium* isolates analyzed.

Group	Assembly	kb	MLST	G+C content (%)	# of CDS	# of Plasmids	# of VFs	# of ARGs
NC	Ef75	2795.7	ST1442	38.08	2651	3	2	3
NC	Ef76	2762.0	ST1527 or ST1567	37.91	2624	5	1	4
NC	Ef77	2915.0	ST841	37.86	2741	4	1	8

Table 5 | Resistance phenotypes among the three tested *E. faecium* isolates.

Drug class	Antibiotic	Ef75	Ef76	Ef77
Aminoglycoside	Kanamycin	Intrinsic resistance	Intrinsic resistance	Intrinsic resistance
	Neomycin	Intrinsic resistance	Intrinsic resistance	Intrinsic resistance
	Streptomycin	Intrinsic resistance	Intrinsic resistance	Intrinsic resistance
Carbapenem	Ertapenem	Intrinsic resistance	Intrinsic resistance	Intrinsic resistance
Cephalosporin	Ceftriaxone	Intrinsic resistance	Intrinsic resistance	Intrinsic resistance
	Cephalexin	Intrinsic resistance	Intrinsic resistance	Intrinsic resistance
Fluoroquinolone	Ciprofloxacin[1]	Susceptible	Susceptible	Resistant
Glycopeptide	Vancomycin[1]	Susceptible	Susceptible	Susceptible
Lincosamide	Clindamycin	Resistant	Resistant	Resistant
Macrolide	Azithromycin	Intermediate	Resistant	Resistant
	Erythromycin[2]	Intermediate	Resistant	Resistant
Penicillin	Amoxicillin[1]	Resistant	Resistant	Resistant
	Ampicillin[1]	Susceptible	Susceptible	Susceptible
Phenicol	Chloramphenicol[2]	Susceptible	Intermediate	Resistant
Sulfonamide	Sulfamethoxazole	Intrinsic resistance	Intrinsic resistance	Intrinsic resistance
Tetracycline	Doxycycline[2]	Susceptible	Susceptible	Resistant

[1] Susceptibility determined via EUCAST breakpoints
[2] Susceptibility determined via CLSI breakpoints

Table 6 | Antimicrobial resistance genes and the percent identity matching to known gene sequences.

ARG	Ef75	Ef76	Ef77	Gene source	Resistance mechanism	AMR Gene Family	Drug Class
aac(6')-Ii	99.5%	99.5%	99.5%	Intrinsic	antibiotic inactivation	AAC(6')	aminoglycoside
catA8	-	-	99.1%	Acquired: plasmid	antibiotic inactivation	chloramphenicol acetyltransferase (CAT)	phenicol
eatA$_v$	96.2%	98.6%	99.0%	Acquired: mutation	antibiotic target protection	ABC-F ATP-binding cassette ribosomal protection protein	lincosamide; streptogramin; pleuromutilin
efmA	-	100%	100%	Intrinsic	antibiotic efflux	major facilitator superfamily (MFS) antibiotic efflux pump	macrolide; fluoroquinolone
ermB	-	-	99.2%	Acquired: *Tn917*, *Tn1545*	antibiotic target alteration	Erm 23S ribosomal RNA methyltransferase	macrolide; lincosamide; streptogramin
msrC	100%	95.1%	95.1%	Intrinsic	antibiotic target protection	ABC-F ATP-binding cassette ribosomal protection protein	macrolide; lincosamide; streptogramin; tetracycline; oxazolidinone; phenicol; pleuromutilin
tetL	-	-	100%	Acquired: plasmid	antibiotic efflux	major facilitator superfamily (MFS)	tetracycline
tetM	-	-	99.5%	Acquired: plasmid	antibiotic target protection	tetracycline-resistant ribosomal protection protein	tetracycline

Table 7 | Changes in survivability under each stressor and t-test results.

Table 7 | Changes in survivability under each stressor and t-test results.

Isolate	Test	*p* value	% change	Log change
Ef75	NaCl	<0.001	-50.20%	-0.30
	HCl	<0.001	-99.90%	-3.06
	Heat	0.120		
	Cold	0.064		
	Oxidation	0.942		
Ef76	NaCl	<0.001	-51.79%	-0.32
	HCl	<0.001	-99.87%	-3.19
	Heat	0.528		
	Cold	0.150		
	Oxidation	0.284		
Ef77	NaCl	<0.001	-28.38%	-0.18
	HCl	<0.001	-99.98%	-3.87
	Heat	0.519		
	Cold	0.889		
	Oxidation	0.239		

Table 8 | Copy number of gene products associated with various stress responses found in the three isolates.

Stressor	Mechanism	Associated enzyme	Ef75	Ef76	Ef77
Oxidative					
	Catalases -catalyze the dismutation of H_2O_2 to H_2O and O_2	Manganese-containing catalase	1	1	1
	Peroxidases – reduce H_2O_2 to H_2O	NADH peroxidase (Npr)	1	1	1
		Alkyl hydroperoxidase (*ahpCF*) complex	1	1	1
		Organic hydroperoxidase resistance (*ohr*) protein	3	3	2
		Glutathione peroxidase	1	1	1
		Thioredoxin	3	3	3
		Thioredoxin reductase	1	1	1
	Convert O_2^- into H_2O_2 and O_2	Superoxide dismutase	1	1	1
	Convert methionine sulfoxide to methionine	Methionine sulfoxide reductase (MsrAB)	MsrA – 2 MsrB – 1	MsrA – 2 MsrB – 1	MsrA – 2 MsrB – 1
	Regulate transcription of oxidative stress mechanisms	LysR family transcriptional regulator	5	7	7
		Transcriptional regulator Spx	4	4	4
		Spx/MgsR family transcriptional regulator	1	1	1
		Redox-sensing transcriptional repressor Rex	1	1	1
Osmotic					
	Transport intake of osmoprotectant	Glycine-betaine ABC transporter	2	2	2
Heat					
	Assist in correct protein folding and assembly	Chaperone protein DnaK	1	1	1
		Chaperone protein DnaJ	1	1	1
		Co-chaperone GrpE	1	1	1
		Chaperonin GroL	1	1	1
		Chaperonin HslO	1	1	1
		ATP-dependent chaperone protein ClpB	1	1	1
		Alpha crystallin family heat shock protein	1	1	1
	Regulate transcription of heat stress mechanisms	Heat-inducible transcription repressor HrcA	1	1	1
	Degrade permanently damaged proteins	ATP-dependent protease ClpX	2	2	2
		ATP-dependent HslVU peptidase	1	1	1
		ATP-dependent Clp protease	1	1	2
Cold					
	Destabilize secondary structure of RNA	Major cold shock protein CspA	3	3	3
		Major cold shock protein CspE	1	1	1
		Cold shock protein CspC	1	1	1
Acid					
	Unknown function	Acid-resistance membrane protein	1	2	2
	Buffer cytoplasmic environment	Arginine deiminase	1	1	1
		Agmatine deiminase	0	0	2
		Carbamate kinase	1	1	2
	Amino acid decarboxylation	Tyrosine decarboxylase	1	2	2

4.7 Figures

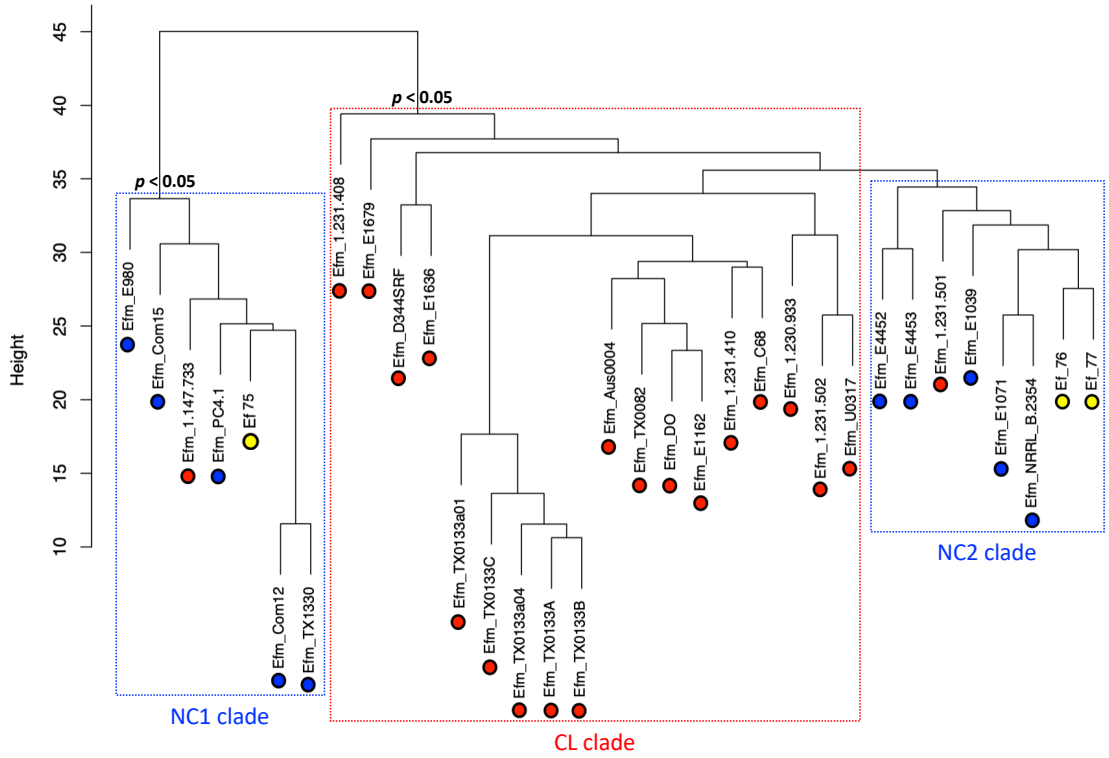

Figure 13 | Hierarchical clustering of *E. faecium* isolates. In the hierarchical clustering of *E. faecium* isolates, NC isolates (blue circles) were enriched in clades NC1 and NC2, and CL isolates (red circles) were enriched in the CL clade. The three study isolates (yellow circles) were associated with the NC clade, with Ef75 among NC1 clade and Ef76 and Ef77 among NC2 clade.

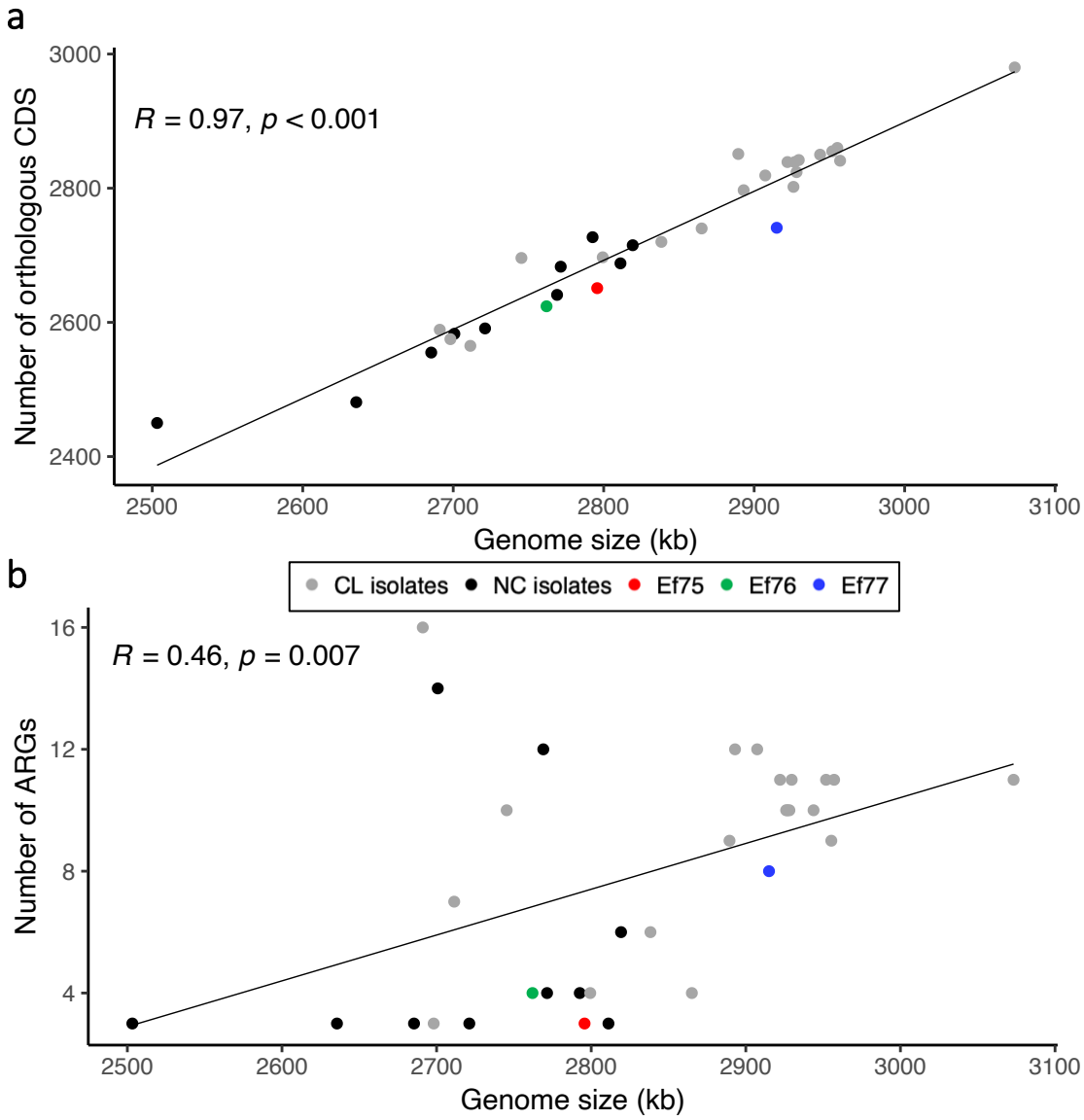

Figure 14 | Correlation of genome size with numbers of orthologous genes and antibiotic resistance genes in *E. faecium*. The numbers of orthologous (a) CDS and (b) ARGs in an individual strain are plotted against the genome size of that strain. Pearson's correlation coefficients and *p* values were calculated and are shown in each scatter plot. The color of each circle indicates CL isolates (gray), NC isolates (black), and the study isolates Ef75 (red), Ef76 (green), and Ef77 (blue).

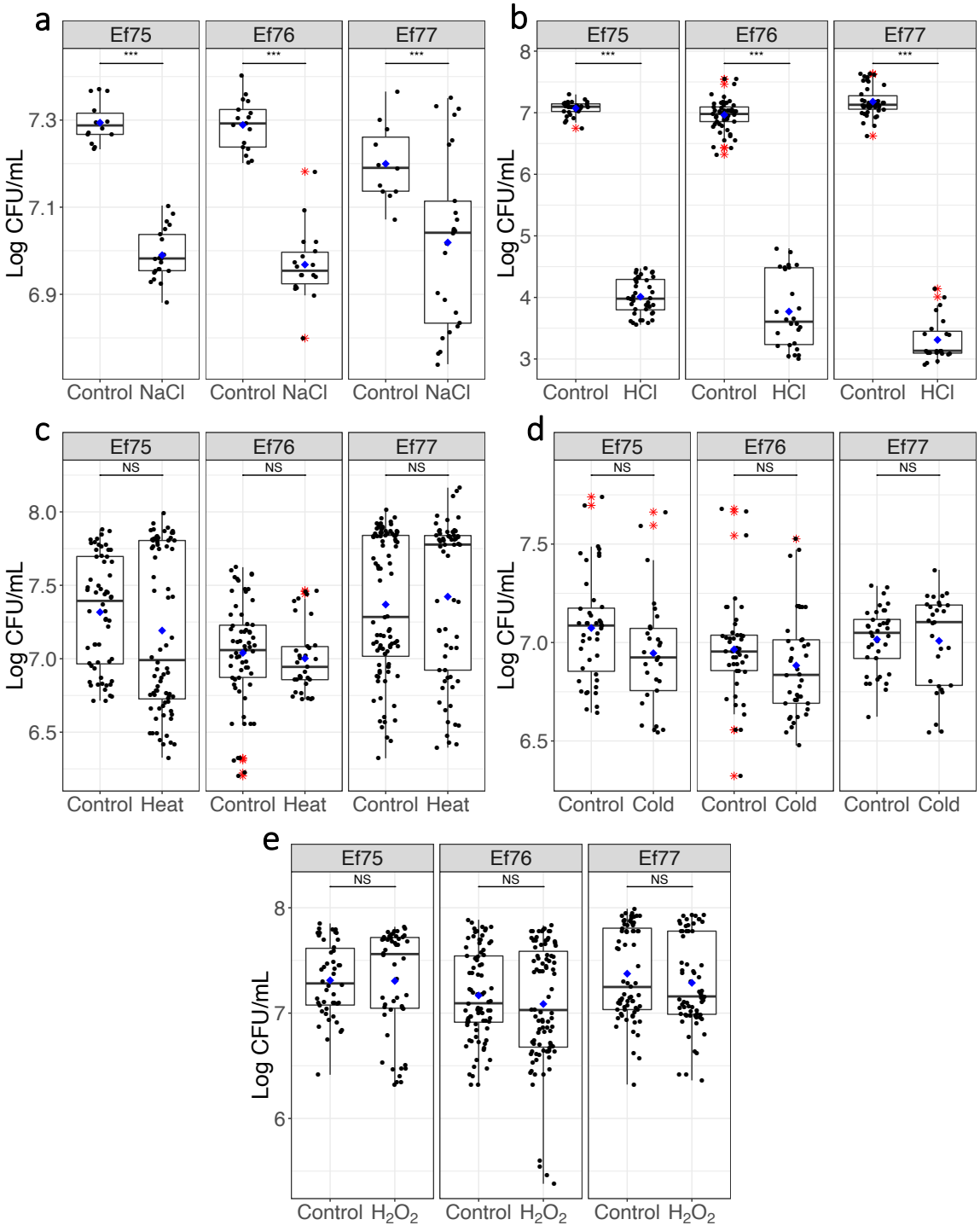

Figure 15 | Survival of *E. faecium* isolates under various stress conditions. Survival of each isolate was assessed with an unmanipulated control and exposure to (a) osmotic, (b) acidic, (c) heat, (d) cold, and (e) oxidative stressors. Significant differences are indicated with bars between the individual tests where "NS" indicates no significance, "*" indicates a *p* value of 0.01 – 0.05, "**" indicates a *p* value of 0.001 – 0.01, and "***" indicates a *p* value <0.001. * identifies outliers; and ◆ indicates the mean.

CHAPTER 5: Analysis of changes in antibiotic-resistant bacterial concentrations from biosolid-applied sediments

5.1 Rationale

A general observation in the study of antibiotic resistance is that environmental compartments with direct influence from human and agricultural activities have higher concentrations of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARG) than those with limited anthropogenic exposure (Verlicchi *et al.*, 2015; Ory *et al.*, 2016; Grenni *et al.*, 2018). It has been suggested that due to the concurrence of growth-promoting conditions of wastewater treatment plants (WWTP) with sub-inhibitory concentrations of antibiotics, there is an increase in the number of antibiotic-resistant bacteria and the associated resistance genes during treatment (Munir and Xagoraraki, 2011). A major route in which antibiotics and ARB are released into the environment is thought to be through wastewater treatment; antibiotic compounds and ARB from WWTPs are continuously being released into various environmental compartments through treated effluent into rivers and streams and through the use of biosolids as fertilizer (Clarke and Smith, 2011).

Biosolids, or sewage sludge, are organic, carbon-rich materials with beneficial uses to enhance soil quality for agricultural yield (Kinney *et al.*, 2006). These biosolids are generated in municipal WWTPs as a byproduct of activated sludge sewage treatment where organic matter is removed from water via aerobic and anaerobic microbial digestion (Auerbach *et al.*, 2007). The use of biosolids in agricultural practices is a promising and sustainable approach to waste management and facilitates a potential reduction in chemical fertilizer use (Sharma *et al.*, 2016). Biosolids act as a suitable fertilizer due to the high content of organic matter (approximately half of the solid fraction), which can provide physical, chemical, and biological improvements to the soil (Kominko *et al.*, 2017). Physical properties include increased retention and movement of water, improved soil aggregation, and soil porosity

(Koutroubas *et al.*, 2014; Kominko *et al.*, 2017). Biosolid amendments can also increase the stability of extracellular enzymes through solid-phase surface properties and provide substrates, such as peptides, to increase microbial propagation (Sharma *et al.*, 2016). These soil enzymes are vital in soil health and agricultural practice by facilitating nutrient cycling, accumulation, and degradation within the soil (Bottomley *et al.*, 2020). Biosolids are rich in nutrients, particularly N and P, that are beneficial for plant growth (Latare *et al.*, 2014). The enhancement of these soil properties ultimately results in increased plant productivity and growth (Sharma *et al.*, 2016). More than half of all WWTP biosolids are applied to farmlands in the United States, yet this use also raises concerns for potential health and environmental effects of ARB (National Research Council, 2002).

The beneficial properties of biosolids are also met with drawbacks, including the presence of toxic heavy metals, pathogenic organisms, and pharmaceuticals, including antibiotic compounds at sublethal concentrations (Kinney *et al.*, 2012; Bondarczuk *et al.*, 2016). A study conducted throughout areas of Sweden, with widespread clinical use of fluoroquinolones, sulfonamides, penicillins, cephalosporins, tetracyclines, and macrolides, found that fluoroquinolones and tetracyclines were the main antibiotic classes detected in sewage sludge and that these chemicals passed unchanged through the WWTPs (Lindberg *et al.*, 2005). Due to this increase in ARB and associated antibiotics, these substances can be detected in sewage sludge and thus within the biosolid-applied soil (Golet *et al.*, 2003). As observed in a similar study from Switzerland, antibiotic compounds could be measured in biosolid-applied soil up to 21 months after application, indicating their persistence and potential for resistance dissemination (Golet *et al.*, 2003).

Antibiotics in soil environments are more persistent than antibiotics found in aquatic locations where degradation can occur in days (Andreozzi *et al.*, 2003). The persistence of

antibiotics in soil environments is likely due to increased sorption to the sludge-soil matrix, reducing antibiotic bioavailability for degradation and leading to longer residence times with the potential for resistance development and propagation (Clarke and Smith, 2011). Nevertheless, the use of biosolids for land application is considered the preferred option for the management of treated sewage sludge as it provides enhancements in nutrient recycling, soil properties, and fertility (European Commission, 2016). This application is also likely to become essential in sustainable nutrient management with the increasing depletion of phosphorus resources (Clarke and Smith, 2011).

The purpose of this study was to quantify the occurrence and potential propagation of antibiotic-resistant bacteria in natural environmental soils applied with biosolids. Soil was collected over one month following the land application of biosolids from two WWTPs in the Charlotte metropolitan area of North Carolina. Antibiotic resistance was assessed using ampicillin, ciprofloxacin, doxycycline, and sulfamethoxazole to monitor changes in ARB numbers over time. The primary focus of this study was to determine soil concentrations of these contaminant ARBs from pre- and post-application of biosolids on an agricultural field relative to background ARB communities.

5.2 Methods & Materials

5.2.1 Sample collection

An agricultural field near Wadesboro, North Carolina, was monitored for one month following the land application of biosolids. Biosolids were collected from the McAlpine Creek Wastewater Management Facility in Pineville and from the McDowell Creek WWTP located in Huntersville, North Carolina. Biosolids from the McAlpine and McDowell WWTPs were applied to two separate sections of the experimental field via tow-behind

manure spreaders. In addition to this experimental field, an adjacent agricultural plot, with no record of biosolid application and uphill from potential runoff, was assessed as a non-applied control site. Land application of the experimental sites took place in March of 2017, and composite soil samples were collected from the experimental sites before biosolid application, as were samples of the biosolids to be applied. Composite samples were created from 16 cores at each site per time-point and mixed in a large bowl until homogenous. Soil samples were also collected from the non-applied control site throughout the study. Samples were collected from all sites at approximately 5 inches below the surface, using soil corers. As the soil was relatively sandy and homogenous, no sieves were needed for debris removal. Triplicate extracts were made from each composite.

5.2.2 Heterotrophic plate counts

The conventional heterotrophic plate count (HPC) method was used to evaluate the concentration of ARB within the samples. The number of ARB was determined by plating samples on media amended with one of four antibiotics: ampicillin, 1000 μg/mL; ciprofloxacin, 50 μg/mL; doxycycline, 100 μg/mL; and sulfamethoxazole, 1000 μg/mL. Extracts pulled from composite samples of biosolids and soil were aseptically weighed out, and 1 g aliquots were placed in 10 mL sterile deionized (DI) water. The soil/water slurry was vortexed until homogenized to disengage microbes from aggregation to the soil. The soil and biosolid samples were then serially diluted 10-fold to achieve a quantifiable concentration of colonies, and 50 μL of the dilution was used for standard spread-plating. The total cultivable heterotrophic bacterial population was determined by plating samples onto media without antibiotics. Colony-forming units (CFU) were visually quantified for count measurements and normalized to CFU per gram.

5.2.3 Statistical analysis

Total heterotrophic growth and antibiotic-resistant abundances were assessed by standard analysis of variance (ANOVA) and Welch two-sample *t*-tests on log normalized count values using the *aov and t.test* functions in R (version 1.1.456).

5.3 Results

5.3.1 Differences in resistant bacterial numbers between biosolid samples

Initial ARB counts were found to vary between the biosolids collected from the McAlpine and McDowell WWTPs (Table 9). Biosolids from the McAlpine WWTP had a greater concentration of total heterotrophic growth ($p<0.001$); however, the McDowell biosolids were more abundant in overall resistant bacterial communities ($p=0.017$). Ampicillin and sulfamethoxazole resistant organisms were found in higher concentrations in McDowell biosolids ($p<0.001$), while ciprofloxacin resistance was observed more in McAlpine biosolids ($p<0.001$). No differences in doxycycline resistant bacteria were found between the two biosolid sampling locations ($p=0.255$).

5.3.2 Background levels of soil ARB differed between sites

Total heterotrophic counts varied among the experimental fields before the application of biosolids. The site where McDowell biosolids were applied had higher total heterotrophic growth than the equivalent field applied with McAlpine sludge ($p=0.015$). A general trend was observed where background ARB levels were higher within the McDowell experimental field ($p=0.002$); however, this variation seems to be driven by the significantly higher numbers of sulfamethoxazole resistant microbes in the McDowell field ($p<0.001$), as no differences in abundance were found among microbes with resistance to the remaining

antibiotics. This trend in higher resistance counts might be attributed to the overall greater microbial abundance observed at the McDowell experimental site.

5.3.3 ARB decreased over the time course after biosolid application

Heterotrophic microbial growth on all four antibiotics displayed changes in abundance over time upon application of McDowell biosolids to the experimental field ($p<0.001$). All antibiotic conditions resulted in an overall difference in ARB abundance over the four weeks ($p<0.001$). By the final time-point, all antibiotic-resistant bacterial counts fell below that of the initial background community (Figure 16c). An initial spike in overall resistant organisms was observed after the first week of application for all antibiotics ($p=0.002$). These levels continued to fluctuate through the second and third weeks until counts dropped below background levels of ARB by the fourth week ($p<0.001$).

The total heterotrophic bacterial counts did not vary from pre-application concentrations after the addition of McAlpine biosolids and remained constant over the four-week time course (Figure D2a). Combined ARB concentrations increased within the first week of McAlpine biosolid application ($p=0.012$). However, this increase appears to be driven solely by the significant increase in sulfamethoxazole resistance as doxycycline-resistant microbes decreased, and ampicillin and ciprofloxacin resistance did not significantly change (Figure D2). Resistance counts decreased to background levels by week two of biosolid application and remained steady for the study duration, despite week four concentrations falling below initial levels (Figure 16b).

5.3.4 Resistance counts relative to the control field

Bacterial abundance was also found to decrease over the one-month timespan within the soil of the control field. Total heterotrophic and combined ARB abundances dropped

significantly from week one to week four (Figure 16a). The combined abundance of antibiotic-resistant organisms from the McAlpine experimental field was no different from that of the control field at any of the time-points. However, the McDowell experimental field was significantly enriched in ARB after one week of application (Table 10, Figure D5b). By the second week of biosolid applications, both experimental fields exhibited ARB levels comparable to those found in the non-applied control field, and each field maintained equivalent resistance concentrations for the duration of the study (Figure D5c-e).

5.4 Discussion

A previous study conducted on a biosolid-applied crop field in Tucson, Arizona over 15 months found that the overall soil ARB concentrations did not deviate from pre-application concentrations over time (Brooks *et al.*, 2007). They also found no difference between ARB concentrations in the control and experimental fields, and unexpectedly found that ARB soil concentrations decreased below pre-application levels, attributing this decrease to the lack of antibiotic selective pressures. A similar trend was observed in the present study as soil concentrations of ARB did not differ from that of the control field, and the final concentrations of ARB were found to be less than that of the pre-application levels.

The immediate increase of ARB within the soil upon the application of biosolids likely reflects the survival of resistant organisms present in the biosolids or an initial enrichment of the indigenous soil ARB caused by the introduction of antibiotic selective pressures (Bondarczuk *et al.*, 2016). After the initial increase in ARB concentrations, resistance levels decreased significantly in both experimental fields. This decrease in ARB concentrations might be due to the diminished selective pressures for antibiotic resistance in the soil environment. As antibiotics within the biosolids degrade, the need for plasmids or

mobile genetic elements carrying ARGs is lost, and the loss of such plasmids in the microbial population could lead to lower overall ARB abundance and, as the experimental fields only experienced a one-time application of biosolids, selective pressures for antibiotic resistance were short-lived (Smith and Bidochka, 1998). The dilution of any biosolid-borne ARB upon application might also account for the decreased concentrations.

As with any environmental study, the regional climate can have a significant effect on bacterial growth. Sample collections took place at the end of winter and beginning of spring. Temperatures steadily rose over the one-month period, with the lowest temperatures occurring during the first week of biosolid application (Table D3). Optimum growth temperatures for soil bacteria have been shown to range from 25 – 30°C (Pietikäinen *et al.*, 2005), and yet this time-point was characterized by the highest cultivable growth during an average temperature of 8.4°C, supporting the notion the additional bacterial load from the biosolids increased ARB growth after application. No precipitation occurred during the final two-week period; however, just before this period, the area experienced high levels of rainfall. This prolonged dry period may have played a role in ARB enumeration; however, total heterotrophic growth was unaltered over the sampling time frame indicating that rainfall might not have had a profound effect (Figure D1-2).

The resistant microbial growth in the virgin soil and control field suggests an abundance of intrinsically resistant microbes within the soil. A vast majority of fungi reside in soil environments and are capable of antibiotic production and are implicated in the selection of fungus-specific antibiotic-resistant bacteria (Boer, Folman, Summerbell, & Boddy, 2005; Bridge & Spooner, 2002). The presence of native resistant microbes in soil environments indicates naturally-developed resistance to antibiotics, independent of exposure to WWTP effluent or byproducts. The return of ARB concentrations to

background levels likely indicates that the addition of biosolids had little effect on the native soil microbial populations and ARB levels.

5.5 Conclusion

Overall, the application of biosolids to these two experimental fields resulted in a reduction of ARB over time and possibly little effect on the indigenous population. However, as no 16S ribosomal RNA (rRNA) gene sequencing or other qualitative analyses were performed, we cannot rule out the possibility of shifts in community composition, which are undetectable with the quantitative analysis performed. Since we have no information on the dominant cultivable ARB native to the fields or the resistant communities after biosolids application, there is no way to determine taxonomic shifts in the soil communities, and additional qualitative studies must be conducted. It is also important to note that this study only demonstrated resistance to four individual antibiotics; thus, multi-drug resistance within the community was not observed. Resistance to one antibiotic often confers resistance to other antibiotics; therefore, further investigation into other classes and combinations of antibiotics is necessary (Brooks *et al.*, 2007). It should also be noted that the heterotrophic growth observed in this study is not indicative of the total viable bacterial community and that viable, non-culturable ARB are not represented. However, this study provides a baseline understanding of shifts in ARB abundance and can be utilized as reference information for future studies on antibiotic resistance propagation due to the use of biosolids as a crop fertilizer.

5.6 Tables

Table 9 | Differential abundance of log normalized bacterial abundances (CFU/g)[a] within biosolids from each WWTP.

	McAlpine	**McDowell**	***p* value**
Total HPC	8.79 ± 0.25	7.85 ± 0.46	<0.001
All ARB	4.42 ± 0.62	4.60 ± 0.64	0.017
Ampicillin	4.37 ± 0.46	4.87 ± 0.34	<0.001
Ciprofloxacin	4.85 ± 0.57	4.30 ± 0.63	<0.001
Doxycycline	3.92 ± 0.61	4.08 ± 0.62	0.255
Sulfamethoxazole	4.71 ± 0.26	5.16 ± 0.14	<0.001

[a] Values are shown as log normalized CFU/g means ± standard deviations.

Table 10 | Differential abundance of log normalized bacterial abundances (CFU/g)[a] between each experimental site at each time-point.

		McAlpine	McDowell	Control [b]	*p* value
Virgin Soil	Total HPC	6.72 ± 0.59	7.04 ± 0.21	-	0.015
	All ARB	4.60 ± 0.59	4.97 ± 0.39	-	0.002
	Ampicillin	5.03 ± 0.05	4.98 ± 0.41	-	0.797
	Ciprofloxacin	5.02 ± 0.25	5.01 ± 0.41	-	0.980
	Doxycycline	4.64 ± 0.36	4.80 ± 0.25	-	0.258
	Sulfamethoxazole	3.65 ± 0.38	5.06 ± 0.44	-	<0.001
Week 1	Total HPC	6.35 ± 0.38	7.10 ± 0.90	7.11 ± 0.98	0.074
	All ARB	5.03 ± 0.57	5.41 ± 0.51	4.91 ± 0.47	<0.001
	Ampicillin	5.36 ± 0.26	5.16 ± 0.45	5.04 ± 0.30	0.057
	Ciprofloxacin	5.21 ± 0.46	6.22 ± 0.09	5.03 ± 0.21	<0.001
	Doxycycline	4.28 ± 0.34	5.11 ± 0.24	4.85 ± 0.15	<0.001
	Sulfamethoxazole	4.98 ± 0.59	5.40 ± 0.22	4.78 ± 0.65	0.006
Week 2	Total HPC	6.36 ± 0.67	6.74 ± 0.43	6.54 ± 0.43	0.040
	All ARB	4.44 ± 0.74	4.48 ± 0.64	4.65 ± 0.75	0.358
	Ampicillin	4.77 ± 0.21	4.81 ± 0.28	5.48 ± 0.54	<0.001
	Ciprofloxacin	4.04 ± 0.21	4.97 ± 0.35	4.71 ± 0.18	<0.001
	Doxycycline	2.56 ± 0.24	3.95 ± 0.34	3.98 ± 0.20	<0.001
	Sulfamethoxazole	4.57 ± 0.70	4.35 ± 0.75	4.29 ± 0.67	0.356
Week 3	Total HPC	6.54 ± 0.40	6.71 ± 0.47	6.83 ± 0.33	0.056
	All ARB	4.69 ± 0.65	4.87 ± 0.60	4.93 ± 0.68	0.142
	Ampicillin	5.25 ± 0.15	5.21 ± 0.32	5.56 ± 0.21	<0.001
	Ciprofloxacin	3.97 ± 0.18	4.79 ± 0.54	4.76 ± 0.24	<0.001
	Doxycycline	3.30 ± 0	3.99 ± 0.44	3.87 ± 0.23	0.018
	Sulfamethoxazole	4.76 ± 0.47	5.07 ± 0.49	5.17 ± 0.14	0.030
Week 4	Total HPC	6.58 ± 0.58	6.54 ± 0.47	6.54 ± 0.47	0.023
	All ARB	4.28 ± 0.48	4.32 ± 0.54	4.32 ± 0.54	0.843
	Ampicillin	4.34 ± 0.47	4.43 ± 0.63	4.43 ± 0.63	0.296
	Ciprofloxacin	4.38 ± 0.17	4.68 ± 0.48	4.68 ± 0.48	0.001
	Doxycycline	4.26 ± 0.22	4.40 ± 0.30	4.40 ± 0.30	0.044
	Sulfamethoxazole	3.98 ± 0.97	3.93 ± 0.40	3.93 ± 0.40	0.322

[a] Values are shown as log normalized CFU/g means ± standard deviations.
[b] No soil was collected from the control site during virgin soil collection.

5.7 Figures

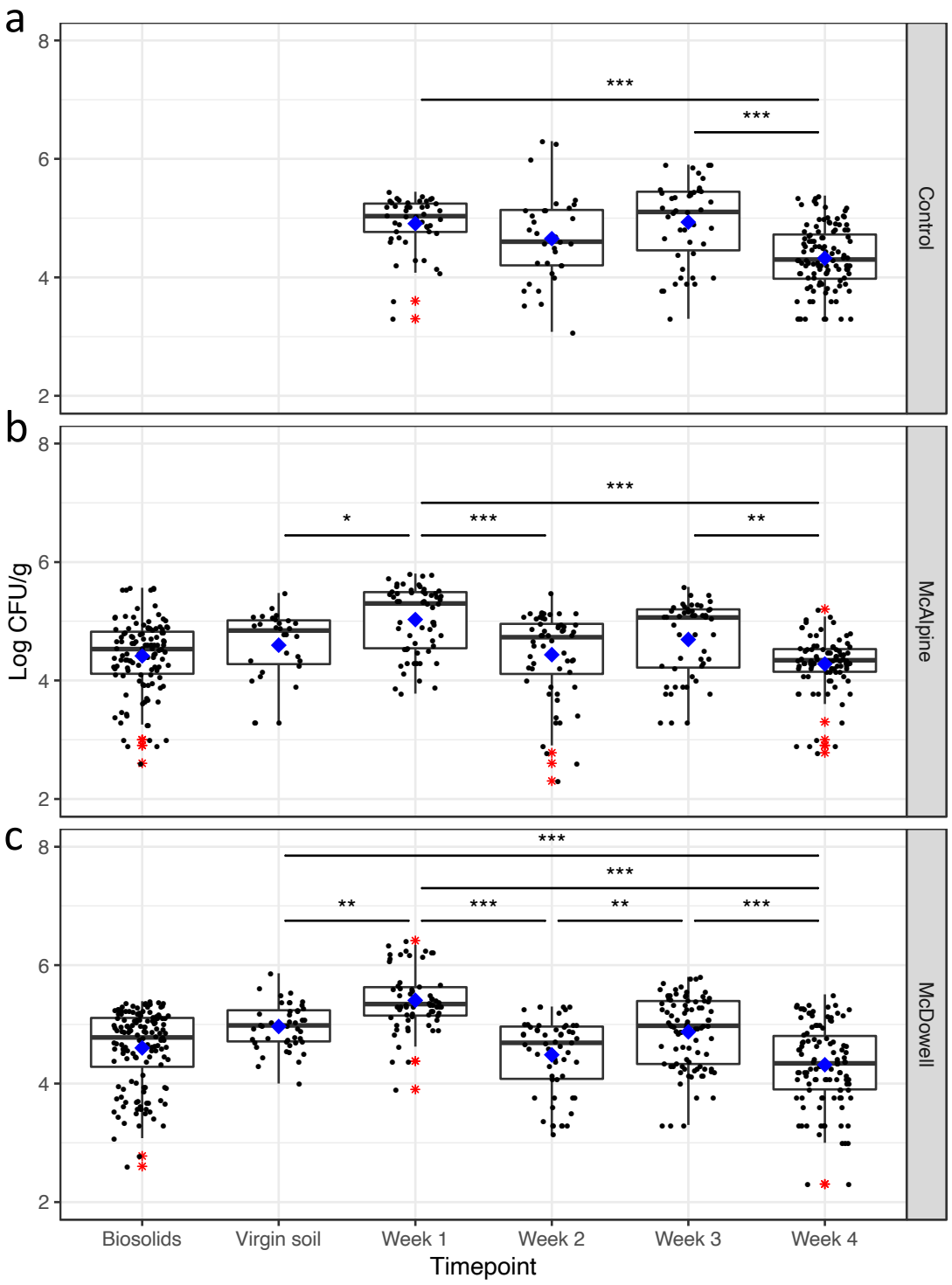

Figure 16 | Antibiotic-resistant bacterial abundance changes upon application of biosolids. Background ARB fluctuations from a (a) control field were monitored in conjunction with experimental fields, (b) McAlpine and (c) McDowell, after application of biosolids. Significant differences are indicated with bars between the individual tests where "*" indicates a *p* value of 0.01 – 0.05, "**" indicates a *p* value of 0.001 – 0.01, and "***" indicates a *p* value <0.001. * identifies outliers; and ◆ indicates the mean.

CHAPTER 6: Summary and Conclusions

6.1 Wastewater treatment plants in the spread of antibiotic resistance

Antibiotic resistance is an increasing concern in the modern age of medicine. With the rapid dissemination of resistance, it is important to understand the ecological implications of antibiotic-resistant bacteria (ARB) from what is currently considered the main route of the spread, wastewater treatment. It is clear that bacteria will continue to develop resistance when in the presence of antibiotic stress due to adaptive fitness. However, as we have shown, the Charlotte Water wastewater treatment plants (WWTP) are effective in removing ARB and their associated resistance genes.

The highest concentrations of resistant bacteria were found in raw sewage and the preliminary treatment stages (Figure 2). These concentrations were significantly reduced with biological aeration treatment. Raw sewage originating from hospital locations had significantly higher ARB abundance relative to primarily residential sewage (Figure A10). The community composition between these sewage sources was largely similar, with no significant differences in diversity or taxonomic abundance (Figure A11 & A12). Bacterial communities collected downstream of the WWTP discharge points showed few differences relative to the effluent-free upstream water communities. No quantitative differences were observed in resistant communities (Figure A6), and overall taxonomic abundance was similar (Figure A8).

However, according to a previous study, the antibiotic compounds themselves are not effectively removed before entry into environmental waters (Lambirth *et al.*, 2018). While the waters located downstream from WWTP effluent were not shown to contain an increase in resistant bacteria, the long-term effects on environmental microbes in the presence of the released antibiotics at sub-inhibitory concentrations could be an important factor in the

spread of resistance. When introduced to effluent-free stream water, sub-inhibitory antibiotic concentrations had little effect on microbial communities (Figure 11 & B3). The primary driver of community change in the water column appeared to be the result of the "bottle effect," with community changes occurring over time (Figure 12), consistent with observations often seen in enclosure experiments. The associated sediment communities showed changes in diversity between controls and antibiotic amendments (Figure B4), though few changes in community composition at the class level were observed over time (Figure B5). Differences in the microbial sediment communities might be the result of antibiotic amendment; however, we cannot definitively attribute these changes to this without additional sequencing methods. While we sought to monitor the changes in bacterial communities exposed to these sub-inhibitory concentrations, no definitive conclusions could be drawn.

The use of biosolids as agricultural fertilizer has also been implicated in the spread of resistance in terrestrial environments (Kinney et al., 2012; Bondarczuk et al., 2016). The application of two ARB-rich biosolid sources onto an agricultural field resulted only in an immediate increase in resistant communities and leveled off over time to pre-application levels (Figure 16). However, such analyses alone offer no insight into the compositional changes occurring within these communities when ARB-rich biosolids are introduced, and further qualitative studies are required.

6.2 The cost of antibiotic resistance

Antibiotic resistance is a natural phenomenon, and this phenotype can be exhibited in bacterial communities that have never encountered the anthropogenic effects of antibiotic use. Although it has been thought that antibiotic resistance genotypes confer fitness costs to

the microbes harboring the ARGs, it has been shown here and in past studies that this is not always the case. More resistant strains of *E. faecium*, strains harboring a higher number of resistance genes, showed no fitness costs to heat, cold, osmotic, acid, or oxidative stressors relative to less resistant strains (Figure 15). Many factors modulate the fitness costs of acquired antibiotic resistance and can even bestow competitive fitness advantages to microbes in the absence of such drugs (Björkman and Andersson, 2000; Trindade et al., 2009; Melnyk et al., 2015).

6.3 Culture-dependent and -independent analyses

While exploring culturable community compositions under various incubation conditions, the incubation temperature appeared to have a greater effect on the enumerated bacterial quantity. Room temperature conditions (~22°C) resulted in significantly more growth than these same samples grown at human body temperature (~37°C) (Figure A15a). At the same time, the media type had a minor effect on overall bacterial enumeration (Figure A15b), though it played a greater role in the composition and diversity of cultured organisms. The reduced nutrient concentrations of R2A agar appeared to promote the growth of more diverse communities than LB agar, which is rich in nutrients.

Overall, several taxonomic groups showed resistance to at least one high-concentration antibiotic (Figure 4). Select culturable taxa were capable of growth at these elevated concentrations throughout the treatment process due to acquired or intrinsic antibiotic resistance. Since a sizeable portion of the microbial community is not easily cultured, resistant communities at these high drug concentrations only offer insight into a small portion of the total population. This cultivability bias may not accurately reflect shifts

in resistant communities since physical, chemical, and biological factors, irrespective of antibiotic amendment, impose selective pressures during cultivation.

While none of the utilized cultivation techniques resulted in growth representative of the overall microbial population within a source sample (Figure 8), the culturable fractions of these populations were higher than previously speculated. On average, 12.8% of the total bacterial population was recovered using standard plating techniques, with some proportions greatly exceeding the widely accepted 1% culturability ideology (Figure A21). Though higher portions of the microbial population were cultivable, the overall makeup of the cultured fractions varied considerably from the natural communities. *Gammaproteobacteria*, particularly *Enterobacteriaceae* and *Pseudomonadaceae*, dominated the cultured communities, while often making up less than 5% of the total environmental source population, whereas the taxonomic makeup of the environmental populations showed greater richness and evenness, comparatively (Figure 8). In general, more operational taxonomic units (OTU) were found to be culturable than previously thought; however, the proportions of these OTUs within their respective communities varied from those identified using culture-independent approaches.

6.4 Future implications

As WWTPs are currently considered the major source for the spread of resistance, understanding how these communities react to treatment conditions provides information that can be used to improve the technology used to treat our water. As multi-drug resistant (MDR) bacterial communities are increasing and have been associated with an increase in fitness, it is imperative to continue developing treatment methods to prevent these microbes from entering our environment.

Additional investigations into the correlation between antibiotic and environmental stress tolerance are needed to elucidate the specific mechanisms conferring fitness advantages. Furthermore, little is known of how these fitness costs observed *in vitro* correlate

with those experienced *in vivo*. With the increasing evidence of fitness compensations in antibiotic-resistant organisms, it is essential to be mindful of the tolerance of these MDR bacteria to environmental stressors to prevent their survival under traditional treatment and disinfection conditions to reduce the dissemination of antibiotic resistance.

Increasing evidence shows the effective removal of antibiotic-resistant organisms in wastewater treatment, but not the actual antibiotic compounds. The development of *in situ* methods to assess native stream communities would provide more reliable depictions of compositional changes upon sub-inhibitory antibiotic exposure as the issues arising from the "bottling effect" and artificial growth conditions are eliminated. Studies, such as this, to determine the effect of antibiotics, alone, on ARB communities is essential for assessment of environmental and human health risks posed by these compounds.

Initiatives have already been put in place in attempts to lessen the spread of resistance, including the reduction of unnecessary prescriptions for these drugs and their use in agriculture (Godman et al., 2017). However, these initiatives alone will not suffice in lessening the burden of antibiotic resistance. Continued investigations into antibiotic resistance outside of clinical settings are of great importance in managing this antibiotic resistance crisis.

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